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Detection of *Listeria monocytogenes* in foods by using genetic and immunological tools.
(Under the direction of JOSEPH FRANK)

A PCR assay targeting the genes encoding internalinAB (*inlAB*) was developed for detecting *L. monocytogenes* in foods. One primer set, targeting a 902-bp region of the *inlAB*, was most specific among those tested. The specific PCR product was detected in 51 *L. monocytogenes* strains belonging to 4 different serogroups. In contrast, the PCR product was not detected in other *Listeria* species and gram positive, indicating that the primer set was highly specific. The detection limit of the PCR assay was 10^5 CFU per ml of pure culture. However, the assay could detect as few as 10 CFU of *L. monocytogenes* in 25 g of frankfurters within 6 h after samples were enriched in modified *Listeria* enrichment broth (LEB) at 37°C. The total assay time, including enrichment, was approximately 24 h.

The developed PCR method was evaluated for detecting *L. monocytogenes* at levels ranging from ca. 1 to 100 cfu/g in with two enrichment procedures. Depending on the food type, i. e. ground beef or Brie cheese, PCR detection was achieved with different enrichment procedures. Ground beef samples enriched in tryptic soy broth (TSB) at 37°C for 6 h, then transferred to LEB with further incubating for 18 h at 30°C was efficient for detecting *L. monocytogenes* cells while we could detect as low as <1 cfu per gram in Brie cheese with enriched in LEB medium for 24 h at 37 °C after PCR detection. Among 8 tested foods, the PCR method could detect as low as ca. <1 cells/g of food sample after enrichment, which had complete agreement with results obtained by using the conventional cultural method. These data suggest that the PCR method is specific and sensitive for detecting *L. monocytogenes* in various foods.

A rapid detection method for *L. monocytogenes* using immuno magnetic separation with flow cytometry was investigated. The efficiency of immuno capturing

using magnetic beads was also determined. None of antibodies tested in our protocol differentiated between negative and positive sample. Attempts to optimize the concentration of magnetic beads or blocking step did not improve the signal difference. Plating results confirmed that cells were captured even with low recovery rate ranging from 7 to 21%. However, when the same antibody (KPL) was used as a fluorescent labeling antibody, the differentiation between negative and positive sample was not obtained.

INDEX WORDS: *Listeria monocytogenes*, PCR, internalinAB, immunomagnetic separation, flow cytometry, rapid detection, foods

DETECTION OF *LISTERIA MONOCYTOGENES* USING GENETIC AND
IMMUNOLOGICAL TOOLS

by

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B. S. Taegu University, South Korea, 1995

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DEDICATION

To my parents, and family for their love and support.

AND

To my wife Alen Kim for her patience, encouragement, and moral support throughout
the years.

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INTRODUCTION

Numerous food-borne outbreaks associated with *Listeria monocytogenes* in the late 1980's prompted the USDA to adopt a zero-tolerance policy for *L. monocytogenes* in cooked, ready-to-eat meats. In addition, the Food and Drug Administration (FDA), and the Food Safety and Inspection Service (FSIS) of the U.S. Department of Agriculture (USDA) recently categorized the risk of foods causing listeriosis depending on various criteria. In this draft risk assessment, risk characterization is based on the origin, composition and process with the relative probability of consuming foods and listeriosis placed into the 20 categories. High-risk categorized foods include smoked seafoods, minced meat, deli meat, fresh soft cheeses and cooked ready-to-eat crustaceans.

In order to provide safe product, there is a need for developing rapid, sensitive, reliable, and specific methods since conventional methods are time-consuming and laborious. Various detection methods have been developed for detecting or enumerating this pathogen based on immunological based methods including enzyme-based (enzyme-linked immunosorbent assay (ELISA), enzyme immuno assay (EIA), and immuno-lift technology. However, antibody-based methods often suffer from low selectivity resulting in false-positive results. Once specific and sensitive antibodies are available, immuno-based methods have a great potential to detect *L. monocytogenes* in food matrix in conjunction with various other sophisticated devices such as biosensor, flow cytometry, or other laser-based devices. Nucleic acid-based methods could give a more specific result than immuno-based assay. One of the promising techniques is the

nucleic acid amplification based method such as polymerase chain reaction (PCR).

Various genes have been targeted for detection of *L. monocytogenes*. Recent development of PCR devices and fluorescent probes makes more rapid or sensitive for this assay. The improvement of PCR methods including sample preparation or more efficient target extraction step is still developing for the application in food industry.

Present objectives of this study are to develop a PCR technique and detect *L. monocytogenes* targeting internalin AB (*inlAB*) in foods. The developed PCR protocol will be tested for its specificity and sensitivity. The developed PCR method will be applied to detect low number of *L. monocytogenes* inoculated in various foods, including hot dog, ground beef, crab meat, smoked salmon, shrimp, deli meat, lettuce, and cheeses (brie and queso fresco). Various enrichment procedures will also be evaluated for the detection of this pathogen.

Flow cytometry will be used to detect *L. monocytogenes* in combination with immuno magnetic separation technique. Various polyclonal antibodies will be evaluated for the feasibility of using immunomagnetic-flow cytometry method.

CHAPTER 1. LITERATURE RIVIEW

Introduction

L. monocytogenes, a gram positive, non-spore forming, intracellular, facultative anaerobic rod, growing at refrigeration temperature, was first recognized by Murray et al. (Murray, 1926). It is catalase positive, oxidase negative, hydrolyses esculin and urea, Voges-Proskauer positive, ferment glucose, and produces a β -hemolysin, which produces zone of clearing on sheep blood agar (Seeliger and Jones, 1986). The hemolysin of this organism acts with the β -hemolysin of *Staphylococcus aureus* resulting in a positive CAMP test. In addition, the sugar fermentation pattern, i. e. L-rhamnose and α -methyl-D-mannoside positive and D-xylose or mannitol negative, is used to differentiate *L. monocytogenes* from the six other *Listeria* spp. (Bille and Doyle, 1991). This organism is ubiquitous in that it has been isolated from many environments including soil, plant, sewage, and water (Weis and Seeliger, 1971) as well as animals and human (Gray and Killinger, 1966). The G + C content of the *L. monocytogenes* DNA is ca. 37 to 39%, and the length of the genome was 3150 kb (Michel and Cossart, 1992). Based on numerical taxonomic, chemical study, DNA homology, and 16S rRNA, genus *Listeria* can be differentiated into six species including *L. monocytogenes*, *L. innocua*, *L. seeligeri*, *L. welshimeri*, *L. ivanovii*, *L. murrayi*, and *L. grayi* (Seeliger, 1984). Among these species, only *L. monocytogenes* is a pathogenic for human. Based on the serotyping, most 33 to 50% of human cases are associated with serovar 4b and other 1/2 serovars also frequently isolated from foods and environment (Boerlin and Piffaretti, 1991).

L. monocytogenes can survive and multiply in macrophages and cause the infection (Mackaness, 1962). Since the T-cell immunity is important to control this intracellular pathogen, lowered immunity is primarily responsible for human infection (Blanden and Langman, 1972; Kaufmann, 1987). The study of listeriosis in the United

States has shown that at least 90 per 100,000 AIDS patients were in this category, which is 150 times more rate than those of the same age group (Gellin, et al., 1991). Another investigation of mortality rate done by Rocourt (1991) showed from 13 to 34% in 1989. The organism usually isolated from the cases of meningitis, encephalitis, septicemia, and stillbirth or miscarriages (Gray and Killinger, 1966). Neonates, elderly individuals, pregnant women, and immunocompromised people, i. e. transplant recipients and AIDS patients, are the most to infection. Listeriosis may cause mortality rates up 50% and annual economic losses in the United States due to disease is more than \$500 million (World Health Organization, 1988).

Virulence factors

Even though the exact mechanisms of pathogenesis of *L. monocytogenes* has not been elucidated, many factors including ability for intracellular growth, production of superoxide dismutase, hemolytic activity and actin polymerization have been observed and identified as potential virulence factors (Chakraborty and Goebel, 1988). The invasion by this organism into the mammalian cell lines has been explored by researchers. A 60 kD extracellular protein (p60) was involved in the invasion of the *L. monocytogenes* since the mutant shows the significant reduced uptake by 3T6 fibroblast cells (Kuhn and Goebel, 1989). In addition, in early stage of invasion, internalin is another important surface protein associated with induced internalization of *L. monocytogenes* by nonprofessional phagocytes. Internalin A (*inlA*) gene, ca. 90 kD protein encoding a 800 amino acid, was identified by screening a library of Tn1545 mutants (Gaillard et al., 1991). They observed that these mutants had lost their invasiveness into epithelial cell line Caco-2. Internalin B, encoding for a 630 amino-acid protein, is located downstream from *inlA* gene and also related to the entry of the

hepatocytes (Dramsi, et al., 1995). Taken together, *inlA* and *inlB* (*inlAB*) are organized as an operon (Dramsi et al., 1993) and its mutants showed less invasive to epithelial cell line indicating the importance of invasion with these two genes.

Once internalized, *L. monocytogenes* escapes from vacuoles and enters the cytoplasm. Two main factors play an important role in this step. Listeriolysin O (LLO), a secreted protein of 58.6 kD and a member of a family of pore-forming thiol-activated cytolysins, is involved (Geoffroy, et al., 1987). LLO is encoded by a single locus, called *hlyA* (Mengaud, et al., 1987). Mutants to which a plasmid bearing this gene is added gain their virulence again. When mutants are injected to mice, they are eliminated from liver and spleen while wild-type hemolytic strain survive for a few hours suggesting the role of virulence of this gene (Karhariou, et al., 1987). In addition, phosphatidylinositol-specific phospholipase C (PI-PLC) encodes a protein of 34 kD with high homology to some gram-positive phospholipase, is another factor in the cell escaping from the vacuoles (Camilli, et al., 1991; Leimeister et al., 1991). Phospholipase deficient strains in various cell types reveal that these bacteria lack ability grow and multiply in the mammalian cell.

The gene called *mpl* located downstream of the *hly* gene could also be involved in pathogenesis (Domann, et al., 1991). The protein encoded by this gene is a zinc metalloprotease similar to thermolysin. The exact mechanism is unclear, however, the transposon mutants of this gene were reduced in pathogenicity but grow normally in mammalian cell lines (Raveneau, et al., 1992).

After escaping from the phagosome, cell-to-cell spread is an important step for attacking other tissues. This intracellular movement of *L. monocytogenes* mediated by actin polymerization encoded by *actA* gene is involved in virulence (Cossart, and Kocks,

1994). Mutation of *actA* gene produced loss of virulence in mice and inability of intracellular movement of *L. monocytogenes* cells (Domann, et al., 1992).

Foodborne-outbreaks associated with *L. monocytogenes*

Most cases of listeriosis have been sporadic; however, recently food-borne outbreaks associated with contaminated foods have increased. Implicated foods in outbreaks include lettuce (Ho, et al., 1986), pasteurized milk (Fleming, et al., 1985), coleslaw (Schlech, et al., 1983), pâté (Morris and Ribeiro, 1989), pork tongue (Bader, 1993), Mexican-style soft cheese (James, et al., 1985), shrimp (Riedo, et al., 1990), and turkey frankfurters (Barners, et al., 1989). Other studies also indicate that the microorganism can be found in various items of fresh produce including lettuce, potatoes, radishes, and cabbage (Schlech et al., 1983; Heisick et al., 1989). Farber (1991) found that more than 75% of smoked salmon in retail store was contaminated with *L. monocytogenes*. Numerous recalls of seafoods including scallop, artificial crab meat, frozen cooked shrimp, and surimi, have been occurred due to *L. monocytogenes* contamination (Dillon and Patel, 1992). These recalls involve variety of foods suggesting that *L. monocytogenes* is a major food borne pathogen that could gain more attention from the public. In addition, processed foods, especially ready-to-eat foods, are faced with cross-contamination problems that could result in listeriosis since this organism can grow at refrigeration temperature (Jacquit, et al., 1995).

A case control study investigated the role of foods in sporadic listeriosis occurring from 1988 through 1990 (Schuchat et al., 1992). They indicated that 7.4 cases per million populations could belong to these incidences and that 23% of these cases could be fatal. In their study (Pinner, et al., 1992), they identified *L. monocytogenes* in ready-to-eat foods and serovar 4b was most likely associated with causing agent of the

listeriosis based on data that collected from patients as well as retail store. Recent investigation show that 106 reports of listeriosis out of 9787 cases including most foodborne disease under surveillance during 1998 were identified and confirmed (CDC, 1999).

Detection methods

Isolation or detection of foodborne pathogens from foods improved in various ways using immunological or molecular approaches. The development of methodology has led to more refining of isolation, early detection, characterization, and enumeration of microorganisms in food, clinical and industrial environmental samples. The purposes of developing microbiological method in the food industry are help to produce safe foods, adequately manage food production, and accurately report the foodborne disease. In order to accurately identify pathogens in foods, the following information can be used: morphology, gram stain, biochemical activity, pigment production, antibiotic sensitivity, metabolites, toxins, nutritional requirement, genetic profile, serotype, phage type, motility, and conductance characteristics. Presently, there is a need in food industry for more rapid method development to produce a safe product.

Coventional detection and enrichment for detecting *L. monocytogenes*

To isolate *L. monocytogenes* from foods and the environment, various chemicals or antibiotics are added to media that inhibit the growth of competing microorganisms. McBride and Girard (1960) developed the first appropriate media for recovering *L. monocytogenes* in the presence of competing miroflora. This medium was further improved by adding more selective agents including phenylethanol, glycine anhydride, lithium chloride, and cycloheximine (Lovett, 1988). Lee and McCain (1986) improved

the medium by adding moxalactam, broad spectrum antibiotic against some of gram-positives and gram-negatives, for detection of *L. monocytogenes* in meats. Other developed selective agars were reported including RAPAMY (van Netten et al., 1988) and PALCAM (van Netten et al., 1989).

There are some comparisons of performance of various media recovering *L. monocytogenes*. Oxford agar developed by Curtis et al. (1989) was more selective than McBride in that this medium was more successfully isolating *L. monocytogenes* from artificially contaminated clinical specimens. Others found that Oxford agar performed better than lithium chloride phenylethanol moxalactam (LPMA) agar in recovering of the cell from foods (Tiwari and Aldenrath, 1990). Al-Zoreky and Sandine (1990) developed a medium (Al-Zoreky Sandine *Listeria* medium) and compared it to other media. They indicated that their medium was as effective as other compared media and achieved 85% recovery of the heat-injured *L. monocytogenes* cells. Buchanan et al (1989) compared LPM and modified Vogel Johnson (MVJ) agars for detection of *Listeria* spp. in meat, poultry, and seafood. However, they encountered false-positives due to the *Staphylococcus* and *Kurthia* spp. An enhanced hemolysis agar was developed by Cox et al (1991) for the isolation of *L. monocytogenes* from foods. A modification of oxford medium by adding sheep blood, 4-methylumbelliferyl- β -D-glucoside, and sphingomyelinase, was made to differentiate colonies by blue fluorescence and zone of hemolysis.

A cold enrichment procedure had been used for isolating *L. monocytogenes* from foods and environmental samples in the 1960's (Gray and Killinger, 1966). However, since this procedure could take up to 3 months, the incorporation of selective agents into enrichment media has been developed to replace it. To date, two enrichment procedures, the Food and Drug Administration (FDA) method (Lovett and Hitchins, 1991) and the U.

S. Department of Agriculture-Food Safety and Inspection Service (USDA-FSIS) method (McClain and Lee, 1989) are commonly used for enriching *L. monocytogenes* in samples in the U. S. Nalidixic acid and acriflavin inhibiting the growth of gram-negative and gram-positives other than *Listeria*, have been added to FDA enrichment broth to isolate *Listeria* in foods other than meats (Lovett, J., 1988; Lovett, et al., 1987). Enrichment broth is incubated for 24 to 48 h at 30°C and then streaked onto two selective media, LPM and oxford agar. In contrast, USDA-FSIS method is used for isolating *L. monocytogenes* from meat and poultry. The enrichment consists of primary enrichment using *Listeria* enrichment broth (LEB) with nalidixic acid and secondary enrichment broth that is fortified with acriflavine-HCL. McClain and Lee (1988) found that up to 42% increase in recovery of *L. monocytogenes* was achieved from meats and poultry products using this procedure. Fraser medium (Fraser and Sperber, 1988) has also been developed by adding acriflavin-HCL, lithium chloride, and ferric ammonium citrate into LEB. Based on the ability of *Listeria* species to hydrolyze esculin and produce black color, this medium provides the indicator responses and shows low false-negative result (1%). Several studies have been done to compare enrichment procedures for the isolation of *Listeria* spp. from cheese (Doyle and Schoeni, 1987), milk (Slade and Collins-Thomson, 1988), or chicken (Pini and Gilbert, 1988). FDA and USDA methods were compared (Warburton et al., 1991) for isolating *L. monocytogenes* from various foods and environmental samples. They observed that the FDA method could reduce the detection time to two days without greatly reducing the number of positive samples. However, the USDA method performed slightly better than the FDA method in isolation of *L. monocytogenes*. However, another study showed that both the FDA and USDA methods were equally appropriate for recovery of heat-injured *Listeria* cells from foods (Bailey, et al., 1990).

Immunological based methods

Immunological based methods can be both simple and rapid. These methods depend on the use of specific antibody-antigen reactions. The development of antibodies for detecting toxin or bacteria in food and clinical samples has been changing for many years. Since assays rely on the specificity of the antibody, a limitation is the potential cross-reactivity of antibodies. The development of monoclonal antibody that is specific for the antigen of interest provides greater specificity. The difficulty of assessing the cell viability is another challenge of using immunological based method, especially in food samples.

Enzyme-linked immnosorbent assay (ELISA) and enzyme immunoassay

Enzyme-linked immnosorbent assay (ELISA) is a heterogeneous enzyme assay in which one of the reactant (antibody or antigen) is immobilized on a solid-phase matrix. In this system, the antigen-antibody reaction does not influence the activity of the enzyme label. The ELISA consists of analyte (antibody or antigen), the labeled enzyme reactant (antigen or antibody), and a method of detecting an enzyme. After incubation with sample, the unbound sample is washed and enzyme-linked reporter antibody is added. After reacting with substrate, the signal such as color, fluorescence, or chemiluminescence from enzyme reaction is developed and detected by an appropriate device (Nakamura and Kasahara, 1992).

A collaborative study conducted by Curiale et al. (1994) evaluated the *Listeria*-Tek™ kit, an ELISA format developed by Organon Teknika Corp. (Durham, NC), for the detection of *L. monocytogenes* in various foods. They also compared ELISA to FDA cultural methods. Their method employed a monoclonal antibody developed by

Mattingly, et al. (1988). Testing indicated a total positive agreement of 85.6% between two methods. However, the authors indicated that some false-positive results were obtained due to the cross reactivity of antibodies to other *Listeria* spp used in this assay. Two other types of immunoassays were compared (Kerdahi and Istafanos, 1997). One is the Tecra *Listeria* visual immunoassay (TLVIA) and another was the Vitek immunodiantic assay system (VIDIS LIS). The former incorporates polystyrene microwell coated with polyclonal antibody specific for *Listeria* antigen. After capturing the *Listeria* antigen, detection antibody labeled with enzyme is added, followed by reacting with substrate developing a green color. All reactions can be completed within 2 h after 42 h selective enrichment (Knight et al., 1996). The latter is a fully automated system for detecting immunofluorescence. The solid-phase receptacle captures the *Listeria* cell from enriched sample and other reagents pre-loaded in the instrument are reacted in the device. Intermediate steps including adding reagents and washings are automated (Gangar, et al., 2000). The authors indicated that both methods appeared reliable for rapid screening of *Listeria* spp.

Several monoclonal (Loiseau, et al., 1995; Kathariou et al., 1994; Mattingly, et al., Gavalchin, et al., 1991; 1988; Farber and Speirs, 1987) or polyclonal (Bubert et al., 1994; Feldsine, et al., 1997) antibodies specific for *Listeria* spp. have been developed and evaluated in conjunction with ELISA format or enzyme immunoassay (EIA) for testing their specificity. Gavalchin et al (1991) have characterized their developed antibodies and screened three hybridomas for detecting *L. monocytogenes* with sandwich ELISA assay. They observed that these hybridomas preferentially reacted with *L. monocytogenes* and did not show any cross-reactivity with *Kurthia* spp. However, these monoclonal antibodies showed some cross reactivity with *L. innocua* and *L. ivanoii* indicating the limitation of usage of these hybridomas as a detection antibody.

Kathariou et al (1994) screened monoclonal antibodies that had strong reactivity with *L. monocytogenes*, especially serotype 4b. But these monoclonal also reacted with *L. innocua*. A synthetic peptide targeting p60 protein of *L. monocytogenes* was used to produce a polyclonal antibodies (Bubert et al., 1994). Based on their result, the antibodies produce from the p60 protein was reacted with native form as well as denatured secreted form, which was confirmed by ELISA assay. To date, most antibodies developed showed limit genus specific or cross reactive with other closely related species.

Immuno-based capture

An antibody-based immnomagnetic separation (IMS) technique has been developed to selectively concentrate and isolate analytes from the sample (Olsvik, et al., 1994). The principle of this tool is the use of specific antibody coated on the magnetic bead. After incubating these beads with sample, the target is recovered via magnetic concentrator. There have been many applications of this technique in conjunction with enzyme or fluorescent immunoassay (Mansfield, et al., 2000; Holt, et al., 1995; Chapman, et al., 1997; Yu, 1998; Cudjoe, et al., 1995), PCR (Rijpens, et al., 1999; Fluit, et al., 1993; Lindqvist, et al., 1998), epifluorescence microscopy (Wang and Sharpe, 1998), and conventional plating (Avoyne, et al., 1997; Mitchell, et al., 1994; Skjerve and Olsvik, 1991).

IMS has been applied to detect *L. monocytogenes* from foods (Skjerve et al., 1990). A monoclonal antibody (MAb) recognizing serotype 4 was coated on the magnetic bead. Authors observed that the quantity of the bead and incubation time was influenced the sensitivity of the assay. The detection limit was less than 10^2 cells/ml in enriched foods. Fluit et al. (1993) coated two kinds of MAbs specific for *L.*

monocytogenes on the magnetic beads to capture *Listeria* cells from enrichment broth containing food samples. After separation, samples were subjected to PCR detection. Even though tested two Mabs exhibited low specificity, this disadvantage was overcome by using a PCR step. They could detect 1 cfu of *L. monocytogenes* per g of cheese after selective enrichment for 24 h in Fraser broth. Cudjoe et al. (1995) developed an immunomagnetic particle based ELISA for detection of *Salmonella* from foods. They indicated that the total analysis was completed within 26 h. The detection limit was 10^5 cells/ml of sample. Mitchell et al. (1994) compared the efficiency of *Listeria* capture using immunomagnetic capture technique to a standard cultural method. They indicated that immunomagnetic technique gave more rapid and sensitive detection than cultural method in that the former could detect an injured cell. The ListerScreen (AES Laboratoire, Combourg, France) method was developed to detect *Listeria* spp. in food (Avoyne, et al., 1997). This test comprises a immunobead and PALCAM agar. After enrichment of samples for 18 h, an immunomagnetic capture was applied and the beads were plated on the agar medium. The accuracy and reproducibility of this method has been evaluated and showed 99% agreement compared to cultural method. However, Uyttendaele et al. (2000) tested the IMS separation method for direct detection of *L. monocytogenes* in cheese. They found that the use of Dynabead anti-*Listeria* magnetic separation did not selectively concentrated the target organism since the result showed that the ratio of *L. monocytogenes* to non-*Listeria* microorganisms was not increased after IMS application.

Some disadvantages associated with the use of larger bead (> 5 micrometer) have been observed (Miltenyi, et al., 1990). The disturbance of the flow and aggregation of cells in the sample that could increase the non-specific binding or attachment of bacteria per bead due to the large magnetic moment, could be a disadvantage of using larger

beads. Small magnetic beads (< 50 nm) were introduced and gave improved characteristics compared to larger beads (Miltenyi, et al., 1990). The binding time is much faster and cells can be quantitatively labeled. In addition, a small magnetic moment causing long separation time can be solved by using a high gradient magnetic field column. Using these microbeads, Jacobsen et al. (1997b) enhanced recovery up to 95% when they used the *L. monocytogenes* cells at concentration from 10^4 to 10^7 . This small microparticle was also applied to detect *Cryptosporidium parvum* oocysts using high gradient columns (Deng, et al., 2000). They achieved over 95% recovery of oocysts using these beads.

Flow cytometry

Flow cytometer is a device that has been used in clinical field for analyzing blood cells. This instrument provides a rapid method for the characterization of individual cells in a mixed population by physical and biochemical aspects. Since the instrument can use multi-parameter analysis such as light scattering and fluorescence, target cells can be discriminated by measuring inherent characteristics, cell size, surface antigenic properties, and nucleic acid content (Davey and Kell, 1996; Shapiro, 1995). The application of flow cytometry in aquatic microbial ecology (Troussellier, et al., 1993; Robertson and Button, 1989), environmental bacteriology (Porter, et al., 1996) and food industry (Laplace-Builhé, et al., 1993) has been reviewed. In addition, the usage of this technique for detecting foodborne microorganisms and limitations for detecting bacteria in mixed cultures have also been discussed (Ueckert, et al., 1995; Phillips and Martin, 1988).

Donnelly and Baigent (1986) used this flow cytometry for detection of *L. monocytogenes* in milk. The immunofluorescent labeling of the target cell in

combination with propidium iodide staining was used for detection. They could differentiate *Listeria* from other microorganisms by measuring immunofluorescence of target cell and the light scattering pattern. These authors also indicated that the assay could be enhanced with selective enrichment. Seo et al. (1998) used this technique in conjunction with immunomagnetic separation for detection of *Escherichia coli* O157:H7 in enrichment culture from ground beef, apple juice, milk. They could detect 4 *E. coli* O157:H7 cells per g of ground beef sample within 7 h including enrichment time. Tortorello et al. (1998) compared two types of flow cytometry systems to the antibody-direct epifluorescent filter technique (Ab-DEFT) for detecting *E. coli* O157:H7 in enriched ground beef samples. They found that the detection limit of flow cytometry analysis was 10^4 cells/ml of sample, however, flow cytometry was more rapid. In addition, *E. coli* O157:H7 cells could be distinguished by using enhanced light scatter. A fluorescence tagged monoclonal antibody against *Salmonella typhimurium* was used to stain the cells and the flow cytometric analysis was applied to detect the pathogen in eggs and milk (McClelland and Pinder, 1994a, 1994b). They found that the detection limit was about 10^3 cells per ml of milk sample after treating with clearing solution since micelles in the sample gave a high false-positive signal. An even lower detection limit (below 10 cells/ml) was achieved after 6 h enrichment.

Another promising approach using flow cytometry is to rapidly provide information about viability of the cells as it relates to membrane potential, integrity or cellular enzyme activity (Shapiro, 1995). Jepras et al (1995) investigated the use of several dyes for assessing the viability of bacteria and found that the anionic membrane potential probe, bis-(1,3-dibutylbarbituric acid) trimethine oxonol [DiBAC₄], was the best candidate for this purpose. Clarke and Pinder (1998) developed a combined protocol using Chemochrome and labeled monoclonal antibodies. They could detect

100 live *S. typhimurium* cell ml⁻¹. Detection of various stages of starved *E. coli* and *Salmonella* was investigated by Lopez-Amoros et al. (1995) using flow cytometry and dyes. They indicated that the intensity of fluorescent dyes, rhodamine 123, propidium iodide (PI), and oxonol, was directly correlated with the viable count and could be observed in a heterogeneous population. They also mentioned that the use of oxonol was the better indicator of dead cells than PI during starvation since the starved cells did not significantly lose their membrane integrity.

Membrane integrity analysis is based on the capacity of the cells to exclude fluorescent intercalating dyes such as propidium iodide (PI) and ethidium bromide, which are not normally membrane permeable. Fluorogenic substrates, which are lipophilic, non-toxic, uncharged and nonfluorescent, are hydrolyzed by esterases to polar fluorescent products that are retained cells with intact membranes. Several dyes used to assess viability of *L. monocytogenes* were evaluated (Jacobsen et al, 1997a). The highest fluorescence intensity of *L. monocytogenes* was observed by using 5(6)-carboxyfluorescein diacetate (CFDA) and Chemochrome B.

Another approach to assess the viability of the cell with flow cytometry is the use of rRNA probe. The number of rRNA copies within a cell to target is proportional to the rate of growth (Amann, et al., 1990; Wallner, et al., 1994).

Some attempts have been tried to enumerate microorganism using flow cytometry (Tortorello et al., 1998; Heidal et al., 1994; Page and Burns, 1991; Pinder et al., 1990; Stewart and Steinkamp, 1982). Since the direct quantitation of bacterial concentration in a sample is not possible, most flow cytometers use an indirect method by using internal standard such as fluorescently-labelled polystyrene beads. The number of a known concentration of beads counted by flow cytometer in a sample can be proportional to

sample volume and then original bacterial concentration can be calculated (Stewart and Steinkamp, 1982).

Nucleic acid based methods

Nucleic acid probe

Nucleic acid probes have been used to specifically detect viruses and bacteria in food, clinical and environmental samples. In order to develop specific probes, the selection of target sequence should be unique and must to be tested to ensure that the probe is specific for the target. Probe sequence usually consists of 15 to 30 base pairs.

Based on the sequences for 16s rRNA of microorganisms, Klinger et al. (1988) (Gene Trak Inc., Framingham, MA) developed a unique probe for detecting *Listeria* in foods. They found that their hybridization assay was superior to the cultural method due to its low false-negative rate. This group has further refined the test using a non-isotope enzyme marker and a dipstick format (King, et al., 1989). This assay could achieved false negative rate as low as 0.8-4.7%. In addition, in situ hybridization using rRNA targeted probe in conjunction with mRNA (targeting *iap* gene) for detecting *L. monocytogenes* has been explored by Wagner et al (1998). By using a multiple labeled probe for mRNA, they could have comparable signal intensity to those obtained from 16S rRNA-targeted probe since the amount of *iap*-mRNA is less than 16S rRNA. Others also developed probes targeting *L. monocytogenes* unique regions and could differentiate from *L. innocua* by appropriate temperature control (Czajka, et al., 1993). A DNA probe developed by Datta et al (1993) was tested to identify *L. monocytogenes* with colony hybridization assay. They observed that their probe targeting the *hlyA* gene and flanking region reacted only with *L. monocytogenes* out of the 150 *Listeria* strains tested. Other

genes including delayed-type-hypersensitivity (DTH) (Notermans, et al., 1989) and listeriolysin O (LLO) (Chenevert, et al., 1989) were also used in hybridization assays.

A type of fluorescent probe, called a molecular beacon, was introduced (Tyagi and Kramer, 1996). This probe consists of short complementary sequence of nucleotides attached to the 5' and 3' ends of the probe sequence resulting in formation of a stem-loop structure in solution. A fluor and a quencher dye are attached to the ends of the stem. When these dyes are in close proximity, fluorescence is quenched; however, when the probe hybridizes with a complementary target sequence, the stem-loop structures is opened and the reporter and quencher dyes are separated resulting the emission of fluorescence (Tyagi and Kramer, 1996; Tyagi, et al., 1998). This probe has been investigated for detection of ruminal bacteria such as *Ruminococcus albus* and *Fibrobacter succinogenes* (Schofield, et al., 1997). They compared the result between membrane and solution hybridization and indicated the result from each method was similar.

The peptide nucleic acid (PNA) probe was first developed by Nielsen et al (1991) and designed to bind double stranded DNA. It is an analog of DNA where the whole negatively-charged sugar-phosphate backbone is replaced with a neutral pseudopeptide backbone consisting of repetitive units of N-(2-aminoethyl) glycin. Four nucleobases are attached to this backbone to allow this PNA to hybridize to complementary DNA or RNA obeying the Watson-Crick base-pairing rules (Egholm, et al., 1993). The relative hydrophobic characters of this probe compared to that of DNA offered this probe easily entered the hydrophobic cell wall of *Mycobacterium* species (Stender, et al., 1999). Other applications using this probe include *in situ* detection of *Escherichia coli* cells in water (Prescott and Fricker, 1999), Epstein-Barr Virus (EBV) in combination with flow cytometry (Just, et al., 1998), and amplified DNA products (Sawata, et al., 1999) or

point mutation in the tumor suppressor gene *p53* (Wang, et al., 1997) with a biosensor.

The authors indicated that the fast response and accuracy of the PNA probe could provide an alternative method to the cultural conventional technique.

Nucleic acid amplification methods

In order to overcome the low sensitivity of hybridization methods that are dependent on the number of copies of the target, the amplification of the nucleic acid target has become an important step for improving the signal. The polymerase chain reaction (PCR) technique has been the most popular method for this. PCR methods have three steps consisting of denaturation of double-stranded DNA, annealing of the target sequence using primer, followed by extension of the primers complementary to single-stranded DNA with a thermostable DNA polymerase. Theoretically, one piece of target can be amplified into millions copies within several hours since amplification is exponential (Saiki, et al., 1988). PCR assay has been used to detect *L. monocytogenes* in raw milk (Herman, et al., 1995), minced beef meat (Duffy, et al., 1999), and various dairy products (Laberge, et al., 1997). Various genes have been employed for detection of *L. monocytogenes* including *hlyA* (Bessesen et al., 1990; Blais, et al., 1997), *iap* (Bubert, et al., 1992, 1999; Manzano, et al., 1998), phospholipase B (*plcB*), and the aminopeptidase gene (Winters, et al., 1999).

Most PCR assays have employed agarose gel electrophoresis and ethidium bromide staining for reading the results due to their simplicity. However, the sensitivity of this staining is low (Laberge et al., 1997), and not compatible with automation. There have been some improvements of this procedure by using a micro-titer format immobilized with a DNA probe (Manzano, et al., 1998; Cano, et al., 1995), RNA-DNA hybrid detection with antibodies (Fliss, et al., 1995; Blais and Phillippe, 1993), fiber-optic

biosensor (Strachan and Gray, 1995) and dot-blot with non-radio active probe (Li, et al., 2000).

The ligase chain reaction is a method that employs a target amplicon for ligation of oligonucleotides designed to match one and another (Wiedmann, et al., 1992). After ligating, each ligated pair serves as a template for subsequent amplification. This method has been used to distinguish *L. monocytogenes* from other *Listeria* species (Wiedmann et al., 1993, 1992). They indicated that this method had high sensitivity in that it could detect single mismatched nucleotide.

Another type of nucleic acid amplification system (Compton, 1991) is the nucleic acid sequence-based amplification (NASBA). This system detects specific nucleic acid sequences including RNA target. It is an isothermal in vitro amplification process containing three enzymes (reverse transcriptase, RNase H and T7 RNA polymerase) with two specific primers. It provides simultaneous amplification of RNA resulting in rapid accumulation of products. As a result, it requires fewer cycles as compared to PCR. After 48 h of enrichment, <10 CFU/g of *L. monocytogenes* in dairy and egg products were detected by targeting *hlyA* gene using NASBA (Blais, et al., 1997). Uyttendaele et al. (1995) also compared NASBA to ELISA result for detection of *L. monocytogenes* and concluded NASBA was more specific for detecting *L. monocytogenes* while ELISA test was best used for *Listeria* spp.

A fluorescence-based 5' nuclease assay, known as TaqMan (Livak, et al., 1995), detects foodborne pathogens including *L. monocytogenes* (Bassler, et al., 1995), *Escherichia coli* O157:H7 (Oberst, et al., 1998; Witham, et al., 1996), and *Salmonella* spp. (Chen, et al., 1997). This assay employs the endogenous 5' → 3' nuclease activity of *Taq* DNA polymerase to cleave a reporter dye from the 5' end of a labeled linear oligonucleotide probe. Upon hybridization of the probe in PCR amplification reaction,

the reporter dye is hydrolyzed and separated from the quencher dye and a quantifiable fluorescence signal is generated (Holland, et al., 1991; Livak, et al., 1995). Norton and Batt (1999) used this format to target the mRNA of *hlyA* gene using reverse transcriptase (RT). They found that a quantitative result was obtained with *in vitro*-transcribed mRNA over a 3 log-unit range of template concentrations. However, the assessment of viability of *L. monocytogenes* using this method was dependent on the primer location since the more internal targeted primer overestimated of the cell viability. A viability assay targeting mRNA was limited because the degradation or the quantity of the RNA can be influenced by environmental factors as well as physiological state of the cell.

A similar approach using a fluorescently labeled oligonucleotide probe (molecular beacon) has been applied to detect *E. coli* O157:H7 in milk (McKillip and Drake, 2000), and apple juice (Fortin, et al., 2001) with PCR assay. Upon hybridization to the target, the probe was opened to a lineal form and incorporated into a PCR reaction. The fluorescence signal correlated with the amount of templates in the reaction. They found that using this format eliminated the agarose gel electrophoresis of amplified products resulting in a real time detection PCR assay.

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CHAPTER 2

PCR DETECTION OF *LISTERIA MONOCYTOGNES* ON FRANKFURTERS USING
OLIGO NUCLEOTIDE PRIMERS TARGETING GENE ENCODING INTERNALIN

AB¹

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ABSTRACT

A PCR assay targeting the genes encoding internalin (*inl*) AB or F was developed for detecting *L. monocytogenes* in ready-to-eat meats. Four sets of primers were evaluated. One set, targeting a 902-bp region of the *inlAB*, was most specific. The specific PCR product was detected in 51 *L. monocytogenes* strains belonging to 4 different serogroups (1/2a, 1/2b, 1/2c, and 4b). In contrast, the PCR product was not detected in other *Listeria* species (*L. innocua*, *L. ivanovi*, *L. geeligeri*, *L. welshimeri*, and *L. grayi*) and gram positive, non *Listeria* bacteria, indicating that the primer set was highly specific for *L. monocytogenes*. The detection limit of the PCR assay was 10^5 CFU per ml of pure cell culture. However, the assay could detect as few as 10 CFU of *L. monocytogenes* in 25 g of hotdog within 6 h after samples were enriched in modified *Listeria* enrichment broth at 37°C. The total assay time including enrichment was approximately 24 h compared to 3-4 days of conventional method. These results suggest that this PCR assay could be used to rapidly detect *L. monocytogenes* from frankfurters, and possibly other types of ready-to-eat meats.

INTRODUCTION

Frequent outbreaks of listeriosis in the late 1980's prompted the USDA to adopt a zero-tolerance policy for *L. monocytogenes* in cooked, ready-to-eat meats (14). Since that time, numbers of recalls of meat product due to *Listeria* contamination have increased (8, 9, 10, 21).

Various detection methods have been developed for detecting or enumerating this pathogen based on ELISA (20) and immuno-lift technology (7). However, antibody-

based methods often suffer from low selectivity resulting in false-positive results (1), since most commercially developed antibodies are not species specific. The polymerase chain reaction (PCR) assay has advantages in that it can be highly specific, sensitive and rapid (2). PCR assays have been used to detect *L. monocytogenes* in raw milk (17), minced beef meat (13), and various dairy products (18). Various genes have been employed for detection of *L. monocytogenes* including *hlyA* (2, 3), *iap* (5, 19), and the aminopeptidase gene (27).

In this study, a PCR assay targeting the genes encoding internalin (*inl*) AB or F was developed for detecting *L. monocytogenes* on frankfurters, ready-to-eat meat product. Previous reports indicated the role of internalin gene associated with entry of *L. monocytogenes* into cultured cell lines, (4, 12). We designed the primers for the *L. monocytogenes*-specific *inlAB* or F gene. The primer sets were tested for their specificity and sensitivity.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *L. monocytogenes* cultures were either from our laboratory or provided by Dr. Tony Hitchins at U.S. Food and Drug Administration (FDA). Cultures tested are listed in Table 2. 1. Fifty-one isolated *L. monocytogenes* strains belonging to serotypes 1/2a, 1/2b, 1/2c and 4b were used in experiments (Table 2. 1). Seven of the isolates were associated with disease outbreaks where remains were environment isolates. All non-*L. monocytogenes* gram positive species were obtained from our lab collections.

Bacteria were grown overnight in brain heart infusion (BHI) broth (Difco, Detroit, MI) at 37°C with shaking. Appropriate ten-fold dilutions were made in sterile 0.1% buffered peptone water (BPW) and plated on BHI or modified Oxford agar (Difco, Sparks, MD). Plates were incubated for at least 24 h at 37°C and colonies were counted. Tryptic soy broth (TSB) (Difco, Detroit, MI) was used for non-selective enrichment while *Listeria* enrichment broth (LEB) (Difco, Detroit, MI) was used for selective enrichment.

Food assay. Frankfurters were obtained from retail grocery store and confirmed by standard microbiological analysis to contain no detectable *Listeria*. Frankfurters samples were inoculated with *L. monocytogenes* Scott A at a level of 10^0 to 10^2 per g of sample. One ml of appropriate diluted cultures in 0.1% BPW was inoculated in prepared sample. For analysis of low populations (10^0 , 10^1 , and 10^2) of *L. monocytogenes* detection after enrichment step, twenty- five grams of sample were added to 225 ml of TSB or LEB and stomached for 1 min at low speed. Each sample was then incubated at 37°C with shaking and 250 μ l aliquots were analyzed at various sampling times for PCR detection. After 24 h, 0.1ml of samples were transferred to 10 ml of Fraser broth (Difco, Detroit, MI) and then further incubated for 24 h at 30°C, and streaked on modified Oxford agar (Difco, Sparks, MD). For confirmation, typical colonies were further tested with biochemical test using API *Listeria* kit (bioMérieux, Hazelwood, MO). Un-inoculated samples served as controls.

Bacterial template preparation. To test specificity, 100 μ l of BHI broth of cultures were centrifuged for 3 min at 12,000 X g (model 5415C microcentrifuge; Eppendorf, Hamburg, Germany). Cells were washed twice with sterile distilled water and then boiled for 10 min. Five μ l of supernatant fluid was used as a bacterial DNA

template. For sensitivity and analysis of foods, a 500 μ l of sample was centrifuged for 10 min at 12,000 X g. The cell pellet was resuspended in 100 μ l of 1 X PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.0) and then incubated for 15 min at room temperature (20°C) after adding lysozyme (Sigma, St. Louis, MO). Proteinase K (200 μ g/ml) (Roche, Indianapolis, IN) was added and the sample was incubated for 1 h at 55°C followed by boiling for 10 min. After boiling, samples were centrifuged and supernatant fluid was used as a template for PCR reaction.

PCR. Oligonucleotide primers used in this study are shown in Table 2. 2. All primer sets were used for amplification of *inlAB* or F-specific. Oligonucleotides were synthesized by Gibco BRL (Rockville, MD). PCR amplification was performed on a DNA Thermal Cycler 480 (Perkin Elmer Cetus, CT, USA). Amplifications were carried out in 50 μ l reaction mixture containing: 1 U *Taq* DNA polymerase (Roche Diagnostics, Indianapolis, IN), 0.2 mM of each deoxynucleotide (dNTP), 1.5 mM MgCl₂, 1 X PCR buffer, 1 μ M of each primer, dH₂O, and 5 μ l of DNA template. DNA templates were heated at 94°C for 5 min and then amplified for 30 cycles, consisting of 94°C for 2 min, 60°C for 1 min and 72°C for 1 min. A final extension step of 72°C for 10 min was followed by holding at 4°C. Amplified products were electrophoresed on 1% agarose gel (Gibco BRL, Gaithersburg, MD) in 1 X TBE buffer (0.089 M Tris-borate, 0.002 M EDTA; pH 8.0), stained with ethidium bromide and visualized with the Gel Doc System 2000 (Bio-Rad Laboratories, Hercules, Calif.). The standard for gel analysis was a 100 bp ladder (Roche Diagnostics, Indianapolis, IN).

RESULTS

Primers and their specificity. Primer sets used in this study are listed in Table 2. 2. We tested four sets of primer, however, AB1 /3 and F1/3 sets were selected for further study since AB2/4 did not amplify the amplicon. Continued testing of 51 *L. monocytogenes* isolates belonging to 4 different serogroups (1/2a, 1/2b, 1/2c, and 4b) indicated all positive bands on agarose gel with AB1/3 primer set, whereas the F1/3 set produced variable results (data not shown). Therefore, we chose AB1/3 primer set for subsequent study. The PCR product was not detected in other *Listeria* species (*L. innocua*, *L. ivanovi*, *L. seeligeri*, *L. welshimeri*, and *L. grayi*) (Fig. 2. 1) and gram positive, non *Listeria* bacteria (Table 2. 1), indicating that the primer set was specific for *L. monocytogenes*.

Detection limit. The minimum detection level without enrichment of this method was determined by serially diluting *L. monocytogenes* cells (from ca. 10^8 to 10^5 cfu/ml) and analyzing the PCR product from each lysate. Without sample treatment with lysozyme and protease K, the direct detection limit was ca. 10^6 cfu/ml of broth culture. However, the detection limit increased 10-fold after cells were treated with lysozyme and protease K. Fig 2. 2 shows the PCR products amplified from the various concentrations of *L. monocytogenes* in BHI broth. The detection limit was ca. 10^5 cfu/ml of broth sample.

Application of the PCR assay with a hot dog. The ability to detect *L. monocytogenes* on frankfurters by using the PCR method was determined. TSB or LEB were evaluated as enrichment broths. We employed low inoculum level (ranging from 10^0 to 10^2 cfu/g) on frankfurters and subjected samples to the PCR method after enrichment. Each sample was withdrawn from the shaker at various times. Positive signal was first detected after 6 h enrichment in TSB. However, the result was not

consistent. In addition, false-negative results were obtained after using TSB enrichment sample. In contrast, LEB enrichment sample did not produce any false-negative results in triplicate testing. LEB produces a consistent result after overnight enrichment whereas TSB enrichment broth did not. After overnight enrichment in LEB, as few as 10 *L. monocytogenes* cfu per 25g of frankfurters sample were detected (Fig 2. 3).

DISCUSSION

The objective of this study was to test a new primer that targets the internalin gene of *L. monocytogenes*. *InlA* and *inlB* (*inlAB*) are organized as an operon (Dramsi et al., 1993) and implicated as a virulence factor for *L. monocytogenes* since this gene is associated with bacterial invasion of mammalian culture cells (11, 15, 16). The few false-negative results obtained using *inlF* targeted primer (F1/F3) could be sequence variation occurring on *inlF* among *L. monocytogenes* strains. The function of this gene is unclear since an *inlF* mutant did not exhibit changes in virulence in a mouse model (12) whereas *inlAB* did (15). Therefore, we chose the *inlAB* targeted primer set for further study.

The sensitivity of the assay was also determined. The assay was applied directly to pure cultures of *L. monocytogenes*. DNA extraction was necessary to achieve sensitivity of 10^5 cfu/ml of broth sample similar to immuno-assay (23) and other PCR methods (24). Considering the contamination level in foods, if there is a below 10^2 cells/g of sample, it is not possible to direct detect this level of contamination by using PCR assay without enrichment. Unknown inhibitory substances in food samples have

been noted (22, 26). Authors indicated that the PCR assay was inhibited depending on food types i. e. those containing high fat and protein, or types of media used for culturing *L. monocytogenes* cells. Therefore, in order to increase the sensitivity of the assay, the efficient removal of PCR-inhibitors as well as concentrating target organisms from the food matrix system is the key to maximize its performance.

To detect low numbers of cells in food samples, two enrichment procedures were evaluated using less than 10^2 cfu/g inoculum. After overnight incubation, consistent positive results were obtained in hot dog samples using LEB broth but not when using TSB. Either TSB supports the growth of background microflora, which may interfere in detection of *L. monocytogenes*, or unknown inhibitory substances in TSB influenced the PCR detection even after washing and enzyme treatment (22). The longer generation time and gram-positive characteristics of these bacteria resulted in low efficiency of DNA recovery and requiring longer enrichment time (6). It is apparent that either the development of enrichment procedures reducing the detection time or DNA purification methods increasing the sensitivity could be further explored for improving the assay.

The total assay time is about 24 h, compared to 3-4 days for conventional method. The assay time could be further minimized by eliminating the gel running procedure by using real-time PCR which employs a fluorescent labeled probe such as molecular beacon (25). The disadvantage of PCR is that it could be subjected to false-positive results due to the DNA amplification from non-viable cells. Using either an enrichment step or RT-PCR could overcome this disadvantage. Further study is underway to test the feasibility of using RT-PCR as well as validity of this assay in various foods.

Overall, the present developed primer set targeting *inlAB* is highly specific for detecting *L. monocytogenes*. Incorporation with overnight enrichment in LEB medium, low number of *L. monocytogenes* on frankfurters could be detected by using our method within 24 h.

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TABLE 2. 1. Bacterial strains tested in this study

Organisms	Total	PCR positive
<i>Listeria monocytogenes</i>	51	51
<i>L. innocua</i>	11	0
<i>L. ivanovi</i>	1	0
<i>L. murrayi</i>	1	0
<i>L. geeligeri</i>	1	0
<i>L. welshimeri</i>	1	0
<i>L. grayi</i>	1	0
<i>Gram positives</i>		
<i>Staphylococcus aureus</i>	2	0
<i>Bacillus cereus</i>	1	0
<i>Lactobacillus bulgaricus</i>	1	0
<i>L. sake</i>	1	0

TABLE 2. 2. Sequences of primer sets tested in amplification of internalin AB or F in *L. monocytogenes*

Target	Primers	Sequence (5' to 3')	Position ^a
Internalin F	F1	CTCATTCCAGAACCTACGAG	1810
	F3	CGCTTGGTAGTTGTTGATGG	2388
	F2	ACGCACCGAACTTGAAGTGG	1610
	F4	TTGGACGGTGATGTTGGTTC	2468
Internalin AB	AB1	CTTCAGGCGGATAGATTAGG	332
	AB3	TTCGCAAGTGAGCTTACGTC	1233
	AB2	GATATAGGCACATTGGCGAG	884
	AB4	CTACCGCCATCGCTAATAGT	1446

^a Positions based on GenBank accession no. M67471 and U77367

Figure 2. 1. Specificity results of PCR assay amplifying 902 bp of *inlAB* gene of *Listeria* species. Lane 1 and 8 contain 100 bp size markers. Lane 2-6: *L. ivanovi*, *murrayi*, *geeligeri*, *welshimeri*, and *grayi*, respectively. Lane 7 is a positive control (*L. monocytogenes* scott A).

Figure 2. 2. Sensitivity of direct PCR assay for various concentrations of *L. monocytogenes* Scott A in LEB broth. An amplified 902 bp product were electrophoresed on 1% agarose gel and visualized with ethidium bromide staining. Lane 1 is the 100 bp ladder. Lane 2-5: *L. monocytogenes* at 1.3×10^8 , 10^7 , 10^6 , and 10^5 cfu/ml, respectively.

Figure 2. 3. Agarose gel electrophoresis of amplified products of PCR of *L. monocytogenes* Scott A cells in hot dog after overnight enrichment with TSB (lane 2-5) or LEB (lane 6-9) at 37°C with shaking. Lane 1 is 100 bp ladder. Lane 2 and 6 were negative control. Lane 3 and 7: 1.2 cfu/g of frankfurters sample; lane 4 and 8: 12.0 cfu/g; and lane 5 and 9: 120 cfu/g.

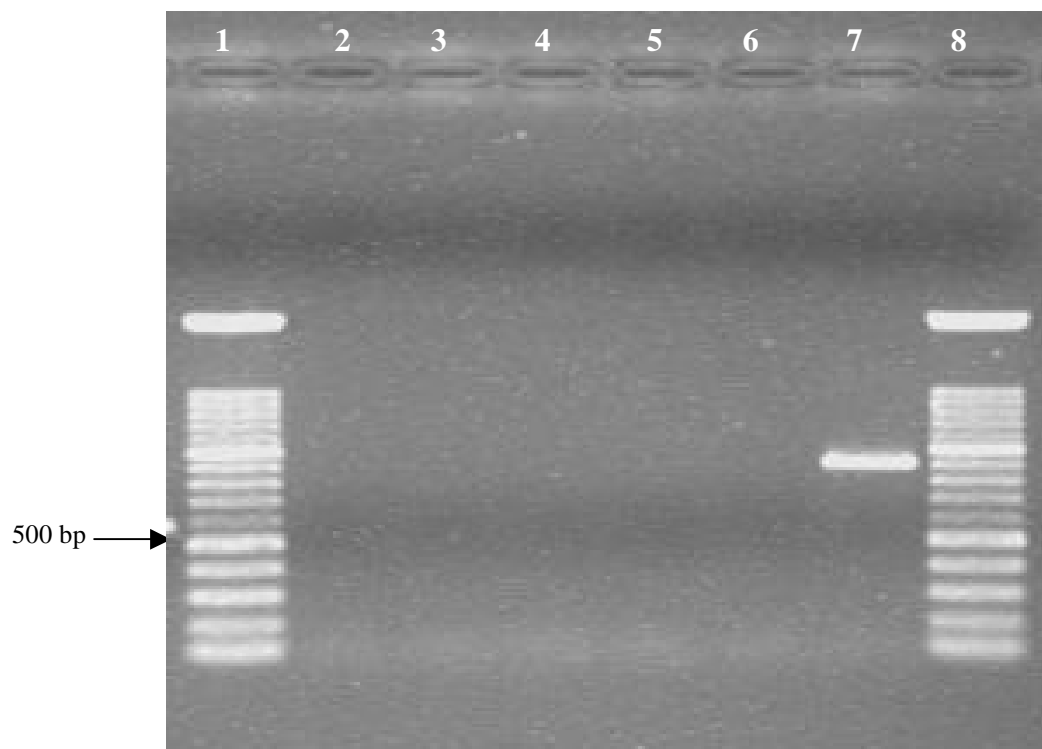


Figure 2. 1. (Jung, et al.)

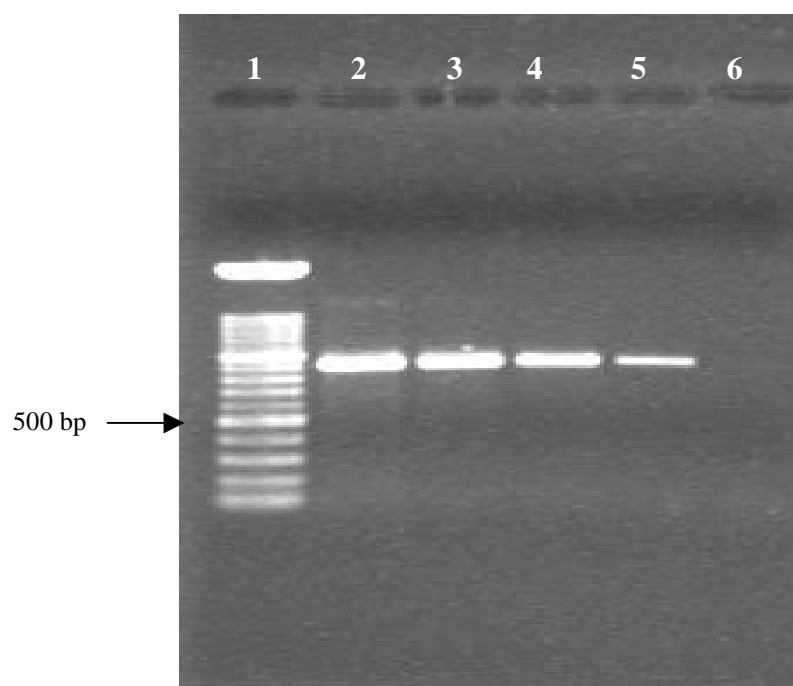


Figure 2. 2. (Jung, et al.)

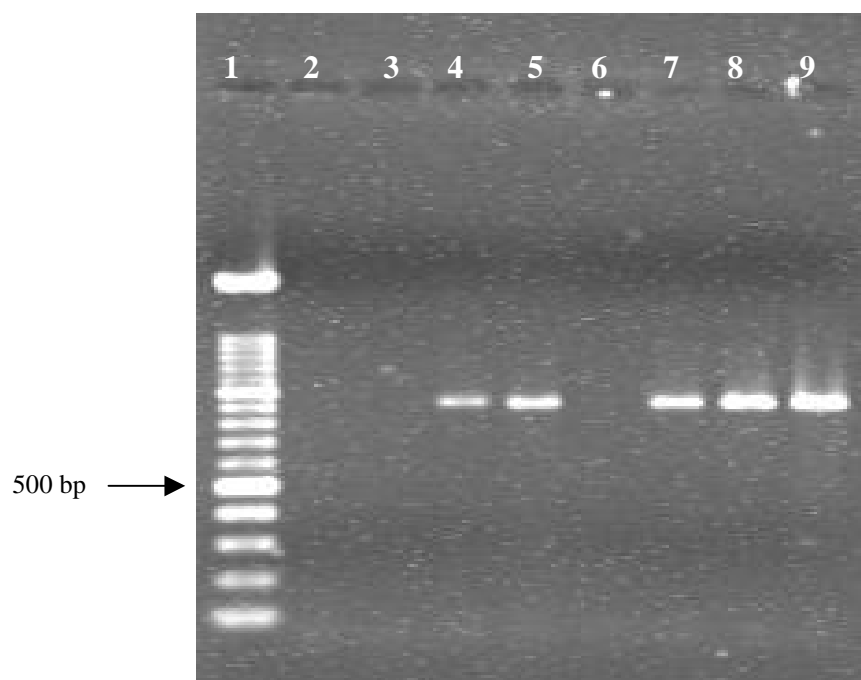


Figure 2. 3. (Jung, et al.)

CHAPTER 3
EVALUATION OF A PCR ASSAY FOR DETECTING *LISTERIA*
MONOCYTOGENES IN VARIOUS FOODS¹

¹Jung, Y. S., J. Chen, J. F. Frank, and R. E. Brackett. To be submitted to International Journal of Food Microbiology

Abstract

Listeria monocytogenes continues to cause food-borne outbreaks leading FDA and USDA to establish a risk assessment. A PCR method targeting internalin AB was evaluated for detecting *L. monocytogenes* at levels ranging from ca. 1 to 100 cfu/g in ground beef, crab meat, smoked salmon, shrimp, deli meat, lettuce, and cheeses (brie and queso fresco) with two enrichment procedures. We could find that depending on the food type, i. e. ground beef or brie cheese case, an efficient PCR detection of *L. monocytogenes* was achieved with different enrichment procedures. Ground beef samples enriched in tryptic soy broth (TSB) at 37°C for 6 h, then transferred to *Listeria* enrichment broth (LEB) with further incubating for 18 h at 30°C was efficient for detecting *L. monocytogenes* cells while we could successfully detect as low as <1 cfu per gram in brie cheese with enriched in LEB medium for 24 h at 37 °C after PCR detection. Among 8 tested foods, our PCR method could detect as low as ca. <1 cells/g of food sample after enrichment, which had complete agreement with results obtained by using the conventional method. These data suggest that the described PCR method is specific and sensitive for detecting *L. monocytogenes* in various foods.

Introduction

Listeria monocytogenes has been involved numerous food-borne disease outbreaks (CDC, 1999, 2000). Pathogen can proliferate at refrigeration temperature producing greater challenges to producers of ready-to-eat foods. The Food and Drug Administration (FDA), and the Food Safety and Inspection Service (FSIS) of the U.S. Department of Agriculture (USDA) recently categorized the risk of foods causing listeriosis depending on various criteria (anonymous, 2001). In this draft risk assessment, the risk characterization has been done based on the origin, composition and process with

the relative probability of consuming foods and listeriosis into the 20 categories. High-risk categorized foods include smoked seafoods, minced meat, deli meat, fresh soft cheeses and cooked ready-to-eat crustaceans.

Determination of *L. monocytogenes* in food samples using conventional methods, requires pre-enrichment, selective enrichment, and biochemical assay. This method is time-consuming and laborious (Lovett and Hitchins, 1988, Donnelly, 1999).

Immunological based methods such as enzyme immunoassay (EIA) and enzyme immunosorbent assay (ELISA), or other immuno-probe based methods are more rapid, but only detect *Listeria* spp. (Mattingly, et al., 1988). There is a need for reliable specific, rapid, and sensitive methods for detecting this organism in foods.

A PCR method targeting the internalin AB (*inlAB*) gene has been developed for this purpose by our group (not published). In this study, we evaluate this method for detecting *L. monocytogenes* in various foods, including ground beef, crab meat, smoked salmon, shrimp, deli meat, lettuce, and cheeses (brie and queso fresco). We also evaluated the enrichment procedures for this method.

Materials and Methods

Bacterial strains and growth media

L. monocytogenes Scott A strain and *Escherichia coli* O157:H7 obtained from our laboratory were test organisms in the experiment. Cultures were grown overnight in brain heart infusion (BHI) broth (Difco, Detroit, MI) at 37°C with shaking. Appropriate ten-fold dilutions were made in sterile 0.1% buffered peptone water and plated on BHI or modified oxford agar (Difco, Sparks, MD). Plates were incubated for at least 24 h at

37°C and colonies were counted. Tryptic soy broth (TSB) (Difco, Detroit, MI) and *Listeria* enrichment broth (LEB) (Difco, Detroit, MI) were used for enrichment in the food assay.

Preparation of food samples

Food samples used in the experiment were ground beef (fat content 25%), processed crab meat, smoked cold salmon, cooked shrimp cocktail, pre-sliced turkey breast, lettuce (iceberg), and cheeses (brie and queso fresco) obtained from local retail store. Each type of foods was purchased at least three different time intervals. Food samples (25 g) were inoculated with dilutions of *L. monocytogenes* Scott A cells achieving levels of ca. 1, 10, and 100 CFU/g. Uninoculated samples served as controls.

Conventional method

After inoculation, twenty-five grams of sample were added to 225 ml of TSB or LEB and stomached for 1 min at low speed using stomacher (Seward, London, United Kingdom). In ground beef, lettuce, and crab meat, 1 ml aliquots from 6 h of TSB enrichment broth at 37°C with shaking were transferred to 9 ml of LEB broth, then further incubated at 30°C for 18 h. All other products were enriched in LEB broth for 24 h at 37°C with shaking. After enrichment, 200 ul aliquots were analyzed via PCR detection while 100 ul of aliquots were transferred to 10 ml of Fraser broth (Difco, Detroit, MI), incubated for 24 h at 30°C, streaked on modified Oxford agar (Difco, Sparks, MD), and then further incubated at 30°C for at least 24 h. For confirmation, typical colonies were further characterized using the API *Listeria* kit (bioMérieux, Hazelwood, MO).

PCR

A 200 µl of enrichment culture was transferred to a microcentrifuge tube and centrifuged for 10 min at 12,000 X g (Eppendorf model 5415C microcentrifuge; Hamburg, Germany). The cell pellet was resuspended in 100 µl of 1 X PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.0) supplemented with lysozyme (3 mg/ml) (Sigma, St. Louis, MO) and then incubated for 15 min at room temperature (20°C). Proteinase K (200 µg/ml) (Roche, Indianapolis, IN) was subsequently added and the sample was incubated for 1 h at 55°C followed by boiling for 10 min. After boiling, samples were centrifuged at 12,000 X g for 10 min. Supernatant fluid was used as a template for the PCR reaction.

The primer set AB1 (5'-CTTCAGGCGGATAGATTAGG-3') and AB3 (5'-TTCGCAAGTGAGCTTACGTC-3') used in this study was developed from our previous study (not published), derived from internalin AB sequence (LISINLAB; GenBank accession no. M67471) (Gaillard et al, 1991). PCR amplification was accomplished by using a DNA Thermal Cycler 480 (Perkin Elmer Cetus, CT, USA). Amplifications were carried out using 50 µl reaction mixtures containing: 1 U *Taq* DNA polymerase (Roche Diagnostics, Indianapolis, IN), 0.2 mM of each deoxynucleotide (dNTP), 1.5 mM MgCl₂, 1 X PCR buffer, 1 µM of each primer, dH₂O, and 5 µl of DNA template. DNA templates were heated at 94°C for 5 min and then amplified for 30 cycles, consisting of 94°C for 2 min, 60°C for 1 min and 72°C for 1 min. A final extension step of 72°C for 10 min was followed by holding at 4°C. Amplified products were electrophoresed on 1% agarose gel (Gibco BRL, Gaithersburg, MD) in 1 X TBE buffer (0.089 M Tris-borate, 0.002 M EDTA; pH 8.0). The gel was stained with

ethidium bromide and visualized with the Gel Doc System 2000 (Bio-Rad Laboratories, Hercules, Calif.).

Results and Discussion

In order to determine the limit of the PCR method for detecting *L. monocytogenes* cells among high concentration of competing microflora, we employed the high numbers of *E. coli* cells (ca. 10^8 cfu/ml) since these cells could outgrow in enrichment at 37°C and attain levels greater than those of *L. monocytogenes* cells. Fig. 3. 1 shows the discriminatory ability of the PCR method to detect *L. monocytogenes* cells in BHI broth mixed with *E. coli* cells. Even though *E. coli* cells were at high concentrations (ca. 10^8 cfu/ml), the detection limit for *L. monocytogenes* did not increase. The lowest detection limit was 10^5 cells in mixed cultures and was similar to the levels in a pure culture system (not published).

Various foods were selected for determining the ability of the PCR method to detect low concentrations of *L. monocytogenes* cells after enrichment. Ground beef samples in TSB were enriched for 6 h at 37°C then transferred to LEB broth with further incubation for 18 h at 30°C. Using this procedure, the lowest inoculum (0.8 cfu/g) was positive after 6 h enrichment in TSB while 4 h enrichment was negative (Fig. 3. 2). Direct sampling from 24 h enrichment in TSB did not show any positive bands on the gel indicating the necessity of enrichment.

Brie cheese produced a different result with samples enriched in LEB for 24 h giving a positive response (Fig. 3. 3). Total indigenous bacterial counts were ca. 10^5 and 10^7 per gram in ground beef and brie cheese but the different results achieved with

enrichment for these samples could be due to the efficiency of increasing the target microorganism among background microflora. In addition, the food matrix system could inhibit the PCR reaction. It is apparent that each food needs the development of specific enrichment procedures for efficient PCR detection of *L. monocytogenes*. Our preliminary study showed inconsistent results for ground pork. Both enrichment procedures did not amplify the gene encoded by the primers in some cases (data not shown). Lantz et al. (1998) observed the PCR inhibition in pork when detecting *Yersinia enterocolitica*. They found PCR inhibitors were the small heat stable molecules. Rossen et al. (1992) investigated various media and their individual substances on the inhibitory effect on PCR. They concluded that complex substances including denaturing DNA compounds or other compounds associated with Mg^{2+} concentrations in PCR reaction played a role in PCR inhibition. Proteinase has also been noted as a PCR inhibitor in milk (Powell et al., 1994). Attempts have been made to eliminate inhibitory factors in PCR food application. Lantz, et al (1994) developed an aqueous two-phase system containing polyethylene glycol to eliminate the inhibitory substances in soft cheese. Washing and concentrating the target organism resulted in the successful detection of *L. monocytogenes* in milk (Cooray, et al., 1994). Other attempts included the use of immuno magnetic capture (Fluit et al., 1993) applied to cheese, centrifugation (Niederhauser, et al., 1992), surface adhesion technique using polycarbonate membrane (Duffy et al., 1999) applied to meat, Triton X-100 treatment for seafoods sample (Agersborg, et al., 1997) and the use of NaI, a chaotropic reagent, removing the inhibitors in PCR detection in soft cheese and minced meat (Makino, et al., 1995). Further study needs to be focused on refining enrichment procedures and sample preparation to enhance the elimination of the false-negative PCR results in testing foods. After successful concentration and extracting of nucleic acid, subsequent detection of

amplified product could further enhance the sensitivity of the assay. Since agarose gel electrophoresis is simple but low in sensitivity (Laberge et al., 1997), and not compatible with automation, there can be improvement of this procedure by using a microtiter format immobilized with a DNA probe (Manzano, et al., 1998), or dot-blot with non-radio active probe (Li, et al., 2000). Therefore, improvement in amplicon detection could also enhance the sensitivity of the assay.

Table 3. 1 and Fig 3. 4 summarize results from food sample co-tested with the conventional method. Our PCR results were in complete agreement with the cultural method. Our PCR method could be completed within 6 h after enrichment as compared to 2-3 additional days required for conventional technique. The use of an immunological-based method could give a result within 1 h; however, it does not give a species-specific result as there is potential for cross-reaction. Since the *L. monocytogenes* is the only a *Listeria* species of concern for human, the false-positives caused by other closely related bacteria are undesirable. Regarding this aspect, PCR method can overcome such low-selectivity.

One disadvantage of using PCR is the potential detection of dead cells. This limitation can be overcome by using reverse transcription (RT)-PCR or enrichment (Herman, 1997, Klein and Juneja, 1997). However, the isolation of RNA from the living cell without DNA contamination is difficult. In addition, the amount of mRNA isolation is dependent on various environmental factors or physiological status of the cell (Uyttendaele, et al, 1996), where there is difficulty in assessing viability and quantitation (Norton and Batt, 1999).

In conclusion, we have evaluated our PCR method against 8 different foods and could detect as few as ca. <1 cell of *L. monocytogenes* per gram of food sample after enrichment. The PCR technique was in total agreement with those obtained with

conventional method. Our results suggest that this PCR method could be used to rapidly detect low-levels of *L. monocytogenes* in foods after appropriate enrichment protocol have been followed.

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Table 3. 1. Detection of *L. monocytogenes* in various foods using PCR and cultural method.

Foods	Inoculum size (cfu/g)	Standard method (modified FDA method)	PCR
Ground beef ^a	Control	0/3	0/3
	0.80 - 1.11	3/3	3/3
	8 - 11.1	3/3	3/3
	80 - 111	3/3	3/3
Crab meat ^a	Control	0/3	0/3
	0.60 - 1.04	3/3	3/3
	6 - 10.4	3/3	3/3
	60 - 104	3/3	3/3
Smoked salmon	Control	0/3	0/3
	0.60 - 1.12	3/3	3/3
	6 - 11.2	3/3	3/3
	60 - 112	3/3	3/3
Shrimp	Control	0/3	0/3
	0.76 - 1.12	3/3	3/3
	7.6 - 11.2	3/3	3/3
	76 - 112	3/3	3/3
Sliced turkey breast	Control	0/3	0/3
	0.60 - 1.00	3/3	3/3
	6 - 10	3/3	3/3
	60 - 100	3/3	3/3
Lettuce ^a	Control	0/3	0/3
	0.60 - 1.04	3/3	3/3
	6 - 10.4	3/3	3/3
	60 - 104	3/3	3/3
Brie cheese	Control	0/3	0/3
	0.84 - 1.08	3/3	3/3
	8.4 - 10.8	3/3	3/3
	84 - 108	3/3	3/3
Mexican cheese (Queso Fresco)	Control	0/3	0/3
	0.60 - 0.76	3/3	3/3
	6 - 7.6	3/3	3/3
	60 - 76	3/3	3/3

^a Samples were enriched in TSB for 6 h at 37°C, then 1 ml was transferred to 9 ml of LEB broth and incubated further for 18 h. All other foods were enriched in LEB for 24 h at 30°C with shaking.

Fig. 3. 1. Ability of the PCR system to detect *L. monocytogenes* in the presence of high number (ca. 10^8 cfu/ml) of *E. coli* O157:H7. Various concentrations of *L. monocytogenes* cells were mixed with 5.5×10^8 cfu/ml of *E. coli* cells. Lane 1 is a 100 bp ladder. Lane 2 to 7, amplified PCR products obtained from total cellular DNA amplification, 1.0×10^9 , 1.0×10^8 , 1.0×10^7 , 1.0×10^6 , 1.0×10^5 and 1.0×10^4 cfu of *L. monocytogenes*, respectively. Lane 8 is a negative control.

Fig. 3. 2. Detection of amplified PCR products from ground beef inoculated with 10^0 cfu/g (lane 3, 7, and 11), 10^1 cfu/g (lane 4, 8, and 12), and 10^2 cfu/g (lane 5, 9, and 13) after enrichment in TSB for 4 h (lane 2 through 5), 6 h (lane 6 through 9), and 24 h (lane 10 through 13), respectively. After ground beef samples were enriched in 4 h and 6 h, 1 ml of aliquots were transferred to 9 ml of LEB. Lane 2, 6, and 10 were negative controls. Lane 1 was a 100 bp ladder.

Fig. 3. 3. Examples of the efficiency of enrichment procedure for detecting various concentrations of *L. monocytogenes* in ground beef (lane 2 through 9) and brie cheese (lane 11 through 18). Samples were enriched in TSB for 6 h and then transferred to LEB medium (lane 2-5 and lane 11-14) compared to enriched in LEB for 24 h (lane 6-9 and lane 15-18). Lane 2, 6, 11, and 15 were negative control. *L. monocytogenes* cells were inoculated at 0.8-1.0 cfu/g (lane 3, 7, 12, and 16), 8-10 cfu/g (lane 4, 8, 13, and 17), and 80-100 cfu/g (lane 5, 9, 14, and 18), respectively. Lane 1 and 19 are 100 bp ladder.

Fig. 3. 4. Examples of PCR amplification of DNA extracted from cultures of various foods inoculated with *L. monocytogenes* Scott A. Lane 1 and 22 is 100 bp ladder. (A) Lane 2, 6, 10, 14, and 18 represent the negative control for each food including ground

beef, crab meat, salmon, shrimp, and deli meat. Lane 3, 7, 11, 15, and 19: *L.*

monocytogenes at ca. 10^0 cfu/g, lane 4, 8, 12, 16, and 21: ca. 10^1 cfu/g, lane 5, 9, 13, 17, and 22 at ca. 10^2 cfu/g of each food. (B) Lane 2, 6, and 10 represent the negative control

for foods including lettuce, brie cheese and queso fresco cheese. Lane 3, 7, and 11: *L.*

monocytogenes at ca. 10^0 cfu/g, lane 4, 8, and 12: ca. 10^1 cfu/g, lane 5, 9, and 13: ca. 10^2 cfu/g of each food.

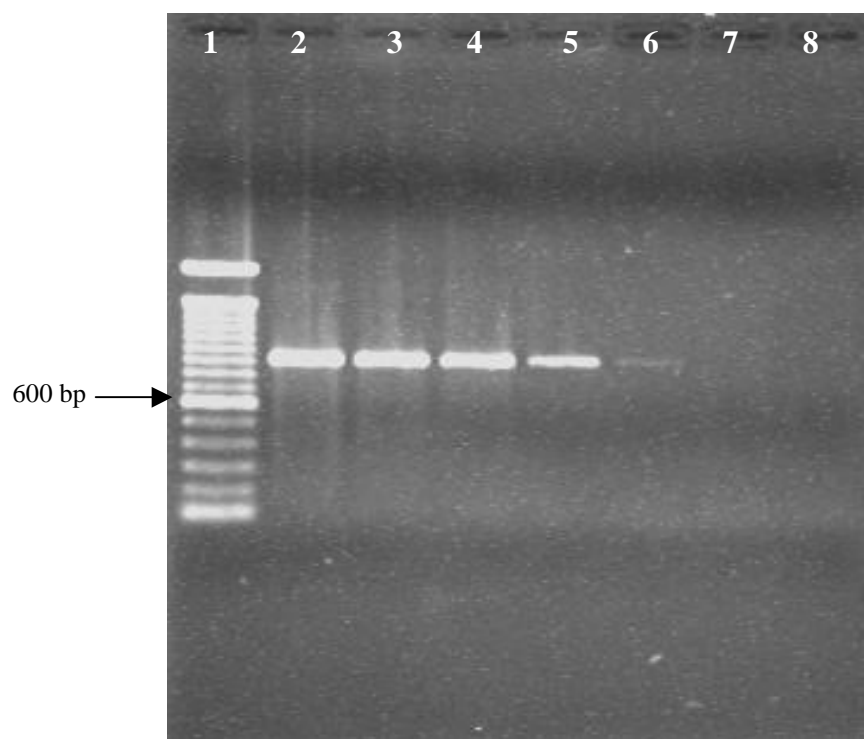


Figure 3. 1. (Jung, et al.)

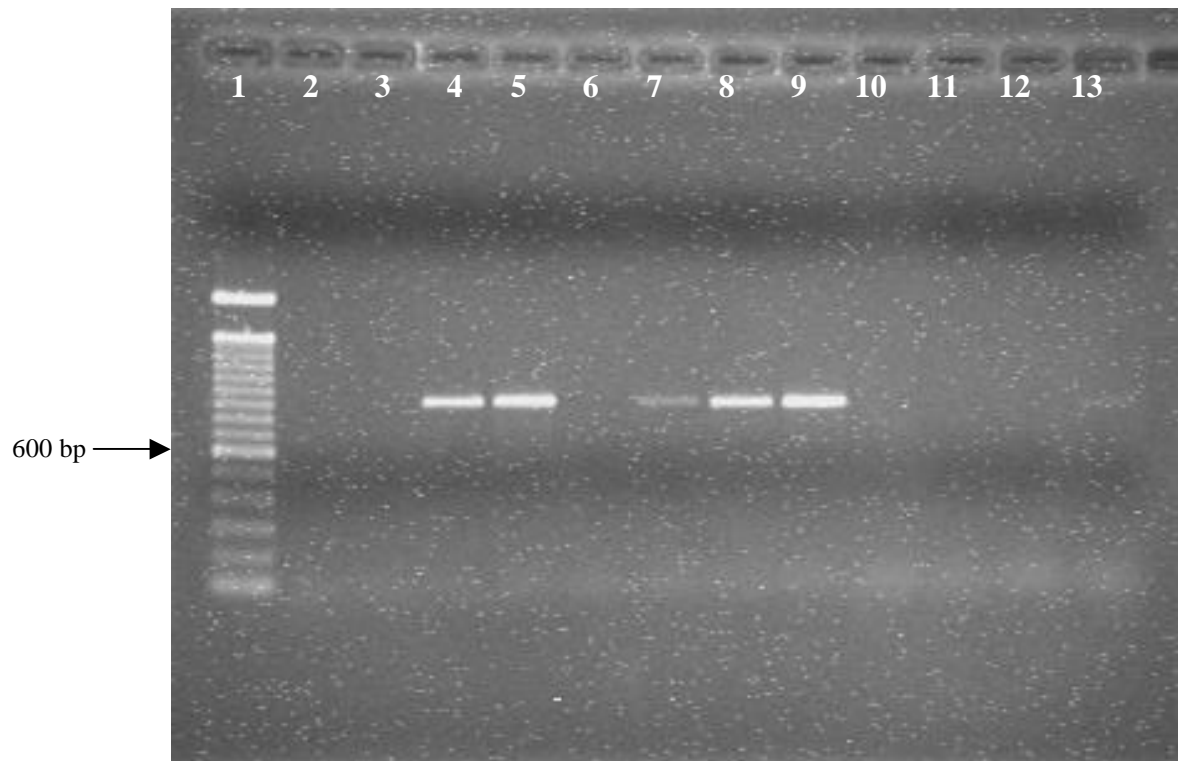


Figure 3. 2. (Jung, et al.)

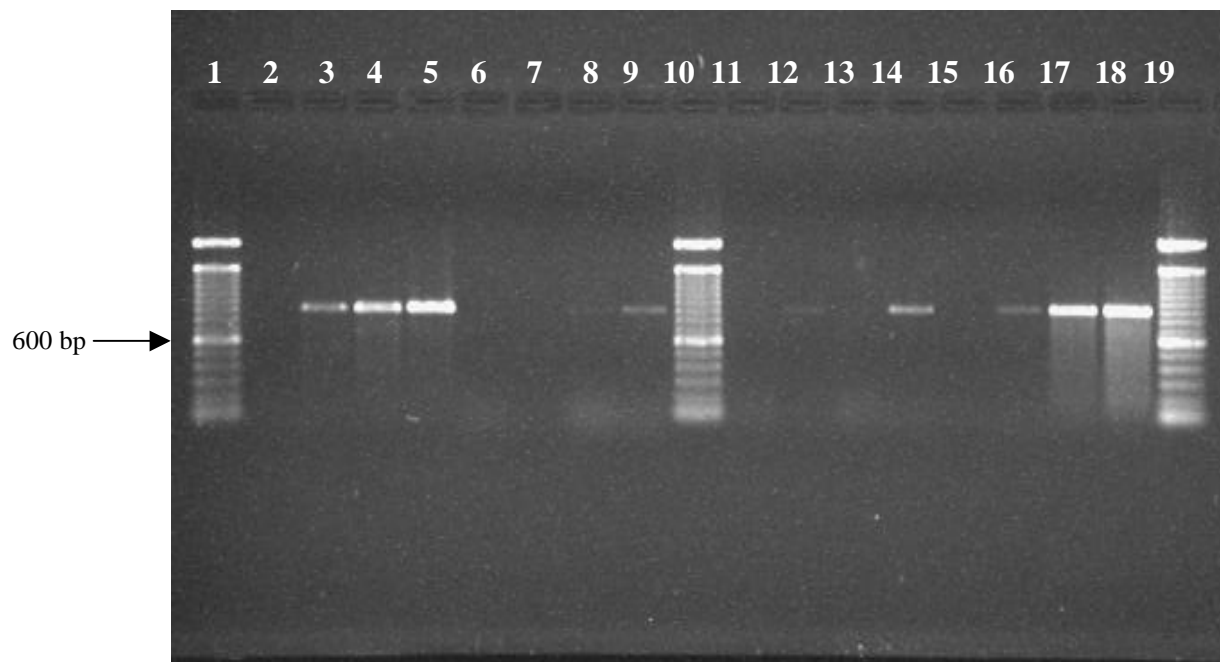


Figure 3. 3. (Jung, et al.)

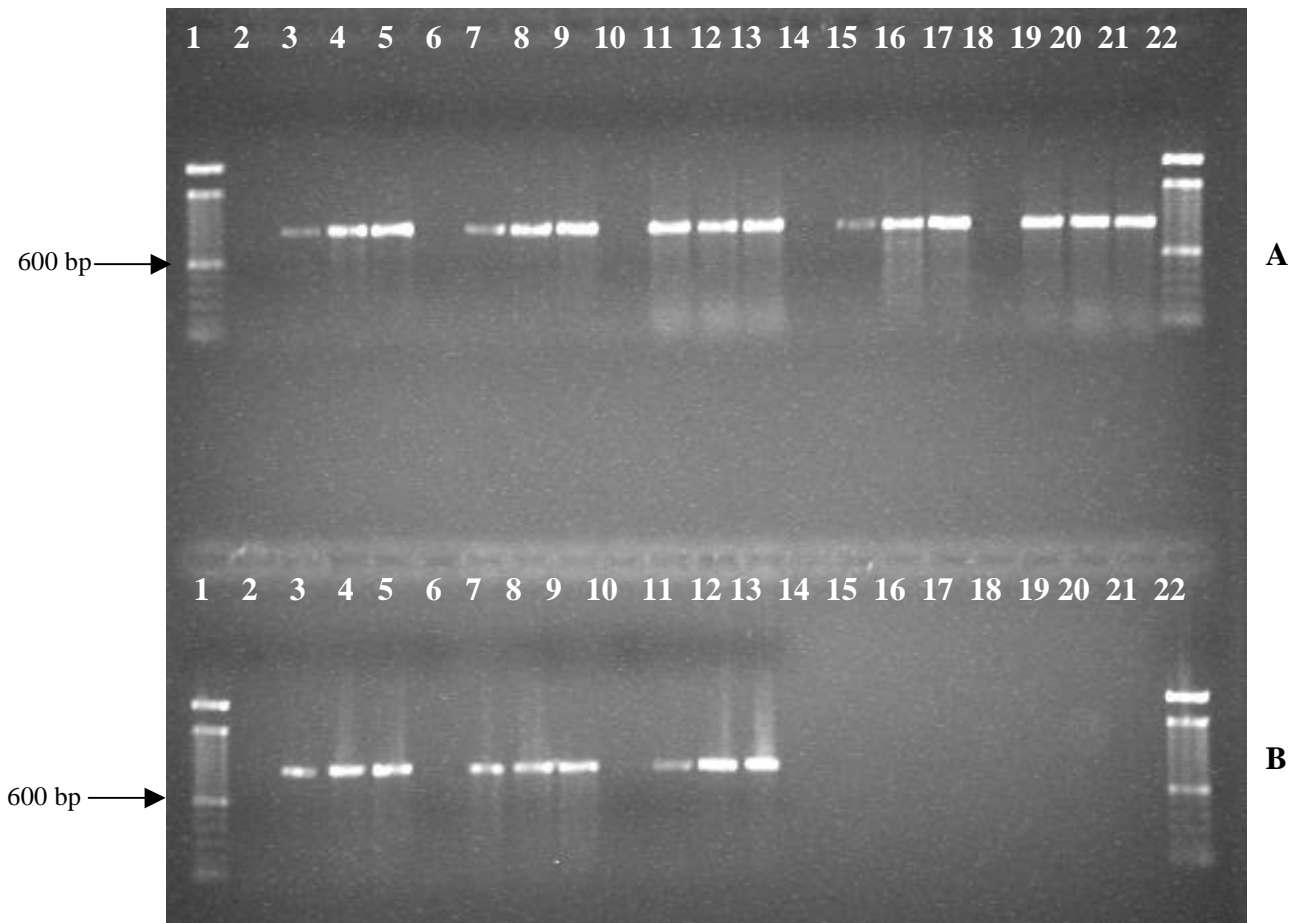


Figure 3. 4. (Jung, et al.)

CHAPTER 4
IMMUNOMAGENTIC SEPARATION AND FLOW CYTOMETRY FOR
DETECTION OF *LISTERIA MONOCYTOGENES*¹

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Abstract

A rapid detection method for *L. monocytogenes* using immuno magnetic separation with flow cytometry was investigated. Four different polyclonal anti-*Listeria* antibodies (Accurate Chemical, Westbury, NY; Kirkeggad & Perry (KPL), Gaithersburg, MD; Maine Biotech., Portland, ME; U. S. Biological, Swampscott, MA) were tested for detection of *L. monocytogenes* as a direct or indirect assay. The efficiency of immuno capturing using magnetic beads was also determined. None of antibodies tested in our protocol differentiated between negative (without cell) and positive (with cell) sample. Attempts to optimize the concentration of magnetic beads or blocking step did not improve the signal difference. The performance of Dynabeads[®] anti-*Listeria* (Dynal, Lake Success, NY) and magnetic bead (Dynal) coated with goat anti-*Listeria* antibody (KPL) was evaluated for capturing efficiency. The recovery rate was even lowered to 0.05% when we used lower concentrations of beads. Plating results confirmed that cells were captured even with low recovery rate ranging from 7 to 21%. However, same antibody (KPL) was used as a fluorescent labeling antibody, the differentiation between negative and positive sample was not obtained.

1. Introduction

Listeria monocytogenes is recognized as one of the major public health concerns in the food industry. This pathogen is ubiquitous within processing plants and is responsible for numerous food-borne outbreaks (CDC, 1999; CDC, 2000). Implicated foods include hot dogs, deli meats, raw vegetables, smoked seafoods, milk, and soft cheese. Traditional detection methods for *L. monocytogenes* are time-consuming as they

require at least 3 to 5 day for completion. Therefore, the development of rapid detection methods is needed to assist in effectively controlling pathogens. Various detection methods have been developed for rapidly detecting or enumerating *L. monocytogenes* based on immunological reaction including enzyme linked immunosorbent assay (ELISA) (Curiale, et al., 1994), antibody-direct epifluorescent filter technique (Ab-DEFT) (Tortorello and Stewart, 1994) and flow cytometry (Seo et al., 1998a).

Flow cytometry has several advantages over these methods in that it can be accurate, sensitive, and semi-automated (Sapiro, 1988). Since the flow cytometer can use multi-parameter analysis of light scattering and fluorescence, target cells can be discriminated by measuring their characteristic, cell size, and surface antigenic properties (Davey and Kell, 1996). This technology has been applied to detect *Salmonella typhimurium* in eggs and milk (McClelland and Pinder, 1994), *L. monocytogenes* from milk (Donnelly and Baigent, 1986) and *Escherichia coli* O157:H7 in enrichment culture from ground beef, apple juice, and milk (Seo et al., 1998b; Tortorello et al, 1998).

Immuno magnetic separation (IMS) has been applied in conjunction with conventional plating (Avoyne, et al., 1997), ELISA (Mansfield, et al., 2000; Holt, et al., 1995), PCR (Rijpens, et al., 1999; Lindqvist, et al., 1998), and flow cytometry (Wang and Slavik, 1999; Seo, et al., 1998a) for detecting *Salmonella* or *E. coli* O157:H7. The advantages of using this method include rapid concentration of bacteria from the food matrix and a reduction in analytical time.

The objective of this study was to detect *L. monocytogenes* by using immunomagnetic separation with flow cytometry. We evaluated several antibodies for their ability to capture and label the cell.

2. Materials and methods

2.1. Bacterial cultures and growth condition

Listeria monocytogenes H7550 (clinical isolates), Scott A (milk outbreak strain), and 12443 (environmental strain) were obtained from the Center for Food Safety (Griffin, GA). Cultures were grown overnight in brain heart infusion (BHI) broth (Difco, Detroit, MI) at 37°C with gentle shaking. Appropriate ten-fold dilutions were made in sterile 0.1% peptone water and plated on BHI or modified oxford agar (Difco, Sparks, MD). Plates were incubated for at least 24 h at 37°C and colonies were counted.

2.2. Immunomagnetic beads and antibodies

Immuno magnetic beads (2.8 um diameter) pre-coated with an affinity-purified polyclonal anti-*Listeria* (Dynabeads[®] anti-*Listeria*, Dynal, Lake Success, NY) were used for capturing *L. monocytogenes*. Uncoated magnetic beads were also obtained from Dynal (Dynabeads[®] M-280 tosylactivated, Dynal). All antibodies used in this study are listed in Table 4. 1. Fluorescein-labeled (fluorescein-4-isothiocyanate, FITC) affinity purified antibodies to *Listeria* (Kirkeggad & Perry (KPL), Gaithersburg, MD; Accurate Chemical, Westbury, NY) were used for detecting *L. monocytogenes* cells after IMS. FITC labeled rabbit anti-*Listeria* (Accurate Chemical, Westbury, NY) was also used for direct detection of *L. monocytogenes*. FITC-labeled goat anti-rabbit affinity purified antibody was used as a secondary antibody (KPL) in our indirect assay.

2.3. Antibody coating of magnetic beads and antibody labeling with fluorescein

Tosylactivated magnetic beads (2.8 um diameter) were coated with antibody according to the manufacturer's instruction. Briefly, beads were vortexed ca. 1 min to

make a homogeneous suspension. Goat anti-*Listeria* IgG antibodies (KPL) in buffer (0.1 M Na-phosphate, pH 7.4) were thoroughly mixed with magnetic beads at the final concentration of ca. 3 ug antibodies/ 10^7 beads as recommended by the manufacture. After blocking with 0.5% (w/v) bovine serum albumin (BSA) (Sigma, St. Louis, MO) for 10 min at 37°C, the mixture was further incubated for 24 h with slow tilt rotation. After incubation, supernatant was removed and then washed twice with PBS (pH 7.4) with 0.1% BSA for 5 min at 4°C, once with buffer (0.2 M Tris, pH 8.5 with 0.1% BSA) for 24 h at 20°C to block free active sites on beads, and then final wash with PBS (pH 7.4) with 0.1% BSA for 5 min at 4°C. Coated magnetic beads were stored at 4°C until use.

Goat anti-*Listeria* antibodies (Maine Biotech., Portland, ME; U. S. Biological, Swampscott, MA) were labeled with fluorescein by using Fluorescein-EX labeling kit (Molecular Probe, Eugene, Oregon) as per company's instruction. Sodium bicarbonate (pH ~8.3, 50 ul of 1 M) was added to 0.5 ml of the ca. 2mg/ml of the antibody solution and stirred for 1 h at room temperature. Hydroxylamine (pH ~8.5) solution was added to stop the binding reaction and then reacted for 30 min at room temperature. The reaction mixture was loaded to the column pre-filled with resin and then eluted with buffer. Labeled antibodies were used in direct assay after IMS.

2.4. *IMS and direct or indirect assay*

One milliliter of cultures was serially diluted in PBS in test tubes, mixed with pre-washed 20 ul of coated-magnetic beads and then incubated for 10 min at room temperature with continuous agitation. After rotation, beads were recovered with a Magnetic Particle Concentrator (Dynal) for 3 min. Supernatants were carefully removed and samples were washed twice with PBS containing 0.1% Tween (PBST) for 10 min.

For our direct assay, 160 ul of serially diluted fluorescen-labeled antibodies were added to each tubes and then incubated for 20 min at 37°C with gentle rotation.

For our indirect assay, rabbit anti-*Listeria* affinity purified antibodies (Maine Biotech) were used as a primary detection antibody. This antibody was incubated with magnetic separated pellets for 20 min at 37°C and then washed twice in PBST. Secondary FITC-conjugated goat anti-rabbit antibodies (KPL) were added, incubated, and then washed twice. After incubating with fluorescein labeled antibodies, the beads were collected with a magnetic concentrator and then resuspended in 200 ul of PBS, which was pre-filtered using 0.2-um-pore-size filter (Millipore, Nalgene, Rochester, NY).

2.5. Flow cytometry

A Coulter Elite Analyzer (Coulter Electronics, Hialeah, FL, USA) equipped with a standard 15 mW argon (488 nm) lamp was used as the excitation source. Forward scatter, side scatter, and log FITC florescence were determined. FITC fluorescence signal was passed through a 525/30 bandpass filter. A gating region was set on the scatter plot to collect fluorescence signals only from the homogenous 2.8 um beads as previously reported (Seo, et al., 1998b). Beads only (without bacteria) or beads (without bacteria) with FITC-conjugated antibody were used as negative controls. Values above the negative control region were set to record the number of positive beads (i. e., greater fluorescence than negative control). Data were acquired for 10,000 particles within the gated region to represent the presence or absence of *L. monocytogenes* antigens.

3. Results and discussion

When heat killed *L. monocytogenes* were used in direct assay, there was no fluorescence signal detected in flow cytometric analysis (FCA). The antibody coated on beads was originally raised to surface and flagella antigen. When cells were boiled, surface structure may be changed resulting in a failure of the antibody/antigen recognition. Live cells were used for our subsequent studies. We had two negative controls, beads only, and beads with FITC-conjugated antibody (FA). Table 4. 2 is data of FCA using different sources of capture antibodies on the magnetic bead. We used the live cell and compared the recovered population after IMS to those before IMS. There was no different fluorescence signal between negative control (Bead + FA) and sample containing cells. Even though the recovery rate was very low, FCM did not discriminate between negative and positive sample, which was confirmed by plate count. Both IMS using different sources of capturing antibodies showed a similar trend.

In order to find the optimum concentration of FA, various dilutions of detection antibodies were used in the assay (Table 4. 3). Based on the plating data, cells were captured but FCA did not differentiate. As detection antibodies were diluted resulting in a reduced signal, there was no difference between negative and positive samples, suggesting detection antibodies may be bound to the bead or some other non-specific site.

Detection antibodies diluted in PBS containing 1% BSA did not improve the signal difference between negative and positive samples (Table 4. 4). Our preliminary results revealed that blocking of the magnetic bead to reduce non-specific binding by incubating beads for 20 min in PBS containing 1% BSA after IMS, did not improve the result (data not shown). In addition, the increase of incubation time for separation (1 h), washing twice (5 min), incubation with FA (40 min), and final washing twice (5 min) did not eliminate the fluorescence signal from the negative control (bead + FA) (data not shown).

Regardless of capturing antibody source, diluting the bead concentrations did not improve the signal differences between negative controls and samples containing the target the target cells (Table 4. 5). Blocking the magnetic beads with increased concentrations (3% and 5%) of BSA for overnight did not improve the signal difference (data not shown). Since the capturing antibody-coated on the Dynal beads was from the goat, rabbit anti-*Listeria* affinity purified antibody (Maine Biotech.) was used as a primary detecting antibody and FITC-conjugated goat anti-rabbit antibody (KPL) was used as a secondary antibody in indirect assay. However, the fluorescent intensity of negative controls (without cells) and positive samples were not different (Fig 4. 1). Plating result confirmed that cells were captured (ca. log 5) into the antibody-coated beads. This indicates that primary antibodies may not be bound adequately to the cell resulting in loss of generating fluorescence signal from secondary antibody. In addition, fluorescence-labeled rabbit anti-*Listeria* (Accurate Chemical, Maine Biotech) and goat anti-*Listeria* (U. S. Biological, KPL) antibodies produced no fluorescence signal difference between negative controls (without the cell) and positive samples in direct assay (Fig 4. 1). For the external control, *E. coli* O157:H7 was subjected to the same protocol except for the antibody sources (i. e. capturing antibody on beads and FITC labeled detection antibody). We could clearly see the large signal shift between negative (with cells) and positive samples (Fig 4. 1). In addition, the recovery efficiency was about 78.6% (data not shown). This external control result revealed that there was no defect with our procedure, technical handling of samples, reagents as well as flow cytometry machine. The only different factor to be considered was an antibody source for capturing and detecting the cell after IMS.

To test if the detection antibody (FA) was binding either on the magnetic bead surface or capturing antibody, Tris-blocked magnetic beads (without capturing antibody),

which differed only in capturing antibody on the surface from antibody-coated magnetic bead, were used to see if these beads also reacted with FA or were able to capture cells. The sources of capturing antibody and detection antibody were the same (goat anti-*Listeria*, KPL). Based on plating results, cells were not captured by Tris-blocked magnetic beads (without antibody coating) while cells were captured with antibody-coated beads (ca. log 5) (data not shown). The signal from the negative control of Tris-blocked (without capturing antibody) beads with FA was not detected indicating FA did not bind to the surface of the bead. This indicates that the capturing antibody did capture the cell when it was incorporated onto the magnetic beads. However, when the same antibody was used as a detection antibody, it also did not discriminate between the negative (without cells) and positive samples.

The use of immuno-magnetic capture has been reported as a useful tool to selectively concentrate target organisms from foods or enrichment broth (Skjerve, et al., 1990). The reported sensitivity was 2×10^2 of *L. monocytogenes* cells/ml in enriched foods. Even lower sensitivity was achieved in ground beef, apple juice, and milk in detection of *E. coli* O157:H7 after enrichment in combination with flow cytometry (Seo, et al., 1998b). The advantages of using this technique would be to selectively concentrate the target organism, changing the ratio of the target to non-target organism (Cudjoe, et al., 1994). However, the low recovery rate (ca. 5%) using magnetic bead separation was observed (Uyttendaele, et al., 2000; Fluit, et al., 1993). Authors also indicated that the use of Dynabead anti-*Listeria* did not selectively recover *L. monocytogenes* in mixtures of non-*Listeria* flora indicating the poor selectivity of the isolation procedure (Uyttendaele, et al., 2000). We observed similar recovery results using Dynabead anti-*Listeria* or dynal bead coated with goat anti-*Listeria* affinity purified antibody (KPL) ranging from ca. 7 to 21% indicating a large loss of cells during

washing. When we were using lower concentrations of beads (Table 5), the recovery rate decreased to 0.05%. Our results clearly implicate that magnetic separation with our protocol may not provides quantitation of *L. monocytogenes*. However, other approaches using micromagnetic beads (<50 nm) have been employed for detecting *L. monocytogenes* (Jacobsen, et al., 1997). They could enhance the recovery rate up to 95% when they used the *L. monocytogenes* cells at concentration from 10^4 to 10^7 /ml. The advantages of using micro magnetic beads over larger beads are rapid reactions, no optical parameters to change, easy to sterilize, and no aggregation of beads (Miltenyi, et al., 1990). In contrast, the larger bead (diameter > 0.5 μ m) has major disadvantages in that the aggregation of beads could non-specifically trap cells resulting in lower specificity of the assay (Jacobsen et al., 1997).

It is apparent that the quality of the antibody is a major important factor in magnetic separation step as is the labeling of the cell. Due to the low specificity and avidity of antibody used in this study, the successful separation and labeling did not result in discriminating cell-containing samples from negative control. Several other *L. monocytogenes* antibodies that have been developed suffer from low specificity (Farber and Speirs, 1987; Sheridan, et al., 1997; Erdenlig, et al., 1999). Since the signal intensity is dependant on the specificity and avidity of antibody, the application of our method is limited. However, the further research should be focused on the improving the magnetic separation using micromagnetic bead as well as using more specific and sensitive antibodies when they become available. Alternatively, the use of nucleic acid probes (i. e. PNA) tagged with fluorescence could provide a higher specificity over antibody probe in a flow cytometry assay. In addition, the assessment of viability by using proper dye in combination with flow cytometry could provide additional valuable information.

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Table 4. 1. Sources of polyclonal antibodies used in this study.

Antibody	Source
Goat anti- <i>Listeria</i> IgG	KPL (Gaithersburg, MD)
Goat anti- <i>Listeria</i> IgG	U.S. Biological (Swampscott, MA)
Rabbit anti- <i>Listeria</i> IgG	Maine Biotech. (Portland, ME)
Rabbit anti- <i>Listeria</i> IgG	Accurate Chemical (Westbury, NY)

Table 4. 2. Detection of live *L. monocytogenes* cells by using different sources of capturing antibodies with flow cytometry

Sample	Fluorescence bead counted (mean \pm SD) ^a	Cells recovered after IMS	% Recovery efficiency	Source of capturing antibody on the bead
Bead	1.5 \pm 2	ND		KPL
Bead + FA ^b	9514 \pm 269	ND		KPL
Bead + 1.1 x 10 ⁶ , + FA	9751 \pm 218	8.7 x 10 ⁴	7.9	KPL
Bead	2.5 \pm 0.7	ND		Dynal
Bead + FA	6721 \pm 87	ND		Dynal
Bead + 9.5 x 10 ⁵ + FA	6141 \pm 193	6.8 x 10 ⁴	7.2	Dyanl

^a The number of fluorescence beads derived from the number of beads with greater fluorescence than the negative control (Bead only) set. A total of 10,000 particles was counted.

^b Goat FITC conjugated antibody (KPL) diluted in PBS (1:10) containing 0.1% BSA.

Table. 4. 3. Detection of *L. monocytogenes* cells by flow cytometry with varying concentrations of FITC-conjugated detection antibody.

Sample	Fluorescence bead counted (mean \pm SD) ^a	Cells recovered afterIMS	% Recovery efficiency	Source of capturing antibody on the bead
Bead	9 \pm 5	ND		KPL
Bead + FA (1:10) ^b	9555 \pm 326	ND		KPL
Bead + 5.6 x 10 ⁵ , + FA (1:10)	9777 \pm 101	6.5 x 10 ⁴	11.6	KPL
Bead + FA (1:20)	9661 \pm 89	ND		KPL
Bead + 1.7 x 10 ⁶ + FA (1:20)	9464 \pm 202	2.5 x 10 ⁵	14.7	KPL
Bead + FA (1:40)	9163 \pm 215	ND		KPL
Bead + 2.8 x 10 ⁶ + FA (1:40)	9297 \pm 12	4.6 x 10 ⁵	16.4	KPL
Bead + FA (1:80)	7487 \pm 243	ND		KPL
Bead + 2.7 x 10 ⁶ + FA (1:80)	7907 \pm 473	2.5 x 10 ⁵	9.3	KPL
Bead + FA (1:160)	5754 \pm 999	ND		KPL
Bead + 2.7 x 10 ⁶ + FA (1:160)	5834 \pm 391	2.5 x 10 ⁵	9.3	KPL

^a The number of fluorescence beads derived from the number of beads with greater fluorescence than the negative control (Bead only) set. A total of 10,000 particles was counted.

^b Goat FITC conjugated antibody (KPL) diluted in PBS (1:10) containing 0.1% BSA.

Table 4. 4. Detection of *L. monocytogenes* cells by using flow cytometry with increasing concentrations of blocking of detection antibody

Sample	Fluorescence bead counted (mean \pm SD) ^a	Cells recovered after IMS	% Recovery efficiency	Source of capturing antibody on the bead
Bead	1 \pm 1.4	ND		KPL
Bead + FA (1:80) ^b	6434 \pm 2078	ND		KPL
Bead + 2.7 x 10 ⁵ , + FA (1:80)	5741 \pm 1581	5.4 x 10 ⁴	20	KPL
Bead + FA (1:160)	3108 \pm 1410	ND		KPL
Bead + 2.7 x 10 ⁶ + FA (1:160)	4930 \pm 523	5.9 x 10 ⁵	21.9	KPL

^a The number of fluorescence beads derived from the number of beads with greater fluorescence than the negative control (Bead only) set. A total of 10,000 particles was counted.

^b Goat FITC-conjugated anti-*Listeria* antibodies (KPL) diluted in PBS containing 1% BSA

Table 4. 5. Detection of *L. monocytogenes* cells with different concentrations of magnetic beads.

Sample	Fluorescence bead counted (mean \pm SD) ^a	Cells recovered after separation	% Recovery efficiency
Bead (1:10) ^b	11 \pm 12	ND	
Bead (1:10) + FA ^c	9897 \pm 35	ND	
Bead (1:10) + 1.3 x 10 ⁶ , + FA	9905 \pm 28	4.5 x 10 ³	0.34
Bead (1:100)	7 \pm 6	ND	
Bead (1:100) + FA	9502 \pm 789	ND	
Bead (1:100) + 1.6 x 10 ⁶ + FA	9914 \pm 95	8.2 x 10 ²	0.05

^a The number of fluorescence beads derived from the number of beads with greater fluorescence than the negative control (Bead only) set. A total of 10,000 particles was counted.

^b Original concentration of the magnetic beads were ca. 6-7 x 10⁸ beads/ml in PBS (pH 7.4) with 0.1% BSA. Beads were diluted in PBS with 0.1% BSA as indicated. There were ca. 1.2–1.4 x 10⁷ (1:10) and 1.2-1.4 x 10⁶ (1:100) beads were used in experiment

^c Goat FITC-conjugated anti-*Listeria* antibodies (KPL) diluted (1:10) in PBS containing 1% BSA

Fig 4. 1. Flow cytometry profiles of direct (a through h) or indirect assay (i and h) by using various sources of antibodies including after IMS. Rabbit anti-*Listeria* antibodies (a through d) obtained from Accurate Chemical (a, b), Maine Biotech (c, d) or goat anti-*Listeria* antibodies (e through h) from U.S. Biological (e, f), KPL (g, through j) were tested. Negative controls (a, c, e, g, i, and k) were compared with positive samples (with ca. 10^5 cfu/ml) (b, d, f, h, j, and l). External control using *E. coli* O157:H7 was used for verifying the detection system protocol (k and l).

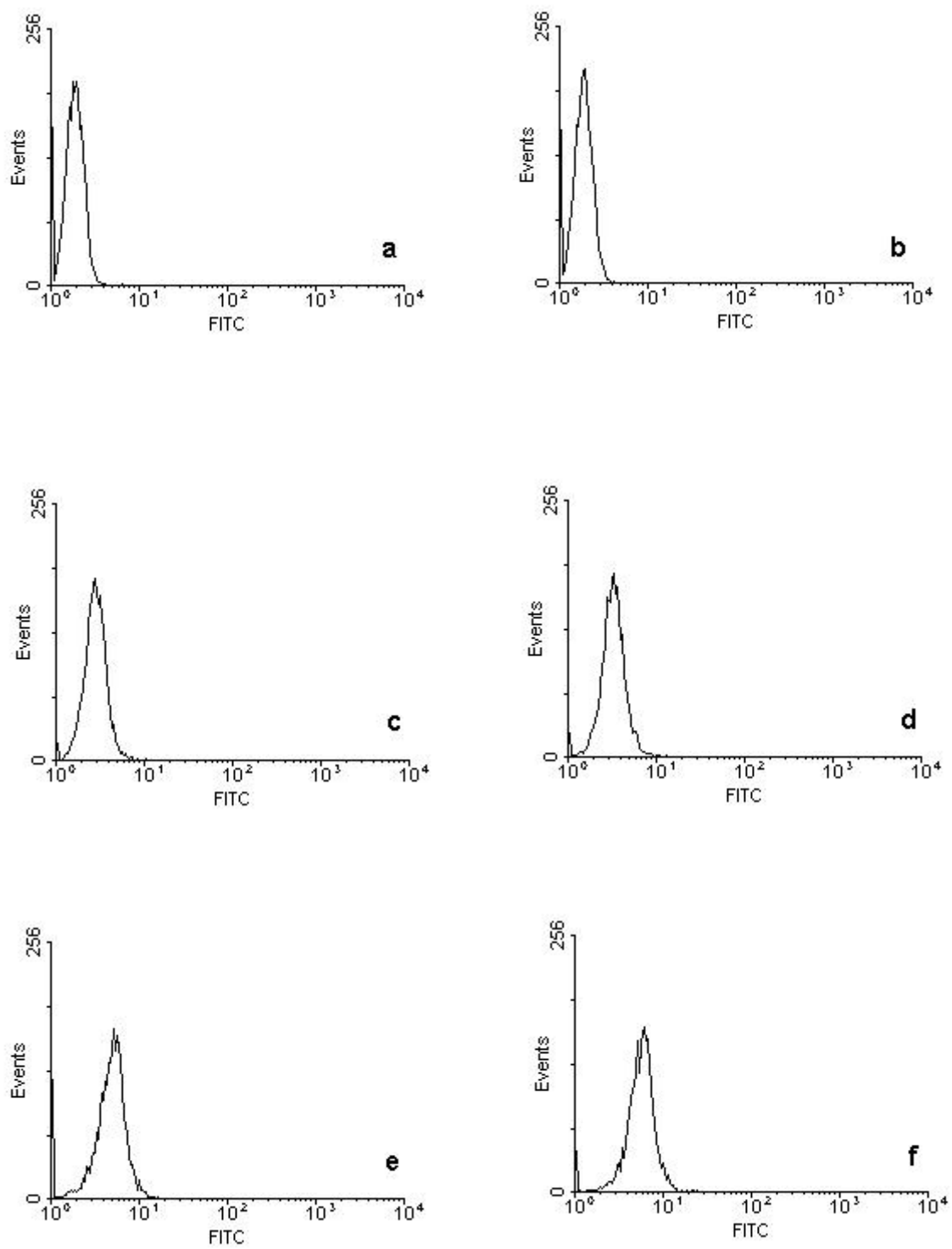
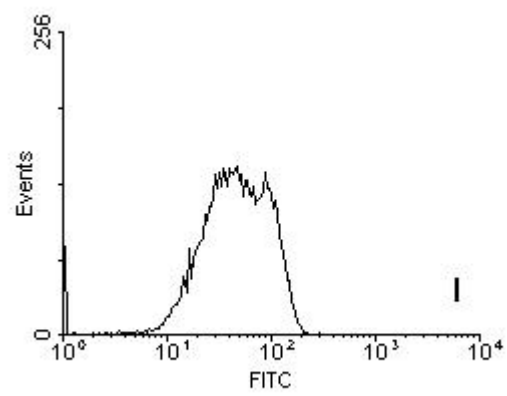
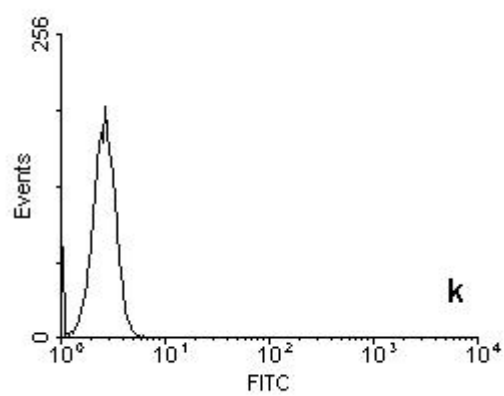
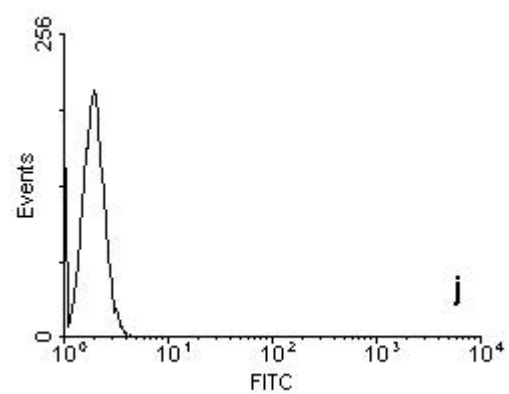
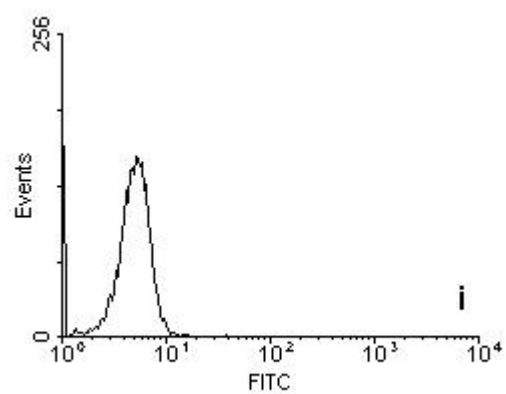
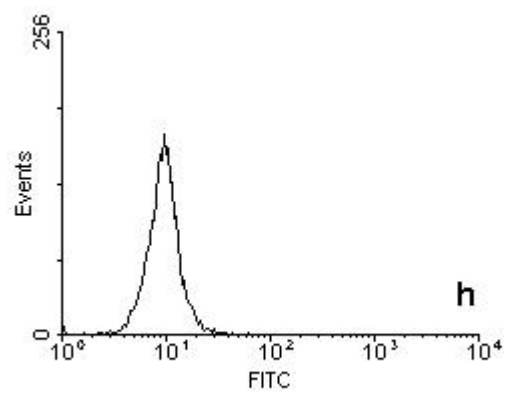
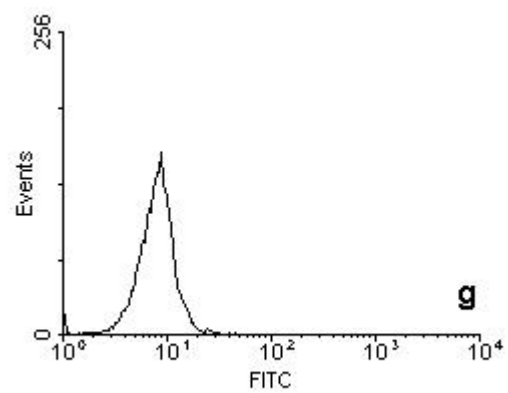


Fig. 4. 1. (Jung, et al.)

Fig. 4. 1., continued



CHAPTER 5
SUMMARY AND CONCLUSION

A PCR detection method for detecting *L. monocytogenes* was developed and applied to various foods in this research. Four sets of primers were evaluated. One set, targeting a 902-bp region of the *inlAB*, was most specific. The specific PCR amplicon was detected in 51 *L. monocytogenes* strains belonging to 4 different serogroups (1/2a, 1/2b, 1/2c, and 4b). In contrast, the PCR product was not detected in other *Listeria* species (*L. innocua*, *L. ivanovi*, *L. geeligeri*, *L. welshimeri*, and *L. grayi*) and gram positive, non *Listeria* bacteria, indicating that the developed primer set was highly specific for *L. monocytogenes*. The minimum detection level without enrichment of this method was ca. 10^5 cfu/ml of broth sample using lysozyme and proteinase K treatment. After overnight enrichment in LEB, as few as 10 *L. monocytogenes* cfu per 25g of frankfurters sample were detected within 24 h.

A developed PCR method targeting internalin AB was evaluated for detecting *L. monocytogenes* at levels ranging from ca. 1 to 100 cfu/g in ground beef, crab meat, smoked salmon, shrimp, deli meat, lettuce, and cheeses (Brie and Queso Fresco) in combination with enrichment procedures. An efficient PCR detection of *L. monocytogenes* was achieved with different enrichment procedures depending on the food type. Ground beef samples enriched in tryptic soy broth (TSB) at 37°C for 6 h, then transferred to *Listeria* enrichment broth (LEB) with further incubating for 18 h at 30°C was efficient for detection of *L. monocytogenes* cells while we could successfully detect as low as <1 cfu per gram in Brie cheese when enriched in LEB medium for 24 h at 37 °C followed by PCR detection. Among 8 tested foods, our PCR method could detect as few as ca. <1 cells/g of food sample after enrichment, which had complete agreement with results obtained by using the conventional method. Our result suggested that the PCR method was specific and sensitive in qualitative study.

A rapid detection method for *L. monocytogenes* using immuno magnetic separation with flow cytometry was also studied. Among four tested polyclonal antibodies, none of them provided a signal difference between negative and positive samples. However, cells were captured when antibodies were incorporated onto the magnetic beads as confirmed by plating result even with low recovery rate ranging from 7 to 21%. An attempt to optimize the bead concentration by serially diluting magnetic beads did not improve a signal. The recovery rate was even lower when fewer beads were used for capturing the cell. It is apparent that the quality of the antibody is a major important factor in magnetic separation step as well as labeling of the cell. Due to the low specificity and avidity of antibody used in this study, the successful separation and labeling did not result in improved recovery rates and failed to discriminate cell-containing samples from negative control using flow cytometry. Future research should be focused on the development of antibody or other sensitive and specific probes to improve the current protocol for the detection of *L. monocytogenes*.