THESIS

Development of a Rapid Detection Assay for *Listeria monocytogenes* on Ready-to-Eat Meat, Food-Contact and Non-Contact Surfaces

Submitted by

Jeffrey Ryan Callaway

Department of Animal Sciences

In partial fulfillment of the requirements

For the Degree of Master of Science

Colorado State University

Fort Collins, Colorado

Spring 2009

COLORADO STATE UNIVERSITY

April 8, 2009

WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR SUPERVISION BY JEFFREY RYAN CALLAWAY ENTITLED "DEVELOPMENT OF A RAPID DETECTION ASSAY FOR *LISTERIA MONOCYTOGENES* ON READY-TO-EAT MEAT, FOOD-CONTACT AND NON-CONTACT SURFACES" BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

Committee on Graduate Work

Advisor Lawrence D. Goodridge

Kendra K. Nightingale

Claudia Gentry-Weeks

John N. Sofos

Department Head William R. Wailes

ABSTRACT OF THESIS

DEVELOPMENT OF A RAPID DETECTION ASSAY FOR *LISTERIA MONOCYTOGENES* ON READY-TO-EAT MEAT, FOOD-CONTACT AND NON-CONTACT SURFACES

Many tests are available to detect foodborne pathogens. Tests have been designed to selectively allow the growth of a target organism to facilitate identification using biochemical methods, while others have incorporated biochemical identification into selective media. The resulting tests are rapid, sensitive, specific and cost-effective. Recently, molecular methods have been employed to increase the speed, sensitivity and specificity of identification. However, current molecular methods require laboratory equipment and trained personnel and are not capable of detecting viable organisms. This thesis describes a set of studies designed to develop and evaluate a new detection method for the foodborne pathogen *Listeria monocytogenes* that is rapid, sensitive, specific, cost-effective and simple enough to be used in the field and does not require expensive equipment or extensive training.

Listeria monocytogenes is ubiquitous and is also an intracellular human foodborne pathogen. The food-processing environment is an optimum reservoir for *L. monocytogenes* where ready-to-eat (RTE) foods can become contaminated due to survival of this pathogen on food contact and non-contact surfaces. Therefore, one objective of this project was to develop and evaluate a rapid detection method for *L.*

monocytogenes on food contact and non-food contact surfaces, including stainless steel, acrylic, and ceramic tile coupons. The test (known as the Phast Swab) is completely integrated, with all test reagents contained within a single device, including a sampling tool (swab), bacterial growth media [Tryptic Soy Broth + 428mM KCl (TSB-KCl)], *Listeria* specific immunomagnetic separation (IMS) beads, and the colorimetric substrate 5-bromo-4-chloro-3-indoxyl-myo-inositol-1-phosphate (X-Inp). This substrate mimics the natural substrate (phosphatidylinositol) of the enzyme phosphatidylinositol-specific phospholipase C (PI-PLC), which when cleaved creates a soluble (and visible) blue precipitate.

The TSB-KCl growth medium was compared to other media and chosen as the media for the assay due to its ability to rapidly increase the number of *L. monocytogenes* cells. The TSB-KCL medium was also evaluated for its ability to increase the concentration of *L. monocytogenes* cells that were subjected to mild stresses as the organisms are likely to be stressed when in processing or food environments. Five strains of *L. monocytogenes* (J1-177, R2-499, C1-056, N1-227 and N3-013) were grown overnight at 37°C, and 5 ml of each strain were transferred into separate flasks containing 45 ml of each of the following: TSB-KCl adjusted to pH 3 (acidic stress), TSB-KCl adjusted to pH 9 (basic stress), and TSB-KCl with 15% NaCl (high osmolarity stress). The flasks were incubated (with shaking) for 2 hours. A heat stress challenge was performed by transferring 5 ml of each strain into 45 ml of TSB-KCl followed by incubation at 37°C for 1.5 hours, and then the flask was placed into a 48°C water bath for 15 minutes once the final temperature (48°C) was reached. After the exposure to the stressing agents, 500 µl of the broth was transferred into 49.5 ml of fresh TSB-KCl (at

room temperature) and then 250 μl of the broth from each treatment was transferred into a BioScreen honeycomb plate placed into a BioScreen machine. The machine was set to incubate at 37°C with shaking for 1,020 minutes with readings every 30 minutes. Each experiment was performed in triplicate.

The assay was then tested on food contact and non-contact surfaces. The five *L. monocytogenes* strains described above were individually grown overnight at 37°C, adjusted to the same optical density (OD_{600nm}) and combined into a cocktail. A series of 10-fold serial dilutions to produce bacterial concentrations in the range of 10^1 to 10^9 CFU/ml were made in lambda diluent. The concentration of the cocktail was confirmed by plate count on Tryptic Soy Agar (TSA). Stainless steel, ceramic and acrylic coupons (10cm x 10cm) were inoculated with 250μl of dilutions that contained 10^5, 10^6 and 10^7 CFU/ml, to simulate low, medium and high (CFU/cm^2) contamination (10^1, 10^2, and 10^3 CFU/cm^2, respectively). Negative controls were made by inoculating a coupon of each type with lambda diluent for each experiment. Each coupon was allowed to dry for 5 hours and was then swabbed with an individual Phast Swab device (horizontally across and back as well as vertically up and down once). The swabs were incubated for 13 hours at 37°C with shaking at 250 RPM. To determine the actual number of cells on the coupons, the surfaces (duplicate coupons) were inoculated as described above and after 5 hours of drying the coupons were placed into individual Whirl-Pak bags with 100 ml of lambda diluent and allowed to incubate at room temperature for 1 hour. The coupons were then massaged by hand and 1ml of the diluent was plated onto a TSA plate and incubated overnight at 37°C. A reduction in the concentration of *L. monocytogenes* was observed on the coupons, regardless of initial concentration or type of coupon. This led to

final concentrations (CFU/cm^2) of 10^1 for the original 10^5 CFU/ml inoculum, 10^2 for the 10^6 CFU/ml inoculum, and 10^3 for the original 10^7 CFU/ml inoculum.

Following the enrichment process, the Phast Swabs were removed from the incubator, vortexed and the IMS beads were concentrated by placing the swabs into a magnetic device. The growth media and swab were then removed and discarded. The beads were resuspended in a mixture of bacterial lysis buffer (100 μl) and X-Inp (50 μl), which was contained in a reservoir at the top of the device. Following a 5 hour incubation at 37ºC with shaking (250 RPM), aliquots of each test sample were transferred to a 96 well plate and the absorbance (OD_{405nm}) was read using a BioTEK Synergy II plate reader. Each experiment was replicated twice.

The results were subjected to statistical analysis. The coupon sensitivity data were analyzed using a 3 x 4 factorial design in PROC GLM of SAS (SAS Inst. Cary, NC). Mean separation was conducted using paired comparisons of LS means with a Tukey adjustment, α=0.05.

The test was analyzed for its specificity for *L. monocytogenes* by growing 77 strains of *L. monocytogenes* and 29 strains of non-*L. monocytogenes* isolates overnight. Two milliliters of each culture were placed into a Phast Swab device, which was analyzed for its ability to correctly distinguish *L. monocytogenes* and non- *L. monocytogenes* isolates as described above.

The TSB-KCL effectively enriched the stressed *L. monocytogenes* cells. The most challenging recovery was observed for the acidic (pH 3) stress where it took nearly 13 hours for recovery to stationary phase with one of the strains not recovering.

The Phast Swab was capable of detecting 10^1 CFU/cm^2 of *L. monocytogenes* on acrylic ($p=0.0229$), 10^2 CFU/cm^2 on ceramic ($p=0.0112$), and 10^3 CFU/cm^2 on steel ($p=0.0028$) compared to the blank coupons. The differences in detection limit may reflect the differential ability of *L. monocytogenes* to survive on the three surfaces.

The Phast Swab correctly identified 74 of 77 (96%) *L. monocytogenes* strains. The 3 strains that were negative were sequenced for the *plc*A gene and upstream region and although no mutations that could explain the lack of activity were found, the isolates were determined to be atypical from a review of literature and phenotypic characterization. Twenty-eight of twenty-nine (96.5%) non-*L. monocytogenes* bacteria produced negative results. The one false positive was identified as *Listeria ivanovii,* which is reported to produce PI-PLC.

L. monocytogenes is easily inactivated via cooking and pasteurization, however RTE food products can potentially be contaminated after heat treatment. Therefore, the second objective of this study was the evaluation of the Phast Swab to qualitatively detect viable *L. monocytogenes* on turkey and ham deli meats.

A cocktail was prepared as described above and inoculated onto turkey (100mm diameter) and ham (100cm^2) deli meat slices by spreading 1 ml of an appropriate bacterial concentration on each slice. The meat slices were individually inoculated with 1 ml of prepared cocktail for each of the specified final concentrations: 10^0 CFU/ml, 10^1CFU/ml, 10^2 CFU/ml, and 10^3 CFU/ml. Three slices of meat were inoculated (using a sterile cell spreader to evenly distribute the inoculum) per concentration, and two of the inoculated slices were swabbed with the Phast Swab device. The third slice was used to determine the concentration of cells on the meat by conducting plate counts on Oxford

media. An individual slice of meat (25g) was placed into a sterile stomacher bag with 225 ml of lambda buffer and stomached for 60 seconds. The resulting liquid was plated onto Oxford plates and incubated for 48 hours at 37°C. In addition, one slice of meat was inoculated with 1 ml of sterile lambda buffer and this was used as a negative control. The inoculated meat was allowed to dry for 10 minutes in a biological safety level II hood. After drying, each slice of meat was swabbed with an individual Phast Swab (horizontally across and back, vertically up and down). The swabs were then incubated for 10 hours with shaking at 37°C. After the enrichment period the swabs were processed as described above. After addition of the bacterial lysis buffer and the X-Inp enzyme substrate, The Phast Swabs were then incubated with shaking (250 RPM) at 37°C for 5 hours, after which, the swabs were visually observed and results were recorded. All experiments were performed in duplicate and repeated three times for a total of 6 readings for each concentration.

The ability of the Phast Swab to detect low levels of *L. monocytogenes* in RTE meats during storage was ascertained. Turkey and ham deli meats were inoculated as described above except each slice of meat was individually vacuum-sealed in vacuum pouches and stored at 5 ºC. The deli meats were then sampled every 3 days for a total of 21 days (Day 0, 3, 6, 9, 12, 15, 18, and 21). Negative controls were made for each day as described above.

When the Phast Swab test was evaluated on artificially inoculated RTE ham, the assay was capable of consistently detecting 10^2 CFU/g, while also capable of detecting levels below 10^2 CFU/g (plate count results were less than 1 CFU), however, not consistently. The Phast Swab was capable of consistently detecting 10^1 CFU/g on

artificially inoculated RTE turkey. The test was capable of detecting samples that may have been less than 10^1 CFU/g (plate count results were less than 1 CFU), although not consistently. When testing the ability of the Phast Swab to detect RTE ham during storage, it was generally capable of detecting 10^2 CFU/g on artificially inoculated and vacuum-sealed RTE ham during the 21-day experiment. The Phast Swab was generally capable of detecting 10^1 CFU/g on artificially inoculated and vacuum-sealed RTE turkey during the 21-day experiment, although the results were not consistent. The difference in detection observed between ham and turkey may be due to the different antimicrobial treatments in the ham versus the turkey deli meat.

The Phast Swab device is capable of sensitively detecting *L. monocytogenes* on food contact and non-contact surfaces as well on RTE ham and turkey. The implementation of this assay in the food industry may lead to production of uncontaminated food through detection of the pathogen in a rapid, specific and sensitive manner.

Jeffrey Ryan Callaway
Department of Animal Sciences
Colorado State University
Fort Collins, CO 80523
Spring 2009

ACKNOWLEDGEMENTS

I would like to thank Lawrence Goodridge for the guidance he provided and his patience and understanding while working with me. I believe that there is genius in your work and I have appreciated the opportunity to work with you.

I also would like to thank Travis Steiner for his time and interest in my project, which helped keep me motivated and inspired. I owe you many thanks, academically and beyond.

Lily, thank you for your support; without you, I may not have finished.

DEDICATION

This thesis is dedicated to my parents. I wouldn't be here (in so many ways) if it were not for you Mom; and Dad, you never let me quit and showed me that hard work pays off.

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OBJECTIVE OF THESIS

I. To develop a rapid detection test, which integrates a sampling method (swabbing), immunomagnetic separation, bacterial enrichment and colorimetric detection in an assay that is simple-to-use, sensitive and specific.

II. To test the assay on food contact and non-contact surfaces.

III. To evaluate and validate the assay on artificially inoculated ready-to-eat deli meat (ham and turkey) slices.

CHAPTER ONE

INTRODUCTION

Listeria monocytogenes is a Gram-positive, non-sporeforming rod that is a ubiquitous, facultative, intracellular pathogen (Bearns, Girard, 1959; Gray, Killinger, 1966; Murray, Webb, Swann, 1926; Schlech et al., 1983). *L. monocytogenes* can be introduced through many routes to food-processing environments and may become established on food-processing equipment (Møretrø, Langsrud, 2004). The organism can be found in nearly all environments, therefore, there are many points-of-entry into the processing environment and pre and post processing contamination risks are possible (Pritchard, Flanders, Donnelly, 1995).

In the last 10 years there have been several outbreaks of listeriosis in the United States and around the world (Table 1). These outbreaks have identified ready-to-eat (RTE) foods as a major vehicle of listeriosis. RTE (deli) meats may become contaminated during slicing at retail, and although large numbers of *L. monocytogenes* may not be transferred to the meat, the pathogen grows at refrigeration temperatures (Sheen, Hwang, 2008), meaning that even low contamination may result in expansion of the bacterial concentration during storage. As such, it is not surprising that many RTE food products have been found to contain *L. monocytogenes* (Lianou, Sofos, 2007; Yang et al., 2006). For example, Cabedo et al. (2008) analyzed the prevalence of *L. monocytogenes* in RTE foods and found the organism in 3.5% of 1,226 samples of RTE meat and dairy products as well as RTE seafood. In some instances, *L. monocytogenes* was isolated from heat-treated products, which suggests

inadequate heat treatment, or more likely, post-processing contamination (Cabedo, Picart i Barrot, Teixido i Canelles, 2008).

Lopes (1986) found that the standard sanitizers used in the food plant environment are effective against *L. monocytogenes* when used as recommended. However, Duffy et al. (2001), concluded that an incidence rate of 19.8% of retail pork products that tested positive for *L. monocytogenes* was most likely due to improperly cleaned grinding and processing equipment (Duffy et al., 2001). A recent outbreak in Canada also demonstrated that proper sanitation is not always enough, as the organism can escape sanitizers by embedding deep within processing equipment (CBC, 2008).

The continued presence of *L. monocytogenes* in food has necessitated the ongoing need for newer, more sensitive and robust analytical systems capable of rapid detection of this pathogen in complex samples. The ideal detection method should be capable of rapidly detecting and confirming the presence of *L. monocytogenes* directly from complex food samples with no false positive or false negative results. Generally, there are three categories of tests that are used to detect *L. monocytogenes*, including traditional or culture-based methods, immunological methods, and molecular based assays. Culture-based methods are based on the inclusion of *L. monocytogenes* specific fluorogenic and chromogenic substrates within solid media. Conventional culture techniques continue to be the gold standard for the isolation, detection, and identification of foodborne pathogens including *L. monocytogenes*. However, a disadvantage of these methods is the fact that they increase detection times by hours to days, causing preliminary test results to be delayed.

The specificity of an antibody for its antigen has been exploited to create many rapid immunological-based assays for detection of *L. monocytogenes* (Gasanov, Hughes, Hansbro,

2005; Karamonova et al., 2003; Olsvik et al., 1994). Immunological methods that have been developed to detect *L. monocytogenes* include enzyme-linked immunosorbent assays (ELISAs) and lateral-flow assays. The production of *L. monocytogenes* specific antibodies has also enabled the development of immunological-based separation and concentration methods for this pathogen (Bilir Ormanci, Erol, Ayaz, Iseri, Sariguzel, 2008; Olsvik et al., 1994; Uyttendaele, Van Hoorde, Debevere, 2000). Immunological methods are often combined with other methods such as immunomagnetic separation (IMS) for increased sensitivity and specificity (Hsih, Tsen, 2001; Olsvik et al., 1994; Shim et al., 2008; Uyttendaele et al., 2000). These methods require trained personnel, lab equipment and are not field-ready, as they require enrichment, which is usually performed externally from the actual test.

A challenge of most rapid detection assays is the ability to differentiate between *L. monocytogenes* and *Listeria* spp. Molecular methods such as the polymerase chain reaction (PCR), provide alternative detection methods that are relatively rapid, sensitive and specific. Molecular methods are capable of identifying and differentiating *L. monocytogenes* to a sub-species level, while ELISA-based methods are generally only capable of identifying genera (Gasanov et al., 2005; Levin, 2003; Rodriguez-Lazaro, Hernandez, Pla, 2004a; Scheu, Gasch, Berghof, 1999). However, molecular methods require an investment in equipment, reagents and trained personnel.

CHAPTER TWO

LITERATURE REVIEW

Listeria monocytogenes

2.1. Introduction

Listeria monocytogenes is the only pathogenic species within the genus *Listeria* (Buchrieser, Rusniok, Kunst, Cossart, Glaser, 2003). *L. monocytogenes* is a Gram-positive, non-spore forming rod that is a ubiquitous, facultative intracellular pathogen (Collins et al., 1991; Schlech et al., 1983). *L. monocytogenes* is not fastidious and can be found in feces, silage, soil, sewage, water, dust, milk and plants. Peritrichous flagella are produced and assembled on the cell surface when the organism is grown between 20 and 25°C and to a much lesser degree at 37°C (Farber, Peterkin, 1991; Peel, Donachie, Shaw, 1988). The organism is capable of growth in a variety of stressful environments including growth over a wide temperature range (-1.5 to 45°C), high osmolarity, as well as a wide pH range (4.0 to 9.6). In addition, *L. monocytogenes* is capable of survival in diverse environments for long periods of time (Hudson, Mott, Penney, 1994; Pearson, Marth, 1990; Petran, Zottola, 1989; Sauders, Wiedmann, 2007; Watkins, Sleath, 1981). The ubiquitous nature, unique growth, survival characteristics and incidence of *L. monocytogenes* in food indicate that this pathogen is well adapted for survival in many foods and food processing environments (Farber et al., 1991).

2.2. Foodborne Disease

L. monocytogenes causes approximately 2500 cases of listeriosis that result in 500 deaths annually (Mead et al., 1999). Those at the highest risk of contracting the disease include pregnant women, their fetuses, newborns, the elderly and immunocompromised persons (Siegman-Igra et al., 2002). Listeriosis is diagnosed when *L. monocytogenes* is isolated from the blood, cerebrospinal fluid or other typically sterile site, such as the brain stem and other components of the nervous system (Ramaswamy et al., 2007). The incubation period and duration of illness for *L. monocytogenes* are not well defined. For example, onset of illness has been recorded within 48 hours to over 90 days from exposure to contaminated food (Linnan et al., 1988; Low, Donachie, 1997; Mead et al., 2006; Olsen et al., 2005).

The majority of human listeriosis cases are caused by lineage I (serotypes 1/2b and 4b) and lineage II (serotype 1/2a) isolates. Serotype 4b isolates are responsible for the majority of outbreaks (Fugett, Fortes, Nnoka, Wiedmann, 2006; Wiedmann et al., 1997). Listeriosis symptoms vary depending on the susceptible population that is infected (Swaminathan, Gerner-Smidt, 2007). For example, pregnant women may present with symptoms such as fever, myalgia, diarrhea, pre-term delivery, abortion or stillbirth, while newborns may experience sepsis, pneumonia or meningitis (Painter, Slutsker, 2007). Immunosuppressed adults and the elderly may also experience sepsis or meningitis but also focal infections. Symptoms for healthy adults include diarrhea and fever (Painter et al., 2007). In addition to listeriosis, febrile gastrointestinal illness, a non-invasive form of infection, is possible, resulting in diarrhea, fever, abdominal pain, chills and myalgia. Febrile gastroenteritis illness generally affects healthy individuals and medical treatment is not usually necessary (Drevets, Bronze, 2008). Isolates from human sources are rarely antibiotic

resistant and are therefore susceptible to the clinically important antibiotics, but are resistant to the cephalosporin class of antibiotics (Drevets et al., 2008). Although listeriosis is rare, the relatively high mortality rate (20%) can be attributed to the virulence of the organism.

Virulence Factors

After consumption of contaminated food, the majority of organisms are inactivated by the conditions in the gastric environment (Kathariou, 2002). Although most adherent cells can be invaded by *L. monocytogenes*, they are not internalized equally. Macrophage and macrophage-like cells can internalize 20 bacteria per cell, whereas other cell types such as fibroblast cell lines internalize less than one bacterium per cell (Portnoy, Auerbuch, Glomski, 2002). Internalization into nonprofessional phagocytic cells, such as human, intestinal cells, is mediated by gene products that enhance the virulence of the organism (Lecuit et al., 2001; Portnoy et al., 2002). Once internalized, *L. monocytogenes* is capable of survival and growth within the host cell and is capable of hijacking host cell actin to move intracellularly as well as intercellularly (Scortti, Monzo, Lacharme-Lora, Lewis, Vazquez-Boland, 2007). Internalization, growth and movement within host cells as well as spread to neighboring cells are facilitated by virulence factors that are regulated by the positive regulatory factor A (PrfA) (Scortti et al., 2007).

PrfA regulon

There are several key virulence factors that facilitate host cell invasion by *L. monocytogenes*. These virulence factors (discussed below) are regulated by PrfA (Scortti et al., 2007). The *inl*AB operon encodes internalins A and B (InlA, InlB), bacterial surface

proteins that mediate the invasion of non-phagocytic cells, including the cells that line the human stomach (epithelial cells). Internalin A promotes binding and internalization into intestinal epithelial cells by binding to E-cadherin, a host cell surface receptor. The binding of InlA to E-cadherin induces internalization of *L. monocytogenes* via a zipper-like mechanism into a host cell vacuole (Lecuit, Ohayon, Braun, Mengaud, Cossart, 1997; Mengaud, Ohayon, Gounon, Mege, Cossart, 1996; Schubert et al., 2002; Swanson, Baer, 1995). Internalin B causes internalization into a wider variety of cell types such as hepatocytes, fibroblasts and epithelioid cells by binding to the Met receptor tyrosine kinase (Cossart, 2001). Another internalin, InlC, encoded by the gene *inl*C, is important for full virulence in mice but its role in human pathogenesis remains unclear (Lecuit et al., 1997; Portnoy et al., 2002).

After internalization, the first step in host cell invasion is the escape of *L. monocytogenes* from the host vacuole (Portnoy et al., 2002). The pore-forming toxin, listeriolysin O (LLO), encoded by the gene *hly*, is the virulence factor primarily responsible for escape from the host vacuole (Birmingham et al., 2008). LLO disrupts the phagocytic vacuole by blocking phagosome-lysosome fusion via generation of small pores in the phagosome membrane that uncouple pH and calcium gradients, resulting in increased pH and decreased calcium in the vacuole (Shaughnessy, Hoppe, Christensen, Swanson, 2006). The pores that are formed disrupt ion gradients across the vacuolar membrane, which inhibits fusion with lysosomes (Shaughnessy et al., 2006). *L. monocytogenes* is able to replicate within the phagosome due to LLO reorganizing the lipid membrane of host cells. Although LLO activity is crucial to vacuolar escape, it is enhanced by phospholipases (PLCs).

The gene *plc*B encodes a broad-spectrum phospholipase C (PC-PLC) and plcA encodes a phosphatidylinositol-specific phospholipase C (PI-PLC), respectively (Portnoy et al., 2002; Vazquez-Boland et al., 2001). The phospholipases, in combination with LLO, facilitate vacuolar escape. PlcB has the ability to react with several enzymes but one of its main roles is assisting in disruption of the phagosome and lysing the vacuole as part of the invasion process. PI-PLC is an enzyme that along with PC-PLC and LLO, facilitates the escape of *L. monocytogenes* from the host cell phagocytic vacuole by hydrolyzing PI and host proteins. Once the vacuolar constituents have been dissolved, the bacteria can enter the host cytosol (Portnoy et al., 2002).

Once inside of the host cell cytoplasmic environment, the bacteria begin intracellular multiplication with generation times of 40 to 60 minutes, very similar to replication in nutrient rich *in vitro* growth (Portnoy, Jacks, Hinrichs, 1988). A hexose phosphate transporter, encoded by the gene *hpt*, is important for rapid growth in the host cytosol (Goldfine, Knob, 1992; Scortti et al., 2007). In addition to replication in the host cytosol, *L. monocytogenes* induces polymerization of host actin filaments, which mediates intracellular movement and then intercellular spread. ActA, encoded by *act*A, is the only bacterial protein that is responsible for hijacking host cell actin and the resulting motility of *L. monocytogenes*. Motility is accomplished when ActA nucleates actin filaments, which catalyzes their elongation by providing multiple binding sites that act as a scaffold for actin polymerization. This polymerization leads to propulsion of the *L. monocytogenes* out of the internalized cell into neighboring cells (Mounier, Ryter, Coquis-Rondon, Sansonetti, 1990; Robbins et al., 1999). This process also requires a metalloprotease (*mpl*), which is involved in the maturation of PlcB and ActA (Portnoy et al., 2002).

Alternative sigma factor B

Another important virulence factor is the alternative sigma factor σ^B, which regulates stress response in low G+C content bacteria including *Bacillus*, *Listeria* and *Staphylococcus* spp. The non-fastidious nature of *L. monocytogenes* is attributed to σ^B, as deletion mutants become more susceptible to osmotic, pH and heat stress as well as carbon starvation, which affect virulence (Hain et al., 2008). The effect on virulence by σ^B is complex due to the interaction with PrfA. Sigma factor σ^B regulates the transcription of *prf*A but also co-regulates the expression of *inl*AB with PrfA. The role of σ^B appears to be related to the adjustment from a saprophytic lifestyle to an infectious one, as it is critically important during the initial stages of infection due to the co-regulation of *inl*AB (Ollinger, Wiedmann, Boor, 2008). The gene products discussed contribute to the virulence of *L. monocytogenes* and are therefore important regarding food that is contaminated and the resulting infection of human hosts.

2.3. Foodborne Outbreaks

Initially, *L. monocytogenes* was recognized as a veterinary disease, but within the last 30 years, foodborne transmission has been identified as the primary route for human disease (Mead et al., 1999; Murray et al., 1926). *L. monocytogenes* was first described in 1926 but has been responsible for retrospective identification that dates back to 1924 with the first confirmed human diagnosis taking place at the end of World War I (Cotoni, 1942; Murray et al., 1926). Because it is ubiquitous in nature, *L. monocytogenes* has many routes to enter the food chain (Ramaswamy et al., 2007). Several food types are more commonly associated

with listeriosis, including ready-to-eat (RTE) meats such as, deli meats, hot dogs, pâtés and other meat spreads (Norton, Braden, 2007). Uncooked and RTE (smoked) fish and dairy products including soft and dairy sliced cheeses and unpasteurized milk are also commonly associated with listeriosis outbreaks (Gombas, Chen, Clavero, Scott, 2003). Raw vegetables have also been linked to outbreaks of listeriosis (Gombas et al., 2003; Ho, Shands, Friedland, Eckind, Fraser, 1986; Schlech et al., 1983).

Dairy products

Cheese

The largest outbreak to date in US history occurred between January and August of 1985 in Los Angeles County, California (Linnan et al., 1988). Mexican-style soft cheese that was contaminated with raw milk was identified as the vehicle of infection where 142 cases of listeriosis resulted in 48 deaths. Of the 48 fatal cases of listeriosis, 20 were fetuses, 10 were neonates and 18 were non-pregnant adults. Eighty-six (82%) of the 105 *L. monocytogenes* isolates available for study were serotype 4b. Of the eighty-six serotype 4b isolates, 63 (73%) were the same phage type. Although the milk was reportedly pasteurized on site, FDA investigators noted that it was possible to bypass the pasteurizer and add raw milk to pasteurized milk. On several occasions the pasteurizer was filled with more raw milk than could be effectively pasteurized. Cheese samples were tested for phosphatase and several of the tests showed excessive levels, which is consistent with unpasteurized or insufficiently pasteurized milk. Environmental samples from an implicated factory (Plant A) were positive for the epidemic phage type as were food samples from another factory (Plant B) that provided the raw milk to Plant A. By-products from Plant B were also positive for the

epidemic strain, however, no cases of listeriosis were epidemiologically linked to Plant B products (Linnan et al., 1988).

This outbreak was noteworthy for several reasons. The incubation period of *L. monocytogenes* had not previously been investigated and Linnan et al. (1988) were able to determine a range for patients with multiple exposure and those with a one-time exposure (1-91 days and 11-70 days, respectively). Additionally, this was the first listeriosis outbreak where the cause was identified and a recall was ordered during the outbreak. Regulatory practices for the fresh cheese industry were instituted that included phosphatase testing, culturing for *L. monocytogenes* and enforced visitations and inspections of processing plants (Linnan et al., 1988). The threat of the organism was recognized and in response, surveillance systems (at the state and national levels) for human listeriosis were created and foodborne listeriosis became a reportable disease in California (Linnan et al., 1988).

Mexican-style cheese has been implicated in other outbreaks of listeriosis. Between October 1, 2000 and January 31, 2001 there was an outbreak in Winston-Salem, North Carolina where 13 people became infected with listeriosis (MacDonald et al., 2005). The outbreak was caused by noncommercial (homemade), fresh, Mexican-style cheese made with raw milk. Twelve of the infected persons were female between 18 and 38 years of age. Eleven of the twelve females were pregnant and the infections resulted in 5 stillbirths, 3 premature deliveries and 3 infected neonates. The other case of listeriosis was a 70-year-old immunocompromised man. It was determined that raw milk from a licensed dairy was being sold to unlicensed cheese makers that sold their products door-to-door, in parking lots and in Hispanic markets. Although this was not the first outbreak due to fresh, Mexican-style cheese, it was the first report of a listeriosis outbreak associated with homemade cheese. This

outbreak increased awareness of the large-scale, illegal importation of noncommercial cheeses from Latin America. As a result of this outbreak, listeriosis became a reportable disease in North Carolina, although it had been a nationally reportable disease since 2001. MacDonald et al. (2005) recommended targeted education and dietary counseling for Hispanic women to inform them of the dangers of eating RTE food while pregnant, as most of the case patients were recent immigrants, spoke no English, were socially isolated and had no access to health care.

Another cheese-related outbreak occurred in May 2002 in Quebec, Canada (Gaulin, Ramsay, Ringuette, Ismail, 2003). Heat-treated cheese (the milk used to make the cheese was heated to temperatures below pasteurization temperatures) was implicated as the source of the outbreak. Seventeen cases of listeriosis were associated with this outbreak. Fifty-six packages of cheese were tested for *L. monocytogenes*, and the results indicated that all of the packages were contaminated with *L. monocytogenes* pulsotype 85 (the same pulsotype isolated from infected individuals). Soil samples taken outside of the factory tested positive for the organism. The *L. monocytogenes* strain discovered in the soil outside of the factory was not identified (pulsotyped) but it is known that construction workers frequently entered the factory to perform renovations. It is possible that the bacteria were introduced to the factory during the renovations, which demonstrates that *L. monocytogenes* can contaminate food via many different routes (Gaulin et al., 2003).

Other dairy

Other dairy products have been implicated in foodborne outbreaks of listeriosis. Pasteurized butter was implicated in an outbreak that occurred in Finland between June 1998

and April 1999 (Lyytikainen et al., 2000). This was the first outbreak of listeriosis caused by a serotype 3a isolate. There were 25 cases of illness that resulted in 6 deaths. The majority of infected individuals were severely immunosuppressed and were hospitalized at a tertiary care hospital. Butter served at the hospital was implicated as the vehicle for the listeriosis infections and was all made at one dairy (Lyytikainen et al., 2000).

During the summer of 1983 there was a listeriosis outbreak associated with consumption of whole or 2% pasteurized milk in Massachusetts (Fleming et al., 1985). The listeriosis outbreak resulted in a total of 49 cases and 14 deaths. The milk implicated in the outbreak was pasteurized at one dairy. FDA inspections revealed that the milk came from a dairy that had an unusual number of animal listeriosis cases and some of the cases were from cows that supplied the whole milk that was implicated in the outbreak. The cause of the outbreak was never definitively determined, however, it was deemed plausible that there was a high inoculum of *L. monocytogenes* in the raw milk and some bacteria were able to survive pasteurization and infect immunocompromised individuals (Fleming et al., 1985).

Meat and meat products

Although cheese and other dairy products are considered high-risk foods for contracting listeriosis, processed meats are also highly correlated with infection (Swaminathan et al., 2007). In particular, RTE meat products present the highest risk because they can be consumed straight from the package without reheating and may appear safe. Although the food may be contaminated with a small number of cells and appear safe, low numbers of *L. monocytogenes* can cause disease (Maijala et al., 2001).

From 1998-1999 the second largest outbreak of listeriosis in US history occurred (Mead et al., 2006). This outbreak affected 24 states with 108 confirmed cases, 14 deaths and four miscarriages/stillbirths. The date of illness onset ranged from January 15, 1998 to February 8, 1999, with most cases occurring between August and January. For this outbreak, the vehicle was identified as contaminated RTE meat (hot dogs and deli meats) processed at a single facility. Several facts make this outbreak unique. For example, two strains of *L. monocytogenes* were isolated from the contaminated food products, but the strain recovered from infected persons was the strain detected at lower levels. This outbreak was the first time that molecular subtyping and an epidemiological approach were used in which patients infected with non-outbreak strains were used as controls. The outbreak was most likely due to a previously colonized ceiling refrigeration fan that when replaced, led to contamination of production equipment. The listeriosis cases declined after hot dogs and deli meats processed at the plant were recalled (Mead et al., 2006).

In the last 10 years, several meat products have been implicated as the vehicles of listeriosis outbreaks (Table 1). For example, in 1999 there was an outbreak from pâté that affected 11 people in three US states (Maryland, Connecticut and New York) (Anonymous, 1999). At the end of 1999 and in early 2000 there were two separate outbreaks in France involving a pâté-like product (rilletes) and pork tongue that affected 10 and 32 people respectively (de Valk et al., 2001). In the United States a multistate outbreak in 2000 affected 30 people and was traced to consumption of contaminated delicatessen turkey meat (Olsen et al., 2005). In 2002, another multistate outbreak in the US caused by delicatessen turkey meat resulted in 54 cases of listeriosis, where 8 died and 3 pregnant women had fetal deaths (Gottlieb et al., 2006). Recently, an outbreak of listeriosis in Canada was linked to RTE meat

produced at a single plant in Toronto, Ontario. The outbreak resulted in 56 confirmed cases with 20 deaths (PHAC, 2008).

Seafood

While *L. monocytogenes* can be frequently isolated from seafood products, few outbreaks of listeriosis have been attributed to these foods (Norton et al., 2007). When outbreaks due to consumption of contaminated seafood have occurred, the resulting number of cases has been small.

Cold Smoked Fish

Smoked fish was categorized as a high-risk food for listeriosis based on a per serving basis by the USDA-FSIS/FDA joint risk assessment in 2003. This classification was primarily due to the high frequency of contamination and the moderate growth rate of *L. monocytogenes* during storage. However, the frequency of listeriosis outbreaks from smoked fish is low (Norton et al., 2007). Cold smoked fish was implicated in an outbreak of listeriosis that occurred in Sweden, between August 1994 and June 1995 (Ericsson et al., 1997; Tham, Ericsson, Loncarevic, Unnerstad, Danielsson-Tham, 2000). Nine cases of listeriosis were associated with this outbreak. Of the 9 total cases, 3 were neonatal and 6 were elderly or immunocompromised individuals with 2 reported deaths (one was a neonate). Cold smoked rainbow trout and gravad (a cured raw fish fillet) were identified as the vehicles for the outbreak. Several subtypes were implicated in this outbreak, however only one (a serotype 4b strain) was identified as the epidemic strain. This outbreak demonstrated that more than one subtype can be spread from a single processing facility and can be present in

one sample. Therefore, during outbreak investigations, several isolates should be identified and typed from food and environmental samples in order to reduce the risk of false negatives (Ericsson et al., 1997; Tham et al., 2000).

A small outbreak of listeriosis occurred in New Zealand in 1992 (Brett, Short, McLauchlin, 1998). Two cases of perinatal listeriosis were reported. Both cases were linked to the consumption of one brand of smoked mussels. Pulsed-field gel electrophoresis (PFGE) analysis indicated that the *L. monocytogenes* isolates recovered from each patient were indistinguishable from *L. monocytogenes* isolated from an unopened packet of mussels (Brett et al., 1998).

Vegetables

As with seafood, vegetables are rarely associated with listeriosis outbreaks (Norton et al., 2007; USDA/FSIS, FDA, 2003). However, they are classified as a moderate risk food by the USDA-FSIS/FDA risk assessment due to the high number of annual servings and moderate frequency of contamination by *L. monocytogenes* (USDA-FSIS/FDA, 2003). Although rare, outbreaks of listeriosis have been linked to contaminated vegetables, including coleslaw (Schlech et al., 1983), as well as celery and lettuce (Ho et al., 1986).

The ubiquitous nature of *L. monocytogenes* enables this pathogen to survive in a variety of foods and food processing environments (Farber et al., 1991). As such, many rapid and sensitive diagnostics have been developed and used to facilitate the detection of *L. monocytogenes* on food products, food-contact surfaces and non-contact surfaces.

2.4. Detection of *Listeria monocytogenes*

The continued presence of *L. monocytogenes* in food has necessitated the ongoing need for newer, more sensitive and robust analytical systems capable of rapid detection of this pathogen in complex samples. The ideal detection method should be capable of rapidly detecting and confirming the presence of *L. monocytogenes* directly from complex food samples with no false positive or false negative results. In general there are three categories of assays for detecting *L. monocytogenes*, including traditional or culture-based methods, immunological methods, and molecular based assays. Culture-based methods are based on the inclusion of fluorogenic and chromogenic substrates that are specific to *Listeria* spp. and *L. monocytogenes,*within solid media. Immunological methods that have been developed to detect *L. monocytogenes* include enzyme-linked immunosorbent assays (ELISAs), lateral-flow assays and immunomagnetic separation (IMS). Molecular based methods include nucleic acid based detection such as the polymerase chain reaction (PCR) as well as gene probes.

Culture-based methods

Although conventional culture techniques increase detection times by hours or days compared to alternative methods, conventional methods continue to be the gold standard for the isolation, detection, and identification of foodborne pathogens including *L. monocytogenes*.

Selective agents have been added to solid bacterial growth media to improve isolation of *L. monocytogenes* from foods. These agents have been added to media to inhibit the growth of competing flora. Gray et al. (1950) described the use of potassium tellurite during

selective isolation and growth of *L. monocytogenes*, and it can also be used for differential purposes, as some *L. monocytogenes* strains reduce tellurite to tellurium, which produces black colonies. However, Olson et al. (1953) later demonstrated that potassium tellurite prevented the growth of some *L. monocytogenes* strains and therefore its use is discouraged (Gray, Stafseth, Thorp, 1950; Olson, Dunn, Rollins, 1953). The combination of lithium chloride (LiCl) and phenylethanol was shown by McBride and Girard (1960) to successfully amplify the numbers of *L. monocytogenes* in the presence of Gram-negative bacteria. These selective agents were combined to form McBride *Listeria* agar (MLA) (McBride, Girard, 1960). Beerens and Tahon-Castel (1966) showed that nalidixic acid was an effective selective agent in the isolation of *L. monocytogenes* from heavily contaminated samples. Nalidixic acid acts by interfering with the DNA gyrase in Gram-negative bacteria (Beerens, Tahon-Castel, 1966). Nalidixic acid can be used alone or in conjunction with other selective agents to reduce the number of streptococci and other background flora (Beerens et al., 1966). For example, the addition of trypaflavine or acriflavine to media containing nalidixic acid was shown to eliminate nearly all other bacteria with only a slight reduction in the growth of *L. monocytogenes* (Olson et al., 1953). MLA agar was modified through addition of the broad-spectrum antibiotic moxalactam and the resulting medium was highly selective for the isolation of *L. monocytogenes* from raw meat and other foods (Lee, McClain, 1986). This new medium was named lithium chloride-phenylethanol-moxalactam (LPM) agar and was recommended by the USDA-FSIS for isolating the organism from raw meat and poultry (Lee et al., 1986; McClain, Lee, 1988).

Various selective and differential media have been developed to affect isolation and detection of *L. monocytogenes* from foods. These media take advantage of biochemical

characteristics that are specific for *L. monocytogenes* or *Listeria* spp., to allow differentiation from closely related bacteria. For example, LPM agar, Oxford, Modified Oxford (MOX) agar, and polymyxin-acriflacine-LiCl-ceftazidime-aesculin-mannitol (PALCAM) agar, make use of the presence of the enzyme β-D-glucosidase in *Listeria* spp. This enzyme acts as an esculinase, and the resulting biochemical reaction results in the formation of black colonies with a black zone surrounding the colonies (Gasanov et al., 2005). The esculinase activity associated with β-D-glucosidase is present in all *Listeria* spp., meaning that *L. monocytogenes* cannot be differentiated from other non-pathogenic species of *Listeria* when grown on the above media (Becker et al., 2006; Greenwood, Willis, Doswell, Allen, Pathak, 2005; Willis, Baalham, Greenwood, Presland, 2006). Several chromogenic media have been developed that can differentiate between pathogenic and non-pathogenic species of *Listeria* (Aragon-Alegro et al., 2008; Becker et al., 2006; Carricajo et al., 2001; El Marrakchi, Boum'handi, Hamama, 2005; Gracias, McKillip, 2004; Greenwood et al., 2005; Manafi, 2000; Reissbrodt, 2004; Vlaemynck, Lafarge, Scotter, 2000; Willis et al., 2006). These media detect the presence of specific bacterial enzymes by the use of chromogenic substrates that are incorporated into the media, allowing for direct identification of colonies by their characteristic color. Phosphatidylinositol-specific phospholipase C (PI-PLC) is an enzyme that is produced only by the pathogenic *Listeria* spp., *L. monocytogenes* and *Listeria ivanovii* (Notermans, Dufrenne, Leimeister-Wachter, Domann, Chakraborty, 1991). Both species can be differentiated from one another via their sugar fermentation profiles (Table 2).

Chromogenic enzyme substrates are mainly phenol-based derivatives (Manafi, 1996). The primary chromogenic substrates are *o*- and *p*-nitrophenols (ONP, PNP), *p*-nitroaniline (PNA), indoxyl-(Y), 5-bromo-4-chloro-3-indolyl-(X), 5-bromo-6-chloro-3-indolyl, 6-chloro-

3-indolyl, N-methylindolyl- and 5-iodo-3-indolyl (iodo) compounds. The enzyme substrates are incorporated into selective media and the resulting colorimetric activity is adsorbed onto the colonies themselves or diffused into the surrounding media (Manafi, 1996). The use of enzyme substrates in selective media can minimize or eliminate the need to subculture or perform biochemical tests to verify the identity of certain target organisms since the identification can be made directly from the plate (Manafi, 1996). Several selective and differential agars have been developed for *L. monocytogenes*, based on incorporation of chromogenic substrates within the media. Several media, including BCM, Agar *Listeria* according to Ottaviani and Agosti (ALOA^{TM}), and Rapid' L. mono agar have been approved for use by the FDA (Hitchins, 2003).

The BCM *L. monocytogenes* detection system (BCM-LMDS, Biosynth, Switzerland) combines selective pre-enrichment and selective plating in one assay for growth and resuscitation of heat-injured *L. monocytogenes* (Manafi, 2000). The pre-enrichment broth contains a fluorogenic substrate (4-methylumbelliferyl-myo-inositol-1-phosphate) that is specific for PI-PLC and allows for a presumptive positive test for pathogenic *Listeria* spp. When plated onto BCM-plating medium (LMPM), *L. monocytogenes* and *L. ivanovii* both form turquoise colonies due to the PI-PLC based hydrolysis of the chromogenic substrate X-Inp (5-bromo-4-chloro-3-indoxyl-myo-inositol-1-phosphate). PI-PLC is a virulence marker for pathogenic strains of *Listeria* (Notermans et al., 1991). In one study, 468 strains of *Listeria* were checked for the presence of PI-PLC by overlaying colonies formed on agar plates with L-α-phosphatidylinositol, a substrate for the enzyme (Notermans et al., 1991). PI-PLC positive isolates exhibited turbid halos around the colonies. None of the 167 non-pathogenic strains of *Listeria* demonstrated PI-PLC activity. Ninety-eight percent (295/301)

of *L. monocytogenes* strains tested could be correctly identified on the basis of their ability to produce PI-PLC, and the 3 most notable serotypes associated with foodborne illness (1/2a, 1/2b and 4b) were detected 99, 98 and 97% of the time, respectively. While *L. ivanovii* strains were not tested in this study, the authors had previously demonstrated PI-PLC activity in this species that is pathogenic to animals (Leimeister-Wächter, Domann, Chakraborty, 1991). *L. monocytogenes* can be distinguished from *L. ivanovii* by plating onto BCM confirmatory plating medium. On this media, both species fluoresce but only *L. monocytogenes* will produce acid due to the fermentation of rhamnose. Other non-*Listeria* bacteria that produce PI-PLC, including *Bacillus cereus*, *Bacillus thuringiensis*, *Staphylococcus aureus*, and yeasts, are eliminated by the presence of selective agents in LMPM (Manafi, 2000). Reissbrodt (2004) evaluated LMPM agar and PALCAM for their abilities to isolate *L. monocytogenes* from meat samples. A total of 1633 samples of raw sausage batters and fresh fermented spreadable sausage were tested, and LMPM was observed to isolate significantly more *L. monocytogenes* than PALCAM (4.8% vs 3.2%). No false positives or false negatives were detected by confirmatory tests (Reissbrodt, 2004).

ALOA™ contains the chromogenic substrate 5-bromo-4-chloro-3-indoxyl-β-D-glucopyranoside (X-β-D-glu) which is cleaved by β-D-glucosidase (Vlaemynck et al., 2000). The media also contains L-α-phosphatidylinositol, which is cleaved by PI-PLC. Pathogenic Listeriaceae form blue/green colored colonies surrounded by a turbid halo when grown on this media (Vlaemynck et al., 2000). Non-pathogenic species of *Listeria* do not produce the halo. When compared to PALCAM and Oxford media, ALOA™ detected *L. monocytogenes* in 31 of 36 (86.1%) samples of naturally contaminated dairy and meat samples, compared to 22 of 36 (61.1%) for PALCAM/Oxford. Five (13.9% of samples tested) false-negative results

were obtained using ALOA™, 2 of which were *Listeria* spp., and the other 3 were completely negative for *Listeria*. Using PALCAM/OXFORD, 14 (38.9%) of the samples falsely tested positive, 8 of which were non-pathogenic *Listeria* spp. and 6 were not *Listeria* (Vlaemynck et al., 2000). Rapid' L. mono medium detects pathogenic *Listeria* spp. on the basis of PI-PLC hydrolysis of the X-Inp substrate, and differentiates *L. monocytogenes* from *L. ivanovii* based on xylose fermentation. *L. monocytogenes* does not ferment the sugar, but *L. ivanovii* does, which causes the production of a yellow halo around the blue colony (Becker et al., 2006). Rapid' L. mono, Oxford, PALCAM and ALOA™ media were evaluated by Becker et al. (2006) for their ability to detect *L. monocytogenes* in 310 samples of naturally contaminated RTE food, including salmon, sausages and salad. Sixty-nine of the samples were confirmed positive for *L. monocytogenes*. ALOA™ and Rapid' L. mono were found to be superior to PALCAM and Oxford after both 24 and 48 hour enrichment periods. For example, after 24 hours Oxford and PALCAM detected 61 and 58 (88% and 84%) positives, respectively and ALOA™ and Rapid' L. mono both detected 61 (88%) samples. However, after 48 hours Oxford and PALCAM both detected 53 (77%) samples while ALOA™ and Rapid' L. mono detected 63 (91%) and 62 (90%) samples, respectively. The authors did not discuss the decrease in positive samples from 24 to 48 hours for Oxford/PALCAM. However, they did mention that the haloes would rapidly increase in size, so that negative colonies could not be distinguished from the positives. This statement only applies to ALOA™ and Rapid' L. mono. The research group noted that the best method was a combination of a standard medium (PALCAM or Oxford) with a chromogenic media (ALOA or Rapid' L. mono), which would have detected 68 of the 69 samples (99%) (Becker et al., 2006).

The cultural methods described above are mainly used to quantify the concentrations of *L. monocytogenes* in food and environmental samples. Enumeration is important in evaluating the degree of *L. monocytogenes* contamination in foods. There are advantages and disadvantages to the various cultural methods used for enumeration, but almost all are laborious, and require at least 24 hours before quantitation can occur. More rapid methods have been developed to effect detection of *L. monocytogenes*. These are qualitative and simply determine the presence of the pathogen in food samples. Many of the rapid tests can be completed within 24 hours, with high throughput, thereby reducing the labor involved in the testing process. These assays can be broadly grouped into three categories including immunological based methods, molecular based assays, and biosensors. It should be noted that many of the rapid tests are presumptive in nature, and the presence of *L. monocytogenes* in a given sample must be confirmed using approved methods.

Immunological detection

The specificity of an antibody for its antigen has been exploited to create many rapid assays for detection of *L. monocytogenes* (Bhunia, 1997; Bilir Ormanci et al., 2008; Gasanov et al., 2005; Gracias et al., 2004; Karamonova et al., 2003; Olsvik et al., 1994; Scheu et al., 1999; Uyttendaele et al., 2000). These immunological based assays include Enzyme Linked Immunosorbent Assays (ELISAs) and immunochromatography assays. The production of *L. monocytogenes* specific antibodies has also enabled the development of immunological based separation and concentration methods for this pathogen (Bilir Ormanci et al., 2008; Olsvik et al., 1994; Uyttendaele et al., 2000).

Enzyme-Linked Immunosorbent Assays

The most commonly used ELISAs for the detection of *L. monocytogenes* are based on the use of whole cells. In this scenario, *L. monocytogenes* cells are enriched from the food sample, heat killed (Comi et al., 1991; Curiale, Lepper, Robison, 1994; Gangar et al., 2000; Mattingly, Butman, Plank, Durham, Robison, 1988; Sewell, Warburton, Boville, Daley, Mullen, 2003; Silbernagel et al., 2005), or formalin-fixed (Solve, Boel, Norrung, 2000), and then detected by an ELISA. One disadvantage to the use of whole cell ELISAs is the fact that many of the cell-surface antigens are genus specific rather than *L. monocytogenes* specific (Durham, Hassard, 1990; Feldstine, Lienau, Forgey, Calhoon, 1997a; Feldstine, Lienau, Forgey, Calhoon, 1997b; Knight et al., 1996). This is problematic, because *L. monocytogenes* is the only pathogenic *Listeria* species in humans (Gray et al., 1966), with the rare exception of other *Listeria* spp. that have caused or have the potential to cause listeriosis (Johnson et al., 2004). In an attempt to develop *L. monocytogenes* specific ELISAs, some researchers have detected the flagella of the bacterium rather than the whole cells (Farber, Sanders, Speirs, 1988; Kim et al., 2005; Skjerve, Bos, van der Gaag, 1991). These assays require sample manipulation prior to testing, including washing steps and extraction of the flagella, and the specificity of these tests is still only genus-specific (Farber et al., 1988; Kim et al., 2005). ELISAs using the O and H antigens have been developed (Palumbo, Borucki, Mandrell, Gorski, 2003). Whole cell protein extracts (Bourry, Cochard, Poutrel, 1997) and detection of LLO have also been employed as detection targets in *L. monocytogenes* ELISAs (Matar, Bibb, Helsel, Dewitt, Swaminathan, 1992; Paoli, Chen, Brewster, 2004).

Karamonova et al. (2003) developed a *L. monocytogenes* specific sandwich ELISA that is based on an antibody raised against an *L. monocytogenes* cell preparation optimized

for extraction of internalin B. Eight strains of *L. monocytogenes* were tested, and all were detected using the ELISA. In contrast, the ELISA did not detect non-*L. monocytogenes* isolates tested, including isolates of *L. ivanovii*, *L. innocua*, *Listeria welshimeri*, *Listeria seeligeri*, or *Listeria grayii*. In pure culture, the ELISA was capable of directly detecting 10^6 CFU/ml of *L. monocytogenes*, and the assay is unaffected by the presence of high numbers (approximately 10^8 CFU/ml) of the other *Listeria* species. The ELISA was capable of detection of artificially contaminated milk samples inoculated with *L. monocytogenes* at an initial concentration of 5 CFU/ml following enrichment (Karamonova et al., 2003).

Other researchers have evaluated foods for the presence of *L. monocytogenes* with the use of ELISAs (Curiale et al., 1994; Mattingly et al., 1988), and a number of commercially available ELISA methods for detection of *L. monocytogenes* in food and environmental samples have been validated by one or more recognized formal validation (Dunbar, Vander Zee, Oliver, Karem, Jacobson, 2003; Sewell et al., 2003). However, because the antibodies used in these tests may cross react with other *Listeria* spp., the ELISAs are only considered presumptive for *L. monocytogenes*. To confirm the presence of *L. monocytogenes* in a respective sample, enrichment cultures found positive by these methods should be streaked onto selective media and suspect colonies biochemically identified as *L. monocytogenes* according to the FDA method (Hitchins, 2003). *Listeria* spp. and *L. monocytogenes* specific immunoassays are summarized in Table 3.

Immunomagnetic Separation

Immunomagnetic separation makes use of antibody specificity towards a pathogen to concentrate that pathogen before other methods are used to amplify and identify the bacteria

(Olsvik et al., 1994). Antibodies are attached to beads, and added to a homogenized sample. Any pathogen with affinity for the antibody should attach to the bead complex. The beads are then separated from the slurry through either the use of a magnet (immunomagnetic separation with magnetic beads (Hudson, Lake, Savill, Scholes, McCormick, 2001)), or through centrifugation (protein-A-linked sepharose beads (Gray, Bhunia, 2005)). In theory, the technique should concentrate pathogens, thus making detection a feasible option without the requirement for the long enrichment incubations required to amplify pathogen numbers to a detectable level.

Hudson et al. (2001) used immunomagnetic separation to isolate *L. monocytogenes* directly from ham. In this procedure, the food was homogenized with some growth media, the particulate matter was removed, and after a number of washes, particles of bacterial size were pelletted and resuspended in a small volume of buffer. Commercial immunomagnetic beads coated with an anti-*Listeria* spp. antibody were added to the buffer solution and incubated to allow binding of the *L. monocyt*ogenes to the beads. The beads were trapped on a magnet, washed, and the DNA extracted for amplification of *L. monocytogenes*-specific genes by the PCR. The immunomagnetic separation and concentration procedures reduced the detection time to about 1 day, but was limited in terms of sensitivity, since the recovery of cells on the beads was only about 20% of those initially added (Hudson et al., 2001). Immunomagnetic separation on average allowed detection of 1–2 CFU/g food sample, but the results were somewhat variable in terms of sensitivity, having a detection limit ranging from 0.1 CFU/g to greater than 5.7 CFU/g (Hudson et al., 2001). This method appears promising, but will not be ready to be used by the food industry until the efficiency of immunomagnetic isolation is improved. IMS separation was compared to conventional

cultivation methods to determine the specificity and sensitivity of *L. monocytogenes* from turkey meat samples (Bilir Ormanci et al., 2008). A total of 180 turkey samples were tested. The conventional methods detected *L. monocytogenes* on 15 (8.3%) of the samples and the IMS method detected *L. monocytogenes* on 22 (12.2%) of the samples. The combination of the conventional and IMS methods revealed that 23 (12.7%) of the samples were contaminated with *L. monocytogones*, which was confirmed by PCR. The IMS method was found to be more sensitive than the conventional culture methods and the authors noted that it is more rapid as well, noting that at least one day was saved in the detection of *L. monocytogenes* from turkey meat (Bilir Ormanci et al., 2008).

A related capture method has been recently developed for isolation of *Listeria* spp. The method employs *Listeria* specific biotinylated phage proteins that attach to the cells. The bacterial cell-biotinylated phage protein complex is mixed with streptavidin coated paramagnetic beads, and the cells bind to the beads, via the biotinylated phage proteins. The cells can then be removed from solution using magnetic separation. The complexes are washed in order to remove possible food residues and can be directly plated on a *Listeria* selective agar, or alternatively detected with the PCR or ELISA. The Profos (Profos AG, Josef-Engert-Strasse 11, D-93053 Regensburg, Germany) capture method was recently evaluated by the Committee on Microbiology and Extraneous Materials (2008). In an inclusivity study, all 60 *Listeria* spp. strains tested were positive with the assay. In the exclusivity study, all 42 non-*Listeria* strains grown in BHI broth were negative with the assay. In internal and independent laboratory studies, the alternative method compared favorably to the International Organization for Standardization (ISO) 11290-1:1996 method (ISO, 1996) for the detection and enumeration of *L. monocytogenes*. Salami, smoked salmon,

and Camembert were naturally and artificially contaminated with different *Listeria* species. No significant difference was detected between the methods. The overall sensitivity rate was 97% and the specificity was 100% (Hammack, 2008).

Immunochromatography

Immunochromatography (ICG) or lateral flow technology uses antibodies immobilized to a membrane surface such that liquid flow (from the sample) is invoked by capillary migration through the membrane transporting soluble antigens (if present) to the immobilized antibodies (Shim et al., 2008). If the target antigens are present, a visually observable precipitate appears. Shim et al. (2008) combined IMS technology with ICG technology and created a test that is capable of rapidly detecting *L. monocytogenes* in a cost-effective and potentially on-site method. A 14-hour enrichment is required as the detection limit of the test is 1 X 10^5 CFU/ml. An ICG test strip was first produced that had a detection limit of 10^5 CFU/ml and was able to detect all 11 *L. monocytogenes* serotypes tested. It took 24 hours to detect 10^2 CFU/10g of meat originally inoculated onto meat samples. The IMS system was shown to be specific for all *Listeria* spp. tested. The combination of IMS and ICG reduced the test time to 14 hours. The developed test is rapid compared to other immunoassays and commercially available kits, as results can be read the same day. Although the test is rapid, the enrichment step requires additional equipment and labor that keeps this test from being field-ready.

Feldstine et al. (1997b) developed an immunoprecipitation method that made use of heat killed *L. monocytogenes* cells to detect contaminated samples. The authors inoculated various food samples with between 0.003 and 11 CFU/ml, and then performed enrichment

culturing. Samples of the secondary enrichment culture were heated to ensure that all *L. monocytogenes* were dead, and the samples were added to the Visual Immunoprecipitate (VIP) device. While the authors claimed that detection of *L. monocytogenes* was equivalent to that of the *Bacteriological Analytical Manual* and the USDA method, they did not separate the detection of *L. monocytogenes* from other *Listeria* spp. and used the two terms interchangeably.

Molecular methods

Differentiating *L. monocytogenes* from *Listeria* spp. has been met with variable success from the previous detection methods discussed. Molecular methods, based on nucleotide base pair matching, namely the PCR, provide alternative detection methods that are relatively rapid, sensitive and specific. Molecular methods are capable of rapidly identifying and differentiating *L. monocytogenes* to a sub-species level, however, these methods require an investment in equipment, reagents and trained personnel (Gasanov et al., 2005).

Nucleic acid based detection

The specificity of base pair matching is at the heart of all nucleic acid detection assays. Any microorganism that contains DNA or RNA can be detected using nucleic acid-based assays, but a limitation of these diagnostics is their inability to detect protein-based agents of disease such as toxins or prions. The most widely used and reported nucleic acid based method is the PCR (Norton, 2002; Swaminathan, Feng, 1994). Many PCR based assays have been developed for *L. monoctyogenes* (Rossen, Holmstrom, Olsen, Rasmussen, 1991; Simon, Gray, Cook, 1996; Sood, Kaur, 1996). Silbernagel et al. (2004) conducted a

multi-laboratory study that compared the *L. monocytogenes* BAX® PCR system (DuPont Qualicon, Wilmington, DE) to standard cultural methods to determine the specificity and sensitivity of the PCR system. The standard methods consisted of AOAC Official method 993.12 for dairy products, the USDA Microbiology Laboratory Guidebook was used for meat and poultry products and the FDA *Bacteriological Analytical Manual* was used for all other food types. A total of 2335 samples representing 6 food types (frankfurters, soft cheese smoked salmon, raw, ground beef, fresh radishes and frozen peas) were tested during this work. The food types were inoculated with high (1-3 CFU/25g) or low (0.5-1 CFU/25g) concentrations of *L. monocytogenes*. Control samples were not inoculated. Although 1109 samples were positive using the BAX® system and 1115 were positive with the standard reference methods, the authors concluded that the BAX® assay performed as well or better than the standard methods, according to Chi-square statistical results, except for radishes. For radishes, the authors reported a significant difference in the detection of both the high and low inoculation levels by the standard reference method, which detected more samples than the BAX® system. Although the authors concluded that the BAX® system performed equivalently or better than the standard methods (except in the case of radishes) in the detection of *L. monocytogenes*, their results suggest otherwise. The standard methods outperformed the assay 11/18 times, which includes all smoked salmon and ground beef trials, as well as both high and low inoculums. The standard methods were more sensitive than the assay (detected 9/18 and 7/18 samples, respectively; 2 samples were detected equally) and the standard method also had a lower false negative rate. The authors concluded that the BAX® assay be adopted for detection of *L. monocytogenes* in dairy products, fruits and vegetables (except radishes), seafood, raw and processed meats, and poultry. Poultry was

not tested in this study and no reference to testing poultry with the BAX® system was given (Silbernagel et al., 2004). The authors did not provide any mention to the inability of the BAX® system to detect the organism on fresh radishes. There was also no mention of the difference in time or cost required for each system. There were several examples of the BAX® system providing indeterminate results.

Hoffman and Wiedmann (2001) compared the BAX® PCR detection method to detection using LMPM. In this work, a total of 512 environmental samples and 315 raw fish samples were tested by both methods to determine which assay could more accurately detect *L. monocytogenes*. Both tests were found to perform equally well at detecting *Listeria* spp. and *L. monocytogenes* in environmental samples. LMPM detected *L. monocytogenes* in raw fish in 97.8% of the samples and 94.8% of the environmental samples. The BAX® system detected 84.8% of the raw fish samples and 94.7% of the environmental samples. LMPM was 100% specific for the environmental and raw fish samples and the BAX® assay was 97.4% and 100% specific, respectively. The primary difference reported by the authors between the two methods was that the BAX® system delivered results in 3 days, while it took 4 to 5 days to obtain a result when using the LMPM detection system. The authors also noted that the BAX® system requires specialized laboratory equipment and does not lead to isolation of pure colonies that can be used for further analyses. The LMPM plating method requires only basic microbiology equipment and does yield a culture isolate (Hoffman, Wiedmann, 2001).

A method to detect *L. monocytogenes* in 25 ml of raw milk by a two-step PCR with nested primers was designed (Herman, De Block, Moermans, 1995). The detection method included a sample preparation method, in which the components of the milk were chemically

extracted prior to the PCR. The detection limit of the assay was observed to be between 5 and 10 CFU/25 ml raw milk.

A possible disadvantage of the use of the PCR to detect bacteria is the fact that dead cells can also be detected. Several PCR assays have been developed based upon detection of messenger RNA (mRNA). These methods employ a reverse transcription step to convert the mRNA to complimentary DNA (cDNA). A method that is based on RT-PCR and targets 3 *L. monocytogenes* genes, *iap, hly* and *prf*A, was developed (Klein, Juneja, 1997). The *iap*-specific product was the most sensitive as it was detected after a 1-hour enrichment in broth with a sensitivity of 10-15 CFU/ml from pure culture. The authors also tested ground meat artificially inoculated with about 3 CFU/g, which they were able to detect after a 2-hour enrichment in broth. Although the *iap* gene is present in most *Listeria* spp., the primer set used was specific to a region only found in *L. monocytogenes*. While the assay was specific for *L. monocytogenes* and only required a 1-hr enrichment, the total time for the assay to be completed was over 50 hours (Klein et al., 1997).

Another disadvantage of the use of conventional PCR to detect microorganisms is the fact that the concentration of the target microorganisms cannot be quantified. Real-time PCR allows the quantification of *L. monocytogenes* (Gasanov et al., 2005). The reaction mixture contains a fluorescent marker that binds specifically to double stranded DNA and as fluorescence increases after each cycle, direct quantitation of target DNA is possible. This method has been used to identify and quantify *L. monocytogenes* in food samples (Bhagwat, 2003; Hough, Harbison, Savill, Melton, Fletcher, 2002; Norton, 2002; O' Grady, Sedano-Balbas, Maher, Smith, Barry, 2008; Rodriguez-Lazaro et al., 2004a; Rodriguez-Lazaro et al., 2004b; Rodriguez-Lazaro, Jofre, Aymerich, Hugas, Pla, 2004c).

DNA Hybridization

DNA hybridization is the simplest molecular method that is used for detection of *Listeria* spp. and specifically, *L. monocytogenes* in foods (Gasanov et al., 2005). A labeled oligonucleotide probe of complementary sequence to a target DNA sequence is used to differentiate *L. monocytogenes* from other *Listeria* by targeting virulence genes. Many commercially available DNA hybridization tests have been extensively tested for their sensitivity and accuracy. The Accuprobe (Gen-Probe Inc., San Diego, Ca) is a version of a DNA hybridization test that targets mRNA for virulence genes, thus ensuring that only viable cells are detected. The detection limit is 10^5 CFU/ml, which precludes the test from direct detection in food or environmental samples and therefore requires enrichment (Ninet, Bannerman, Bille, 1992). The DNA hybridization technology has proven to be robust and adaptable. It has been combined with other forms of detection including the PCR and is capable of detecting many different genes to specifically target *L. monocytogenes* in a high-throughput format (Cocolin, Manzano, Cantoni, Comi, 1997).

Emerging Molecular Detection Methods

Two of the emerging methods for molecular detection of foodborne pathogens include nucleic acid sequence-based amplification (NASBA) and microarray technology.

Nucleic acid sequence-based amplification (NASBA)

Nucleic acid sequence-based amplification (NASBA) is a sensitive, isothermal, transcription-based amplification system specifically designed for the detection of RNA

targets. In some NASBA systems, DNA is also amplified though very inefficiently and only in the absence of the corresponding RNA target or in case of an excess (>1,000-fold) of target DNA over RNA (Lim, Simpson, Kearns, Kramer, 2005). During NASBA, a primer binds to the target RNA sequence and a reverse transcriptase step produces a cDNA strand. RNase is used to digest the template RNA and a second primer binds to the cDNA, which the reverse transcriptase uses to form a double stranded cDNA molecule. The addition of bacteriophage T7 RNA polymerase facilitates the production of RNA transcripts via the amplification process. Deiman and colleagues (2002) have described the applications, advantages and disadvantages of NASBA (Deiman, van Aarle, Sillekens, 2002).

Several NASBA based methods have been described for foodborne pathogens including *Campylobacter* spp., *L. monocytogenes* and *Salmonella enterica* serovar Enteritidis in various foods, and for *Cryptosporidium parvum* in water as reviewed by (Cook, 2003). Both 16S rRNA and various mRNAs have been used as target molecules for detection. Most of the methods to detect pathogens in foods have employed enrichment in nutrient medium prior to NASBA, as this can ensure sensitivity of detection and encourage the detection of only viable target cells. Although a relatively recent method, NASBA has the potential for adoption as a diagnostic tool for foodborne pathogens.

Nadal et al. (2007) recently described a molecular beacon-based real-time NASBA (QNASBA) assay for detection and identification of *L. monocytogenes*. The assay targeted a sequence from the mRNA transcript of the *hly* gene, and included an internal amplification control to detect failure of the reaction. The assay consistently detected as few as 100 target molecules and 40 exponentially growing *L. monocytogenes* cells per reaction. The researchers also demonstrated the accurate quantification of target RNA molecules in the

presence of DNA in the sample. In combination with a short RNase treatment prior to nucleic acid extraction, the QNASBA assay specifically detected viable *L. monocytogenes* cells. The authors demonstrated the successful application of the assay to rapid detection of *L. monocytogenes* in meat and salmon products, and the authors concluded that the assay could be a useful tool for the study of the growth of *L. monocytogenes* in food samples (Nadal, Coll, Cook, Pla, 2007).

DNA microarrays are powerful tools that allow for high-throughput analysis of gene expression, unique genes associated with a target organism, phylogenetic relationships between serogroups and species-specific polymorphisms for every gene in an organism (Abu Al-Soud, Radstrom, 1998; Borucki, Kim, Call, Smole, Pagotto, 2004; Borucki, Krug, Muraoka, Call, 2003; Brehm-Stecher, Johnson, 2007; Volokhov, Rasooly, Chumakov, Chizhikov, 2002). Modern microarray hybridization protocols involve immobilization of the probe on a solid support using chemical methodologies. In a typical array, single strands of known sequences (the probe) are placed at specifically known sites on the solid support. This is accomplished using optical or electrical methods. The optical approach involves selective de-protection of sites where known sequences of single strands can be built base by base (Bhattacharya, Jang, Yang, Akin, Bashir, 2007). The electrical method takes advantage of the net negative charge of a DNA molecule, which can be electrophoretically transported to specified locations on chip surfaces as previously described (Bashir, 2004). To analyze a sample of unknown DNA target sequences, the DNA in the sample is first labeled with a fluorophore and added to the microarray chip, where hybridization will occur if the target and probe are complentary. The non-hybridized DNA is washed off of the array, and the surface of the array is analyzed using a microarray scanner to detect the fluorescent signal.

There are many advanced applications for DNA microarrays, however, for foodborne detection of pathogens, this technique is most useful in combination with the PCR. The combination of the PCR with DNA-DNA hybridization allows for more sensitive PCR amplifications. When traditionally viewed via gel electrophoresis, the highly sensitive amplifications as well as multiplex PCR tend to generate non-specific DNA products and complicate detection (Volokhov et al., 2002). Therefore, the use of DNA microarrays that contain thousands of DNA probes specific to desired gene targets, allows for accurate characterization of sensitive and multiple PCR products. Volokhov et al. (2002), combined oligonucleotide microarray technology with PCR for the rapid detection and identification of the six *Listeria* species. The authors noted that previously developed PCR-based protocols could easily take advantage of the specificity and speed of microarray technology for more powerful, robust and high-throughput monitoring of the food supply (Volokhov et al., 2002).

Emerging methods

The continued presence of pathogenic microorganisms and their toxins in food and drinking water has necessitated the ongoing need for newer, more sensitive and robust analytical systems capable of rapid detection of these contaminants in complex samples. There are significant challenges to the detection of foodborne pathogens. The ideal detection method should be capable of rapidly detecting and confirming the presence of foodborne pathogenic microorganisms directly from complex samples with no false positive or false negative results. Furthermore, these assays should be user-friendly, cost effective, and capable of testing for multiple pathogens simultaneously. Finally, as foodborne pathogen testing increasingly moves from the laboratory to the food-processing environment, such

methods should be portable and amenable to testing with minimal equipment. With respect to assays for detection of *L. monocytogenes*, efforts have been specifically directed at decreasing the detection time and increasing the sensitivity and specificity of detection of the whole cell. Two of the emerging assays include bacteriophage based methods and biosensors.

Bacteriophage-based Assays

Reporter bacteriophage assays have been developed for rapid detection of *L. monocytogenes* (Loessner, Rees, Stewart, Scherer, 1996; Loessner, Rudolf, Scherer, 1997). In this method, DNA carrying a reporter gene is introduced into target bacteria by bacteriophages with specificity for the target (Willford, Goodridge, 2008). The reporter gene is not detected until the phage multiplies in the host, after which their numbers will have increased to visible levels (Willford et al., 2008). These assays have several advantages over previously described methods. For example, since bacteriophages require the host bacteria to be metabolically active for infection, detection of the expressed reporter protein is indicative of viable bacteria. Non-viable bacteria will not be capable of producing a positive test result. Also, the sensitivity of reporter bacteriophage assays is increased due to the self-amplifying nature of the bacteriophages.

Lux^+ reporter bacteriophages have been used to detect *L. monocytogenes*. Loessner and coworkers (1996) constructed a recombinant bacteriophage with a *lux*AB gene, which encodes a fused *Vibrio harveyi lux*AB protein. The phage, A511, is a genus specific, virulent myovirus, capable of infecting 95% of *L. monocytogenes* cells belonging to serovars 1/2 and 4. In the recombinant phage (A511::*lux*AB), the *lux* gene was inserted immediately downstream of the major capsid protein gene, by homologous recombination between phage

A511 DNA and a plasmid carrying *lux*AB. The recombinant phage A511::*lux*AB was tested for its ability to detect *L. monocytogenes* in pure culture, and a variety of foods. To determine lower detection limits for the A511::*lux*AB assay, low numbers of six different strains of three *Listeria* species were directly challenged with A511::*lux*AB without a prior enrichment step. As few as 500 cells were detected, and a positive result was defined as those samples that resulted in luminescent signals that were two-fold or more greater than the background signal (Loessner et al., 1996). An experiment was carried out using artificially contaminated salad in order to determine whether specific enrichment prior to the A511::*lux*AB assay could improve detection limits in samples with extremely low numbers of cells. Following a 16 hour pre-incubation, the initial presence of less than one *L. monocytogenes* Scott A cell per gram of salad could be identified. The total assay time, including incubations and luciferase measurement, was 22 to 24 hours.

Other foods have been tested using phage A511::*lux*AB. Loessner et al. (1997) tested both naturally and artificially contaminated food samples to determine detection limits for this assay. Foods tested included meat and poultry samples, as well as dairy products such as pudding, soft, semi-soft and cream cheeses. All samples were also analyzed by the International Dairy Federation (IDF) standard plating method for detection of *Listeria*. In the artificially contaminated food samples tested, the detection limit for *L. monocytogenes* was found to be dependent on the individual food item. For example, foods that possessed a large endogenous microflora, such as minced meat and soft cheese, typically inhibited detection in samples inoculated with less than 10 cells/g. This effect was seen with both the IDF method and the *lux*$^+$ phage assay. The researchers concluded that the decreased sensitivity of the *lux*$^+$ phage assay in the artificially contaminated foods was most likely not due to a light

quenching effect, and more likely due to the inhibitory action of the competing microflora on the *Listeria* population (Loessner et al., 1997). For the naturally contaminated foods, slightly different results were reported between the IDF standard procedure and the *lux*$^+$ phage assay. Among the poultry samples, the plating method detected *Listeria* in seven more samples (18 of 35) than did the *lux*$^+$ phage assay (11 of 35). Of these isolates, five were identified as *L. innocua*, and two were *L. monocytogenes*. These seven strains were insensitive to bacteriophage A511::*lux*AB infection, and therefore, could not be detected in the assay. This result was also observed for the one strain recovered by the plating method from raw meats, which was identified as a phage resistant *L. innocua* strain. These limits (i.e. false negatives) could possibly be overcome by the construction of phage mutants that can infect these strains (Loessner et al., 1997), or with the use of a cocktail of phages possessing the required host range.

In contrast to the meat and poultry samples, where the plating procedure proved superior to the *lux*$^+$ phage assay, the dairy samples produced two additional positive samples (15 of 121) when tested with the *lux*$^+$ assay. Dairy samples that were tested included milk, cheese, and pudding. The *lux*$^+$ assay also detected four more positive results among environmental samples tested (27 of 158), than did the standard plating procedure. One possible explanation for this is the fact that the selective compounds inhibit certain *Listeria* strains used in isolation media, and cannot grow on these agars (Curtis, Lee, 1995), while luciferase expression by the *lux*$^+$ phage assay requires only the presence of viable *Listeria* cells (Loessner et al. 1997). Overall, the standard plating method and the *lux*$^+$ assay were able to detect similar numbers of samples that were contaminated with *Listeria*. However, the advantage of the *lux*$^+$ assay is the minimum time required for detection of *Listeria*, 24 hrs,

which is much shorter than the 4 days needed by the standard plating method. Additionally, samples that yield positive bioluminescence signals can then be further investigated by colony isolation, species differentiation and typing of *Listeria* strains, if required.

Biosensors

Biosensors have been defined as the offspring of the combination of biology and electronics (DeYoung, 1983). Modern biosensors have effectively combined both disciplines, with electronics/information technology exemplified by microcircuits and optical fibers, and biology exemplified in the form of enzymes or affinity probes (Richter, 1993). The basic principle of a biosensor employs electronic or optical transduction technology to monitor a parameter of the reaction between an affinity probe and an analyte, and to display the parameter as a quantifiable electrical or optical signal (Griffiths, Hall, 1993). The signal can be related to concentration. Analytes that are not recognized by the respective affinity probe will not produce a signal. Various biosensors are currently being developed that make use of affinity probes.

For example, a piezoelectric immunoassay has been developed as a biosensor for *L. monocytogenes* (Jacobs, Carter, Lubrano, Guilbault, 1995). The piezoelectric transducer is a quartz crystal disk with a gold electrode on each face. The electrodes are coated with antibody, antigen from the target will attach and the electrode mass increases. The test takes approximately one hour, however, the detection limit is 10^5 CFU/ml, making an enrichment step necessary. Gupta and coworkers (2004a,b) have applied the use of resonant cantilever biosensors to detection of bacteria, and were able to detect a single *L. innocua* cell in pure culture using this technique (Gupta, Akin, Bashir, 2004a; Gupta, Akin, Bashir, 2004b).

An antibody-based, fiber-optic biosensor to detect low levels of *L. monocytogenes* cells following an enrichment step was developed by Geng and coworkers (2004). The principle of the sensor is a sandwich immunoassay where a rabbit polyclonal antibody was first immobilized on polystyrene fiber waveguides through a biotin-streptavidin reaction to capture *Listeria* cells on the fiber. A cyanine 5-labeled murine monoclonal antibody, C11E9, was used to generate a specific fluorescent signal, which was acquired by laser based excitation at 635-nm and emission at 670 to 710 nm. The immunosensor was specific for *L. monocytogenes* and showed a significantly higher signal strength than for other *Listeria* spp. and non- *L. monocytogenes* bacteria in pure or mixed-culture. Fiber-optic results could be obtained within 2.5 hours of sampling. The sensitivity threshold was about 4.3×10^3 CFU/ml for a pure culture of *L. monocytogenes* grown at 37°C. When *L. monocytogenes* was mixed with lactic acid bacteria or grown at 10°C with 3.5% NaCl, the detection threshold was 4.1×10^4 or 2.8×10^7 CFU/ml, respectively. In less than 24 hours, this method could detect *L. monocytogenes* in naturally or artificially contaminated hot dog or bologna following enrichment in buffered *Listeria* enrichment broth (Geng, Morgan, Bhunia, 2004).

Nanduri et al. (2007) recently developed a surface plasmon resonance (SPR) biosensor for the detection of *L. monocytogenes*. Whole cells of *L. monocytogenes* were detected with a compact SPR sensor using a phage-displayed scFv antibody to the virulence factor ActA for biorecognition. Phage Lm P4:A8, expressing the scFv antibody fused to the pIII surface protein was immobilized to the sensor surface through physical adsorption. The specificity of the sensor was tested using common foodborne bacteria and a control phage, M13K07 lacking the scFv fusion on its coat protein. The detection limit for *L.*

monocytogenes whole cells was estimated to be 2×10^6 CFU/ml (Nanduri, Bhunia, Tu, Paoli, Brewster, 2007).

2.5 Summary

No perfect methodology exists to detect *L. monocytogenes*. All methods have advantages and disadvantages. Due to the nature of detection, no one method will fulfill all needs. However, modern methods have become very sensitive, specific and rapid (less than 24 hours including enrichment). The advent of molecular biology, and the combination of biology and electronics have resulted in the development of emerging novel, sensitive and rapid methods for the detection of foodborne pathogens. A discussion of methodologies cannot be complete without the mention of sample preparation. While the emerging rapid detection techniques discussed above have progressed to the point where single cell detection may be possible, the methods are limited by the inherent problems (time and labor intensiveness, well trained staff, expensive laboratory equipment) of sample processing. The major challenge in preparing an appropriate sample comes from the high probability of components of the food matrix such as fat and proteins interfering with the ability of the detection method to reliably identify the target organism. The new frontier for detection of *L. monocytogenes* should combine the advantages from the various methods to create a single test that is rapid, sensitive and specific. Currently, there is no test that has these qualities but is so simple that it can be used in the field or processing facility with little to no expensive equipment or training. An example of a method that would address these new concerns would be a test that is vertically-integrated, where sampling, enrichment and the test itself occur in one device. The objective of this project was to develop and evaluate a vertically

integrated rapid method for detection of *L. monocytogenes* on food contact surfaces including stainless steel, acrylic, and ceramic tile coupons. The assay was also assessed for its ability to rapidly detect *L. monocytogenes* in RTE deli meat (turkey and ham).

CHAPTER THREE

Development of a Rapid Detection Assay for *Listeria monocytogenes* on Food Contact and non-Contact Surfaces

3.1. Abstract

The objective of this study was to evaluate a newly developed rapid detection method for *L. monocytogenes* on stainless steel, acrylic, and ceramic tile coupons. The Phast Swab is a self-contained test device that contains a sampling tool, growth media, specific immunomagnetic separation beads, lysis buffer and a colorimetric substrate. Individual stainless steel, ceramic and acrylic coupons ($100cm^2$) were chosen to simulate food contact and non-contact surfaces and were inoculated with different concentrations of inoculum. The coupons were allowed to dry and then were swabbed with a Phast Swab device. The results of this study indicate that the Phast Swab is capable of detecting 10^1, 10^2 and10^3 CFU/cm^2 of *L.* monocytogenes on acrylic, ceramic and stainless steel, respectively, on food contact and non-contact surfaces within 18 hours.

3.2. Introduction

Listeria monocytogenes is a Gram-positive, non-sporeforming, facultative intracellular foodborne pathogen (Bearns et al., 1959; Gray et al., 1966; Murray et al., 1926; Schlech et al., 1983). *L. monocytogenes* is not fastidious and can be found in feces, silage, soil, water, dust, milk, plants and is capable of growth in a variety of stressful environments such as a wide range of temperatures (1-45°C), high osmolarity (able to tolerate up to 20%

salt concentration), as well as at high and low pH (Hudson et al., 1994; Pearson et al., 1990; Petran et al., 1989; Sauders et al., 2007; Watkins et al., 1981). The ability of this pathogen to survive in diverse environments enables it to survive in many different foods. Mead et al. (1999) has estimated that *L. monocytogenes* causes approximately 2500 cases of listeriosis and 500 deaths each year in the U.S (Mead et al., 1999). *L. monocytogenes* has been isolated from many ready-to-eat (RTE) food products including pasteurized and raw milk, a variety of different cheeses (Lopes, 1986), as well as pre-packaged salads (Little et al., 2007), cabbage and coleslaw (Francis, O'Beirne, 2006) and meat products and seafood (Cabedo et al., 2008). Gombas et al. (2003) surveyed 8 categories of RTE food products and reported that, out of 31,705 samples obtained over a period of 14 to 23 months, 1.82% of the samples were positive for *L. monocytogenes*. Among those samples with higher levels of contamination were deli meats and smoked seafood (Gombas et al., 2003). One route by which deli meats can become contaminated with *L. monocytogenes* is through transfer from contaminated food processing equipment and food contact suraces, and although large numbers of *L. monocytogenes* may not be transferred to the meat, the pathogen can grow during storage of the product at refrigeration temperatures (Gray, Stafseth, Thorp, Sholl, Riley, 1948). A major food safety concern is that of post-process contamination. Many RTE food products have been implicated in outbreaks of listeriosis around the world (Lianou et al., 2007; Yang et al., 2006), and post process contamination has been suspected in several outbreaks. For example, in 1998-1999, a large outbreak of listeriosis occurred. The outbreak affected 24 states with 108 confirmed cases, 14 deaths and 4 miscarriages/stillbirths. The vehicle of infection was contaminated RTE meat (hot dogs and deli meats) products processed at a single facility. An investigation determined that the outbreak was likely

caused due to a previously colonized ceiling refrigeration fan that, when replaced, led to contamination of production equipment, environmental surfaces and the meat produced on the equipment. The listeriosis cases declined after hot dogs and deli meats processed at the plant were recalled (Mead et al., 2006).

During the late summer and fall of 2008, an outbreak of listeriosis in Canada was linked to RTE deli meat produced at a Maple Leaf Foods plant in Toronto, Ontario. The outbreak caused 56 illnesses and 20 deaths (PHAC, 2008). Subsequent analysis of the outbreak was accomplished to determine the exact source of contamination. Following careful analysis of records, the physical plant and product test results, it was determined that the most likely contamination source was two meat slicing machines at the plant. While the slicers were sanitized on a daily basis in accordance with or exceeding the equipment manufacturer's recommendations, full disassembly of the equipment revealed areas deep within the equipment where bacteria could accumulate, thereby avoiding the sanitization process (PHAC, 2008).

Due to the possibility of *L. monocytogenes* contamination of equipment and food contact and non contact surfaces, the Food Safety Inspection Service (FSIS) has mandated environmental testing of non-food contact surfaces, as well as testing food contact surfaces for the presence of *L. monocytogenes* or *Listeria* spp, including the routine risk-based testing of food contact surfaces such as conveyor belts, cooler storage racks, luggers, slicers, peelers, loaders, table tops and routine risk-based testing of non-food contact surfaces in the RTE production areas such as floors, drains, walls, air-vents, overhead structures (FSIS, 2009).

Many different procedures have been developed for detection of *L. monocytogenes* including cultural, immunological and molecular methods (Bansal, McDonell, Smith,

Arnold, Ibrahim, 1996; Bearns et al., 1959; Beumer, Brinkman, 1989; Bilir Ormanci et al., 2008; Brehm-Stecher et al., 2007; Curiale et al., 1994; Curtis et al., 1995; Gasanov et al., 2005; Hitchins, 2003). Cultural methods require intensive labor and time, immunological methods suffer from a lack of sensitivity. Molecular methods, while being sensitive, are expensive, require operator training, and may or may not detect viable organisms. In addition, all of the currently available methods are not designed to be used to detect *L. monocytogenes* directly in the food plant environment. The objective of this study was to develop and evaluate an integrated method for rapid detection of viable *L. monocytogenes* on food contact and non-contact surfaces in food processing plants.

3.3. Materials and methods

3.3.1. Bacterial strains and cultivation

Seventy-seven strains of *L. monocytogenes* (Table 4) and 29 strains belonging to species other than *L. monocytogenes* (Table 5) were used in this study. Fifty-seven of the *L. monocytogenes* strains were from the International Life Sciences Institute (ILSI) collection at Cornell University (www.pathogentracker.net) and the other 20 strains were wild type isolates from RTE meat processing plants. Of the 77 strains of *L. monocytogenes*, 5 strains (C1-056 (serotype 1/2a, human isolate), J1-177 (serotype 1/2b, human isolate), N1-227 (serotype 4b, food isolate), N3-013 (serotype 4b, food isolate) and R2-499 (serotype 1/2a, human isolate) were combined in a cocktail for inoculation experiments. These strains were chosen because they are representative of the genetic diversity of human disease–associated *L. monocytogenes,* and they have been recommended as the strains of choice when

developing cocktails for inoculation experiments (Fugett et al., 2006). Stock bacterial cultures were maintained in 20% glycerol and were frozen at -80°C.

Prior to all experiments, fresh bacterial host cultures were produced by inoculating frozen stock cultures onto Tryptic Soy agar (TSA) plates followed by overnight incubation at 37°C. For growth experiments, the inocula consisted of stationary phase cells that were obtained by inoculating Tryptic Soy Broth (TSB) supplemented with 428 mM potassium chloride (KCl) with a swath of cells (3-5 well isolated colonies) from an overnight TSA plate and incubating the preparations overnight with shaking (250 RPM) at 37°C. The overnight cultures were combined into a five-strain cocktail by equalizing the OD_{600nm} of each individual culture, followed by the combination of an equal volume of each culture into a sterile test tube. Inoculum levels used in all experiments were determined by serial (10-fold) dilution in lambda diluent, followed by plate count analysis (on TSA) to verify the *L. monocytogenes* concentration.

3.3.2. Evaluation of different growth media

Six different media were evaluated to determine the best growth medium to use during enrichment of *L. monocytogenes*. The media evaluated were Buffered Peptone Water (BPW), Universal Preenrichment Broth (UPB), Brain Heart Infusion (BHI) broth, BHI supplemented with 428 mM potassium chloride (KCl), Tryptic Soy Broth (TSB) and TSB supplemented with 428 mM KCl. To evaluate each growth medium, the *L. monocytogenes* isolates that comprised the cocktail were individually subjected to growth curve experiments in each broth. To conduct the growth curves, overnight broth cultures were prepared as described, and the following day, 1 ml (10%) of the broth culture was subcultured into 9 ml

of the growth media and incubated for 3 hours at 37°C and 250 RPM in a shaking incubator. After the three hours, 100 µl of the broth culture was subcultured into a test tube containing 10 ml of the growth media to be evaluated and 250 µl of this bacterial suspension was transferred to an individual well of a honeycomb BioScreen plate (Growth Curves USA, Piscataway, NJ). In addition, 250 µl of the growth media to be evaluated was added to another well to serve as a blank and background control. The honeycomb plate was placed into a BioScreen C device (Growth Curves USA), incubated at 37°C, and shaken continuously for the duration of the experiment (on medium setting). Optical Density (OD_{600nm}) readings were taken at 30-minute intervals for a total time period of 12 hours, and each growth curve was performed in triplicate.

3.3.3. Recovery of enriched stressed cells

Preliminary results from the growth curve assay indicated that TSB supplemented with 428 mM KCl resulted in the fastest enrichment of the *L. monocytogenes* cells. Since it is possible that *L. monocytogenes* cells present in the food production environment would be sublethally stressed, an experiment was conducted to determine if *L. monocytogenes* cells could be enriched in TSB (KCL) after being subjected to several types of stresses, including high pH (pH 9.0), low pH (pH 3.0), heat, and high osmolarity.

During this experiment, all bacterial strains were evaluated individually. Each strain of *L. monocytogenes* that comprised the cocktail was grown separately on TSA plates overnight. A well isolated colony was transferred from the TSA plate into 50 ml of TSB (KCl), followed by overnight incubation for 18 hours at 37°C and 250 RPM. After the incubation, 5 ml of the broth culture was subcultured into 45 ml of TSB (KCl), which was

previously adjusted to reflect the type of stress to be evaluated. These methods were based on the study conducted by McClure et al. (1991). For the pH studies, the TSB (KCL) was adjusted to the required pH (3 or 9) with the use of hydrochloric acid (HCl) or sodium hydroxide (NaOH). For the osmolarity studies, the TSB (KCL) broth was supplemented with sodium chloride (NaCl) to a final concentration of 15% (w/v) (McClure, Kelly, Roberts, 1991). Each flask was incubated (37°C at 250 RPM) for 2 hours. The heat stress challenge was performed by transferring 5 ml of the overnight culture into 45 ml of TSB (KCl), followed by incubation for approximately 1.75 hours at 37°C with shaking (250 rpm). Next, the flask was placed into a 48°C water bath for 15 minutes (once the final temperature of 48°C was reached). After the various incubations described above, 500 µl of each broth culture was transferred into an individual flask containing 49.5 ml of TSB (KCl), vortexed, and then 250 µl of these suspensions were transferred into separate wells of a BioScreen honeycomb plate. The plate was placed into the BioScreen C device, which was set to take OD_{600nm} readings at 30-minute intervals for 1020 minutes. The other operating parameters of the Bioscreen C device were as described. Each growth curve was repeated in triplicate.

3.3.4. Development of the integrated test method

To develop the integrated test method, Snap Valve Devices (Hygiena, Camarillo, CA) were modified to contain all of the diagnostic reagents necessary for rapid detection of *L. monocytogenes.* One milliliter of TSB (KCl) was placed in the bottom of the device, along with 40 µl of *Listeria* specific IMS beads (Invitrogen, Carlsbad, CA). One hundred microliters of Reporter Lysis Buffer (Promega Corporation, Madison, WI) and 50 µl of the colorimetric phosphatidylinositol-specific phospholipase C (PI-PLC) substrate, 5-bromo-4-

chloro-3-indoxyl-myo-inositol-1-phosphate (X-Inp) (VWR International Incorporated, Aurora, Co) were added to the reservoir in the cap of the device. Prior to introduction to the Snap Valve device, the X-Inp substrate was prepared by adding the powdered substrate to 0.1 M monobasic sodium phosphate buffer (ph 7.5) to a final concentration of 83.3 mg/ml. The integrated test is known as the Phast Swab (Figure 1). The Phast Swab procedure is explained in Figure 2.

3.3.5. Specificity tests

Seventy-seven strains of *L. monocytogenes* (Table 4) and 29 strains belonging to species other than *L. monocytogenes* (Table 5) were tested to determine the specificity of the Phast Swab assay. The non-*L. monocytogenes* strains that were tested included: 3 strains of *E. coli* O157:H7; 5 strains of non pathogenic *E. coli*; 1 strain each of *Salmonella enterica* serovars Typhimurium, Anatum, Enteriditis and Newport; 2 strains of *Enterococcus faecalis;* 1 strain of *Pseudomonas aeruginosa*; 1 strain of *Staphylococcus aureus*; 1 strain each of *Listeria ivanovii* and *Listeria grayi,* 7 strains of *Listeria innocua* and 4 strains of *Listeria welshimeri.* The specificity of the substrate was tested on the *L. monocytogenes* strains by adding 50 µl of the X-Inp substrate to an overnight culture of each isolate. After incubation for 5 hours at 37°C and 250 RPM in a shaking incubator, the cultures were evaluated for a color change (to indigo) indicating a positive result. The non-*L. monocytogenes* strains were analyzed by placing 2 ml of an overnight culture into an integrated test device, followed by incubation for 20 minutes to allow the bacteria time to attach to the anti-*Listeria* IMS beads. Next, the devices were placed on a magnet (Invitrogen), allowing the beads (and any bound bacterial cells to be attracted to the magnet) and the broth was removed. The lysis buffer and

substrate were then simultaneously added and the device was incubated for 5 hours at 37°C with shaking at 250 RPM.

3.3.6. Sensitivity tests

The five-strain cocktail of *L. monocytogenes* was produced as described, and adjusted to a final concentration of 10^9 CFU/ml. The cocktail was serially diluted (10-fold)in lambda buffer in a range from 10^0-10^9 CFU/ml. One milliliter of each dilution was placed into an individual test device, and incubated for 15-20 minutes to allow any *L. monocytogenes* cells to attach to the IMS beads. Each device was placed on a magnet (Invitrogen) to separate the beads from the broth, which was removed with a disposable transfer pipette. The IMS beads were resuspended in 100 µl of lysis buffer and 50 µl of X-Inp as described. The devices were incubated as described and then 100 µl from each device was removed and pipetted into a separate well of a 96 well microtiter plate and evaluated at OD_{450nm} in a BioTEK Synergy II plate reader (BioTek Instruments Incorporated, Winooski, VT) to determine the detection limit of the assay.

3.3.7. Artificial inoculation of coupons

The five strain *L. monocytogenes* cocktail was prepared, and serial (10-fold) dilutions of the cocktail were prepared as described above. Individual stainless steel, acrylic and ceramic tile coupons (4"×4") were chosen to simulate food contact and non-contact surfaces, and were inoculated with 250 µl of the 10^7, 10^6, 10^5, CFU/ml dilutions. The coupons were allowed to dry for 5 hours and were swabbed with an individual test device. The devices were incubated for 13 h. Following completion of the test, 100 µl of each sample was

removed from the respective test device and placed into an individual well of a 96 well microtiter plate. The plate was placed into a BioTEK II Synergy Plate reader (BioTek Instruments Incorporated) and the absorbance was read at OD_{405nm}.

To determine the actual number of cells on the coupons, the surfaces were inoculated as described above and after 5 hours of drying the coupons were placed into individual Nasco Whirl-Pak (Modesto, CA) bags with 100ml of lambda diluent and allowed to incubate at room temperature for 1 hour. The coupons were then massaged by hand and 1ml of the diluent was plated onto a 100 x 15mm TSA plate and incubated overnight at 37°C. A reduction in the concentration of *L. monocytogenes* was observed on the coupons, regardless of initial concentration or type of coupon. This led to final concentrations (CFU/cm^2) of 10^3 for the original 10^7 CFU/ml inoculum, 10^2 for the 10^6 CFU/ml inoculum, and 10^1 for the original 10^5 CFU/ml inoculum. Materials were chosen based on principles described by AOAC Official Methods of Analysis (AOAC, 2002).

3.3.8. Sequence analysis of three *L. monocytogenes* isolates

In testing the specificity of the assay, 3 strains of *L. monocytogenes* (J1-094, J1-110, and J1-012) did not yield the indicative indigo color when incubated with the X-Inp substrate. Therefore, PCR amplification and sequencing of the *plc*A gene and the upstream promoter region for these isolates were accomplished to probe mutations that could explain the lack of enzymatic activity. The PCR amplification of *plc*A was performed in a total volume of 100 µl. The following concentrations of reagents were used in all PCR reactions: *Thermus aquaticus* (Taq) DNA polymerase [4 units (0.8 µl)], 1X PCR buffer (20 µl), $MgCl_2$

at a final concentration of 1.5mM, deoxynucleotide triphosphates (DNTPs) at final concentration of 0.2mM (Promega, Madison, WI), DNA template (2 µl of lysate produced from a single *L. monocytogenes* colony), and forward and reverse oligonucleotides (2 µl of each) (Table 6). Sterile water (47.2 µl) was added to produce a final volume of 100 µl. A touchdown and hotstart PCR protocol for amplification of *plc*A was used with annealing temperatures decreasing at a rate of 0.5°C/cycle from an initial annealing temperature of 55°C to a final annealing temperature of 45°C after 20 cycles. The initial 20 cycle amplification was followed by another 20 cycles with the annealing temperature set at 45°C. Following PCR, amplicons (1617 bp) were visualized on (1.5%) agarose gels. Amplicons were purified from the gels using a Qiaquick® PCR Purification Kit (Qiagen Inc., Valencia, California), and submitted for sequence analysis. DNA sequencing was performed at the Macromolecular Resources Lab at Colorado State University (Fort Collins, CO) using the ABI 3700 DNA analyzer. A set of 6 primers (Table 6) were designed to amplify overlapping 500bp sections of the *plc*A gene during sequence analysis. Proofreading and alignment of nucleotide sequences were completed with Seqman and Megalign (part of the DNAStar software package, Lasergene, Madison, WI). Additional sequence data for the isolates are available through the PathogenTracker database (www.pathogentracker.net).

3.3.9. Statistical analysis

The coupon sensitivity data were analyzed using a 3 x 4 factorial design in PROC GLM of SAS (SAS Inst. Cary, NC). Mean separation was conducted using paired comparisons of LS means with a Tukey adjustment for multiple comparisons, α=0.05. This method was used to determine p-values for evaluating the 3 different initial inoculum levels

on each surface compared to the blank (surfaces inoculated with sterile lambda diluent). P-values were also generated to determine if there were any concentration to surface interactions and to compare the different inoculum levels to each other, in general, regardless of surface type.

3.4. Results

3.4.1. Evaluation of different growth media

Several broths were evaluated for their ability to rapidly increase the numbers of *L. monocytogenes* during enrichment. These broths included Buffered Peptone Water (BPW), Universal Preenrichment Broth (UPB), Brain Heart Infusion (BHI) broth, BHI supplemented with 428 mM potassium chloride (KCl), Tryptic Soy Broth (TSB) and TSB supplemented with 428 mM KCl. The results are shown in Figure 3 (A-F) and indicated that TSB had shorter lag phase and more rapid log phase growth than BHI. We determined that TSB (KCl) allowed for the best enrichment of *L. monocytogenes* (Figure 3F). While the lag phases of *L. monocytogenes* cells grown in TSB (KCL) were slightly extended compared to growth in TSB, the TSB (KCl) resulted in a slightly higher concentration of cells after the enrichment (as compared to TSB), and Myers et al. (1993) demonstrated that production of PI-PLC is enhanced when the cells are grown in media supplemented with KCl (Myers, Dallmier, Martin, 1993). Both BPW and UPB resulted in very poor enrichment of *L. monocytogenes* (Figures 3A and B).

3.4.2. Recovery of stressed cells

The results of the growth experiments showed that TSB supplemented with 428 mm KCl resulted in the best growth of *L. monocytogenes* (Figure 3F). To assess the ability of the media to recover stressed *L. monocytogenes* cells, 5 strains were subjected to stresses that are reasonably expected to occur in a food plant, including high (pH 9) and low pH (pH 3), heat (48°C), and salt (15%w/v). The results are shown in Figure 4 (A-D). When the *L. monocytogenes* cells were subjected to low pH, all of the strains reached stationary phase within 13 hours, with the exception of strain C1-056, which did not grow (Figure 4A). At high pH, all 5 strains entered stationary phase within 8 hours (Figure 4B). Under high osmolarity conditions the cells recovered and entered stationary phase within 9.5 hours (Figure 4C), and the heat stressed cells recovered within 7.5 hours (Figure 4D). These results indicate that the TSB (KCl) growth media effects recovery of mildly stressed *L. monocytogenes* within 13 hours. Therefore, the enrichment time of the assay was set at 13 hours for all tests.

3.4.3. Specificity tests

The Phast Swab was tested for its specificity by assaying 77 *L. monocytogenes* isolates (Table 4) and 29 non-*L. monocytogenes* isolates (Table 5). The Phast Swab correctly identified 74/77 (96.1%) *L. monocytogenes* isolates. Three false negative results were obtained, and these isolates (J1-012, J1-094, J1-110) were biochemically and genetically characterized to determine the presence or absence of PI-PLC. Specifically, sequence analysis of *plc*A (which encodes PI-PLC) and its promoter region was accomplished to determine the functionality of the gene. Isolate J1-094 showed little to no growth on Oxford

media and exhibited little to no bile esculinase activity. The isolate produced white colonies on LMPM, and displayed hemolytic activity on blood agar. Sequence analysis of *sig*B confirmed that J1-094 isolate is *L. monocytogenes* (data not shown), but its biochemical characteristics indicate that it is an atypical isolate. Isolates J1-012 and J1-110 showed similar biochemical results on all media; they produced black colonies on Oxford media, white colonies on LMPM and had little to no hemolytic activity on blood agar. All isolates had identical point mutations in the *plc*A gene and their *prf*A sequences did not reveal any mutations that could explain the lack of PI-PLC activity (data not shown). Although these strains did not produce positive results in the Phast Swab, previous work has shown that these isolates are avirulent, or exhibit reduced virulence. For example isolate J1-110 has previously been described to carry several authentic mutations that could lead to truncated gene products, including *inl*B and a DNA polymerase (Nightingale et al., 2007). Isolate J1-094 is a serotype 1/2c isolate and this serotype has been associated with premature stop codons in *inl*A (Orsi, Ripoll, Yeung, Nightingale, Wiedmann, 2007; Van Stelten, Nightingale, 2008). The mutated genes lead to reduced invasiveness and therefore limited virulence. Finally, the secreted PI-PLC of *L. monocytogenes* plays a role in the bacterium's ability to escape from phagosomes and spread from cell to cell, and isolates lacking PI-PLC have been shown to be avirulent (Camilli, Goldfine, Portnoy, 1991). Therefore, in this study it is likely that the false negative results produced by the Phast Swab are the result of *L. monocytogenes* isolates that are attenuated in virulence, which may be due to reduced expression of *prf*A or *plc*A.

When the Phast swab was tested against non-*L. monocytogenes* isolates, 28/29 (96.6%) of the isolates were correctly identified as non-*L. monoctyogenes*. One false positive

result was obtained. This isolate was identified as *Listeria ivanovii*, which is known to produce PI-PLC (Leimeister-Wächter et al., 1991). While *L. ivanovii* is considered to be an animal pathogen, it can cause human illness in rare cases (Karunasagar, Krohne, Goebel, 1993).

3.4.4. Sensitivity tests

In pure culture 10^6 CFU/ml of *L. monocytogenes* were directly detected within 3 hours. The addition of 428 mm KCl increased the sensitivity to 10^5 CFU/ml (data not shown). Charcoal also enhanced sensitivity of the assay but made the test difficult to read and was therefore not used further.

3.4.5. Detection limit on artificially inoculated coupons

The ability of the assay to detect *L. monocytogenes* on food contact and non-food contact surfaces was evaluated, by artificially inoculating stainless steel (food contact), acrylic (food contact) and ceramic (non-food contact) 100 cm^2 coupons with low levels of *L. monocytogenes*. The coupons were allowed to dry for 5 hours at room temperature following inoculation with the cocktail and then each coupon was swabbed with an individual test device. The Phast Swab was capable of detecting 10^1 CFU/cm^2 of *L. monocytogenes* on acrylic ($p=0.02$), 10^2 CFU/cm^2 on ceramic ($p=0.01$), and 10^3 CFU/cm^2 on stainless steel ($p=0.003$) (Figure 5). The differences in detection limit may reflect the differential ability of *L. monocytogenes* to survive on the three surfaces or ability to recover bacteria by swabbing.

3.5. Discussion

Listeria monocytogenes is a ubiquitous pathogen and a major cause of food related illness. The scientific literature indicates that food (meat) samples may become contaminated with *L. monocytogenes* through improperly cleaned grinding and processing equipment (Borch, Nesbakken, Christensen, 1996; Duffy et al., 2001). The contamination of processing equipment is a serious concern, because *L. monocytogenes* is a foodborne pathogen that possesses the ability to grow at refrigerated temperatures, meaning that even when *L. monocytogenes* is present at low levels in biofilms in the processing plant, the pathogen can survive in food products and become enriched during subsequent storage at refrigerated temperatures. Borch et al. (1996) has suggested that because bacteria such as *L. monocytogenes* can be endemic in the meat processing environment, and since these bacteria are effectively controlled with proper sanitation, *L. monocytogenes* would be useful as an indicator of the success of processing equipment cleaning and disinfection protocols (Borch et al., 1996). As such, rapid, integrated methods that allow for detection of this pathogen should be developed. Current methods of *L. monocytogenes* detection require either a long detection time (24 to 48 hours for cultural methods), are technically challenging (PCR), expensive, or require dedicated laboratory facilities and trained personnel (PCR). In addition, these methods do not integrate sampling with the testing method.

In this study, a rapid, easy to use test to detect *L. monocytogenes* was developed, that requires little to no equipment or technical training. The Phast Swab is a self-contained test device, containing a sampling tool (swab), growth media, immunomagnetic separation (IMS) beads, and a colorimetric substrate, which when cleaved by the enzyme PI-PLC, forms a visible indigo reaction. The development of an integrated assay has obvious advantages over

currently available test methods. For example, the fact that all test reagents are contained within a single device means that reagents do not need to be added to complete the assay, thereby decreasing the chances of any errors due to improper use of the test. Also, the colorimetric nature of the Phast Swab means that the results can be determined visually, without the need for instrumentation. The assay is capable of detecting *L. monocytogenes* on food contact and non-food contact surfaces within 18 hours. Collectively, the results of this study indicate that that the Phast Swab can detect *L. monocytogenes* on food contact and non-contact surfaces in the food processing environment in a rapid and specific manner without the need for instrumentation to read the test result.

CHAPTER FOUR

Development of a Rapid Detection Assay for *Listeria monocytogenes* on Turkey and Ham Deli Meat Slices

4.1. Abstract

The purpose of this study was to develop a test to detect *L. monocytogenes* in RTE meat that is rapid, easy to use, and requires little to no equipment or scientific technical training. The Phast Swab is a self-contained test device that contains a sampling tool (swab), bacterial growth media (TSB-KCl), *L. monocytogenes* specific immunomagnetic separation (IMS) beads, and a colorimetric substrate. A five-strain cocktail of *L. monocytogenes* was prepared so that the final concentration of the cocktail was 10^9 CFU/ml. The cocktail was serially diluted in a range from 10^0-10^{-9}, and individual slices of ready-to-eat (RTE) deli meat (turkey and ham) were inoculated with 1 ml of each dilution. The meat was allowed to dry and then each piece was swabbed with a Phast Swab device, followed by a 10-hour enrichment. The beads were then concentrated by IMS, the broth was discarded and the lysis buffer and substrate were added. After 5 hours, the tests were read. A second experiment was conducted where inoculated deli meat samples were individually vacuum-sealed and sampled over a 21-day period. The results of this study indicate that the Phast Swab can detect low levels of *L. monocytogenes* on RTE meats directly or after storage within 15 hours. These data show that the Phast Swab can detect *L. monocytogenes* on deli meat in a rapid and specific manner without the need for instrumentation to read the test result.

4.2. **Introduction**

Listeria monocytogenes is a non-sporeforming, Gram-positive, facultative intracellular, foodborne pathogen that can be found in feces, silage, soil, water, dust, and other environmental niches (Bearns et al., 1959; Gray et al., 1966; Murray et al., 1926; Schlech et al., 1983). The organism is capable of growth in a variety of stressful environments including high osmolarity (able to tolerate up to 20% salt concentration), over a wide pH range and a wide range of temperatures (1-45°C), (Hudson et al., 1994; Pearson et al., 1990; Petran et al., 1989; Sauders et al., 2007; Watkins et al., 1981). The survival of *L. monocytogenes* in diverse environments helps explain the survival of *L. monocytogenes* in many different foods.

Mead et al. (1999) has estimated that *L. monocytogenes* causes approximately 2500 cases of listeriosis and 500 deaths each year in the U.S., of which, 99% are foodborne (Mead et al., 1999). *L. monocytogenes* has been isolated from many ready-to-eat (RTE) food products including pasteurized milk, a variety of cheeses (Lopes, 1986), pre-packaged salads (Little et al., 2007), cabbage, coleslaw (Francis et al., 2006) and meat products and seafood (Cabedo et al., 2008). Gombas et al. (2003) surveyed 8 categories of ready to eat (RTE) food products over a period of 14 to 23 months and reported that 1.82% were positive for *L. monocytogenes*. Among those samples with higher levels of contamination were deli meats and smoked seafood (Gombas et al., 2003). Yang et al. (2006) noted that although at-home food handling practices of deli meat contribute to the risk of listeriosis, retail contamination is a larger factor and has a greater correlation with mortality (Yang et al., 2006). The 2001 FDA/FSIS risk ranking model indicated that deli meats posed the greatest risk for listeriosis

of all RTE foods, where nearly 80% of all deaths and cases are caused by deli meats (Gallagher, 2003).

One route by which deli meats become contaminated with *L. monocytogenes* is through transfer from contaminated food processing equipment (Lin et al., 2006). Although large numbers of *L. monocytogenes* may not be transferred to the meat, the pathogen can grow during storage of the product at refrigeration temperatures (Gray et al., 1948). The transfer of *L. monocytogenes* from a slicer to deli meat has been recently demonstrated (Sheen et al., 2008). A 2008 outbreak of listeriosis in Canada was linked to RTE deli meat produced in Toronto, Ontario. The outbreak caused 56 illnesses and 20 deaths (PHAC, 2008). Subsequent analysis of the outbreak was accomplished to determine the exact source of contamination. It was determined that the most likely source of contamination, after careful analysis of records, the physical plant and product test results, was two meat-slicing machines at the plant. While the slicers were sanitized on a daily basis in accordance with or exceeding the equipment manufacturer's recommendations, full disassembly of the equipment revealed areas deep within the equipment where bacteria could accumulate, thereby escaping the sanitization process (PHAC, 2008).

In 2002, turkey deli meat was the source of a large multistate outbreak of listeriosis that affected 54 individuals and resulted in 8 deaths and 3 fetal deaths (Gottlieb et al., 2006). Investigation of the deli turkey meat consumed by the patients led to several turkey processing facilities. The outbreak strain was found in two of the facilities. In one facility, *L. monocytogenes* was found in the processing environment and in another facility the organism was present in turkey breast products. Following the outbreak, the FSIS increased federal

regulations for microbial testing of RTE foods and poultry products and completed a risk assessment of *L. monocytogenes* in deli meat (FSIS, 2009).

RTE meats (deli meats) are among the food products that have been mandated by the FSIS to undergo regular sampling for the presence of *L. monocytogenes*. A FDA/FSIS ranking model was developed to identify the relative risk of illness or death caused by various categories of RTE foods (FDA/FSIS, 2001). The model showed that of all RTE foods examined, deli meat posed the greatest public health risk for contraction of listeriosis. For example, approximately 80% of all cases and deaths were shown to be caused by deli meats.

Many different procedures have been developed for detection of *L. monocytogenes* including cultural, immunological and molecular methods (Bansal et al., 1996; Bearns et al., 1959; Beumer et al., 1989; Bilir Ormanci et al., 2008; Brehm-Stecher et al., 2007; Curiale et al., 1994; Curtis et al., 1995; Gasanov et al., 2005; Hitchins, 2003). Cultural methods require intensive labor and time, immunological methods suffer from a lack of sensitivity, and molecular methods, while being sensitive, are expensive, require operator training, and do not detect viable organisms. In addition, all of the currently available methods are not designed for direct detection of *L. monocytogenes* in the food plant environment. The objective of this study was to develop and evaluate an integrated method for rapid detection of viable *L. monocytogenes* on RTE meat products.

4.3. Materials and Methods

4.3.1. Bacterial strains and cultivation

Bacterial strains were maintained and cultivated as previously described in section 3.3.1.

4.3.2. Evaluation of different growth media

Several different growth media, Buffered Peptone Water (BPW), Universal Preenrichment Broth (UPB), Brain Heart Infusion (BHI) broth, BHI supplemented with 428 mM potassium chloride (KCl), Tryptic Soy Broth (TSB) and TSB supplemented with 428 mM KCl were evaluated as described in sections 3.3.2 and 3.41.

4.3.3. Recovery of enriched stressed cells

The ability of *L. monocytogenes* to recover from mild stresses was evaluated as described in sections 3.3.3 and 3.4.2.

4.3.4. Specificity and sensitivity tests

Specificity and sensitivity tests were conducted as described in sections 3.3.5, 3.3.6 and 3.4.3, 3.4.4 respectively.

4.3.5 Development of the integrated test method

The Phast Swab was developed and described in section 3.3.4.

4.3.6. Evaluation of the Phast Swab on artificially contaminated delicatessen meat

The Phast Swab was tested on artificially contaminated turkey and ham deli meat that was purchased from a local grocery store and refrigerated at 4°C until use. Both types of meat were produced by the same manufacturer. The inoculation, storage, and testing procedures were the same for each type of deli meat. Serial (10 fold) dilutions of the *L. monocytogenes* cocktail was made, and meat slices were individually inoculated with

dilutions containing 10^1, 10^2, 10^3, and 10^4 CFU/ml by adding 1 ml of each dilution to an individual deli slice followed by the use of a sterile cell spreader to evenly distribute the inoculum. For each bacterial concentration, 3 deli slices were inoculated. One of the slices was used to determine the concentration of cells on the meat via plate count using Oxford media (Oxoid, Cambridge, UK), and the other two slices were tested using the Phast Swab. All inoculation took place in a BSL II biological Safety Cabinet. The inoculated meat was allowed to dry for 10 minutes, and after drying, each slice of meat was swabbed with an individual Phast Swab device in a vertical up and down motion and side-to-side horizontal fashion. The swabs were then incubated for 10 hours with shaking (250 RPM) at 37°C. After the enrichment period, the swabs were removed from the incubator, vortexed and the IMS beads were concentrated by placing the swabs on Dynal magnet (Figure 2 Step 2). The growth media and swab were then removed and discarded. The beads were resuspended in lysis buffer (200 μl) and X-Inp (50 μl) from the reservoir at the top of the device, and each Phast Swab was incubated with shaking (250 RPM) at 37°C for 5 hours. The results were visually determined. Negative controls consisted of deli meat slices that were inoculated with 1 ml of lambda diluent. All experiments were performed in duplicate, and repeated three times.

4.3.7. 21-day deli meat experiment

The ability of the Phast Swab to detect low levels of *L. monocytogenes* in deli meats during storage was ascertained. Turkey and ham deli meats were inoculated as described above except each slice of meat was individually placed into Prime Source Vacuum Pouches (Model 75001816, KOCH Supplies Inc., Kansas City, MO) and vacuum-sealed in a Hollymatic vacuum sealer (Hollymatic Corporation, Countryside, Illinois) followed by

storage at 5ºC. The deli meats were then sampled every 3 days for a total of 21 days (sampling occurred on days 0, 3, 6, 9, 12, 15, 18, and 21). Negative controls consisted of deli meat slices that were inoculated with 1 ml of lambda diluent, and these samples were vacuum packaged and stored in an identical manner to the inoculated samples. Plate counts were conducted on each day of sampling to determine the actual number of *L. monocytogenes* cells on the deli slices. Plate counts were performed as described in section 4.3.4. Each experiment was completed in duplicate.

4.3.8. Measurement of pH and water activity

The water activity (a_w) was measured for both types (ham and turkey) of RTE meat. Five saturated salt solutions (K_2SO_4, KCl, NaCl, $Mg(NO_3)_2$, and MgCl) were used to calibrate an Aqua Lab series 3 (Design Devices, Inc., Pullman, WA) instrument per manufacturer's instructions. A total of 9 samples of each RTE meat (3 slices from 3 different packages) were analyzed in the instrument. The resulting data was fitted to a standard curve produced from the salt solutions and analyzed in Excel (Microsoft, Redmond, WA) using the regression analysis tool.

The pH of each RTE meat was also measured. A total of 6 samples of each deli meat (2 samples from 3 different packages) were analyzed. Each slice of meat was placed in a stomacher bag and suspended in a 10-fold volume of distilled water, which was then stomached in an IUL Masticator (Barcelona, Spain) for 120 seconds. The pH of the resulting liquid suspension was taken using a Thermo Scientific Orion Star Series™ Benchtop pH meter (Cole-Parmer Instrument Company, Vernon Hills, Illinois).

4.4. Results

4.4.1. Direct detection limit of the Phast Swab on artificially inoculated deli meat

Individual deli meat (turkey and ham) slices were artificially inoculated with 4 different dilutions of (10^1 to 10^4 CFU/ml) of a 5 strain *L. monocytogenes* cocktail. Following 10 min of drying, each slice was swabbed with an individual Phast Swab. On both types of meat, the Phast Swab consistently detected the original 10^3 CFU/ml inoculum, which translated to 10^1 to 10^2 CFU of *L. monocytogenes* per gram of meat (approximately 20-25 g) (Table 7). The test was also capable of detecting the original 10^2 and 10^1 CFU/ml inoculums, but not consistently (Table 7). The total time needed for the assay was 15 hours.

4.4.2. Detection of *L. monocytogenes* on artificially inoculated deli meat over 21 days

The Phast Swab was evaluated for its ability to detect artificially inoculated deli slices following vacuum packaging and storage for 21 days at 4ºC. For turkey slices, the assay consistently detected the original 10^4 CFU/ml inoculum on all sampling days, which translated to 10^2 CFU of *L. monocytogenes* per gram of turkey (Table 8). The assay also detected the original 10^3 CFU/ml inoculum (10^1 or lower CFU/g of meat) up until day 18. Detection of *L. monocytogenes* at initial inoculum levels below 10^3 CFU/ml tended to be sporadic (Table 8).

The Phast Swab was less sensitive when tested on ham samples. For example, the Phast Swab detected the original 10^4 CFU/ml inoculum on each day that the ham was sampled (Table 8). This concentration translated to a concentration of 10^2 CFU/g of ham for everyday but Day 0. The assay detected the original 10^3 CFU/ml inoculum on days 0 and 3

(10^1 and 10^2CFU/g, respectively). Detection of lower concentrations of *L. monocytogenes* was unreliable.

4.5. **Discussion**

Contaminated ready to eat meat has been implicated in recent large outbreaks of listeriosis (Olsen et al., 2005; PHAC, 2008). As such, there is a continuing need for the development of rapid and sensitive methods to detect *L. monocytogenes* in food. Conventional methods are labor and time intensive, and rapid methods such as PCR and biosensors are sensitive and specific but often expensive, complex, and nonportable, which limit their usefulness in the food production environment. Finally, all of the above methods require manipulation of the food sample prior to analysis.

The objective of this study was to develop an integrated test for rapid detection of *L. monocytogenes* that could be accomplished in the food production environment, with minimal training and equipment. To develop the assay, testing reagents (growth media, IMS beads, bacterial lysis buffer and an enzyme substrate) were combined into a single test device, which also contained a sampling tool. The addition of the sampling tool is noteworthy, because it allows for integration of sample preparation into the test. This significantly decreases time and also the cost of sampling food.

In pure culture, the Phast Swab detected 10^5 CFU/ml of *L. monocytogenes* (data not shown). The ongoing outbreaks associated with production of RTE meat dictated the evaluation of the assay to detect *L. monocytogenes* in these foods. The Phast Swab was able to detect low numbers of *L. monocytogenes* on RTE turkey and ham samples (Tables 7 and 8). For example, the Phast Swab directly detected as few as 10^2 CFU/g of meat within 15

hours. Lower concentrations were detected, but positive test results tended to be sporadic. The sensitivity of the Phast Swab compares well with other published studies. Geng and colleagues (2004) described the development of a fiber-optic immunosensor, and showed that the biosensor could detect between 10^1 and 10^3 CFU/g of artificially contaminated hot dog or bologna in less than 24 hours (Geng et al., 2004). Recently Shim et al. (2008) evaluated the use of IMS combined with ELISA and an immunochromatography test strip to effect detection of *L. monocytogenes* (Shim et al., 2008). The results of the study indicated that 100 CFU/10g of *L. monocytogenes* would be detected within 15 hours in food samples (pork, beef, chicken, fish and processed meat), although the test produced false positive reactions with *Staphylococcus aureus* strains. *S. aureus* tested negative using the Phast Swab in this study.

We were interested in evaluating the ability of the Phast Swab to detect low numbers of *L. monocytogenes* in RTE meats stored over time, since the shelf life of slice cured and uncured RTE meats can be as long as 60 to 90 days (Pal, Labuza, Diez-Gonzalez, 2008). The Phast Swab detected 10^1 CFU/g of turkey meat for up to 18 days, and 10^2 CFU/g of ham for all 21 days of the experiment. Plate counts showed that the concentration of *L. monocytogenes* did not increase during the sampling period, which was likely due to the fact that both RTE meats were cured with sodium nitrite ($NaNO_2$). For example, Nyachuba et al. (2007) determined the impact of nitrite on detection of *L. monocytogenes* in various RTE meats and seafoods, and these researchers showed that the concentrations of *L. monocytogenes* in cured products remained fairly constant for 3 weeks (Nyachuba, Donnelly, Howard, 2007). Following depletion of $NaNO_2$, the concentration of *L. monocytogenes* increased. Nyachuba et al. (2007) also showed that the presence of 100 to 200 ppm $NaNO_2$

resulted in 83 to 99% injury to *L. monocytogenes*, and this observation may account for the inability of the Phast Swab to detect *L. monocytogenes* inoculated at lower than 10^1-10^2 CFU/g of RTE meat. Finally the authors assessed the effect of $NaNO_2$ on the ability of 5 test methods including the USDA/FSIS method using University of Vermont-modified Listeria enrichment broth (UVM) as the primary enrichment medium; the FDA method using Listeria Enrichment Broth (LEB) as the primary enrichment medium; the USDA/FSIS method using Listeria Repair Broth (LRB) as the primary enrichment medium; the modified USDA/FSIS method using both UVM and LRB as primary enrichment media, and the PCR-based BAX® System. The genetic-based BAX® System and modified USDA/FSIS methods detected 98% to 100% of *L. monocytogenes* food samples and were consistently significantly superior ($P < 0.05$) to conventional cultural methods in recovering *L. monocytogenes* from cured samples.

In this study, the Phast Swab detected 100% of all RTE turkey samples that were positive by plate count, except on days 6 (75%) and 9 (50%) (Figure 6). In contrast, the assay detected lower percentages of ham samples that were shown to contain *L. monocytogenes* by plate count. For example, the assay detected 100% of plate count positive ham samples on day 0, but the percentage of ham samples that tested positive decreased steadily to 50% by day 12. These results, when coupled with the sensitivity data, indicate that the assay consistently detected lower numbers of *L. monocytogenes* in RTE turkey than ham. The a_w (0.977 for turkey and 0.966 for ham) and pH of both RTE meats were comparable, although the pH of the ham was lower (6.43 for turkey and 6.22 for ham). The ham contained 30 mg/slice more sodium than the turkey, and also contained sodium lactate and sodium diacetate, which are included as ingredients in RTE meat products for control of *L. monocytogenes* contamination during storage (Barmpalia et al., 2005). In contrast, the

turkey samples contained only sodium diacetate. Combinations of sodium lactate and sodium diacetate have been shown to inhibit growth at temperatures below 7ºC in wiener or bratwurst formulations (Glass et al., 2002), and Barmpalia and colleagues (2005) reported that the combination of both antimicrobials was more effective in controlling the growth of *L. monocytogenes* in pork bologna, than when either antimicrobial was used individually. Therefore, in this study, it is possible that the combination of the two antimicrobials led to increased injury, and less survival and recovery of *L. monocytogenes* in the RTE ham samples.

The results of this study indicate that the Phast Swab can detect low levels (10^1-10^2 CFU/g) of *L. monocytogenes* on RTE meats within 15 hours. The integrated nature of the test, combined with visual detection, makes it possible for the Phast Swab to be used to assess RTE meats for the presence of *L. monocytogenes* in the food production environment, with minimal effort and equipment. This is an advantage over currently available test methods. Future efforts will include further optimization of the assay to allow for detection of *L. monocytogenes* from different types of RTE food.

CHAPTER FIVE
APPENDIX

Table 1. Selected listeriosis outbreaks in the last 10 years (Adapted from Norton and Braden (2007).

Year	Food	Country	Number of Cases (Deaths)	Reference
1998-1999	Processed RTE meat	USA (24 states)	108 (18)[a]	(Mead et al., 2006)
1999	Pasteurized butter	Finland	26 (6)	(Lyytikainen et al., 2000)
1999	Pâté	USA (Maryland, Connecticut and New York)	11	(Anonymous, 1999)
1999	Rilletes (pâté-like meat product)	France	10	(de Valk et al., 2001)
2000	Pork tongue (jellied)	France	32	(de Valk et al., 2001)
2000	Homemade Mexican-style cheese	USA (North Carolina)	13 (5)[b]	(MacDonald et al., 2005)
2000	RTE turkey meat	USA (11 states)	30 (7)[c]	(Olsen et al., 2005)
2002	RTE turkey meat	USA (9 states)	54 (8)	(Gottlieb et al., 2006)
2002	Raw milk cheese	Quebec, Canada	17	(Gaulin et al., 2003)
2008	RTE Meat	Ontario, Canada	56 (20)	(PHAC, 2008)

[a]14 deaths, 4 miscarriages
[b]4 deaths, 3 miscarriages
[c]5 stillbirths

Table 2. Differential sugar fermentation profile of *Listeria monocytogenes* and *Listeria ivanovii* (adapted from Gasanov et al., 2005).

Sugar Fermentation	*L. monocytogenes*	*L. ivanovii*
L-Rhamnose	+	-
D-Xylose	-	+
α-Methyl-Mannoside	+	-

TABLE 3. Selected *Listeria* spp. and *L. monocytogenes* commercial immunoassay kits for food and environmental samples (adapted from Gasanov et al., 2005).

Test	Specificity	Sample type	Detection Limit	Reference
Listeria-Tek™ (Organon Teknika Corp.)	*Listeria* spp.	Dairy, meat and fish products	1 cell/25g ice-cream	(Comi et al., 1991; Walker, Archer, Appleyard, 1990)
Dynabeads® anti-*Listeria* (Invitrogen)	*Listeria* spp. *L. monocytogenes*[a]	Broth enrichment (food and environmental samples)	N/A	(Gasanov, et al., 2005; Jung, Frank and Brackett, 2003; Nexmann Jacobsen, Fremming and Jakobsen, 1997; Uyttendaele, et al., 2000)
Listeria Unique® (TECRA International)	*Listeria* spp.	Food and environmental	N/A	(Gasanov, et al., 2005)
VIDAS® LIS (bioMerieux)	*Listeria* spp.	Dairy, vegetables, seafood, raw and processed meats and poultry	10^4-10^5 CFU/ml	(Gangar, et al., 2000; Silbernagel, et al., 2005)
VIDAS® LMO (bioMerieux)	*L. monocytogenes*	Food (mixed cut vegetable salad, smoked salmon, and sterile smoked salmon)	1-10 CFU/25g	(Kerdahi and Istafanos, 2000)
VIDAS® LDUO (bioMerieux)	*Listeria* spp. and *L. monocytogenes* [b]	Food	0.2-2.7 cells/25g	(Janzten, Navas, Corujo, Moreno, Lopez and Martinez-Suarez, 2006)
Listertest (Vicam)	*Listeria* spp.	Food (seafood) and environmental	<10 CFU/g	(McCarthy, 1997)
Singlepath® L'mono (Merck)	*L. monocytogenes*	Food and environmental	N/A	http://www.rapidmicrobiology.com/news/1054h10.php
PATHATRIX *Listeria* species test system (Matrix)	*Listeria* spp.	Food	1-10 CFU/g	(Andrews and Hammack, 2006)

[a]when combined with another method such as chromogenic plating media or the PCR
[b]simultaneous detection

Table 4. Strains of *L. monocytogenes* used in specificity studies.

Species	Strain	Serotype	Source	Origin	Test Result
Listeria monocytogenes	J1-119	4b	ILSI, Cornell[a]	Human epidemic	Y
Listeria monocytogenes	J1-123	4b	ILSI, Cornell[a]	N/A	Y
Listeria monocytogenes	J1-049	3c	ILSI, Cornell[a]	Human sporadic	Y
Listeria monocytogenes	J1-108	4b	ILSI, Cornell[a]	Human epidemic	Y
Listeria monocytogenes	J1-220	N/A	ILSI, Cornell[a]	N/A	Y
Listeria monocytogenes	J1-169	3b	ILSI, Cornell[a]	Human sporadic	Y
Listeria monocytogenes	J1-020	4b	ILSI, Cornell[a]	N/A	Y
Listeria monocytogenes	J2-035	1/2b	ILSI, Cornell[a]	Caprine	Y
Listeria monocytogenes	J1-031	4a	ILSI, Cornell[a]	Human sporadic	Y
Listeria monocytogenes	J1-225	4b	ILSI, Cornell[a]	Human epidemic	Y
Listeria monocytogenes	J2-054	1/2a	ILSI, Cornell[a]	Ovine	Y
Listeria monocytogenes	J1-177	1/2b	ILSI, Cornell[a]	Human sporadic	Y
Listeria monocytogenes	J1-126	4b	ILSI, Cornell[a]	Human epidemic	Y
Listeria monocytogenes	J2-020	1/2a	ILSI, Cornell[a]	Bovine	Y
Listeria monocytogenes	J1-168	4a	ILSI, Cornell[a]	Human sporadic	Y
Listeria monocytogenes	R2-501	4b	ILSI, Cornell[a]	Human epidemic	Y
Listeria monocytogenes	N1-225	4b	ILSI, Cornell[a]	Human epidemic	Y
Listeria monocytogenes	C1-122	4b	ILSI, Cornell[a]	Human sporadic	Y
Listeria monocytogenes	J2-064	1/2b	ILSI, Cornell[a]	Bovine	Y
Listeria monocytogenes	R2-591	4b	ILSI, Cornell[a]	N/A	Y
Listeria monocytogenes	W1-110	4c	ILSI, Cornell[a]	N/A	Y
Listeria monocytogenes	R2-764	4b	ILSI, Cornell[a]	Sliced deli meat	Y
Listeria monocytogenes	W1-111	4c	ILSI, Cornell[a]	N/A	Y
Listeria monocytogenes	C1-115	3a	ILSI, Cornell[a]	Human sporadic	Y
Listeria monocytogenes	R2-568	1/2a	ILSI, Cornell[a]	N/A	Y
Listeria monocytogenes	J2-066	1/2a	ILSI, Cornell[a]	Ovine	Y
Listeria monocytogenes	R2-765	4b	ILSI, Cornell[a]	N/A	Y

Listeria monocytogenes	N3-008	4b	ILSI, Cornell[a]	Coleslaw	Y
Listeria monocytogenes	C1-056	1/2a	ILSI, Cornell[a]	Human sporadic	Y
Listeria monocytogenes	R2-500	4b	ILSI, Cornell[a]	Cheese	Y
Listeria monocytogenes	J1-101	1/2a	ILSI, Cornell[a]	Human sporadic	Y
Listeria monocytogenes	J1-107	4d	ILSI, Cornell[a]	N/A	Y
Listeria monocytogenes	J1-112	4b	ILSI, Cornell[a]	N/A	Y
Listeria monocytogenes	J1-116	4b	ILSI, Cornell[a]	Human epidemic	Y
Listeria monocytogenes	J1-129	4bx	ILSI, Cornell[a]	N/A	Y
Listeria monocytogenes	J1-158	4b	ILSI, Cornell[a]	Caprine	Y
Listeria monocytogenes	J2-031	1/2a	ILSI, Cornell[a]	Bovine	Y
Listeria monocytogenes	J2-063	1/2a	ILSI, Cornell[a]	Ovine	Y
Listeria monocytogenes	J1-012	4b	ILSI, Cornell[a]	N/A	N[b]
Listeria monocytogenes	M1-004	N/A	ILSI, Cornell[a]	Human sporadic	Y
Listeria monocytogenes	N1-227	4b	ILSI, Cornell[a]	Food epidemic (Hot dog)	Y
Listeria monocytogenes	N3-013	4b	ILSI, Cornell[a]	N/A	Y
Listeria monocytogenes	N3-022	4b	ILSI, Cornell[a]	N/A	Y
Listeria monocytogenes	N3-031	1/2a	ILSI, Cornell[a]	Turkey franks	Y
Listeria monocytogenes	R2-499	1/2a	ILSI, Cornell[a]	Human epidemic	Y
Listeria monocytogenes	R2-502	1/2b	ILSI, Cornell[a]	Chocolate milk	Y
Listeria monocytogenes	R2-503	1/2b	ILSI, Cornell[a]	N/A	Y
Listeria monocytogenes	R2-559	1/2a	ILSI, Cornell[a]	N/A	Y
Listeria monocytogenes	R2-575	4b	ILSI, Cornell[a]	N/A	Y
Listeria monocytogenes	R2-584	4b	ILSI, Cornell[a]	N/A	Y
Listeria monocytogenes	R2-597	1/2b	ILSI, Cornell[a]	N/A	Y
Listeria monocytogenes	R2-598	1/2b	ILSI, Cornell[a]	N/A	Y
Listeria monocytogenes	R2-600	4b	ILSI, Cornell[a]	N/A	Y
Listeria monocytogenes	R2-763	4b	ILSI, Cornell[a]	Human epidemic	Y
Listeria monocytogenes	W1-112	4a	ILSI, Cornell[a]	N/A	Y
Listeria monocytogenes	J1-094	1/2c	ILSI, Cornell[a]	Human sporadic	N[b]
Listeria monocytogenes	J1-110	4b	ILSI, Cornell[a]	Mexican style cheese	N[b]
Listeria monocytogenes	SW1-091	1/2b	Nightingale Collection	Meat Plant	Y
Listeria monocytogenes	SW1-113	1/2b	Nightingale Collection	Meat Plant	Y

Listeria monocytogenes	SW1-215	1/2b	Nightingale Collection	Meat Plant	Y
Listeria monocytogenes	SW1-211	1/2a	Nightingale Collection	Meat Plant	Y
Listeria monocytogenes	SW1-167	1/2b	Nightingale Collection	Meat Plant	Y
Listeria monocytogenes	SW1-163	1/2b	Nightingale Collection	Meat Plant	Y
Listeria monocytogenes	SW1-159	1/2b	Nightingale Collection	Meat Plant	Y
Listeria monocytogenes	SW1-137	1/2a	Nightingale Collection	Meat Plant	Y
Listeria monocytogenes	SW1-129	1/2b	Nightingale Collection	Meat Plant	Y
Listeria monocytogenes	SW1-121	1/2b	Nightingale Collection	Meat Plant	Y
Listeria monocytogenes	SW1-087	1/2b	Nightingale Collection	Meat Plant	Y
Listeria monocytogenes	SW1-111	1/2b	Nightingale Collection	Meat Plant	Y
Listeria monocytogenes	SW1-107	1/2b	Nightingale Collection	Meat Plant	Y
Listeria monocytogenes	SW1-099	1/2b	Nightingale Collection	Meat Plant	Y
Listeria monocytogenes	SW1-083	1/2b	Nightingale Collection	Meat Plant	Y
Listeria monocytogenes	SW1-075	1/2b	Nightingale Collection	Meat Plant	Y
Listeria monocytogenes	SW1-061	1/2b	Nightingale Collection	Meat Plant	Y
Listeria monocytogenes	SW1-057	1/2b	Nightingale Collection	Meat Plant	Y
Listeria monocytogenes	SW1-049	1/2a	Nightingale Collection	Meat Plant	Y
Listeria monocytogenes	SW1-041	1/2c	Nightingale Collection	Meat Plant	Y

Y= positive (blue)
N= negative (brown or clear)
N/A= Not Available
[a]International Life Sciences Institute (ILSI) collection at Cornell University
[b] These isolates were characterized using phenotypic and genotypic approaches

Table 5. Strains belonging to species other than *L. monocytogenes* used in specificity studies.

Species	Strain	Serotype	Origin	Test Result
Listeria ivanovii	SPM0000479	N/A	Goodridge Strain Collection	Y
Listeria innocua	SW-171	N/A	Meat Plant	N
Listeria innocua	SW-151	N/A	Meat Plant	N
Listeria innocua	SW-179	N/A	Meat Plant	N
Listeria innocua	SW-139	N/A	Meat Plant	N
Listeria innocua	SPM0000480	N/A	Goodridge Strain Collection	N
Listeria innocua	SPM0000481	N/A	Goodridge Strain Collection	N
Listeria innouca[a]	J1-023	3a	ILSI, Cornell	N
Listeria welshimeri	SW-187	N/A	Meat Plant	N
Listeria welshimeri	SW-155	N/A	Meat Plant	N
Listeria welshimeri	SW-147	N/A	Meat Plant	N
Listeria welshimeri	SW-203	N/A	Meat Plant	N
Listeria grayi	N/A	N/A	Goodridge Strain Collection	N
Escherichia coli	SPM0000011 6	O157:H7	Goodridge Strain Collection	N
Escherichia coli	SPM0000011 9	O157:H7	Goodridge Strain Collection	N
Escherichia coli	SPM0000012 0	O157:H7	Goodridge Strain Collection	N
Escherichia coli	Famp	N/A	Goodridge Strain Collection	N
Escherichia coli	MS2	N/A	Goodridge Strain Collection	N
Escherichia coli	G3	N/A	Goodridge Strain Collection	N
Escherichia coli	SPM0000025 8	N/A	Goodridge Strain Collection	N
Escherichia coli	SPM0000351	N/A	Goodridge Strain Collection	N
Salmonella enterica	SPM0000437	Typhimurium	Goodridge Strain Collection	N
Salmonella enterica	SPM0000438	Anatum	Goodridge Strain Collection	N
Salmonella enterica	SPM0000445	Enteriditis	Goodridge Strain Collection	N
Salmonella enterica	SPM0000485	Newport	Goodridge Strain Collection	N

Pseudomonas aeruginosa	ATCC 15692	N/A	Goodridge Strain Collection	N
Enterococcus faecalis	ATCC 29212	N/A	Sofos Collection	N
Enterococcus faecalis	ATCC 33186	N/A	Sofos Collection	N
Staphylococcus aureus	ATCC 12600	N/A	Sofos Collection	N

[a] hemolytic
Y= positive (blue)
N= negative (brown or clear)
N/A=not available

Table 6. Primer sequences used in analysis of the *L. monocytogenes plc*A gene.

Name	Direction	Sequence	Use
*plc*AF	Forward	5' TAGGACTTFCAGGCGGAGATG 3'	PCR and Sequencing
*plc*AR	Reverse	5' TCATGTCTCATCCCCCAATCG 3'	PCR and Sequencing
*plc*A1	Forward	5' GGAATAAGCCAATAAAGAACTC 3'	Sequencing
*plc*A2	Reverse	5' GATAAGCAGTCTGGACAATCTC 3'	Sequencing
*plc*A3	Forward	5' CAATGGTCCGAGTGTGAAAAC 3'	Sequencing
*plc*A4	Reverse	5' GCTAGGTTTGTTGTGTCAGGTAG 3'	Sequencing

Table 7. Ability of the Phast Swab assay to detect an artificially inoculated five-strain cocktail of *L. monocytogenes* on ready-to-eat ham and turkey slices.

Sample	Inoculum[a]	Phast Swab results[b,c]					
		Sample	Trial 1	Trial 2	Trial 3		
Turkey[d]	10^1	1	–	(<1)	–	(<1)	–	(<1)
		2	–		–		–	
	10^2	1	–	(<1)	+	(<1)	+	(<1)
		2	–		–		–	
	10^3	1	+	(6 x 10^1)	+	(8 x 10^1)	+	(1.2 x 10^2)
		2	+		+		+	
	10^4	1	+	(5 x 10^2)	+	(7.2 x 10^2)	+	(6.8 x 10^2)
		2	+		+		+	
Ham[d]	10^1	1	–	(<1)	+	(<1)	+	(<1)
		2	–		–		–	
	10^2	1	–	(<1)	+	(<1)	+	(<1)
		2	–		–		–	
	10^3	1	+	(1 x 10^2)	+	(1.2 x 10^2)	+	(1.2 x 10^2)
		2	+		+		+	
	10^4	1	+	(8.3 x 10^2)	+	(1.2 x 10^3)	+	(1.2 x 10^3)
		2	+		+		+	

[a] *L. monocytogenes* five–strain cocktail. Cell counts are expressed as cells per milliliter.
[b] (–) indicates a negative test result and (+) indicates a positive test result for the corresponding meat sample (each concentration was tested in duplicate for each trial).
[c] Values in parentheses are cell counts (CFU/g) recovered from the artificially inoculated deli meats.
[d] Turkey (25g) and Ham (20g) samples were artificially inoculated with varying concentrations of the *L. monocytogenes* five–strain cocktail.

Table 8. Ability of the Phast Swab assay to detect an artificially inoculated five-strain cocktail of *L. monocytogenes* on ready-to-eat ham and turkey when stored at 5ºC over a 21-day period.

Sample	Inoculum[a]	Phast Swab results[b,c]													
		Sample	Day 0		Day 3		Day 6		Day 9		Day 12		Day 15		Day 18		Day 21
Turkey[d]	10^1	1	–	(<1)	–	(<1)	–	(<1)	–	(<1)	+	(<1)	–	(<1)	+	(<1)	–	(<1)
		2	–		–		–		–		–		–		–		–	
	10^2	1	+	(<1)	–	(<1)	–	(<1)	–	(<1)	+	(<1)	–	(<1)	+	(<1)	–	(<1)
		2	–		–		–		–		–		–		–		–	
	10^3	1	+	($8x10^1$)	+	($1.2x10^2$)	+	($1x10^2$)	–	($6x10^1$)	+	(<1)	+	($7x10^1$)	–	(<1)	–	(<1)
		2	+		+		–		–		+		+		–		–	
	10^4	1	+	($7.2x10^2$)	+	($6.8x10^2$)	+	($7.6x10^2$)	+	($8.2x10^{2)}$	+	($5.6x10^2$)	+	($5x10^2$)	+	($9.2x10^2$)	+	($7.2x10^2$)
		2	+		+		+		+		+		+		+		+	
Ham[d]	10^1	1	+	(<1)	–	(<1)	+	(<1)	–	(<1)	+	(<1)	–	(<1)	–	(<1)	–	(<1)
		2	–		–		–		–		–		–		–		–	
	10^2	1	+	(<1)	–	(<1)	–	(<1)	–	(<1)	–	(<1)	–	(<1)	–	(<1)	–	(<1)
		2	–		–		–		–		–		–		–		–	
	10^3	1	+	($1.2x10^2$)	+	($7x10^1$)	–	($1x10^2$)	–	($4x10^1$)	–	($3x10^1$)	–	($9x10^1$)	–	($2.5x10^2$)	–	($1x10^0$)
		2	+		–		–		–		–		–		–		–	
	10^4	1	+	($1.2x10^3$)	+	($3.7x10^2$)	+	($6.7x10^2$)	+	($6.6x10^2$)	+	($6.8x10^2$)	+	($6.7x10^2$)	+	($4.4x10^2$)	+	($5.5x10^2$)
		2	+		+		–		–		+		+		+		+	

[a] *L. monocytogenes* five–strain cocktail. Cell counts are expressed as cells per milliliter.

[b] (–) indicates a negative test result and (+) indicates a positive test result for the corresponding meat sample (each concentration was tested in duplicate for each day).

[c] Values in parentheses are cell counts (CFU/g) recovered from the artificially inoculated deli meats.

[d] Turkey (25g) and Ham (20g) samples were artificially inoculated with varying concentrations of the *L. monocytogenes* five–strain cocktail.

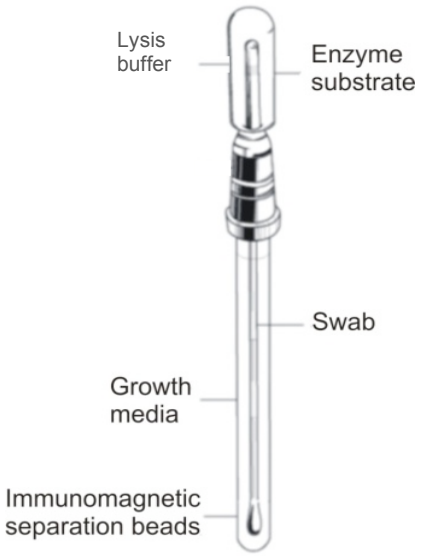


Figure 1. The integrated Phast Swab device. The device contains a (cotton) swab, Trypic Soy Broth (TSB) supplemented with 428mM potassium chloride (KCl) as the growth media and 40 μl anti-*Listeria* immunomagnetic beads. In the top of the device is a reservoir that contains a lysis buffer and the phosphatidylinositol-specific phospholipase C (PI-PLC) enzyme substrate, 5-bromo-4-chloro-3-indoxyl-myo-inositol-1-phosphate (X-Inp).

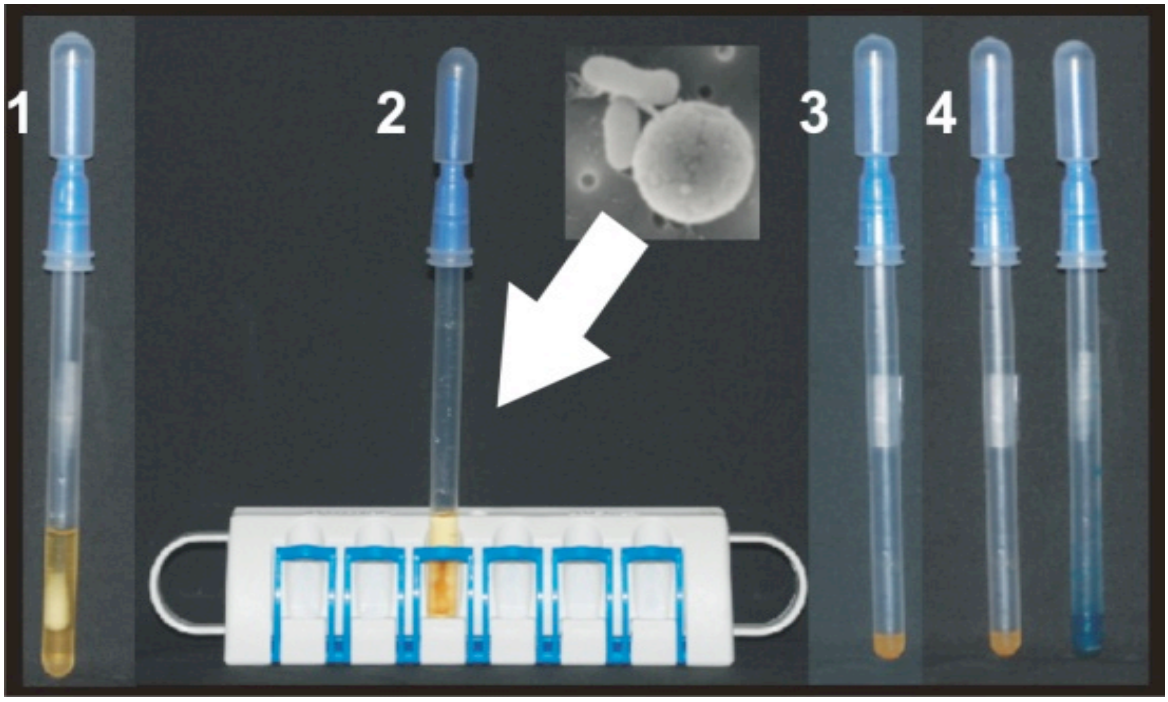
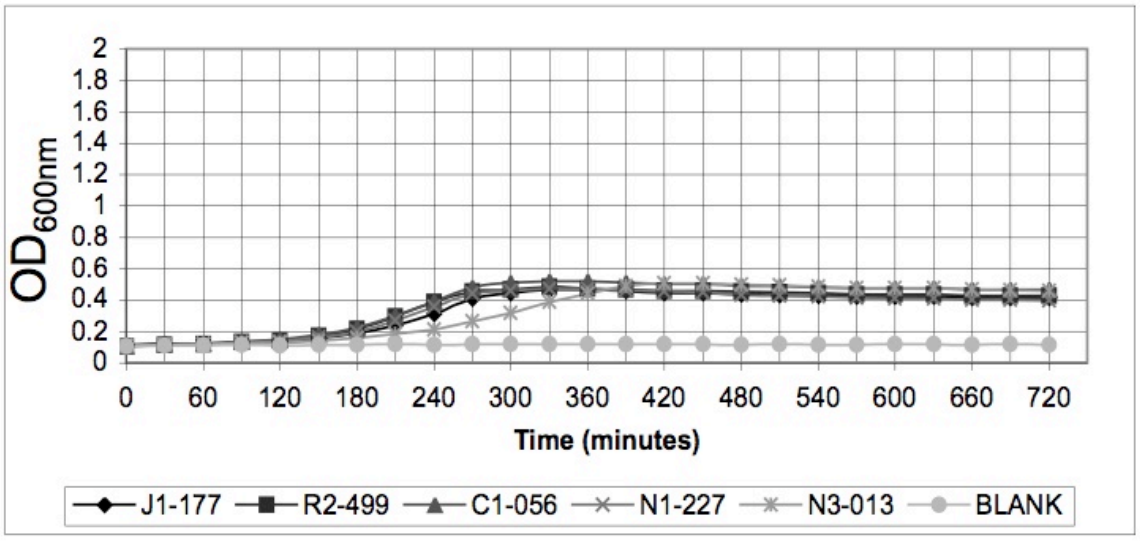


Figure 2. The Phast Swab procedure. 1. The cotton swab is removed, the desired surface is swabbed and the swab is returned to the broth for enrichment. 2. After enrichment, the device is placed on a magnet and the beads are pulled out of solution allowing the broth to be discarded. The captured cells are now concentrated onto the beads. 3. Lysis buffer (100 μl) and the substrate 5-bromo-4-chloro-3-indoxyl-myo-inositol-1-phosphate (X-Inp) (50 μl) are then added to the beads, followed by a 5-h incubation. 4. The liquid in the device will turn blue if PI-PLC is present and will remain brown (if beads are in solution) or clear (if the beads are removed from the solution with a magnet) if the enzyme is not present.

A)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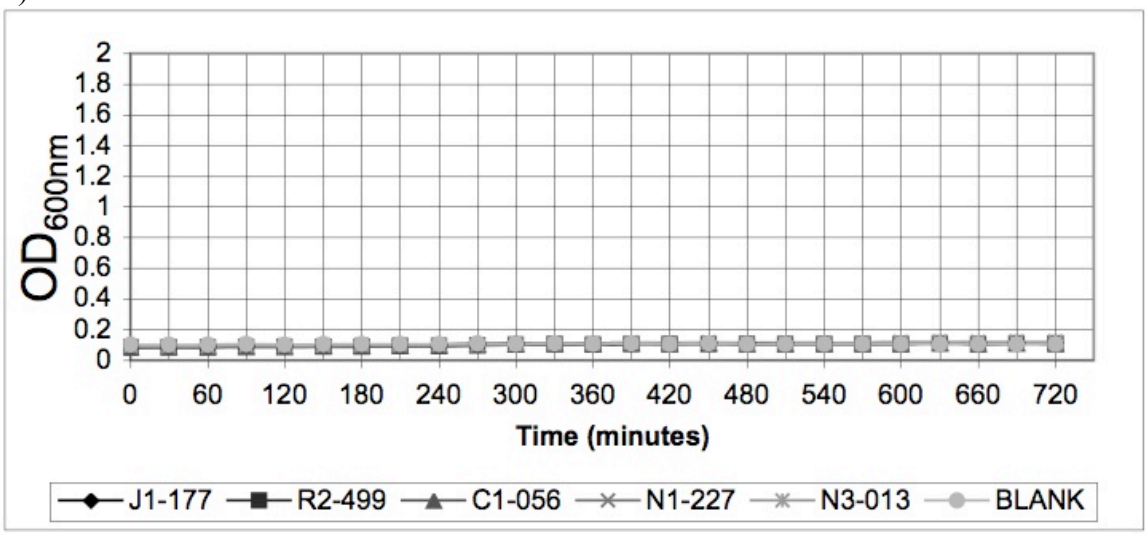
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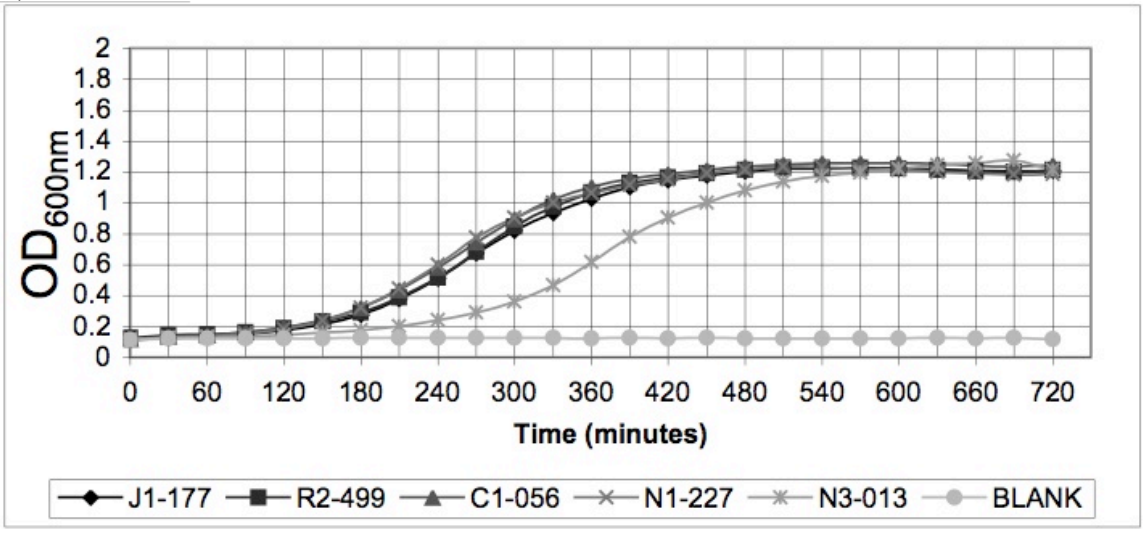
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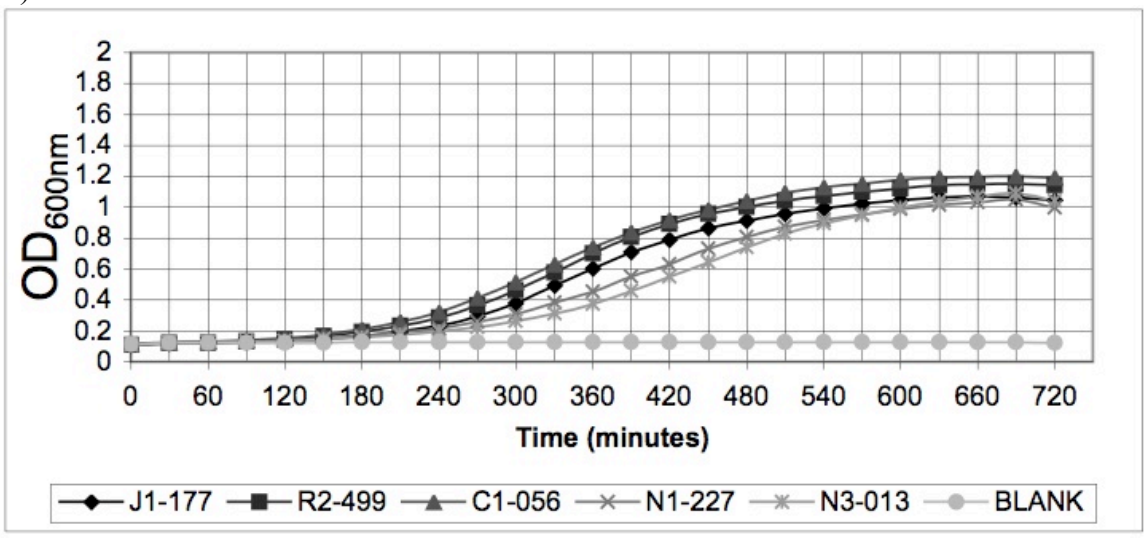
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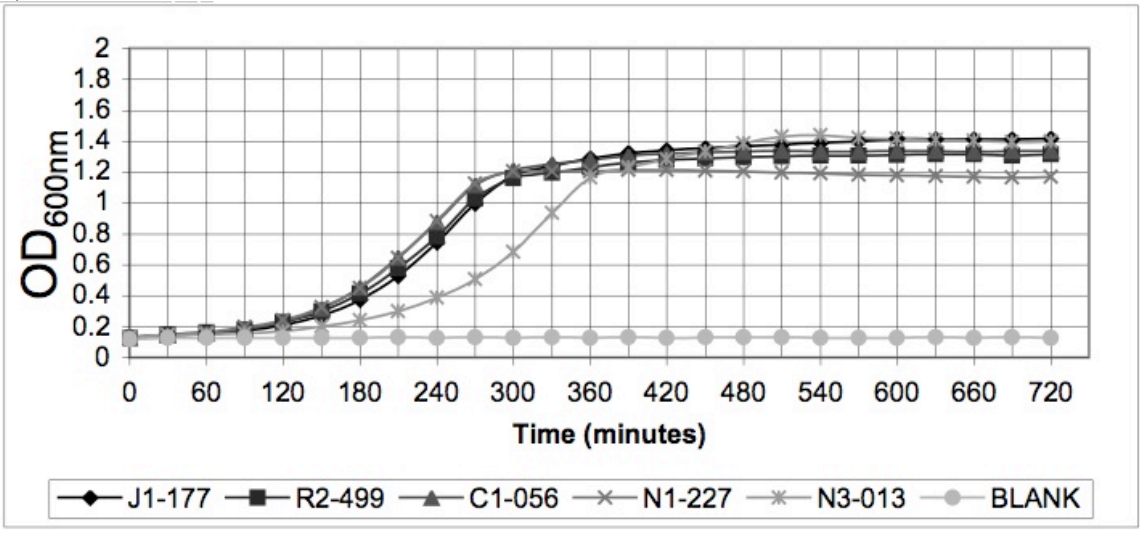

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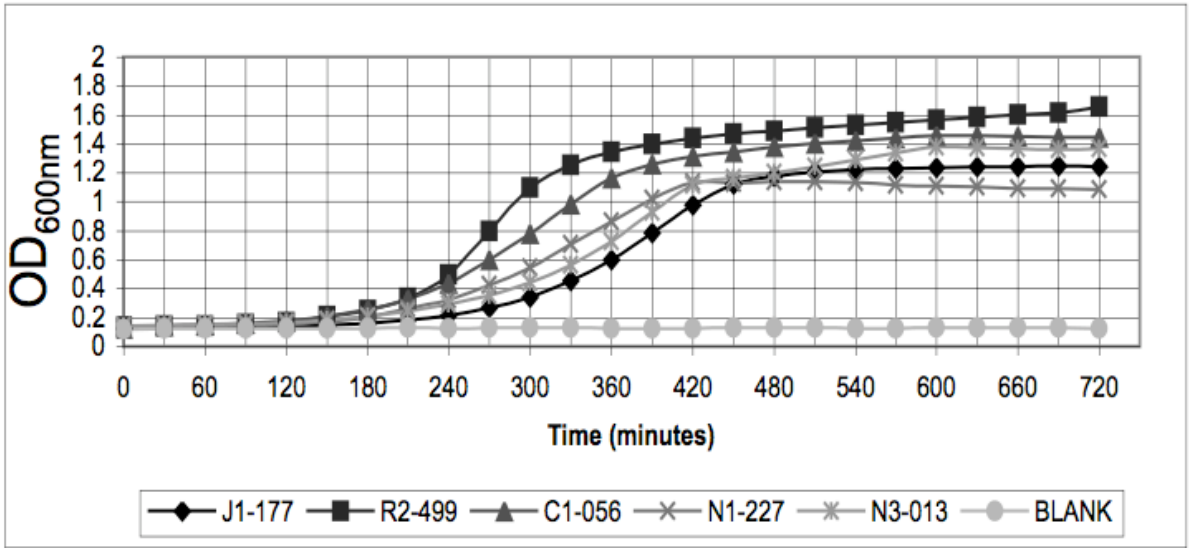

Figure 3. Evaluation of six growth media to determine the best growth medium to use for enrichment of *L. monocytogenes* in the Phast Swab. Five strains of *L. monocytogenes* (J1-177, R2-499, C1-056, N1-227 and N3-013) were used in this experiment. The media evaluated were: A) Buffered Peptone Water (BPW) B) Universal Preenrichment Broth (UPB) C) Brain Heart Infusion (BHI) D) BHI supplemented with 428 mM potassium chloride (KCl) E) Tryptic Soy Broth (TSB) F) TSB supplemented with 428 mM potassium chloride (KCl).

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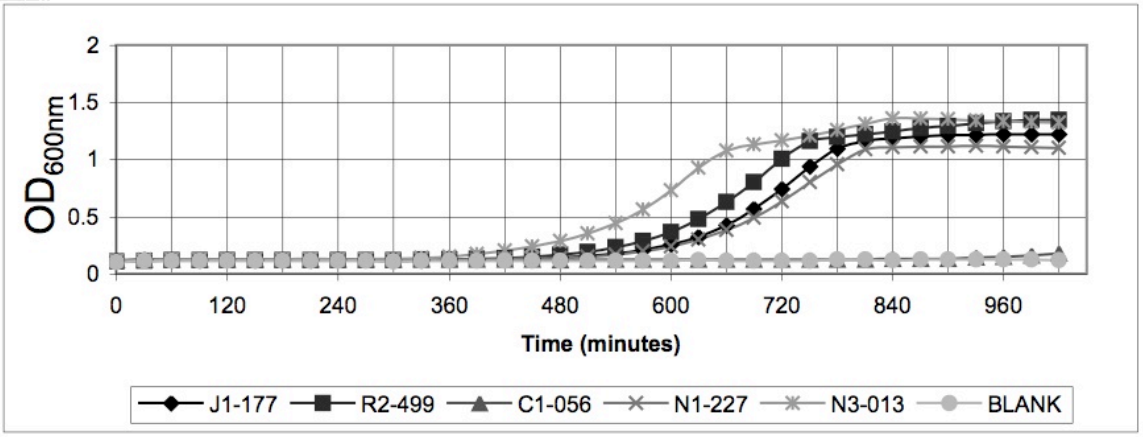
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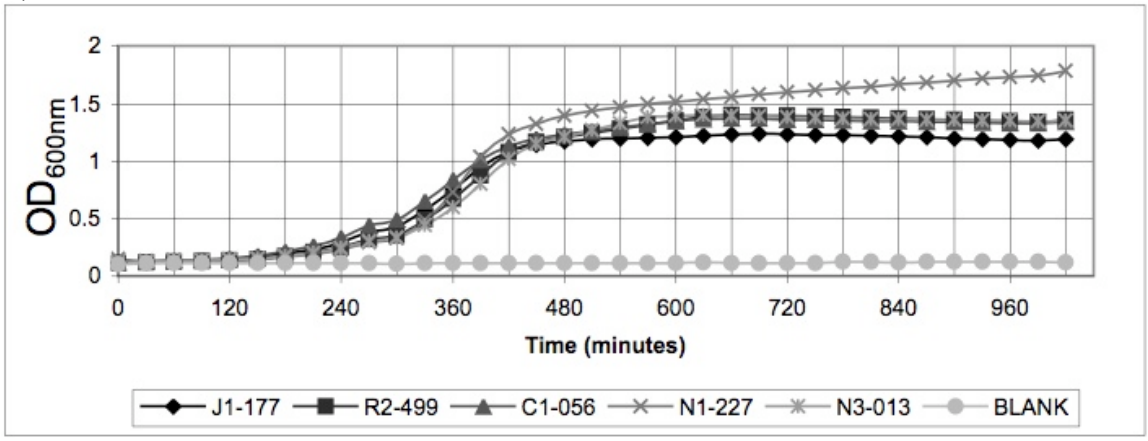
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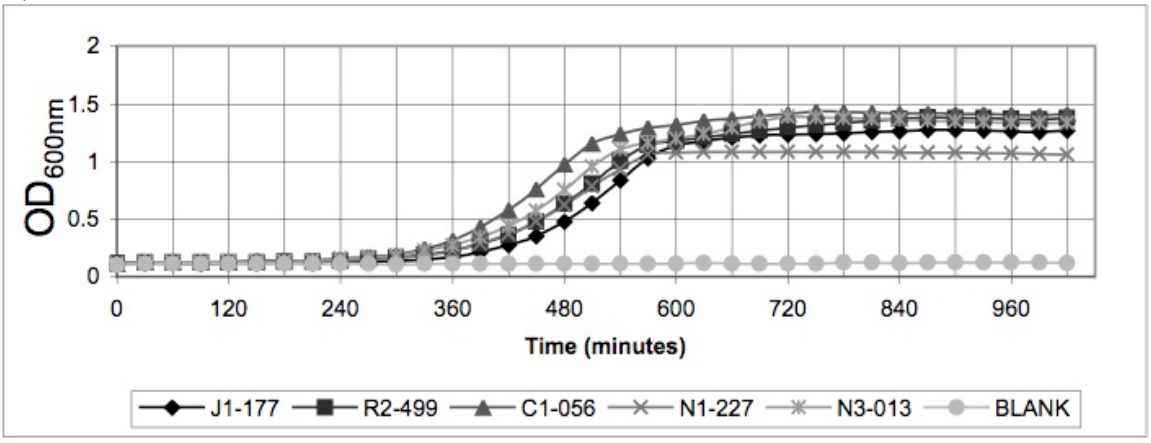
C)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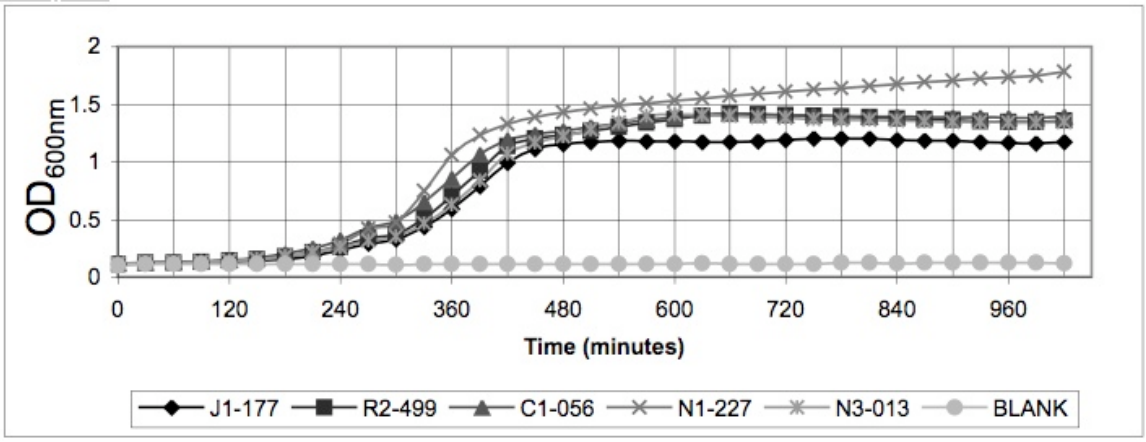
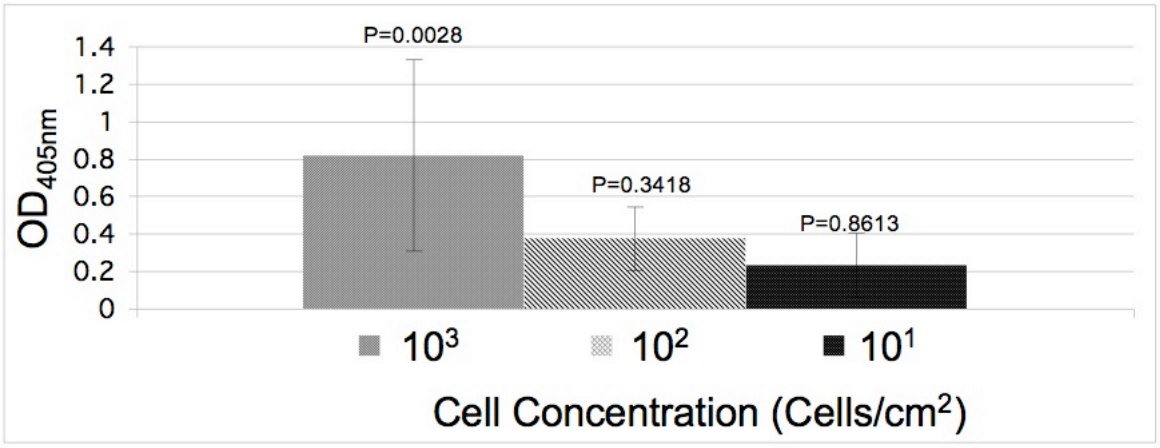


Figure 4. Recovery of *L. monocytogenes* strains (J1-177, R2-499, C1-056, N1-227 and N3-013) in Tryptic Soy Broth (TSB) supplemented with 428mM potassium chloride (KCL) following exposure to sublethal stress. A) low pH (pH 3.0), B) high pH (pH 9.0), C) osmolarity (15% NaCl) and D) heat (47°C).

A)



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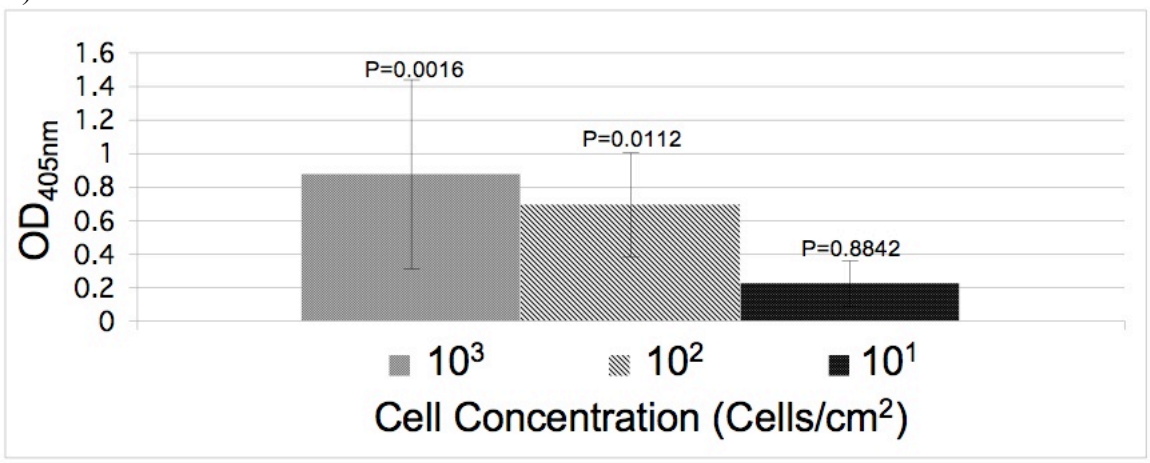

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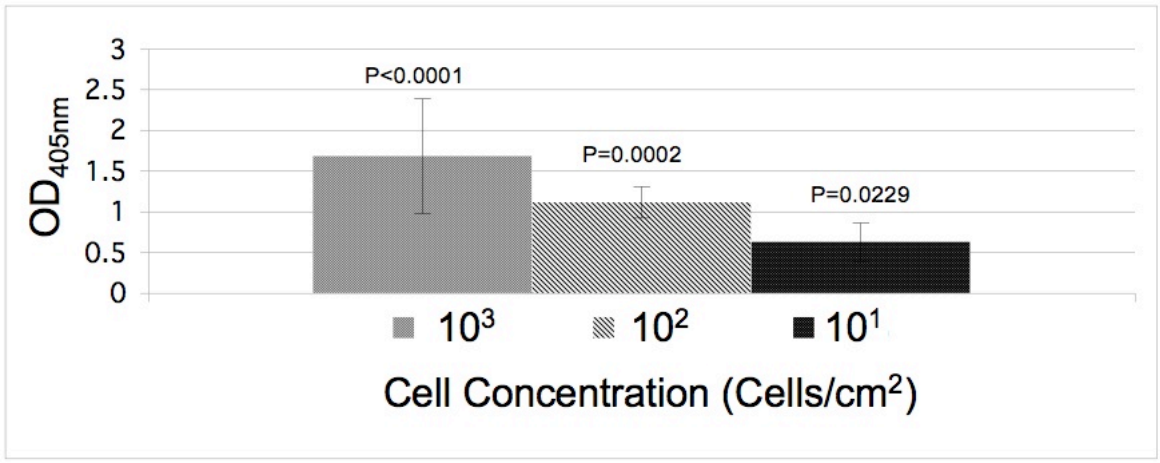

Figure 5. Detection limits of the Phast Swab following inoculation and recovery of *L. monocytogenes* from 3 food contact and non-food contact surfaces. A) Stainless steel, B) Acrylic and C) Ceramic tile.

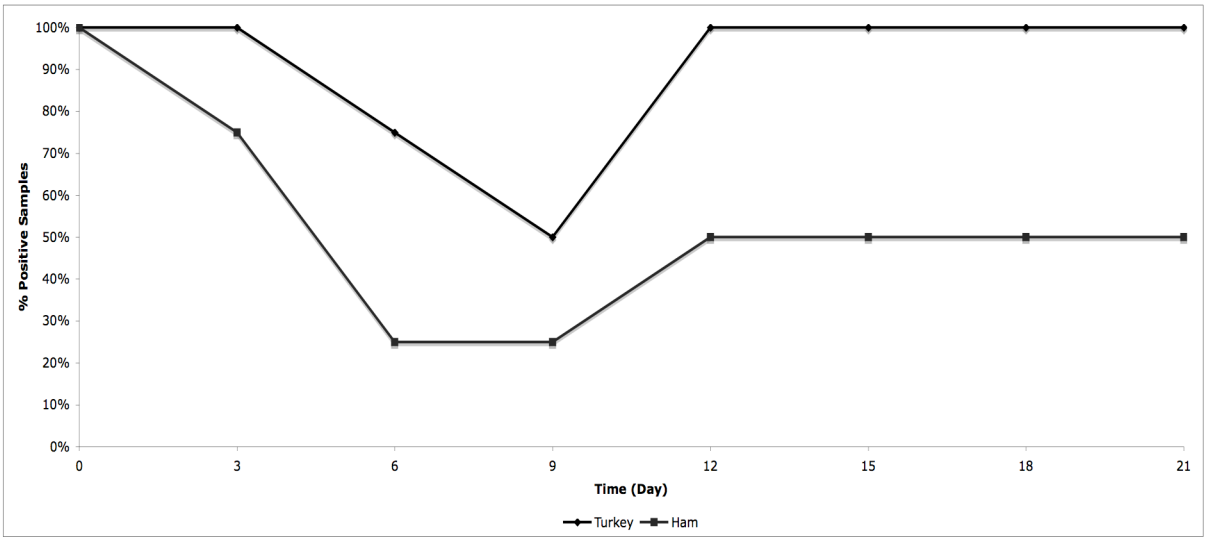

Figure 6. Percent of *Listeria monocytogenes* artificially contaminated ready-to-eat cured meat (turkey and ham) samples that tested positive on each sample day during the 21-day storage period at 5°C.

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