RAPID DETECTION OF *LISTERIA MONOCYTOGENES* IN SALAD BY POLYMERASE CHAIN REACTION

Ken R. Walker

A Thesis

Submitted to

the Graduate Faculty of

Auburn University

in Partial of the

Requirements for the

Degree of

Master of Science

Auburn, Alabama
August 8, 2005

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Except where reference is made to the work of others, the work described in this thesis is my own or was done in collaboration with my advisory committee. This thesis does not include proprietary or classified information.

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I'm Ken Robbin Walker son of Dorsey and Brenda Walker. I was born on March 19, 1979 in Huntsville Hospital. I graduated from Section High School in the spring of 1997. I furthered my education by attending Northeast State Community College were I received an Associates in Science in the spring of 2000. In the fall of 2000 I enrolled in at Auburn University were I received a Bachelors of Science in the field of Food Science in December of 2002. I began by graduate work in Food Science in the spring of 2003.

THESIS ABSTRACT

RAPID DETECTION OF *LISTERIA MONOCYTOGENES* IN SALAD BY POLYMERASE CHAIN REACTION

Ken R. Walker

Master of Science, August 8, 2005

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The development of rapid detection methods for the testing of ready-to-eat products is an area of high importance, not only for the food industry but also for consumers. The USDA standard for zero tolerance of *Listeria monocytogenes* in ready-to-eat products causes massive recalls that can be fatal to small food industries. In order to combat this growing problem, it is important for food industry to find the presence of *L. monocytogenes* in their products as soon as possible to avoid these potentially hazardous impacts.

The development of the polymerase chain reaction (PCR) technique has been a huge leap forward in the development of rapid, specific and sensitive methods for the detection of food borne pathogens. The principle behind the PCR method is the amplification of a

gene sequence that is specific to the targeted pathogen. After amplification, the sample can be analyzed through agarose gel electrophoresis.

In this study, a set of primers were developed to amplify 400 base pairs of DNA, a segment of the internalin A gene. This protein is link to the pathogenesis of *L. monocytogenes*, which causes listeriosis in humans. Therefore, this gene is only found in the pathogenic strain of *L. monocytogenes*. The specificity of the primers was tested against 34 different bacteria and only the pathogenic *L. monocytogenes* isolates showed positive results on the agarose gel. Five different isolates of *L. monocytogenes* were tested to determine the detection sensitivity of PCR, and the results showed that it was able to detect as low as 298 cfu.

Six different enrichment broths of modified Penn State University (mPSU), Listeria enrichment broth (LEB), Tryptic® soy broth plus 0.6% yeast extract (TSBYE), half Fraser's broth, University of Vermont medium (UVM), and half Tryptic® soy broth plus 0.6% yeast extract (1/2 TSBYE) were tested to determine the growth rate of the bacterium in 6 hr. The TSBYE, LEB, mPSU, and UVM were chosen and tested to determine the recovery level of *L. monocytogenes* cells from inoculated salad. From the results, TSBYE, mPSU and LEB were chosen to represent both selective and non-selective enrichment media for *L. monocytogenes* enrichment on inoculated salad in PCR detection.

In this study, 25 grams of ready-to-eat salad were inoculated with *L. monocytogenes* at 200 cfu/g for use. After blended with a stomacher, the sample was filtered through

glass wool to remove large food partials, and then the filtrate was centrifuged to concentrate the bacteria and enriched in selected broths. Following 6 hr enrichment, the samples were centrifuged again to concentrate bacteria for PCR amplification. Samples were also spread plated on modified Oxford medium for actual bacterial counts. The final bacterial concentrations of TSBYE, LEB, and mPSU were 4.0×10^4, 8.2×10^3, 6.8×10^2 cfu/g, respectively. Positive PCR results were shown only on the LEB sample. This study showed that by using the internalin A based primers, filtration, centrifugation, and a 6 hour enrichment process; the PCR technique can detect *L. monocytogenes* at 10^3 cfu/g of ready-to-eat salad.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to Dr. Tung-Shi Huang, my major advisor, for his guidance, support, and encouragement throughout the course of this study. I would also like to thank Dr. Jean Weese and Dr. Tom McCaskey for their willingness to serve on my committee and their invaluable support. I would also like to express my heartfelt gratitude to my parents for their steadfast moral and fiscal support.

Style manual of journal used Journal of Food Science

Computer software used Microsoft Word

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

I. INTRODUCTION

Currently the USDA has a zero tolerance standard for the presence of *Listeria monocytogenes* in ready-to-eat food products. This bacterium is also the leading cause of ready-to-eat product recalls. *Listeria monocytogenes* can cause listeriosis in humans particularly in certain well-defined high-risk groups, including pregnant women, neonates, and immunocompromised individuals. According to the Center for Disease Control (CDC) it is estimated that there are 2,500 cases and 500 fatalities every year, due to listeriosis (CDC, 2004). Over the past decade, the level of foodborne illness associated with fresh fruits and vegetables has increased throughout the United States (Beuchat and other, 1997). A study was done to determine the occurrence of pathogens in vegetable salads. Out of 63 vegetable salads tested 14% were positive for potentially pathogenic bacteria (Lin and others, 1996). The survivability of *Listeria monocytogenes* in a variety of environments and on processing equipment makes this bacterium a food safety risk and a serious threat to the consumer as well as the food industry (Fenlon, 1999). Therefore, the development of rapid and accurate methods for the detection of this foodborne pathogen is necessary for the food industry in order to reduce the number of ready-to-eat food recalls.

There have been many types of rapid detection methods developed, and many use the PCR technique. However, these methods are the collaboration of the PCR technique

along with various other forms of extraction and concentration methods. The use of immunochemical technology, such as immunomagnetic beads in order to extract the targeted bacterial cells from the food sample matrix, is popular. Others may use DNA probes to concentrate the DNA material and extract it from the food samples. Another technique that is commonly used is the chemical extraction and purification of the DNA before PCR analysis. By incorporating these methods and techniques together, rapid detection of *L. monocytogenes* can reach a very specific and highly sensitive level. However, with the introduction of these methods, additional costs for labor and training of laboratory personnel will be required.

The goal of this study was to develop a rapid, simple, accurate and cost effective method for the detection of *L. monocytogenes* in food samples, specifically for ready-to-eat salads. The study used the primers that were designed to amplify the internal A protein gene. The study involved a simple glass wool filtration process of a blended salad sample, followed by centrifugation, and then the sample was enriched in either modified Penn State University or Listeria enrichment broth. After enrichment another centrifugation step was employed to concentrate the bacteria for PCR amplification. Agarose gel electrophoresis was used to view the results. The objective of this study was to develop a PCR combination protocol for detection of *L. monocytogenes* in ready-to-eat salads within 6 hr at a level of below 100 cfu per gram salad.

II. LITERATURE REVIEW

Listeria monocytogenes' Morphology

Listeria monocytogenes is a catalase-positive, oxidase-negative, facultative anaerobic and gram-positive rod-shaped bacterium (CDC, 2004). This bacterium is motile when grown at 20 to 25°C (Rocourt, 1999). *Listeria* is not a spore forming bacterium, however it is moderately tolerant to freezing, drying, as well as heating (FDA, 1992). This bacterium grows well on a majority of common media. However, media that contains fermentable carbohydrate, such as glucose, improves its growth. When *Listeria* is cultured on an agar plate, a strong acid odor can be observed, which is thought to be due to the production of alcohols, hydroxy acids, and carboxylic acids. When grown in enrichment broth media, 8-24 hr at 37°C is needed for visual turbidity to be observed. Some of the primary growth factors that are need for *Listeria* growth are: leucine; cystine; methionine; arginine; valine; cysteine; isoleucine; biotin; riboflavin; thiamine; thioctic acid; as well as glucose and glutamine as primary carbon and nitrogen sources (Rocourt, 1999). Under aerobic conditions, all *Listeria* produce lactate, acetate, and acetoin from glucose (Rocourt, 1999; Romick and others, 1996). *L. monocytogenes* metabolizes glucose by importing it through a high-affinity phosphoenolpyruvate-dependent phosphotransferase system as well as having a low-affinity proton motive force-mediate system (Christensen and others, 1994; Parker and others, 1997). *Listeria monocytogenes* is beta-hemolytic, causing the lysis of hemoglobin cells in humans,

sheep, cows, horses, piglets, and guinea pigs (Rocourt, 1999). This particular characteristic of *L. monocytogenes* is caused by its production of listeriolysin O. Research into the characteristics of *Listeria monocytogenes* tapered off until several outbreaks of *Listeria* foodborne disease were reported which stimulated new interest in the epidemiology and molecular biology of *L. monocytogenes* (Rocourt, 1999).

Natural Environment

The natural habitat for *L. monocytogenes* is considered to be in soil, water, and plant material (Fenlon, 1999). This bacterium has also been reportedly found present in animal feed. However, its presence in feed is not considered to be of high concern due to the low water activity of feeds, which prevents growth of the bacterium to a level that would present a risk to the animals. *L. monocytogenes* in spoiled vegetation has been determined as the source of listeriosis in numerous cases involving farm animals and possibly attributed to contamination throughout the food chain. *L. monocytogenes* can also be found in the fecal material of a large variety of healthy animals. *L. monocytogenes* is a non-spore forming bacteria, that has the ability to survive moderately harsh environmental conditions, more so than most other non-spore forming bacteria of importance in food safety. Having this ability as well as being able to survive on processing equipment, makes *L. monocytogenes* a serious threat to the food industry (Fenlon, 1999).

Listeria monocytogenes' Effect on Humans

Listeria monocytogenes can be found in cheeses, raw milk, ice cream, raw and cooked poultry, raw vegetables, raw milk, raw sausages, and raw as well as smoked fish. The ability to grow at 3°C, puts most refrigerated, further-processed foods at risk for *Listeria*. *Listeria monocytogenes* causes an acute disease in humans known as listeriosis. Those who are most susceptible to infection are immunocompromised individuals, pregnant women and their fetuses, and the elderly. According to a surveillance program in place by the Center for Disease Control, the hospitalization rate for listeriosis (94%) was higher than any other foodborne disease (Dr. Joseph F. Smith Medical library, 2005). Pathogenic *Listeria* can cause meningitis, encephalitis, septicemia, or cervical infections in pregnant women, resulting in stillbirths or spontaneous abortions (FDA, 1992). *L. monocytogenes* has the ability to migrate through the intestinal wall of the host and into the blood stream. Once in the blood stream, the bacterium can travel to any part of the host body, usually the central nervous system and placenta of pregnant women. *L. monocytogenes* has this ability because it can survive in white blood cells, known as macrophages. These macrophages hide the bacteria from the immune response system of its host, as well as preventing access of antibiotics to the bacteria. While in the macrophage cells, *L. monocytogenes* can replicate and spread to other macrophages. Once *L. monocytogenes* is ingested, symptoms of infection can appear from 11-70 days (Dr. Joseph F. Smith Medical library, 2005). Symptoms in pregnant women with listeriosis may not be specific, but usually develop into a mild flu-like illness (Schuchat,

1997). Transplacental transmission tracking maternal bacteremia or the spreading from vaginal colonization is thought to be the means by which *Listeria monocytogenes* spreads to the fetus. This intrauterine infection can cause amnionitis, spontaneous abortions, preterm labor, stillbirths, or the early onset of neonatal infection (Hume, 1976; Linnan and others, 1988). Fetuses infected with *L. monocytogenes* have only a 50% chance of survival (Medline Plus, 2003). Although meningitis is the most common form of listeriosis in non-pregnant adults, current studies show that the bacteremia is becoming more common (Gellin and others, 1991). Meningitis is a condition in which the tissues surrounding the brain and spinal cord become inflamed and can lead to brain damage or death, even if trearted (Medline Plus, 2003). Bacteremia is where the bacterium enters the blood stream and produces a focal infection or if allowed to progress into septicemia (multiple organ failure, shock, intravascular coagulation, and death) (Holland, 2005). The principle symptoms for most hosts, who become infected with *Listeria monocytogenes*, include gastrointestinal problems as well as a fever (Dalton and others, 1997). Although the dose level is unknown, it is believed to vary according to strain as well as the susceptibility of the host (FDA, 1992). Unlike the infections of other foodborne pathogens, listeriosis has a mortality rate of 20% (Gellin and others, 1989). According to the Center for Disease Control (CDC) it is estimated that there are 2,500 cases and 500 fatalities every year, due to listeriosis (CDC, 2004). In 1985, the largest outbreak in North America occurred in California. There were 142 cases reported with a fatality rate of 32% among pregnant patients and 37% among the non-pregnant adults.

The cause of this outbreak was traced to a particular brand of Mexican-style cheese (Linnan, 1988). Between 1989 and 1993 there was a 44% decrease in listeriosis cases and between 1996 and 2002 a 38% decrease. However outbreaks still continue to occur, such as in 2002 when 9 states were affected by contaminated turkey, leading to 54 illnesses, 8 deaths, and 3 fetal deaths (CDC, 2004). Avoiding interactions with *Listeria monocytogenes* can be difficult due to its presence in the environment. However, there have been recommendations made to the public on how to reduce the risk of contamination: wash raw vegetables before eating; keep uncooked meat separate from cooked, raw, and ready-to-eat foods; avoid raw milk; cook meat thoroughly; and wash hands as well as cooking utensils after working with raw foods (CDC, 2004). Due to the severity of *Listeria monocytogenes,* researchers are constantly working to develop methods of detecting this bacterium more rapidly, accurately, and economically than current methods.

The affect of *Listeria monocytogenes* on the Food Industry

Listeria monocytogenes can be a serious problem for food manufacturers, especially for ready-to-eat products and the poultry industry. Food processing plants have surfaces, such as stainless steel and rubber, which *L. monocytogenes* grow on and produce biofilms. Since *L. monocytogenes* is able to survive in harsh environmental conditions, it is frequently found in food processing plants (FSIS, 1999). Since the mid-1980s food manufactures have been active in developing strategies to control *L. monocytogenes*

(Bernard and other, 1994). The industry uses programs such as: hazard analysis critical control point (HACCP) plans, good manufacturing practices (GMP) and sampling programs (environmental and end product) to identify and reduce the presence of *L. monocytogenes* in their products as well as in their processing plant (FSIS, 1999). The USDA passed a "zero tolerance" policy for the presence of *Listeria monocytogenes* in ready-to-eat products. Under 9CFR417.4 the Food Safety and Inspection Service requires that all establishments that produce poultry or ready-to-eat products, must reassess their hazard analysis critical control point (HACCP) plan to see if *L. monocytogenes* is reasonably likely to occur in their products. If so then *L. monocytogenes* must be addressed in the HACCP plan. However, with these preventative measures in place, if a product is released to the market containing *L. monocytogenes* it is adulterated and under the meat and poultry act the producer has broken the law. In these cases the contaminated product undergoes a class I recall and the manufacturer is liable. A recent example of this took place in 1998, when a recall was placed on hotdogs and deli meats that where produced by Sara Lee Corporation. More then 15 million pounds of product that was manufactured had to be recalled. In the end Sara Lee Corporation paid out $200,000 in fines, agreed to pay $3 million in food safety research funding, and a civil settlement of over $1 million (Sara Lee, 2001). This is an example of a large recall that involved a familiar name brand. There are cases of *L. monocytogenes* recalls that take place every month. Not only can the results of a recall destroy small food companies, their brand names are damaged, thus reducing their profits. Due to the

presence of *L. monocytogenes*, in 1999 there were 35 million pounds of Thorn Apple Valley products recalled, resulting in the company going bankrupt and in 2001, 14.6 million pounds of Bar-S Foods brand were also recalled (Marsden and others, 2001). These are all just a few examples of why the food industry takes *L. monocytogenes* testing very seriously.

Methods of *Listeria monocytogenes* Detection

Traditional Methods

The traditional methods for determining the presence of *L. monocytogenes* in a food product are considered accurate and highly sensitive. These methods involve the use of differential or selective media as well as enrichment broths, in order to isolate and verify the presence of a targeted bacterium. The United States Department of Agriculture (USDA) and the Food and Drug Administration (FDA) have developed two protocols that have become the standard method for detecting the presence of *L. monocytogenes*. These protocols can be used with products such as seafood, vegetables, dairy foods, meat and poultry products, etc. (Donnelly, 1999). The two protocols can vary slightly according to the particular food product that is being tested. However, the following is a general protocol for the two methods.

The FDA method begins with 25g or ml of sample (testing product) to be added to 225 ml of Listeria Enrichment Broth (LEB). The sample is then stomached or blended and incubated at 30°C for 4-hr. Next, selective agents (acriflavine, nalidixic acid, and

cycloheximide) are added to the LEB, then incubated at 30 °C for 20 hr and 44 hr. After 20 and 44 hr the sample is streaked onto oxford agar (OXA) and LiCl-phenylethanol-moxalactam agar (LPM) (with or without esculin/$Fe3^{+}$) or polymyxin acriflavine lithium chloride ceftazidime aesculin mannitol agar (PALCAM). OXA and PALCAM plates are then cultured for 24 and 48 hr at 35 °C and LPM at 30°C. The plates are examined for *Listeria*-like colonies. For OXA and PALCAM the colonies have a black halo around them, as will the LPM plates if esculin/$Fe3^{+}$ is added. Once the colonies are identified, five of them from each plate are removed and transferred to tryptic soy agar plus 6% yeast extract (TSAYE) for confirmation. The TSAYE is incubated at 35 °C for 24-48 hr, followed by a series of biochemical tests to obtain confirmation. The whole process takes up to 10-11 days to complete (Hitchins, 1995).

The method, developed by USDA, for detection of *L. monocytogenes* is used primarily for meat and poultry products. Twenty-five grams of a meat sample is removed and added to 225ml of University of Vermont broth (UVM), and stomached for 2 minutes. The sample is incubated at 30 °C for 20-24 hr. Next, 1ml of the primary enrichment broth is added to 10 ml of the secondary enrichment broth, Fraser broth. The secondary enrichment broth is incubated for 26 ± 2 hr at 35 °C. A sample from the secondary enrichment is streaked on to modified oxford media (MOX) and the secondary enrichment broth is incubated for another 24 hr. The MOX plates are incubated for 24 hr at 35 °C. If the initial MOX plate is negative for black colonies then another sample is

taken from the secondary enrichment broth after a total of 48 hr of incubation time and streaked on to MOX. The plate is then incubated and checked for black colonies after 24-48 hr (Johnson, 1998).

While these methods are the current standards for confirming the presents of *Listeria monocytogenes* in food products, the methods are labor intensive, costly, and time consuming. That is why researchers are constantly hard at work developing accurate, relievable, cost efficient, less labor intensive, as well as rapid means of detecting this bacterium.

Biosensors

The traditional methods have been developed since the 1980's and though they have been updated for types of growth media used, the methods are not rapid enough in order to ensure safety before perishable products are consumed. The current regulations that require a "zero-tolerance" for ready-to-eat products have necessitated the need for rapid detection methods. These methods can act as a surveillance tool for monitoring possible foodborne outbreaks. The release of a product, containing *L. monocytogenes*, on to the market primarily leads to a class I recall.

A biosensor is an analytical device composed of a biological detection element (probe/receptor) and a transducer (platform) system. The biological sensing element has the ability to bind the targeted biological agent (by conventional methods of using monoclonal or polyclonal antibodies, or more recent methods of using phages) to a sensory platform. The platform/transducer transforms a change, whether it is physical or

chemical, into a signal that can be measured. The type of platform/transducer used categorizes biosensors: electrochemical, optical, and mass sensor.

An electrochemical biosensor receives its information through an electrical signal that indicates an interaction between the target and the biological sensing element. Electrochemical biosensors are classified into three major groups conductimetric/impedimetric, potentiometric, and amperometric.

The conductimetric/impedimetric method measures the change in the amount of electrical impedance. For example, bacteria metabolize uncharged or weakly charged materials: carbohydrates, proteins, or fats. The end products of the metabolic process are highly charged substances, such as amino acids, organic acids, and fatty acids, thus causing a change in the impedance of the medium.

The potentiometric method is based on the reaction of the targeted material with that of the biologically active material, in reference to a standard. The immobilized electrode and the standard electrode have a difference in potential. This difference in potential is proportional to the logarithms of the target concentration (Patel, 2002).

Amperometric biosensors measure the level of current produced by the chemical reaction between electroactive species. Compared to the potentiometric system, the amperometric system produces a linear relationship, making it more suitable for bacterial detection (Ivnitski and others, 1999). However, the amperometric system's selectivity can be affected due to current produced by various other chemical species.

Optical biosensors are highly desirable, due to their direct and rapid detection of bacteria. One form of the optical biosensor is the use of fluorescence. This technique utilizes the individual types of fluorescence, produced by various bacteria, and measures it under a UV source. This method is limited to the bacterium that produces a fluorescent component (Ivnitski and others, 1999).

Another form of optical biosensor technology is that of bioluminescence. Bioluminescence operates on the principles that some enzyme-catalyzed reactions produce photons. This type of biosensor is highly specific but requires an extensive response time (Ivnitski and others, 1999). Another optical testing study was performed on the rapid detection of *L. monocytogenes* in eggshells, milk, ground beef, and some ready-to-eat products. This detection method was based on *L. monocytogenes'* ability to hydrolysis esculin into 6,7-dihydroxycoumarin and its reaction with ferric ions. The process involved a 6 hr pre-enrichment step, to amplify low numbers of bacteria, followed by overnight incubation in modified Listeria broth. The overnight incubation process took place in an optical sensor device (BioSys). This device recorded the changes in light transmittance. With the color of the broth changing, the level of light transmittance decreased. This process was able to detect the presents of 10-50 cells per 25 grams of sample within 18 hr (Peng, 2000). The most popular optical biosensor is based on the surface plasmon resonance technology or SPR. This technology is based on energy produced by photons that can excite electrons on the surface of metal. SPR biosensors are capable of detecting slight changes in the refractive index, brought on by

the binding of antigens to antibodies (Homola and others, 1999). The draw back to these biosensors is the interference of ambient light as well as the narrow range of the testing area (Leonard and others, 2003).

Acoustic wave devices have become highly intriguing platforms for biosensors, due to their cost, simplicity, and real-time detection (Leonard and others, 2003; Tamarin and others, 2003; Raiteri and others, 2001). All acoustic wave platforms operate on the same basic principle, the frequency shifts when there is a change in the mass on the sensor. The amount of mass on the sensor can be measured according to the shift in fundamental resonance frequency (Thundat, 1997; Raiteri and others, 2001). However, the sensitivity of these sensors is based on having a high quality merit factor and a platform with high sensitivity (Raiteri and others, 2001).

Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) is a molecular method developed for the *in vitro* amplification of DNA. The PCR technique is the amplification of DNA segments by using a heat stable polymerase and two synthetic oligonucleotides (primers) (Gasanov and others, 2005). For pathogen identification in food products, the method is the amplification of gene sequence that is specific to the targeted pathogen. PCR is a three step cycling process: denaturation of targeted DNA at a high temperature, the annealing of two synthetic oligonucleotides at opposite strands to allow hybridization of the targeted DNA sequence, and polymerization with the oligonucleotides as primers (Olsen and others, 1995). After the segment is amplified, the amplified DNA is detected

primarily by an agarose gel electrophoresis (Gasanov and others, 2005). This method has been proven to be highly accurate, sensitive, and specific.

The problems with PCR based detection of foodborne pathogens are the extraction of food components that inhibit the PCR process, and that process does not distinguish between live or dead cells (Hill, 1996; Gasanov and others, 2005). However in an effort to overcome these obstacles, many researchers use PCR in conjunction with various other methods, such as enrichment, purification, extraction, and/or filtration methods.

The types of PCR methods that are used to detect *L. monocytogenes* in food are: direct, multiplex, nested, ligase, antigen capture, and real-time. There has been a lot of research done on the detection of *L. monocytogenes* in a wide variety of food products. With each method there is a variation, whether it be the enrichment media or process, the extraction of bacteria cells or DNA, or the gene that is amplified.

Direct PCR detection is the basic process of sample preparation and the use of a single set of primers to detect the targeted pathogen. In a study that combined the PCR technique with the technology of the Dcode Universal Mutation Detection system, not only was the detection of *L. monocytogenes* possible in a wide variety of meat products but also it was distinguished from other strains of *Listeria.* After an over night enrichment process, in brain heart infusion broth, and a DNA extraction process, *L. monocytogenes* could be detected as well as distinguished from other *Listeria* species by the agarose gel electrophoresis migration point (Cocolin and others, 2002). When determining the detection level of *L. monocytogenes* in pork, a set of primers based on

the *act*A gene was employed. After a 16 hr enrichment, in Listeria enrichment broth and a DNA extraction, it was determined that 10 cfu per 25 grams of pork could be detected by agarose gel electrophoresis (Zhou, and other, 2005).

A study was conducted to identify *L. monocytogenes* in seafood by a PCR method. Listeriolysin O (*hly*) gene fragments, as well as, invasion-associated protein (*iap*) gene were used as primers. The study showed a detection level of 1 to 5 cells of *L. monocytogenes* per 5 grams of sample after a 55 hr incubation time. The seafood samples were primarily enriched in either universal pre-enrichment broth (UPB) or tryptic soy broth (TSB) for 24 hr, then transferred to UPB for secondary enrichment for 24 hr. The DNA was then extracted from the cells and the PCR method was employed using primers specific to listeriolysin O gene. The results were then determined by the use of an agarose electrophoresis gel (Agersborg and others, 1997). The primers designed from the listeriolysin O were also used to detect *L. monocytogenes* in milk and ground beef. The procedure consisted of a two-step enrichment process, first a non-selective enrichment followed by a selective enrichment step. With this method 0.1 cfu/ml or gram could be detected after less then 3-days (Thomas and others, 1991). PCR detection of *Listeria monocytogenes* in meat and poultry has also been investigated. Geraldine Duffy et al. (1999) combined PCR with surface adhesion to isolate and detect the presence of *L. monocytogenes* in meat and poultry. The method involved the addition of a polycarbonate membrane to the enrichment broth to collect the bacteria cells. Once the membrane was collected, it was dissolved and the DNA was extracted from the cells.

The DNA was then processed through the PCR procedure (using primers associated with the lysteriolysin O gene) to detect the present of *L. monocytogenes*. This method was able to detect 10^4 cfu/ml detection level, within 29 hr (Duffy and others, 1999). Studies on fish products, such as salmon and catfish, compared the use of the Probelia PCR kit to the current International Organization for Standards (IOS) method in the detection of *L. monocytogenes*. The results showed that the Probelia PCR method produced a sensitivity of 20 cfu/ml within 48 to 50 hr, and the IOS method took 5 to 6 days detection level (Wan and others, 2003). The detection level of *L. monocytogenes* in channel catfish was 1-2 cfu/g for less than 2 days. However, the primers used were sequenced from the invasive-associated protein (*iap*) and did not detect the presents of *L. monocytogenes* serotype 4c. This was thought to be due to the recent discovery that the protein (*iap*) is likely a "murein hydrolase with bacteriolytic activity" (Wang and others, 1999). PCR can be used to detect *L. monocytogenes* on beef shoulders using a single enrichment medium and PCR. The samples were swabbed with sponges, then the sponges where enriched in universal pre-enrichment broth for 18 hr. Samples were then taken from the pre-enrichment broth and the DNA was extracted. For this study the BAX™ PCR amplification kit was used. This produced a 0.5 cfu/cm^2 sensitivity level (Bhaduri and others, 2001). In a study by Lilach Somer and Yechezkel (2003) the PCR technique was used in dealing with a diverse range of food products: a ready-to-eat (RTE) pasta dish, meat and fish, potato salad, ice cream, vegetable salad, and various dairy products. The method involved the use of enrichment broths: Listeria enrichment broth for the milk

samples, University of Vermont medium for the meat samples, and Fraser broth for the secondary enrichment. The primers used were sequenced from the 16S subunit of the rRNA gene. The method resulted in a detection level of 1-5 cfu/25g of sample after 48 hr of enrichment time (Somer and others, 2003). H. Ericsson and P. Stalhandske (1997) studied the detection of *L. monocytogenes* in Rainbow trout. After a 4-hour enrichment (Listeria enrichment broth + supplements) period and DNA extraction, it was possible to detect 10-100 cfu/g in the samples after 12 hr. If the samples were left to incubate for 24 hr the detection level increased to 1-10 cfu/g (Ericsson and other, 1997).

Multiplex PCR is the ability to check for the presence of more than one pathogen in a single sample. This is achieved by the use of more than one set of primers being added to PCR samples. This type of PCR testing is intriguing to the food industry because of its reduction in labor and reagent cost as well as a reduction in testing time for multiple bacterial pathogens (Gasanov and others, 2005).

By using the multiplex PCR method *L. monocytogenes* could be detected in a wide range of products, such as seafood, vegetables, dairy products, processed meats and raw poultry. The multiplex PCR method uses an overnight incubation time in a primary enrichment broth. Afterwards the samples are subjected to a series of centrifugations; 500 x *g* for 10 minutes at 4 °C and 6000 x *g* for 20 minutes at 4 °C. The samples are denatured for 12-15 minutes at 96 °C, followed by another centrifugation at 12,000 x *g* for 10 minutes. The supernatant is added to the PCR reagents along with primers that are specific to the ribosomal DNA sequence of the *Listeria* species and to the primers

specific to the listeriolysin O gene. The results are identified by agarose gel electrophoresis. The detection level is estimated at 100 cfu/ml of sample and the overall process takes 48 hr to complete (Bansal and others, 1996). For the detection of *L. monocytogenes* and *Salmonella* spp. in ham by the multiplex method, the samples are enriched with either buffered peptone-water (BPW) or half Fraser broth and incubated for 48 hr at 37 °C. The DNA is then purified using a commercial DNeasy tissue kit. The primers to detect *L. monocytogenes* by this method are the *prf*A gene and the primers for the *Salmonella* spp. are the *inv*A gene. The detection level achieved in ham by the multiplex method was 1 cfu per 25 grams of sample (Jofre and others, 2005). In another study multiplex PCR was combined with laser-induced fluorescence detection (LIF) and capillary gel electrophoresis (CGE) was used to determine the results. Meat samples were incubated, after filtration, in BPW for 0-6 hr. The samples were centrifuged at 4000 rpm for 10 minutes, followed by DNA extraction by the DNeasy tissue kit. The PCR process contained primers for amplifying the *Hly*A gene in *L. monocytogenes* as well as two other primer sets for detecting *Salmonella* and *Staphylococcus aureus.* Analysis of the samples was preformed by PACE-MDQ instrument containing an Ar^{+} laser set at 488nm and 520nm. By using the CGF-LIF technology the size of the amplified fragments can be determined accurately and sensitivity of the multiplex PCR method can be increased by 10 to 1000 fold. This method detected 79 cfu/ml for *L. monocytogenes* after 6 hr of enrichment (Alarcon and others, 2004). When the multiplex PCR method was combined with immuno-magnetic beads for cell extraction, and the slot blot assay

was used for identifying the results, detection level for both *L. monocytogenes* and *Salmonella* was 40 cfu/ml in milk after 7 hr (Li and others, 2000).

Nested PCR is the use of two sets of primers designing to amplify the targeted sequence. Two successive PCR treatments are carried out instead of one single PCR application (Hill, 1996). This PCR method is used primarily to increase specificity and sensitivity (Gasanov, 2005). The nested PCR is used to identify *L. monocytogenes* in raw milk. The milk is separated from the bacterial cells by a chemical extraction method. The cells are then concentrated by centrifugation and the DNA is extracted from the cells. In order to increase the sensitivity of detection, a two-step PCR method along with two sets of nested primers are used. A detection level of 1cfu per 25ml of milk was achieved after the second PCR step. The key factors for this method are the efficiency in purification, concentration, lysis of the bacterial cells, and the use of a two-step PCR procedure with the nested primers (Herman and others, 1995).

Ligase PCR uses two oligonucleotides, which are designed to be complementary to an adjacent targeted DNA sequence. In the presence of the targeted sequence the two oligonucleotides bind together with a DNA ligase. When two sets of oligonucleotides are used, both of the targeted DNA strands become ligated and the amount increases exponentially (Hill, 1996). The advantage to this technique compared to other PCR methods is that there is no newly synthesized DNA, thus no amplification of non-targeted sequences takes place (Benjamin and others, 2003).

Antigen capture PCR uses a combination of the PCR method and an immunochemical purification process. As discussed before, the problem with PCR detection of pathogens is the extraction of the cells from the food matrix and the existence of PCR inhibitors in the food. This method uses antibodies to capture the desired bacterial cells and then allows the washing away of food components (Hill, 1996).

Magnetic beads consist of an inorganic core of iron oxide, maghemite, or other insoluble ferrites. The core is coated with a polymer, which can be coated with antibodies for binding with cells, proteins or DNA. The magnetic beads can be mixed with a food sample. The beads then bind to the targeted biological material in the sample. By using a magnetic field, the biological material can be removed from its surrounding matrix. This particular technique is becoming popular for the purification step used by many rapid detection methods. With this technique, magnetic beads are coated with monoclonal antibodies that are specific to *L. monocytogenes*, and the bacterial cells are isolated from the food samples and then lysed for the DNA detection by the PCR method. In a study conducted by Fluit and others (1993) for the detection of *L. monocytogenes* in cheese by PCR, the cheese samples were enriched in Listeria enrichment broth for 24 hr at 30 °C. Followed by a secondary enrichment in Fraser broth for 24 hr at 30 °C. Then the immunomagnetic beads were added for separation of the *L. monocytogenes* cells from the enrichment broth and cheese. After separation, the bacterial cells and beads were washed for PCR amplification. Agarose gel electrophoresis was used for the detection of the listeriolysin O-specific gene. This study

was able to detect 1 cfu of *L. monocytogenes* per gram of cheese after a 55 hr analysis and enrichment (Fluit and others, 1993). The use of immunomagnetic beads in conjunction with the PCR method detected 1 to 2 *L. monocytogenes* cells in a 25 gram sample of ham. A 25 gram sample of autoclaved ham was inoculated with *L. monocytogenes* and incubated for 24 hr at 14-6 °C for bacterial attachment. After incubation, 50 ml of peptone water was added and the sample was stomached for 2 minutes. The sample was decanted and centrifuged for 2 minutes at 1560 x *g*. The supernatant was decanted again and centrifuged for 2 minutes at 1560 x *g*. The pellet was re-suspended in buffer with the anti-*Listeria* immunomagnetic beads and agitated for 10 minutes. The beads were trapped by a magnetic device and the beads with the captured *Listeria* were added to an enzymatic lysis buffer. The DNA was processed through a multiplex PCR method, which amplified both the listeriolysin O gene sequence, and a region of the 23S rRNA gene. Agarose gel electrophoresis was used to detect the presence of the genes. The entire method was completed within 24 hr. This method was also preformed without the use of the immunomagnetic beads, and the results were negative (Hudson and others, 2001). This methodology was also applied to cheese in a similar methodology. However, the study was done to determine the level of *L. monocytogenes* recovery for the immunomagnetic beads. The samples were inoculated with *L. monocytogenes* and processed through a series of decants as well as centrifugations, followed by the addition of immunomagnetic beads to separate the bacterial cells. The study showed that after a 24 hr enrichment time, that low levels of *L.*

monocytogenes (<10 cfu/g) could be detected by the use of immuno-magnetic separation (Uyttendaele and others, 2000).

DNA Hybridization

DNA is a double helix structure that is composed of four nucleotides: adenine (A); guanine (G); cytosine (C) and thymine (T). This structure can be denatured if it is exposed to a high pH or temperature. When the structure denatures, the stable double helix pulls apart into two single strains. But if the temperature or pH is restored then the two single strains will reform the double helix structure, this process is called "hybridization". Hybridization is made possible by the nucleotide's formation of hydrogen bounds, only with the complementary bases (A to T; C to G). However, if one or more of the base pairs are mis-matched then the level of instability in the hybridization increases. DNA probes generally consist of a short sequence of nucleotides, with a label for detection, which binds perfectly with a specific targeted sequence. DNA probes consist of a sequence that can hybridize with a sequence that controls a cell specific function. This sequence dictates the specificity of the probe to the targeted pathogen as well as some environmental factors. In order for the probe to be efficient it must not hybridize with nucleotides from other microorganisms or with the food product being tested. Another factor, which will affect the DNA probe efficiency, is the stringency. Stringency consists of various aspects, such as high salt concentration or low temperature that can allow for mismatching of the nucleotides between the probe and target (Wolcott, 1990).

One study evaluated the use of the oligonucleotide array technology for the rapid detection of foodborne pathogens. A fragment of the 23S rDNA gene sequence was used as the oligonucleotide probe. The primer pair was incorporated into the 5'-end of a digoxigenin compound for an ELISIA based color change. The material used for detection of the pathogens was a positively charged nylon membrane that was spotted with the oligonucleotide probes. The samples were added to the membrane and allowed to hybridize. The membrane was then added to anti-digoxigenin antibodies and given time to react. Color development was produced by the digoxigenin-linked ELISA method. When testing the pure samples, nine species of pathogens presented a high sensitivity and specificity of hybridization, with *L. monocytogenes* being one of the species. When the pathogens were tested in actual food samples, a positive result could be achieved at anywhere between 10^6 and 10^2 cfu/ml of sample. However, this method is not without some flaws. In the actual food samples that were tested, there was some cross-reaction between some of the cultures. The use of different labeling methods also changed the results of detection. The proper design of the probes is an important factor that determines the success of the oligonucleotide array hybridization (Hong and others, 2004).

Immunochemical Assay

A study was done to determine the detection level of *L. monocytogenes* in various food samples using the rapid enzyme linked fluorescent assay (ELFA). After a primary enrichment in Palcam for 26 hr and a secondary enrichment in UVM for 26 hr, it was

determined that the ELFA method had a 98.1% sensitivity and a 97.0% specificity level for the detection of *Listeria* spp. in food samples (Sewell and others, 2002).

However, with new molecular and immunochemical tests emerging, culture-based testing is still dominating the microbiological laboratories for two reasons. One the molecular methods were introduced before the reproducibility and massive scale technology was available to meet the needs of high standard labs. Secondly, the people who work in the culture-based labs are not familiar with and lack training for molecular and immunochemical base testing.

Rapid Detection Methods

In the food science field a rapid detection method is considered to be a method that can produce result after several hr. The production of commercial rapid detection can help food microbiologist reduce human error, increase quality control and lower labor cost (Wolcott, 1990). Due to the lengthiness of microbial detection by tradition methods and the increase in production of short shelf life products there has been more pressure placed on the development of rapid detection methods. As a result, there has been an increase in the development of rapid tests based on molecular techniques (PCR or DNA hybridization) or immunoassays (ELISA). Recently methods have been developed to identify RNA rather then DNA of a pathogen, such as real-time polymerase chain reaction (RT-PCR) and nucleic acid based sequence amplification (NASBA). By identifying the RNA rather than the DNA, the quantitative amount of pathogen can be

determined as well as whether or not the bacteria that are viable cells (Gasanov and others, 2005).

A new development in media production has become a form of rapid detection. The media is referred to as chromogenic media. This form of media has a substrate added, so that it may identify specific bacteria according to the types of enzymes that it produces. For example, phosphatidylinositol-specific phospholipase is produced solely by *L. monocytogenes* and *L. ivanovii*, when either of these bacteria grow on a commercial (Rapid'L.mono®) agar plate the colonies are blue. These chromogenic culture plates have advantages over other rapid detection methods: simplicity, high sensitivity, cost effective, easy interpretation, large sample throughput, specificity and competitive processing time with other rapid methods (Gasanov and others, 2005).

Immunoassay methods have been used for testing in the food industry for years. These methods for testing have been well received by the industry because of their sensitivity, accuracy, simplicity, and limited sample preparation. These immunoassay kits are also available commercially and approved by regulatory agencies. A good example of an immunoassay method is ELISA. The ELISA method consists of an immobilized antibody, specific to *L. monocytogenes*, attached to a microtitre well plate. The antibodies capture the antigen, *L. monocytogenes* cell, and then a secondary antibody coupled with an enzyme attaches to the antigen. A substrate is then added to the microtitre well plate to produce a color change, if *L. monocytogenes* is present. This method is currently the most widely used due to its ability of performing with difficult

sample matrices (Gasanov and others, 2005). Another form of immunoassay testing is radioimmunoassay. This method operates on the same basic principle as the ELISA method, except by using a radioistope instead of an enzymatic color change. However, due to the expense as well as safety issues, this method has been phased out of the industry setting. A few other immunoassay methods are also available, such as aggutination and immunomobilization (Wolcott, 1990).

III. MATERIALS AND METHODS

Culture Preparation

Two days before the experiment, each culture used in this experiment was transferred into 15 ml Tryptic® soy broth (TSB) and incubated at 37 °C in gyratory water bath (100 rpm) for 24 hr. On the second day, a loopful of each bacterium was taken from the overnight culture broth and transferred into 15ml of new TSB, and incubated at the previous holding conditions for 24 hr. The bacteria were washed twice with Butterfield's phosphate buffer (BPB) by performing centrifugation at 3,000 x *g* for 10 min and resuspended in the original volume of BPB as a bacterial stock suspension for use. The concentration of the bacterial stock suspension was estimated by using the O.D. at 640 nm of the bacterial suspension in a pre-determined equation. The bacterial concentration for sample inoculation was prepared by a dilution from stock suspension.

Comparison of Growth Rate on Different Media

Before the correct method can be developed, the proper growth media must be determined. In order to do so the various media must be researched to establish which would be appropriate for the ready-to-eat salads, as well as, produce a high level of strictly *Listeria monocytogenes* cells. It was determined through previous research, that the different medias to use were modified Penn State University (mPSU), University of Vermont (UVM), tryptic soy broth plus 0.6% yeast extract (TSBYE), half strength tryptic

soy broth plus 0.6% yeast extract (1/2 TSBYE), Listeria enrichment broth (LEB), and half Fraser's broth. All six media were tested to determine the one that is the optimal medium for *Listeria monocytogenes* growth within 6 hr.

Six media were inoculated with 5 isolates of *L. monocytogenes*, and then the samples were incubated in a gyratory water bath (100 rpm) at 37 °C. An aliquot was removed from each sample at 1, 2, 3, 4, 6, and 8 hr after incubation. The aliquots were diluted and spread plated onto modified Oxford medium plates (mOX). The bacterial numbers on the plates were recorded after 24 hr incubation at 37 °C for further analysis.

Recovery of *Listeria monocytogenes* from inoculated salad

Two isolates of *L. monocytogenes* were grown in Tryptic® soy broth plus 0.6% yeast extract (TSBYE) for 18 hr in a gyratory water bath (100 rpm) at 37 °C. The bacteria were washed twice with Butterfield's phosphate buffer (BPB) through centrifugation at 3000 x *g* for 10 min. The bacterial number was then estimated using a pre-determined growth equation along with the optical density at 640 nm of the bacteria solution. Then, the bacterial solution was diluted to 2.5×10^3 and 50 cfu/ml for the inoculum. The 50 grams of ready-to-eat salad purchased from local grocery stores were put into a stomacher bag, and 1 ml of the inoculum was added to the salad. The BPB was used to inoculate the salad as a negative control. The inoculated samples were then placed at 4 ^{o}C refrigerator temperature for 2 hr, and then 225 ml of the half strength TSBYE or half Fraser Broth were added to the stomacher bag. The samples were blended for 2 min at

260 rpm using a Seward® 400 Circulator stomacher (Seward Co., Seward, England). The blended samples were filtered through glass wool to collect the solution in a sterile 1-liter flask containing 225 ml of corresponding broth to make 1:10 dilution of salad to enrichment broth. The samples were spread plated on modified oxford media with 100 ppm of nalidixic acid and 15 ppm streptomycin (mOA-NA-S) plates for bacterial enumeration. Another 100 ml of the filtrate were placed in a 250 ml sterile centrifuge tube, and centrifuged at 8,000 x *g* for 10 min. The supernatant was discarded and the precipitate was re-suspended in 300 µl of Bufferfield's phosphate buffer and spread plated on mOA-NA-S plates for bacterial enumeration. The rest of the filtrate was placed in a gyratory water bath (100 rpm) at 37 °C for incubation. At the incubation times of 2, 4, and 6 hr, 100 ml of the samples were removed to check the bacterial numbers through centrifugation and resuspension procedures mentioned previously. All of the plates were placed into an incubator at 37 °C for 24 hr and the bacterial numbers were recorded for further analysis.

Primer Design

To design primers for this experiment, the internalin-A coding sequence (Acession #AL591975 REGION: 94534.96991) from *Listeria monocytogenes* strain EDG (Glaser, Frangeul et al. 2001) was bioinformatically analyzed. This coding sequence was input into NCBI database and BLAST against the entire bacterial nucleotide sequences. A segment (760.1160 of the ORF), which exists in all Listeria monocytogenes strains, was

chosen as candidates for PCR targets. This region of *Listeria monocytogenes* genomic sequence was highlighted and searched for primers with the help of Veter NTI (version 9.1, Invitrogen Co., Carlsbad, CA). Theoretically, this pair of primers will recognize chromosomes from 95392 to 95738 forms of *Listeria monocytogenes* EGD, and yield a 347 bp fragment.

PCR protocol

In a sterile 0.5 ml amplification tube, the following materials were mixed in order:

1. Sterile H_2O	30 µl
2. 10X amplification buffer	10 µl
3. Mixture of 4 dDTPs (1.25 mM each)	16 µl
4. Primer 1 (20 pmoles/µl)	5 µl
5. Primer 2 (20 pmoles/µl)	5 µl
6. Polymerase (5 units/µl)	0.4 µl
7. Template DNA (bacteria)	2 µl

8. H_2O to final volume of 100 µl

The denaturation, annealing, and polymerization times and temperatures used for amplifying the nucleic acids are listed below:

Cycle number	Denaturaiton	Annealing	Polymerization
35 cycles	30 sec at 95^{o}C	30 sec at 55.3^{o}C	30 sec at 72^{o}C
Last cycle	1 min at 95^{o}C	30 sec at 55.3^{o}C	3 min at 72^{o}C

After the amplification was completed, the sample from the test reaction mixture was withdrawn and analyzed by electrophoresis through an agarose gel (Sambrook and Russell, 2001a).

Preparation and Examination of Agarose Gels

One gram of agarose was added to 50 ml of TAE buffer and heated in a microwave oven until the agarose dissolved. The agarose solution was cooled to 60 ^{o}C, and 25 µg of ethidium bromide were added to the solution and mixed thoroughly. The solution was poured into the mold to make an agarose gel 5 mm thick, and the comb was placed 1.0 mm above the plate. After the gel was completely set for 45 min at room temperature, the comb was removed to make a well for sample loading. The gel was put in to the electrophoresis unit and the electrophoresis buffer was added to cover the gel to a depth of about 1 mm. The 12 µl DNA sample was mixed with 2 µl 6X Loading buffer (Promega, Madison, WI) and 10 µl of the mixture were loaded into the slots of the submerged gel (Sambrook and Russell, 2001b). The DNA markers of an appropriate size were also loaded into the gel. The gel was run at 7.0 volt/cm of electric field for 45 min. DNA was visualized with ethidium bromide at UV 254 nm and the image was captured by a Kodak Gel Logic 200 Imaging System (Eastman Kodak Co., New Haven, CT). The size of the target DNA was analyzed by comparing the markers on the same gel.

Primer Specificity

To determine the specificity of the chosen primer, the following bacteria were used: *Listeria monocytogenes* ATCC 19111, *L. monocytogenes* Scott A, *L. monocytogenes* H7738, *L. monocytogenes* G3928, *L. monocytogenes* G3990, *L. monocytogenes* G6005, *Listeria inocua*, *Staphylococcus aureus* ATCC 12600, *E. coli* O157:H7 204p, *E. coli* O157:H7 301C, *E. coli* O157:H7 505B, *E. coli* O157:H7 ATCC 43895, *E. coli* K12 SA1332, *E. coli* K12 VSM1692, *E. coli* O157:H7 932, *E. coli* O157:H7 LC40, *E. coli* O157:H7 7121583, *E. coli* BD2399, *E. coli* 48-2, *E. coli* GM 2163, *Salmonella enteritidis* H2292, *S. enteritidis* H4267, *S. enteritidis* H4638, *S. enteritidis* H4639, *S. typhi* CT18, *S. typhi* TY2, *S. paratyphi* SA2723, *S. paratyphimurium*, *S. motevideo*, *S. dublin*, *S. panama*, *S. typhimurium* ATCC1311, *S. typhimurium* PP1002, *S. typhimurium* SA 2317, and *S. mission*. These bacteria were grown in both TSBYE for the *Listeria* spp and TSB for the remaining bacteria in a gyratory water bath (100 rpm) at 37 ^{o}C for 24 hr. The bacterial solutions proceeded to the PCR amplification and the amplified DNA was analyzed by agarose gel electrophoresis. The image of the gel was recorded using Kodak Gel Logic 200 Imaging System (Eastman Kodak, New Haven, CT) system for analysis.

Sensitivity

After the 5 isolates of *Listeria monocytogenes* were grown in TSBYE in a gyratory water bath (100 rpm) at 37 °C for 24 hr, the bacteria were removed and washed twice in PBS buffer through centrifugation at 3000 x *g* for 10 min. The bacteria number was

estimated by the optical density reading at 640 nm of the bacteria suspension through the calculation in a predetermined growth equation. Once the bacterial numbers were estimated, these bacterial suspensions were then diluted in PBS to 10^6, 10^5, 10^4, 10^3, 10^2, 50, 10, 5, 1 cfu per 2 µl for PCR amplification. Each sample was run through the PCR process in duplicates, as well as negative and positive controls. The negative control was the DNA template substituted with the same volume of distilled water, and the positive control is the DNA template of internalin gene cloned in plasmid pCRscript (Stratagene). The bacterial suspension was adequately diluted and spread plated onto modified oxford agar plates to obtain actual bacteria numbers in the bacterial solutions. The plates were incubated at 37 °C for 24 hr, and bacterial numbers were recorded for further analysis. After the PCR amplification process was completes, the samples were analyzed by agarose gel electrophoresis to determine the detection level of *Listeria monocytogenes* in pure culture.

PCR Detection on Inoculated Ready-To-Eat Salad

Listeria monocytogenes H7757 and G3982 were grown in TSBYE in a gyratory water bath (100 rpm) at 37 °C for 24 hr. The bacteria were then removed and washed twice in Butterfield's phosphate buffer (BPB) through the centrifugation at 3000 x *g* for 10 min. The bacteria number was then estimated using a pre-determined growth equation along with the optical density of the bacteria suspension at 640 nm. Then the bacterium was diluted to $2.5x10^3$cfu/ml.

The ready-to-eat salad experiment was completed in triplicate samples and run at 2 and 72 hr of incubation at 4 ^{o}C. First 25 grams of ready to eat salad was weighted out into a sterile stomacher bag. The samples where labeled: BG2 and BG72 (background samples); A2, B2, C2, A72, B72, and C72 for 2 and 72 hr incubation. Each sample, except for the background samples, was inoculated with 1 ml of the 2.5 x 10^3 cfu/ml bacteria solution. After inoculation, BG2, A2, B2, and C2 were stored at 4 ^{o}C for 2 hr. Then 225 ml of ½ TSB+6% yeast extract were added to each sample and stomached for 2 min at 260 rpm. After blending, the samples were filtered through glass wool into a 500 ml sterile centrifuge tube. The bacterial numbers in the filtrates were enumerated by spread plating on modified oxford agar plates. The filtrates were then centrifuged at 8,000 x *g* for 10 min, and the supernatants were discarded and the precipitates were re-suspended in 500 µl of TSBYE. Each sample was equally dispersed in the following media: TSBYE, mPSU, and LEB. The mPSU medium was sealed in test tubes and placed in a dry incubator at 37 °C and the TSBYE, as well as, LEB media were placed in a gyratory water bath (100 rpm) at 37 °C for enrichment. After 6 hr, each medium from different samples were removed and spread plated onto MOX for actual bacteria enumeration. The remaining sample medias were centrifuged at 8,000 x *g* for 10 min. The supernatants were discarded and the pellet was re-suspended in 100 µl of BPB for PCR amplification. The PCR results were analyzed by agarose gel electrophoresis.

IV. RESULTS AND DISCUSSIONS

PCR is frequently combined with other methods such as immunochemical, biochemical, and/or enrichment for *Listeria monocytogenes* detection. Many studies employ DNA extraction methods (DNA probes and/or chemical) to remove the DNA from the food matrix and/or the bacterial cells, these methods can result in a detection level of 10-100 cfu/g of food sample after 12 hr (Ericsson and others, 1997). The extraction of DNA can be tedious, costly, and time consuming. Some procedures involve the use of immunochemical methods (immunomagnetic beads) to remove the bacterial cells or DNA from the food sample matrix. By combining PCR techniques with immunomagnetic beads a detection level of 1 to 2 cfu/25g of sample could be achieved after 24 hr (Hudson and others, 2001). However, the use of immunomagnetic beads can be costly as well as labor intensive. Other methods incorporate a lengthy enrichment process that can range from 16 to 55 hr of incubation time in order to produce a 1 to 2.5 cfu/g detection level (Zhou and other, 2005; Agersborg and others, 1997). The method developed here processes the sample through a set of common steps, in order to extract the bacterial cells from the ready-to-eat salad. By using this simple process, a positive result could be obtained after a 6 hr enrichment process and the implementation of this PCR technique.

There were six different media tested to determine the best growth rate for the use with PCR for the rapid test of *Listeria monocytogenes* in salad. These media were

modified Penn State University (mPSU), Listeria enrichment broth (LEB), Tryptic® soy broth plus 0.6% yeast extract (TSBYE), half Fraser's broth, University of Vermont medium (UVM), and half Tryptic® soy broth plus 0.6% yeast extract (1/2 TSBYE). Each of the six different broths was inoculated with 130 cfu of *L. monocytogenes* H7757 and incubated in a gyratory water bath at 100 rpm, except for mPSU, which was incubated in a incubator without shaking at 37 °C for 6 hr. After incubation, samples from the broths were spread plated onto MOX, in order to obtain the bacterial count. Table 1 shows the growth rate of *Listeria monocytogenes* among these six different enrichment broths. After 6 hr of incubation, *Listeria monocytogenes* grew the most in TSBYE and ½ TSBYE, followed by LEB, mPSU, UVM. In half Fraser's broth, the *Listeria monocytogenes* count decreased substantially after 6 hr enrichment. Based on these results LEB, mPSU, UVM, and half Fraser's broth produced either a slight increase or an actual decreases the in the level of bacterial presence in the broth. This can be due to the four broths being selective media, which contain inhibitors that can inhibit the growth of the bacteria that are not the targeted bacterium. However, these inhibitors can also prevent or inhibit the growth of damaged targeted cells, as well as, increase the time of the bacterial lag phase, by forcing the bacteria to build up a tolerance level to the inhibitors before the growth is resumed. From these results, four broths of TSBYE, LEB, mPSU, and UVM were chosen and tested to determine the recovery level of *L. monocytogenes* cells from the salad sample. A 25g sample of salad was inoculated with $1.1x10^3$ cfu and stomached with 225 ml of buffer. The sample was filtered through

fiberglass wool into a centrifuge tube and centrifuged at 8,000 x *g* for 10 min. The supernatant was removed and the pellet was resuspended in 500 µl BPB buffer. The 500 µl of the bacterial suspension was evenly dispersed at 125 µl in 5 ml each of the four broths for enrichment. Samples were spread plated on MOX at 1, 2, 3, 4, 6, and 8 hr of incubation for bacterial numeration. Table 2 shows the results of the four broths that were used and the level of bacterial growth achieved after 1, 2, 3, 4, 6, and 8 hr of incubation. In the table, time 0 represents the inoculation level. From 1 to 4 hr, there was a limited amount of growth in the selective broths, but after 6 hr of enrichment the level increased dramatically. The selective broths may have inhibited the bacteria in the initial growth stage resulting in the slow growth. However, in this experiment, the indigenous bacteria in the salad used esculin in the modified Oxford medium (MOX) and produced black halos around the colonies the same as *L. monocytogenes*. This factor made it difficult to differentiate the *L. monocytogenes* from the background bacteria. In order to overcome this challenge, *L. monocytogenes* H7757 was grown in TSBYE media with streptomycin and nalidixic acid and the concentration of these antibiotics were gradually increased over time to build antibiotic resistance in *L. monocytogenes*. After 10 days, the bacteria had built-up a resistance to 15 ppm streptomycin and 100 ppm of nalidixic acid. By adding those levels of antibiotics to MOX media, the background bacteria were inhibited. The results in table 2 indicates that TSBYE could produce a large number of bacteria within a short period of time and the mPSU, as well as, LEB were highly selective for the growth of *L. monocytogenes* but they still were able to produce high bacterial numbers

for PCR detection in 6 hr. Therefore, TSBYE, mPSU and LEB were chosen to represent both selective and non-selective enrichment media for *Listeria monocytogenes* enrichment on inoculated salad in PCR detection.

The primers that were used in this methodology are based on the amplification of a segment of the internalin A protein gene (Figure 1). The primers are highlighted on the sequences, and the amplified DNA sequence has 348 base pairs of nucleic acids. This protein gives the *L. monocytogenes* cells the ability to attach to the intestinal wall of the host, thus allowing the development of listeriosis. From the NCBI BLAST output for internal-A sequence alignment, the segment of the gene for PCR amplification is very specific to *L. monocytogenes* and a good target for detection (Figure 2). If the internalin A protein gene segment in a sample was amplified, the sample should include the *L. monocytogenes* (Figure 3), otherwise samples must not be the pathogenic *L. monocytogenes*. There are 34 bacteria including *Listeria monocytogenes* ATCC 19111, Scott A, H7738, G3928, G3990, and G6005, *Listeria inocua*, *Staphylococcus aureus* ATCC 12600, *E. coli* O157:H7 204p, O157:H7 301C, O157:H7 505B, O157:H7 ATCC 43895, K12 SA1332, K12 VSM1692, 932, LC40, 7121583, BD2399, 48-2 and GM 2163, *Salmonella enteritidis* H2292, H4267, H4638, and H4639, *Salmonella typhi* CT18 and TY2, *S. paratyphi* SA2723, *S. paratyphimurium*, *S. motevideo*, *S. dublin*, *S. panama*, *Salmonella typhimurium* ATCC1311, PP1002, and SA 2317, and *S. mission* at 2 x 10^6 cfu per PCR tube. These were used to test the specificity of the designed primers. Only

Listeria monocytogenes isolates were positive in the agarose gel (Figure 1). This indicates that the primers were very specific for *Listeria monocytogenes*.

Five different isolates of *L. monocytogenes* were tested to determine the level at which their presence could be detected by the PCR technique. Each isolate was grown overnight in TSBYE and washed twice in BPB. The bacterial number of each isolate was obtained by their O.D. reading and determined by a predetermined growth equation. The isolates were then diluted and 10^6, 10^5, 10^4, 10^3, 10^2, 50, 10, 5 and 1 cfu in 2 µl were added to PCR tubes. The actual bacterial number was also obtained by spread-plating the bacterial suspensions on MOX. PCR detection sensitivity varied with different isolates of *Listeria monocytogenes* in pure culture (Table 4). Among the tested 5 isolates, *Listeria monocytogenes* H7738 4b had the highest detection sensitivity at 298 cfu and the lowest sensitivity was *Listeria monocytogenes* G3990 at 2,570 cfu. These data were used to determine the proper bacterial number to use for inoculating the salad samples.

Once the primers, bacterial isolate, recovery level, enrichment media and incubation time were selected, the study was prepared to determine the level of detection of *L. monocytogenes* on ready-to-eat salad. *Listeria monocytogenes* H7757 was grown overnight and washed twice in BPB. The O.D. reading and a pre-determined growth equation were used to determine the bacterial number. Then the bacterial suspension was diluted to 1,000 cfu/ml of inoculum and 25 g of ready-to-eat salad were inoculated with 1 ml of the inoculum. Three 25 g of ready-to-eat salad samples were inoculated. The samples were diluted with 225 ml of half strength TSBYE and blended with a stomacher.

After blending, the samples were filtered through fiberglass wool into a centrifuge tube, and then centrifuged at 8,000 x *g* for 10 min. Once centrifuged, the supernatant was removed and the pellet was resuspended in 500 µl BPB; then equally distributed among the three enrichment broths of TSBYE, LEB, and mPSU. The enrichment broths were incubated for 6 hr at 37 °C. After which the broths were removed and centrifuge again at 8,000 x *g* for 10 min, the supernatant was removed and the pellet was resuspended in the least possible amount of BPB, and 2 µl of each sample was used for the PCR amplification. After amplification the results were analyzed by agarose gel electrophoresis. Samples were spread plated onto MOX before centrifugation to determine the actual bacterial number used in PCR. The final bacterial concentrations of TSBYE, LEB, and mPSU were 4.0×10^4, 8.2×10^3, 6.8×10^2 cfu/g, respectively (Table 4). A positive PCR result was shown only on the LEB sample (Figure 5). Although TSBYE had a higher bacterial level than mPSU and LEB, the PCR result was negative. This may be explained in that TSBYE was not adequately selective and the background bacteria dominated the growth in the media and interfered with the PCR amplification. In the mPSU enrichment broth, the negative result of PCR amplification may have been caused by the lack of bacterial cells or interference of mPSU broth. This study showed that by using the internalin A based primers, filtration, centrifugation, and a 6 hr enrichment process; the PCR technique detected *L. monocytogenes* at 10^2 to 10^3 cfu/g in ready-to-eat salad. Compared to other studies, in which the enrichment process was used for concentration, as high as 55 hr of incubation time was recorded for the detection of 1

to 5 cells per 5 grams of seafood sample (Agersborg and others, 1997). As far as ready-to-eat salads, a detection level of 1-5 cfu/g of sample could be detected but only after a primary and secondary enrichment process that would take 48 hr of enrichment time to complete (Somer and others, 2003).

Table 1. The growth of *L. monocytogenes* in different enrichment broths for 6 hr.

Enrichment Broth	Bacterial number (cfu/ml)*
mPSU	6.1×10^2
Half Fraser	1.0×10^1
UVM	2.3×10^2
TSB + 0.6% Yeast Extract	2.0×10^5
Half TSB + 0.6% Yeast Extract	2.0×10^5
Listeria Enrichment Broth	2.1×10^3

*The Inoculum is 1.3×10^3 cfu/ml .

Table 2. Recovery of *L. monocytogenes* cells from inoculated salad.

	Time (hr)						
Enrichment Broth	0	1	2	3	4	6	8
mPSU	92	25	10	50	1.0×10^2	5.7×10^2	2.1×10^3
TSBYE	92	30	75	2.5×10^2	7.1×10^2	TNTC	TNTC
LEB	92	25	20	35	65	3.2×10^2	2.0×10^3
UVM	92	35	40	30	20	3.6×10^2	5.6×10^2

Table 3. The detection level of different *Listeria monocytogenes* isolated by PCR.

Microorganisms	Detection Level (cfu)
L. monocytogenes H7738 4b	2.98×10^2
L. monocytogenes G3982 4b	5.27×10^2
L. monocytogenes Scott A	1.43×10^3
L. monocytogenes G3990	2.57×10^4
L. monocytogenes H7757	4.44×10^2

Table 4. The detection of *L. monocytogenes* on inoculated ready-to-eat salad by PCR in different enrichment broths.

Enrichment broth	Bacterial Count	PCR Detection
TSB + 0.6% Yeast extract	4.0×10^4	-
LEB	8.2×10^3	+
mPSU	6.8×10^2	-

Inoculium 2.2×10^2 cfu/g

```
   1 gtgagaaaaa aacgatatgt atggttgaaa agtatactag tagcaatatt agtatttggc
  61 agcggagtat ggattaacac gagtaacggg acaaatgctc aggcagctac aattacacaa
 121 gatactccta ttaatcagat ttttacagat acagctctag cggaaaaaat gaagacggtc
 181 ttaggaaaaa cgaatgtaac agacacggtc tcacaaacag atctagacca agttacaacg
 241 cttcaggcgg atagattagg gataaaatct atcgatggag tggaatactt gaacaattta
 301 acacaaataa atttcagcaa taatcaactt acggacataa cgccacttaa aaatttaact
 361 aagttagttg atattttgat gaataataat caaatagcag atataactcc gctagctaat
 421 ttgacgaatc taactggttt gactttgttc aacaatcaga taacggatat agacccgctt
 481 aaaaatctaa caaatttaaa tcggctagaa ctatccagta acacgattag tgatattagt
 541 gcgctttcag gtttaactag tctacagcaa ttatcttttg gtaatcaagt gacagattta
 601 aaaccattag ctaatttaac aacactagaa cgactagata tttcaagtaa taaggtgtcg
 661 gatattagtg ttctggctaa attaaccaat ttagaaagtc ttatcgctac taacaaccaa
 721 ataagtgata taactccact tgggatttta acaaatttgg acgaattatc cttaaatggt
 781 aaccagttaa aagatatagg cacattggcg agtttaacaa accttacaga tttagattta
 841 gcaaataacc aaattagtaa tctagcacca ctgtcgggtc taacaaaact aactgagtta
 901 aaacttggag ctaaccaaat aagtaacatc agtcccctag caggtttaac cgcactcact
 961 aacttagagc ttaatgaaaa tcagctggaa gatattagcc caatttctaa cctgaaaaat
1021 ctcacatatt taactttgta ctttaataat ataagtgata taagcccagt ttctagttta
1081 acaaagcttc aaagattatt tttctataat aacaaggtaa gtgacgtaag ctcacttgcg
1141 aacttaacaa atattaattg gctttcagct gggcataacc aaattagcga tcttacacca
1201 ttggctaatt taacaagaat cacccaacta gggttgaatg atcaagcatg gacaaatgca
1261 ccagtaaact acaaagcaaa tgtatccatt ccaaacacgg tgaaaaatgt gactggcgct
1321 ttaattgcac cagctactat tagcgatggc ggtagttaca cagagcctga tataacatgg
1381 aacttaccta gttatacaaa tgaagtaagc tataccttta gccaacctgt cactattgga
1441 aaaggaacga caacatttag tggaaccgtg acgcagccac ttaaggcaat ttttaatgtt
1501 aagtttcatg tggacggcaa agaaacaacc aaagaagtgg aagctgggaa tttattgact
1561 gaaccagcta agcccgtaaa agaaggtcac acatttgttg gttggtttga tgcccaaaca
1621 ggcggaacta aatggaattt cagtacggat aaaatgccga caaatgacat caatttatat
1681 gcacaattta gtattaacag ctacacagca acctttgata atgacggtgt aacaacatct
1741 caaacagtag attatcaagg cttgttacaa gaacctacgg caccaacaaa agaaggttat
1801 acttttaaag gctggtatga cgcaaaaact ggtggtgaca agtgggattt cgcaactagc
1861 aaaatgcctg ctaaaaacat caccttatat gcccaatata gcgccaatag ctatacagca
1921 acgtttgatg ttgatggaaa atcaacgact caagcagtag actatcaagg acttctaaaa
1981 gaaccaaagg caccaacgaa agccggatat actttcaaag gctggtatga cgaaaaaaca
2041 gatgggaaaa aatgggattt tgcgacggat aaaatgccag caaatgacat tacgctgtac
2101 gctcaattta cgaaaaatcc tgtggcacca ccaacaactg gagggaacac accgcctaca
2161 acaaataacg gcgggaatac tacaccacct tccgcaaata tacctggaag cgacacatct
2221 aacacatcaa ctgggaattc agccagcaca acaagtacaa tgaacgctta tgacccttat
2281 aattcaaaag aagcttcact ccctacaact ggcgatagcg ataatgcgct ctacctttg
2341 ttagggttat tagcagtagg aactgcaatg gctcttacta aaaaagcacg tgctagtaaa
2401 tagaagtagt gtaaagagct agatgtggtt ttcggactat atctagcttt tttatttt
```

Figure 1. Internalin-A coding sequence (Acession #AL591975 REGION: 94534..96991) from Listeria *monocytogenes* strain EDG .

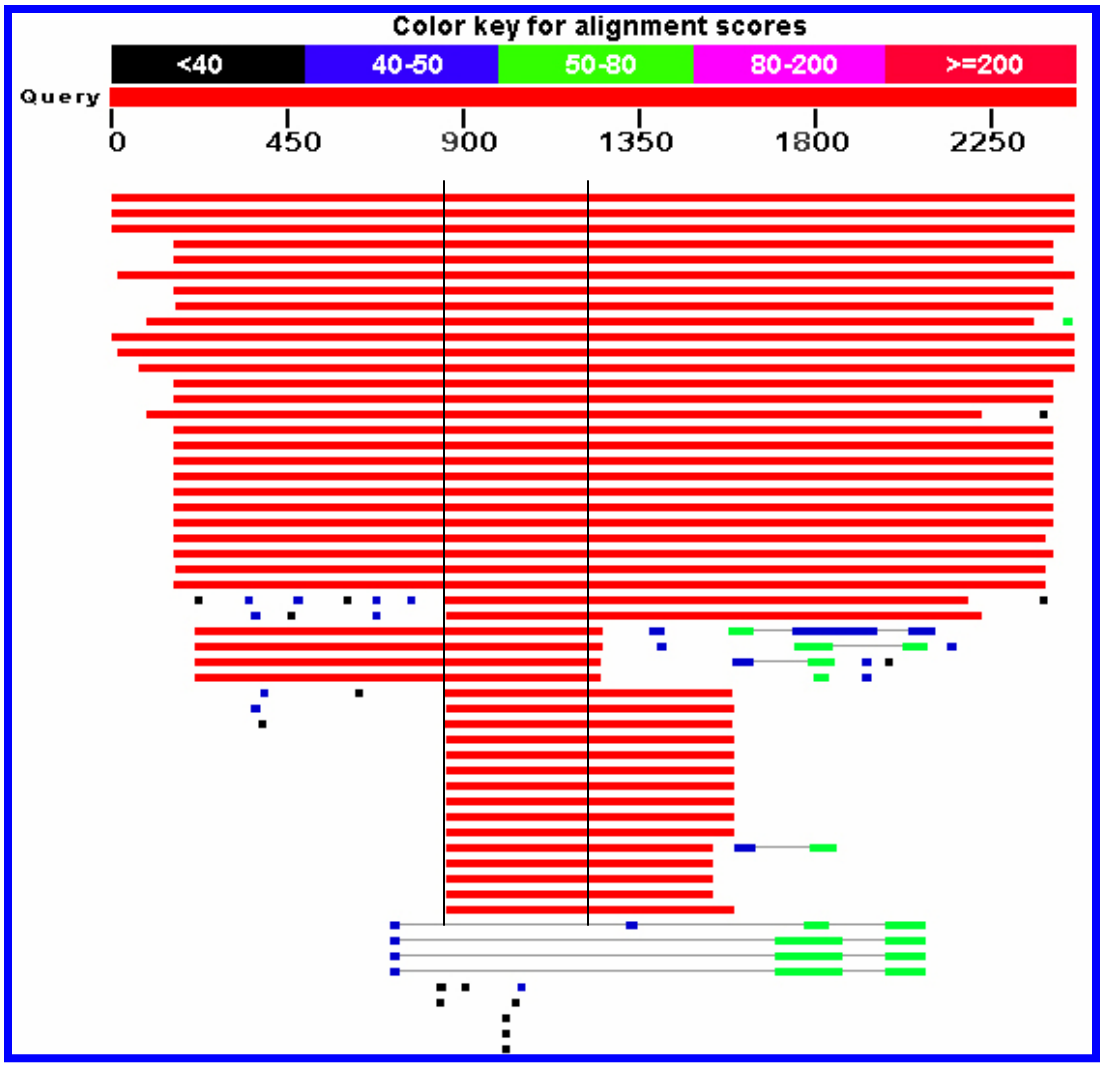

Fig2. NCBI BLAST output for internal-A sequence alignment. Those lines with red (>=200 aliment scores) are all *inlA* from *Listeria monocytogenes.* The closest relative sequence is *inlC2* from *Listeria monocytogenes*. Sequences between 2 vertical lines are very conserved and are good targets for detection.

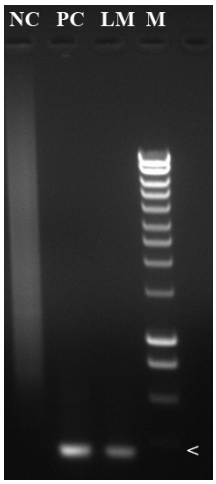

Figure 3. DNA Electrophoresis analysis of PCR products in test primer for *Listeria monocytogenes* detection. The samples are lanes (M) DNA ladder, (NC) negative control, (PC) positive control, and (LML) *Listeria monocytogenes* H7738. The arrow indicates the position of 400bp in makers.

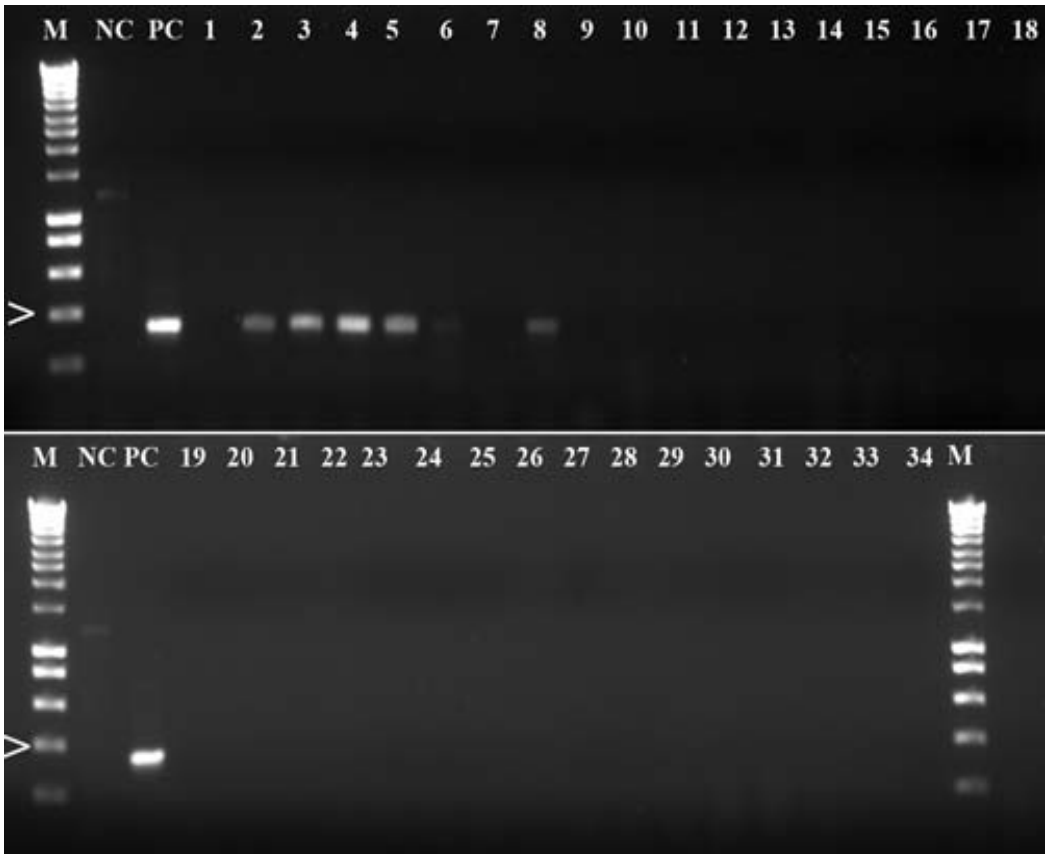

Fig. 4. PCR products analysis in the specificity test of *Listeria monocytogenes* by gel electrophoresis. The samples are lanes (M) DNA ladder (Bioline Hyperladder Type I, Bioline), (NC) negative control-DNA template substituted with same volume of distilled water, (PC) positive control-DNA template of internalin gene cloned in plasmid pCRscript (Stratagene), (1-6) *Listeria monocytogenes* isolates ATCC19111, H7738, G3928, G3990, G6005, (7) *Listeria inocua*, (8) *Listeria monocytogenes*, (9) *Staphylococcus aureus* ATCC 12600, (10-19) *E. coli* O157:H7 204p, O157:H7 301C, O157:H7 505B, O157:H7 ATCC 43895, K12 SA1332, K12 VSM1692, 932, LC40, 7121583, BD2399, 48-2 and GM 2163, (20-21) *Salmonella entertidis* H4267 and H2292, (22-23) *Salmonella typhi* CT18 and TY2, (24) *S. paratyphi* SA2723, (25) *S. paratyphimurium*, (26) *S. motevideo*, (27) *S Dublin*, (28) *S. panama*, (29) *S. missio*, (30-32) *Salmonella typhimurium* ATCC1311, PP1002, SA 2317, and (33-34) *Salmonella entertidis* H4639 and H4638. The arrow indicates the position of 400bp in makers.

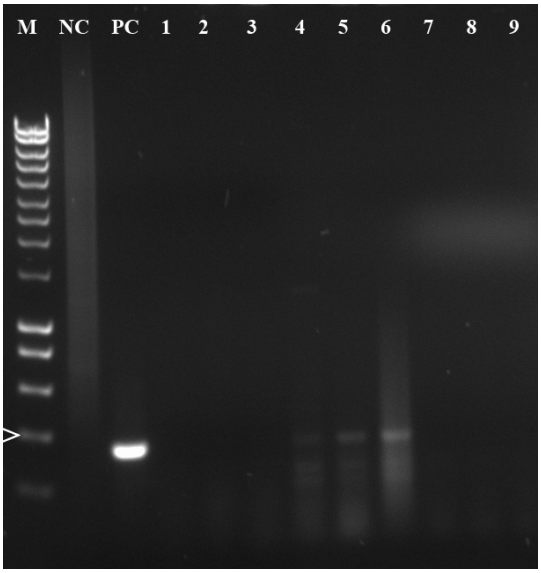

Figure 5. Electrophoresis analysis of PCR products in detection of *Listeria monocytogenes* in salads. The samples are lanes (M) DNA ladder, (NC) negative control, (PC) positive control, (1) background in TSBYE, (2-3) inoculated salad in TSBYE, (4) background in LEB, (5-6) inoculated salad in LEB, (7) background in mPSU, and (8-9) inoculated salad in mPSU. The arrow indicates the position of 400bp in makers.

V. CONCLUSIONS

The development of the polymerase chain reaction (PCR) technique has been a huge leap forward in the development of rapid, specific and sensitive methods for detection of food borne pathogens. The current PCR methods available for the detection of *Listeria monocytogenes* in food samples are either time consuming due to the enrichment of the samples or to the use of other costly and time-consuming methods. The goal of this study was to develop a rapid, specific, sensitive and cost efficient means to detect *L. monocytogenes* in food samples by a PCR method.

The targeted enrichment method in this study was to produce the highest level of *L. monocytogenes* cells in the least amount of time. The TSBYE broth supported good growth of *L. monocytogenes* in 6 hr. The mPSU, as well as, LEB were highly selective for the growth of *L. monocytogenes,* and they supported good bacterial growth, which was adequate to detect the bacterium in 6 hr. Therefore, TSBYE, mPSU and LEB were chosen to represent both selective and non-selective enrichment media for *L. monocytogenes* enrichment on inoculated salad in PCR detection. The LEB produced the best growth rate and did not interfere with the PCR detection of *L. monocytogenes.*

The primers used were developed to amplify a segment of the internalin A gene. This gene only exists in the pathogenic strain of *L. monocytogenes*. The specificity of the primers was very high which was specific only for *L. monocytogenes* isolates.

By using the PCR with the filtration, centrifugation, and enrichment process, this PCR method can detect as low as 10^2 cfu of *L. monocytogenes* in ready-to-eat salad.

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