APPLICATION OF FLUORESCENT-TAGGED PRIMERS FOR PCR-BASED NON-GEL DETECTION OF *LISTERIA MONOCYTOGENES*

By

HANNAH NAYAKANTI

Bachelor of Science
Osmania University
Hyderabad, India
1996

Master of Science
Madras University
Madras, India
1998

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Thesis Approved:

Ulrich Melcher

Thesis Adviser

Richard C Essenberg

Peter Muriana

Timothy J. Pettibone

Dean of the Graduate College

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NOMENCLATURE

μL	microliter
μg	microgram
μM	micromolar
bp	base pair
CFU	colony forming unit
dNTP	deoxyribonucleoside triphosphate
ddH2O	double-distilled water
gm	gram
hr	hours
Kb	Kilo base pair
mg	milligram
mM	millimolar
ml	milliliter
ng	nanogram
PCR	polymerase chain reaction
RFU	relative fluorescence units

Literature Review

1. *Listeria monocytogenes* and *Listeria* spp

1.1 General Characteristics

The genus *Listeria* consists of small, short, gram-positive, rod-like bacteria that are 0.4 μm to 0.5 μm in diameter and 0.5 μm to 2.0 μm in length (Broome et al. 1991) Members of the genus are facultatively anaerobic, non-spore-forming microbes that demonstrate characteristic motility by means of flagella when cultured at 20 ^{0}C to 25 ^{0}C (Broome et al 1991). *Listeria* spp. can grow within a pH range of 6 to 9 and a temperature range of 1.0 ^{0}C to 45 0 ^{0}C. The optimum growth of these bacteria occurs between 30 ^{0}C and 37 ^{0}C The genus *Listeria* previously consisted of *L. monocytogenes, L. innocua, L. ivanovii, L. seeligeri, L. welshemeri, L. grayi, L. murrayi,* and *L. denitrificans*. However, based on genomic and phenotypic studies, *L. grayi, L. murrayi,* and *L. denitrificans* have been excluded from the genus (Donachie and Low, 1997). In general *Listeria* spp. are catalase-positive. Voges-Proskauer-positive, oxidase-negative and methyl-red-positive (Donachie and Low, 1997)

1.2 Biochemical Characteristics

Different *Listeria* spp. can be distinguished on the basis of simple biochemical tests that are listed in Table 1 The characteristic hemolysis that is shown by some *Listeria* is important for species differentiation, as only *L. monocytogenes, L. ivanovii* and *L. seeligeri* are hemolytic (Donachie and Low, 1997) *L. monocytogenes* is β-hemolytic and forms a narrow zone of hemolysis around colonies that are grown on blood agar In contrast, *L. ivanovii* when grown

Property	*L. monocytogenes*	*L. ivanovii*	*L. innocua*	*L. welshimeri*	*L. seeligeri*	*L. grayi*
Tumbling motility						
Catalase	+	+	+	+	+	+
Oxidase	-	-	-	-	-	-
Urea	-	-	-	-	-	-
NO_3	-	-	-	-	-	-
β-haemolysis	+	+	-	-	-	-
CAMP test *S. aureus*	+	-	-	-	-	-
CAMP test *R. equi*	-	-	-	-	+	-
Acid production from						
L-rhamnose	+	+	d	d	+	+
D-xylose	+	-	-	d	-	+
α-methyl-D-mannoside	-	+	-	-	+	-
Mannitol	+	+	-	+	+	-
Hydrolysis of Hippurate	+	+	+	+	-	+
Hydrolysis of Esculin	+	+	+	+	+	+
Polysaccharide formation	-	-	+	+	+	-

Symbols: + = 90% or more of strains positive; - = 90% or more of strains negative; d = 11-89% of strains positive

Adapted from Seeliger & Seeliger (1991)

Table 1. Biochemical identification and differential characteristics of the *Listeria* genus.

on sheep or horse blood agar produces a large well-defined zone of clearing around the bacterial colony (Broome et al. 1991). The CAMP test (named after Christie, Atkins and Munch-Peterson who first described this reaction in streptococci) is used to differentiate among the hemolytic species of *Listeria*. It enhances the hemolysis shown by *L. monocytogenes* and *L. ivanovii* using *Staphylococcus aureus and Rhodococcus equi*, respectively (Donachie and Low, 1997). The

CAMP test is performed by streaking (single streak) *S. aureus* and *R. equi* horizontally onto a blood agar plate and streaking (single streak) the different *Listeria* spp. perpendicularly to the *S. aureus* and *R. equi* streak, going up to it but not crossing it. The β-toxin of *S. aureus* binds to the erythrocytes and sensitizes them to the lytic activity of *L. monocytogenes* (Donachie and Low, 1997). Thus β-hemolysis of *L. monocytogenes* synergistically combines with the β-hemolysis of *S. aureus* producing complete hemolysis which is seen as arrowhead-shaped clearings on the agar plate (Fig. 1) (Donachie and Low, 1997). The CAMP test also forms an important basis for separating *L. innocua* from *L. monocytogenes*, since they react similarly to most of the biochemical tests (Table 1). Clinical infections are caused primarily by *L. monocytogenes*, with *L. ivanovii* being the only other pathogenic organism in the genus. *L. ivanovii* is known to be particularly associated with abortions in ruminants (Donachie and Low, 1997)

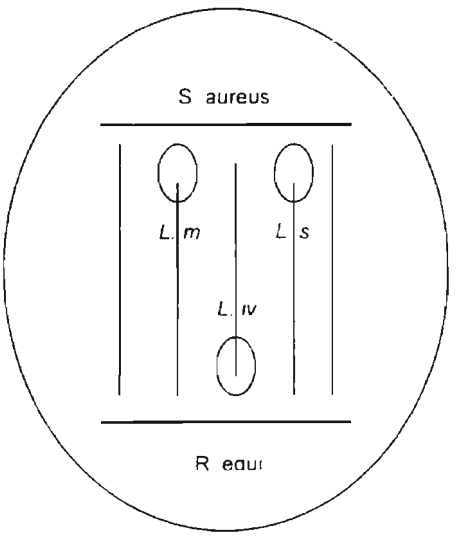


Figure 1. CAMP test used to differentiate *Listeria* spp

The diagram shows *S. aureus* and *R. equi* streaked horizontally on the blood agar plate and the different species of *Listeria* streaked vertically, not touching the horizontal streaks. The enlarged areas represent the typical hemolysis of *L. monocytogenes*, and *L. seeligeri* that is enhanced by hemolysis due to *S. aureus* and that of *L. ivanovii* whose hemolysis is enhanced by *R. equi*. The outer non-enlarged streaks represent *L. innocua* (Redrawn from Silliker Laboratories, Chicago Heights, IL)

1.3 History

In the last two decades, *L. monocytogenes* has emerged from relative microbial obscurity to become an important food-borne pathogen in humans (Seeliger, 1988). In the early 1920s, Pirie described listeriosis as a cause of an epizootic in veld rodents from South Africa known as the "Tiger River disease". In 1926 Murray et al. described a septic illness in laboratory rabbits that was characterized by peripheral monocytosis leading to the name *Bacterium monocytogenes* (Schlech III. 2000). The genus name was later changed to *Listerella* and then to *Listeria* (Seeliger, 1988). Since its initial description, *L. monocytogenes* has been shown to be of worldwide prevalence and has been associated with serious disease in a wide variety of animals including man (Donachie and Low, 1997).

1.4 Growth and survival conditions:

L. monocytogenes multiplies readily in aerobic and microaerophilic conditions at pH values as high as 9.6 though the normal pH range for the survival of *Listeria* spp is 6 to 9 Growth is absent or scant in completely anaerobic conditions At pH values lower than 5.6 the organism can survive but cannot multiply (Donachie and Low, 1997). *L. monocytogenes* can survive at temperatures approaching 0 ^{0}C The organism can also tolerate salt concentrations as high as 30% sodium chloride (Klima and Montville, 1995) *L. monocytogenes* is killed by pasteurization and by various thermal processes that are used to destroy food-borne pathogens However, the organism has been found in processed foods including cheese and milk indicating the presence of either a heat resistant strain of *L.monocytogenes* or the occurrence of post-process contamination (Doyle et al. 2001). In addition to the above mentioned characteristics, the ability to form biofilms helps the bacteria to survive longer under adverse environmental conditions and thus makes it even more difficult to eliminate the organism from food processing environments

1 5 Occurrence

Studies suggest that 1% to 10% of all healthy humans may be intestinal carriers of *L. monocytogenes* and that it can be found in at least 37 mammalian species, 17 species of birds and possibly some species of fish and shellfish (Center for Food Safety and Applied Nutrition, 2001). It is also commonly found in soil, water and decaying plant material Thus, human exposure to *L. monocytogenes* is common, yet invasive listeriosis occurs rarely Factors that influence the occurrence of the invasive disease include the virulence of the infecting organism, the susceptibility of the host and the size of the inoculum.

1.6 Pathogenesis

1.6.1 Invasion and Virulence

After ingestion of contaminated food, the bacteria breach the intestinal barrier and travel throughout the body, crossing the blood-brain barrier and the placenta of pregnant women In order to produce illness, *L. monocytogenes* must survive the acidity of the stomach, penetrate the intestinal lining and grow in the host. The virulence factors that contribute to the pathogenicity of the organism include a hemolysin (Listeriolysin O), a monocyte stimulating agent (an anionic material present in the cell membrane of *L. monocytogenes* that possesses monocytosis producing activity) and several undefined toxins (Marth and Ryser, 1991)

L. monocytogenes attaches and enters into the intestinal endothelial cells and macrophages by a process known as parasite-directed phagocytosis in phagocytic cells and parasite-directed endocytosis in non-phagocytic cells (Faber et al 1991). In non-phagocytic cells, the entry of the bacteria involves the activity of its membrane protein called internalin (Portnoy and Smith, 1997). The binding of internalin to an epithelial transmembrane protein called E-cadherin enables the entry of *Listeria* into the gut epithelial cells (Finlay, 2001) Internalin belongs to a family of bacterial surface proteins called invasins A variety of intracellular bacterial

pathogens use invasins to invade nonphagocytic host cells such as those of the gut epithelia. E-cadherin is expressed on the basolateral epithelial cell surface and enables tight junctions to form between epithelial cells. *Listeria* strains that do not express internalin are noninvasive and cannot invade host epithelial cells, but when forced to express internalin, they become invasive (Finlay, 2001). A single amino acid at position 16 in E-cadherin was found to be responsible for the host specificity of the virulent forms of *Listeria*. Evidence for this was given by Lecuit et al. 1999, who with their mice models confirmed the presence of a proline residue at position 16 in human E-cadherin and a glutamic acid residue in mouse and rat E-cadherin. When the glutamic acid at position 16 was converted to a proline residue, rodent epithelial cells became susceptible to Listerial infection (Babinet et al. 2001). This amino acid difference also explains the observation that mice cannot be orally infected with *Listeria* even though oral infection is a normal route in humans (Babinet et al. 2001).

After internalization, the bacteria are enclosed in the host phagolysosome. The bacteria soon escape from the phagolysosomal vacuole and enter the cytoplasm where they multiply rapidly A pore-forming hemolysin known as listeriolysin O (LLO) helps the bacteria escape the host vacuole LLO is the first identified virulence factor in *L. monocytogenes*. (Gedde et al. 2000). It is a 58.6 KDa protein and belongs to a family of pore-forming thiol-activated cytolysins of which streptolysin O is the prototype. Once in the vacuole the toxin binds to the vacuolar membrane causing oligomerization that leads to pore-formation and membrane lysis (Dangl, 1994). The isolation and genetic analysis of nonhemolytic transposon mutants established the role of LLO in the lysis of the phagosomal membrane (Moors and Portnoy, 1995) In these analyses, insertion of various transposons into the LLO structural gene (*hly*) resulted in the production of inactive truncated proteins. These mutants could not escape the host vacuole and remained avirulent The *hly* gene functioned as a monocistronic unit and when introduced into such LLO-defective strains, restored the wild-type phenotype Further evidence for the role of LLO in the lysis of the phagosomal membranes was obtained when the *hly* gene was cloned into the noninvasive soil bacterium *Bacillus subtilis* and was expressed under the control of an IPTG-inducible promoter In the presence of IPTG, this strain exhibited hemolytic activity and

lysed the phagosomal membrane of a macrophage-like cell line, and grew rapidly and extensively in the host cytoplasm.

1.6.2 Cell-to-Cell Spread of *L. monocytogenes*

Cell-to-cell spreading of *L. monocytogenes* is a complex biological process. After the escape from the host phagocytic vacuole the bacterium is coated with a large population of actin filaments. These filaments rearrange to form a "tail" that is 5 μm long This complex then moves to the surface of the host cell and produces a pseudopodial projection of the cell membrane with the bacterium in its tip. The pseudopodium touches a neighboring cell and the bacterium is released by the action of the LLO and is phagocytosed by the neighboring macrophage. In the second cell, *L. monocytogenes* is again surrounded by the phagosomal membrane. Thus the encapsulated bacterium escapes into the cytoplasm by dissolving the membrane with its hemolysin and the cycle is repeated (Fig. 2).

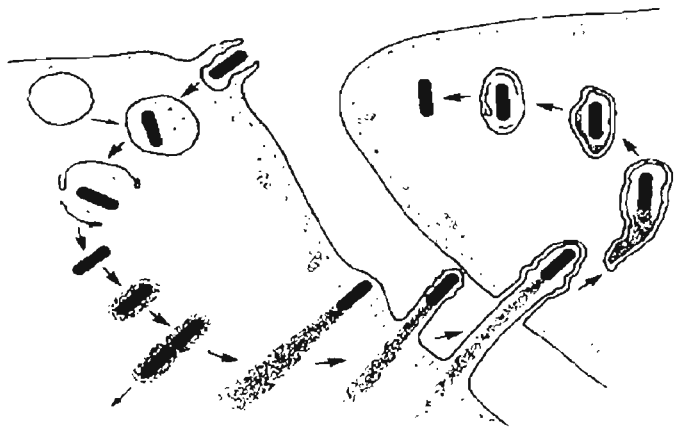


Figure 2. Cell-to-cell spread of *L. monocytogenes*.

After phagocytosis the phagosome fuses with the lysosome to form the phagolysosome. The phagolysosomal membrane is dissolved by the listeriolysin O of the bacterium The organism is then surrounded by actin filaments, which orient into a tail after the bacterial replication A pseudopod extension of the macrophage enables the bacterium to enter an adjacent macrophage (From Portnoy and Tinley, 1989)

It is now known that while in the cytoplam of a host cell *L. monocytogenes* nucleates the assembly of actin off a fine fibrillar material that appears on its surface (Connelly et al. 1990). The *actA* gene of *L. monocytogenes* has been identified as the primary factor required for actin-based motility (Connelly et al. 1990). Though mutants lacking ActA protein successfully enter and grow in the cytosol of eukaryotic cells, they are unable to polymerize host actin and thus cannot spread from cell-to-cell. It was also shown that the *actA* null mutants are 1000-fold less virulent than wild type in a murine model of infection (Connelly et al. 1990). The expression of *actA* gene in *L. innocua*, a nonpathogenic strain that naturally lacks the *actA* gene, resulted in a phenotype of actin-based motility in these bacteria.

The *actA* gene appears to control at least four functions that collectively lead to actin-based motility. These include, initiation of actin polymerization, polarization of ActA function, transformation of actin polymerization into a motile force and acceleration of movement mediated by the host protein profilin (Fig. 3) Profilin is a 15 Kda actin-binding protein that was first implicated in ActA function in 1994 (Connelly et al. 1990). It has a unique ability to bind actin monomers without inhibiting their addition to an actin filament that is kinetically active. It was observed through indirect immuno-fluorescence microscopy that profilin was concentrated at the bacterium-tail interface indicating it to be the site for rapid filament elongation This rapid filament elongation may in turn increase the velocity of the intracellular bacterial motility (Connelly et al 1990)

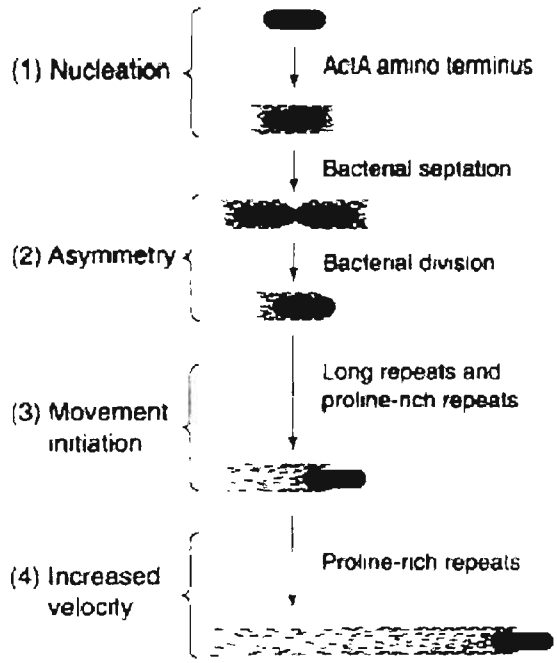


Figure 3. The role of ActA protein in the mobility of *Listeria* spp.

Four steps contributing to bacterial motility are shown. (1) Initiation of actin polymerization. (2) Directional motility, suggesting that ActA only functions at one pole of the bacterium. The asymmetric nature of actin remains controversial though bacterial septation and division are implicated in this process. (3) In addition to actin polymerization at least one more step is essential for mobility to begin. Nothing is known about this step, although separate roles for the ActA long repeats and ActA proline-rich repeats are implicated. (4) The bacterial velocity is increased by action of ActA proline-rich repeats, which recruit vasodilator-stimulated phosphorylation (VASP) and profilin to the site of actin filament assembly (From Portnoy and Smith, 1997)

2. Listeriosis

2.1 History

Serious infection by *L.monocytogenes* causes a general group of disorders known as listeriosis. Listeriosis is clinically defined when the organism is isolated from blood,

cerebrospinal fluid, or an otherwise sterile site (e.g. placenta & fetus) (Broom et al. 1991). The history of listeriosis dates back to 1929 when Gill and Nyfeldt made the first confirmed isolation of the bacterium from sheep and humans respectively (Farber and Peterkin, 1988). It was initially recognized as a disease in animals and was generally regarded as a veterinary disease (Broom et al. 1991). Infected animals were considered to be the primary reservoirs for the organism and it was thought to spread to humans who were in direct contact with them. The first reported case of food-borne listeriosis occurred in 1953. Consumption of unpasteurized milk from a cow with listerial mastitis led to the stillbirth of twins in this case (Food Safety and Inspection Service, U.S. Department Of Agriculture, 2001). In 1966, Gray and Killinger observed many other cases of listeriosis in urban residents who had only an indirect contact with the infected animals (Broom et al. 1991). It was this observation that focused attention on the possibility of food-borne listeriosis.

2.2 Prevalence and risk groups

Though listeriosis has a worldwide prevalence, it occurs more commonly in countries with cold climate such as New Zealand, certain parts of Australia, North America Europe, and the United Kingdom and to a lesser extent in countries with tropical or subtropical climate (Schneider, 1994). Occurrence of clinical disease is usually during late winter or early spring (Schneider, 1994). Most food-borne pathogens cause significant morbidity but little mortality. *L. monocytogenes*, however, causes commonly fatal infection of the bloodstream and the CNS (Schlech III, 2000). In the United States, listeriosis affects an estimated 2500 people each year causing 400 adult and almost 100 fetal deaths (Food Safety and Inspection Service, U.S. Department of Agriculture, 2001). It often occurs in people considered to be at a higher risk This includes new-born babies, pregnant women and immunocompromised individuals (Bellon-Fontaine et al. 1999). Active surveillance of sepsis and meningitis shows the attack rate of listeriosis as approximately 0.7 cases per 100,000 population The rate of infection was 10 cases per 100,000 population in infants and approximately 1 4 cases per 100,000 population in the elderly (Food Safety and Inspection Service. U S Department of Agriculture, 2001)

Neonatal listeriosis is divided into early- and late-onset listeriosis. The early-onset listeriosis develops from intrauterine infection such as maternal sepsis and chorioamnionitis. The characteristics of this syndrome include pustular skin lesions or "granulomatosis infantiseptica" The syndrome usually results in abortion. stillbirth, or premature delivery of a severely infected infant. In late-onset listeriosis the infant is borne healthy with the disease symptoms occuring 7 to 20 days after delivery. Infants show physical signs of meningeal irritation (Schlech III, 2000; Deaver et al. 1995). Case fatality rate of late-onset listeriosis is 26% whereas that of early-onset listeriosis is 38% (Deaver et al. 1995). But residual neurological damage is usually observed in late-onset listeriosis. While transmission of the infection from mother to fetus accounts for early-onset listeriosis, modes of transmission for late-onset disease are not well understood. Nosocomial transmission may be the cause for some postnatal infections (Deaver et al 1995)

In pregnant women, listeriosis may develop at any time during pregnancy, although most infections are detected during the third trimester (Schlech III, 2000). Mothers who deliver infants with early-onset listeriosis often have influenza-like symptoms including persistent fever. Intrauterine infection most likely results from maternal bacteremia followed by transplacental transmission. In some cases a vaginal colonization of *Listeria* spp. may spread to cause intrauterine infection. Occasionally, an infant can be treated in utero if a blood culture performed before the onset of labor is positive for *Listeria* If left untreated, the infant is born with severe infection while maternal listeriosis is resolved spontaneously (Schlech III, 2000)

In immunocompromised individuals, such as patients on immunosuppressive drugs during organ transplantation, AIDS patients and elderly people, the disease includes the occurrence of meningoencephalitis with accompanying ataxia and multiple cranial nerve abnormalities (Schlech III, 2000). Abscesses of the brain and the spinal cord, endocarditis, endophthalmitis, osteomyelitis, bacterial meningitis and septic arthritis may also occur (Schlech III, 2000).

detection of *L. monocytogenes* during routine sampling (Farber and Peterkin, 1991). Another outbreak associated with soft cheese occurred in Switzerland between 1983 and 1987 Soft cheeses have since been involved in outbreaks and several sporadic cases and are thus considered as risk products. According to the European council directive 92/46/EC *L monocytogenes* must not be present in 25g samples of soft cheeses and a positive finding will lead to a recall procedure. USA and Canada have also adopted such a zero tolerance policy for *L. monocytogenes* in soft cheeses (Haario et al. 2000).

In the United Kingdom a massive increase in the incidence of listeriosis occurred in the period between 1987 and 1989, which was in part attributed to contaminated pate (Donachie and Low, 1997) A similar food product was incriminated in an outbreak that occurred in western Australia in 1990. In 1992 a major outbreak in France was traced to the consumption of jellied pork tongues and a more recent outbreak to pork rillettes (Donachie and Low, 1997). The contact of cooked products with soiled surfaces, cross-contamination between raw and cooked channels and the inadequacy of cleaning and disinfecting procedures were the major causes of contamination (Colin et al. 1994). In 1998, an outbreak in Michigan was traced to hot-dogs produced by a manufacturer in the state The events that led to the contamination of the finished products by *L. monocytogenes* were never known. But the hypothesis was that the building construction in the post-processing environment led to contamination of the product contact surfaces and ultimately to the contamination of the finished product (Gombas and Mazzotta, 2001)

According to the U.S Food and Drug Administration (FDA), during the fiscal years 1994 to 1998, *L. monocytogenes* accounted for the greatest number of food products recalled (813) because of microbial contamination (Delgado et al 2000). The frequency with which *L monocytogenes* was detected during environmental surveys has raised doubts as to whether the food manufacturers can effectively eliminate the organism from the processing environment (Deaver et al. 1995). Depending on the shelf-life and the handling of a particular product, even a low initial inoculum in a food during manufacturing can translate into a substantial dose of listeriae

for the consumer. The ability of the organism to grow in refrigeration conditions enables it to grow on processed food before use by the consumer. Considering this the FDA stated that any ready-to-eat (RTE) food products contaminated with *L. monocytogenes* will be considered as adulterated under the federal Food, Drug and Cosmetic Act and will be recalled (Delgado et al 2000). The USDA (United States Department of Agriculture) also, in 1989 instituted a zero tolerance policy for *L. monocytogenes* in RTE products (Deaver et al. 1995).

2.4 Outbreaks caused by other agents

In addition to the large community outbreaks, several nosocomial infections of neonates by *L. monocytogenes* have been reported. Early-onset listeriosis was detected in most of the cases and one or more cases of late-onset listeriosis were detected (Deaver et al 1995). A large nosocomial outbreak of listeriosis occurred in Costa Rica in 1989. More than 3% of infants born during this period developed the disease. Investigation into the outbreak suggested that transmission occurred when contaminated mineral oil was used to bathe the newborns. The outbreak occurred following the delivery of an infant with early-onset listeriosis and presumably, the oil became contaminated during the delivery of the infant Although all newborns were bathed with the contaminated oil, the infants borne by cesarian section were more likely to develop disease. Transmission may have occurred when oil came in contact with the mucous surfaces or when infants aspirated the contaminated oil applied on the face (Deaver et al. 1995).

2.5 Treatment

The time taken for treatment of listeriosis may range from 2 weeks for cases involving uncomplicated sepsis and meningitis to 4-6 weeks for cases involving endocarditis or nonmeningitic disease in immunocompromised patients (Deaver et al 1995) Though *L monocytogenes* is said to be susceptible to various anibiotics like erythromycin, trimethoprim-sulfamethoxazole, chloramphenicol, rifampin, tetracyclins and aminoglycisides, the most commonly used antibiotics for invasive listeriosis are penicillin and ampicillin In some cases it has been observed that chloramphenicol and rifampin antagonize the effect of penicillin In

conditions where the patient is allergic to penicillin, sulfamethoxazole is used for therapy (Deaver et al. 1995).

3. Detection of *L. monocytogenes*

Some authors have mentioned that *L. monocytogenes* has the ability to grow over a temperature range of –1.5 ^{0}C to 50 ^{0}C and a pH range of 4.3 to 9.6. It can also survive up to 25.5% sodium chloride (Donnelly, 2001). In addition to these characteristics, the ubiquitous nature of the organism makes it difficult to control *L. monocytogenes* by using classical food preservation techniques. Thus it continues to be a challenge for the food processing plants (Donnelly, 2001). Though the current stringent procedures in the food processing plants are able to reduce the incidence of bacterial contamination in food, they could not prevent the occurrence of serious outbreaks of listeriosis (Food Borne Pathogens, Center for Biological Nanotechnology 2001). As a result the total elimination of pathogenic bacteria from food has been stated as "impractical and may be impossible" by the World Health Organization (food borne pathogens, Center for Biological Nanotechnology 2001). The detection of the pathogen thus plays an important role in prevention of listeriosis.

3 1 Traditional detection methods

In the United States the two major agencies that are responsible for inspecting food-processing plants have adopted a zero tolerance policy for *L. monocytogenes* These agencies include the USDA's Food Safety and Inspection Service (FSIS) which is responsible for inspecting plants that slaughter and/or process meat, poultry and egg products and the FDA, which oversees the safety of most other foods. The USDA-FSIS has devised an isolation scheme to detect the presence of *Listeria* in meats and meat products (Table 2) According to this procedure it takes 6-10 days to confirm the presence of the pathogen The FDA has also devised a scheme which takes almost the same time period as the USDA procedure but differs in the selective enrichment and the plating media used along with the size of the sample tested (Donnelly 1988). It is difficult to store the food products for such long periods of time, especially

3.2 Rapid methods of detection

A fast and simple method that was developed for detection and identification of *L. monocytogenes* was Polymerase Chain Reaction (PCR) (Cantoni et al 1997). This technique permits the rapid amplification of specific DNA sequences by a factor of up to 10^7 (Border et al. 1990). The sensitivity of the system is typically compared to the detection of a single bacterium (Border et al. 1990). Even though carrying out the PCR involves difficulties such as initial optimization, these difficulties are more than overshadowed by the sensitivity and the wide dynamic range of the technique (Hermann et al. 1998). During the past 10 years PCR has been used to detect *L. monocytogenes* from various food products like milk, cheese, eggs, meat and meat products, fish and fish products etc. Analysis of the PCR product has usually been done using ethidium bromide-stained agarose gels (Bassler et al. 1995). The making and the running of the agarose gels are considered as an additional time consuming and cumbersome procedure by the food industry.

3.2.1 TaqMan PCR (5' Nuclease assay)

A new fluorogenic PCR-based assay that allows homogeneous quantification of the initial template concentration has been developed. This assay is known as the TaqMan PCR or the 5' nuclease PCR assay (Bassler et al. 1995). The assay takes advantage of the endogenous 5'→3' nuclease activity of the Taq DNA polymerase (Bassler et al 1995) A probe (TaqMan probe) that is specific to the target sequence is designed such that it has a fluorescent reporter dye (6-carboxyfluorescein, tetrachloro-6-carboxyfluorescein, or hexachloro-6-carboxyfluorescein) covalently attached to its 5' end and a fluorescent quencher dye (6-carboxy tetramethyl rhodamine) attached two or more bases down stream from the reporter dye The proximity of the quencher to the reporter results in reduction of the emission intensity of the reporter dye (Bassler et al. 1995) As the Taq DNA polymerase enables the PCR primers to extend it also cleaves the TaqMan probe only when the probe is hybridized to the target sequence. This separates the reporter dye from the quencher dye resulting in an increase in the fluorescence intensity at 518nm This increase in intensity is quantitative of the initial amount of target template present

(Bassler et al. 1995). Repeated cycles of denaturation, annealing and extension result in exponential amplification of the PCR product and of the fluorescent intensity (Fig. 4) (Bassler et al. 1995).

The position of the reporter and the quencher moieties on the TaqMan probe is critical. It is essential that they are not too close to each other, as the 5'→ 3' hydrolysis by the Taq DNA polymerase should be performed between the fluorophore and the quencher (Bhatnagar et al. 1997). However this requirement is a major drawback of the assay because the efficiency of the energy transfer decreases with the inverse sixth power of the distance between the reporter and the quencher. As a result the background emission from the unhybridized probe can be very high (Bhatnagar et al. 1997).

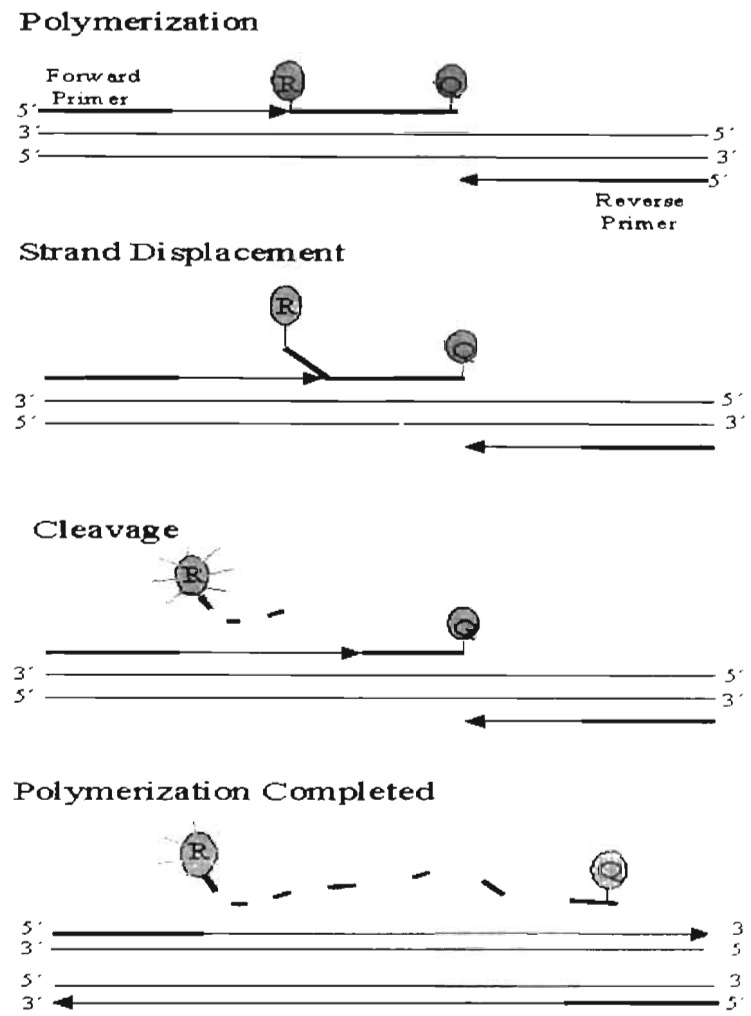
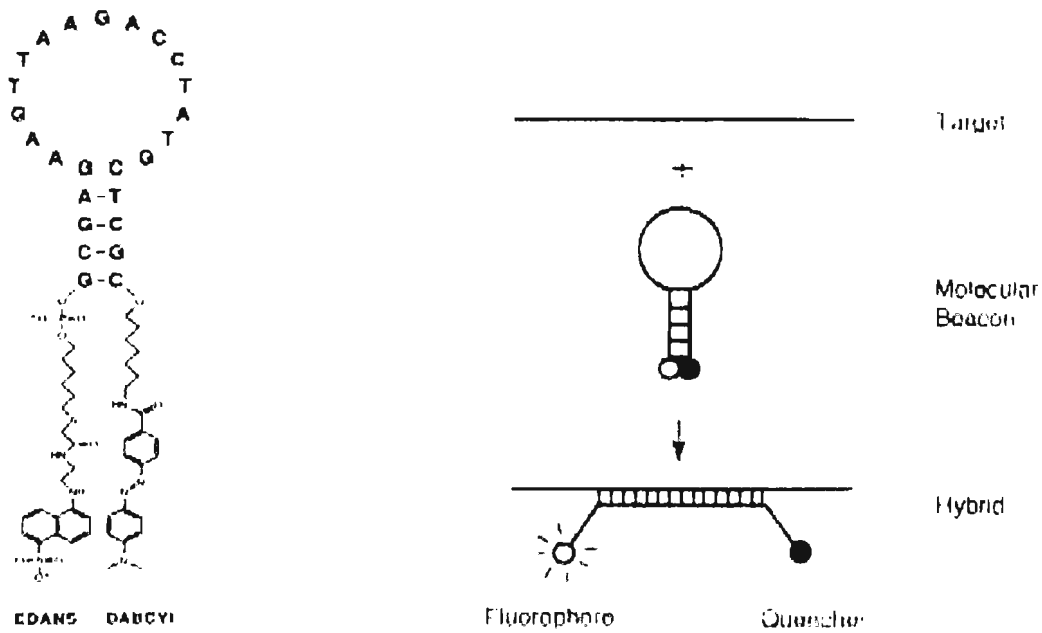


Figure 4. Fluorogenic 5' nuclease assay

Step 1 shows a probe with the fluorescent moieties attached to an internal complementary sequence As in normal PCR the forward and the reverse primers are extended In step 2 the probe is displaced as the polymerase extends the primers. Step 3 shows that the 5' nuclease activity of the polymerase cleaves the fluorophore from the probe In step 4 the polymerization is completed and a fluorescent signal is generated (From Core facility, Department of International Medicine, University of Freiburg Medical Center Freiburg, Germany).



EDANS: 5-(2'-aminoethyl) aminonaphthalene-1-sulfonic acid
DABCYL: 4-(4'-dimethylaminophenylazo) benzoic acid

Figure 5. The structure and the principle of the Molecular Beacons

Panel (A): The basic structure of the molecular beacon.

It is a hairpin-shaped dual-labeled oligonucleotide with a fluorescent reporter group (EDANS) on one end and a fluorescence quencher group (DABCYL) at the other end (From Kramer and Tyagi, 1996).

Panel (B): The principle by which molecular beacons operate

In the presence of the complementary target sequence, the probe hybridizes to the target increasing the distance between the fluorescent reporter and the quencher and therefore resulting in emission of detectable fluorescence (From Kramer and Tyagi, 1996)

PCR can also be used for real-time quantification of the initial amount of nucleic acid present in the sample being tested. The Light Cycler™ is an instrument that has been designed for this type of assay. The instrument features rapid thermal cycling for PCR and on-line detection of the reaction kinetics occurring in the same reaction vessel (De Silva et al. 1998). The instrument can use fluorescent probes such as the molecular beacon and the taqman probe but most commonly, it uses the SYBR green I dye and the hybridization probes.

3.2.3 SYBR green I dye

The SYBR green I dye, like ethidium bromide, binds to double stranded (ds) DNA. The dye is thought to bind to the minor groove of the ds DNA (Hermann et al. 1998) In it's unbound state the dye has relatively low fluorescence but once it is bound to the DNA it fluoresces very brightly and the fluorescence increases in proportion to the amount of PCR product (Fig. 6) (Hermann et al. 1998). SYBR green is easy to use in quantitative PCR as it binds to any PCR product. However, this virtue has a major drawback in that the dye does not recognize specific ds DNA (PCR product) and can bind to non-specific ds DNA such as the primer dimers (Hermann et al. 1998). This non-specific binding of the dye makes quantification of low copy numbers very difficult. However the Light Cycler™'s continuous monitoring capability has been used to overcome this problem (Hermann et al 1998). The specific PCR product melts at a higher temperature than the smaller non-specific primer dimers. A sharp reduction in the level of fluorescence can be observed at the melting temperature (Tm) of the specific PCR product. Thus the melting curves obtained from the thermal cycles can be monitored continuously to verify and confirm the specific product (Hermann et al 1998) Though this technique is simple the confirmation of the specific product may require additional knowledge and expertise. When considered for a large industrial application this requirement may be regarded as a drawback

(A)

(B)

(C)

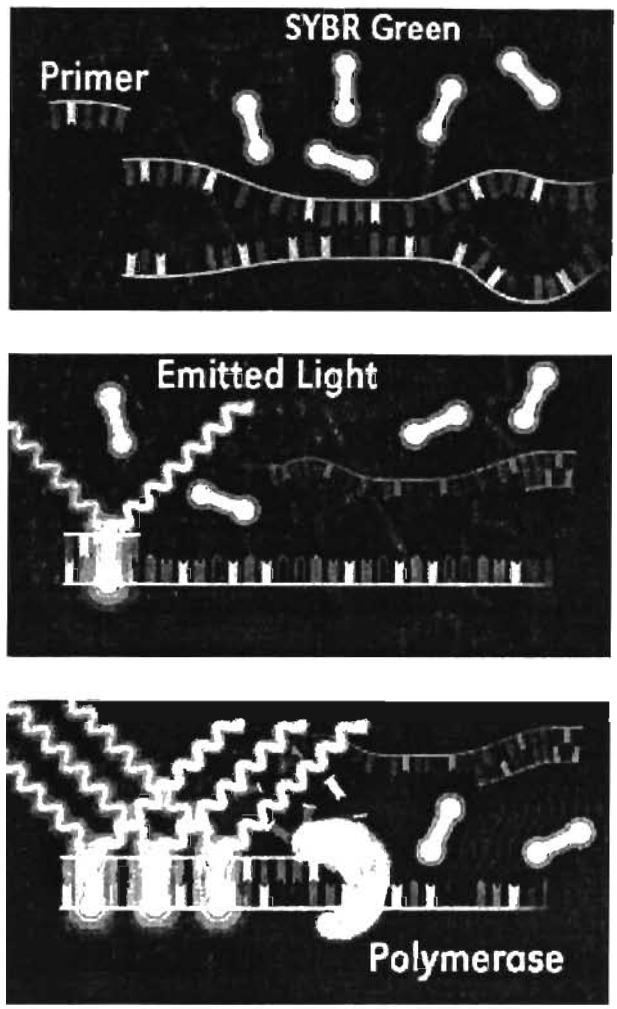


Figure 6. Fluorescence detection using SYBR Green I dye

(A) The PCR mixture containing the denatured DNA, the primers and the dye. A minimal background fluorescence signal is generated by the unbound dye

(B) When the primer anneals to the target sequence the dye is incorporated into the resulting double strand

(C) Increase in fluorescence is observed which is proportional to the amount of DNA amplified (From Boehringer Mannheim, Switzerland)

3.2.4 Hybridization probes

The Light Cycler™ can also use hybridization probes for more specific detection of the product (De Silva et al. 1998). The Light Cycler™ uses the concept of Fluorescence Resonance Energy Transfer (FRET) wherein fluorescence energy transfer occurs between two adjacent fluorophores (De Silva et al. 1998). The probes are designed such that they are specific to an internal sequence within the target. The 3' end of one probe is labeled with a donor fluorophore (fluorescein) and the 5' end of the adjacent probe is labeled with an acceptor fluorophore (LC Red 640) (Bernard et al. 1999). In order to prevent the extension of the probe labelled with LC Red 640, its 3' end is blocked by dephopsphorylation (Fig. 7) (Bernard et al. 1999). The Tm of the probes should be 5.0 ^{0}C to 10.0 ^{0}C higher than the Tm of the primers (Bernard et al. 1999). The donor fluorophore absorbs light from the blue light-emitting diode (LED) of the Light Cycler™ instrument, the resonance energy from this fluorophore is absorbed by the adjacent acceptor fluorophore and the fluorescence emitted by it is detected and measured (De Silva et al. 1998). This energy transfer occurs only when the two probes are in close proximity (i.e bound to the target). The FRET signal increases with each thermal cycle and is proportional to the amount of specific PCR product available for hybridization (Fig. 7) (De Silva et al 1998) Though the sensitivity of the technique is high the method requires designing, synthesis and optimization of the hybridization probes in addition to the optimization of the PCR.

(A)

(B)

(c)

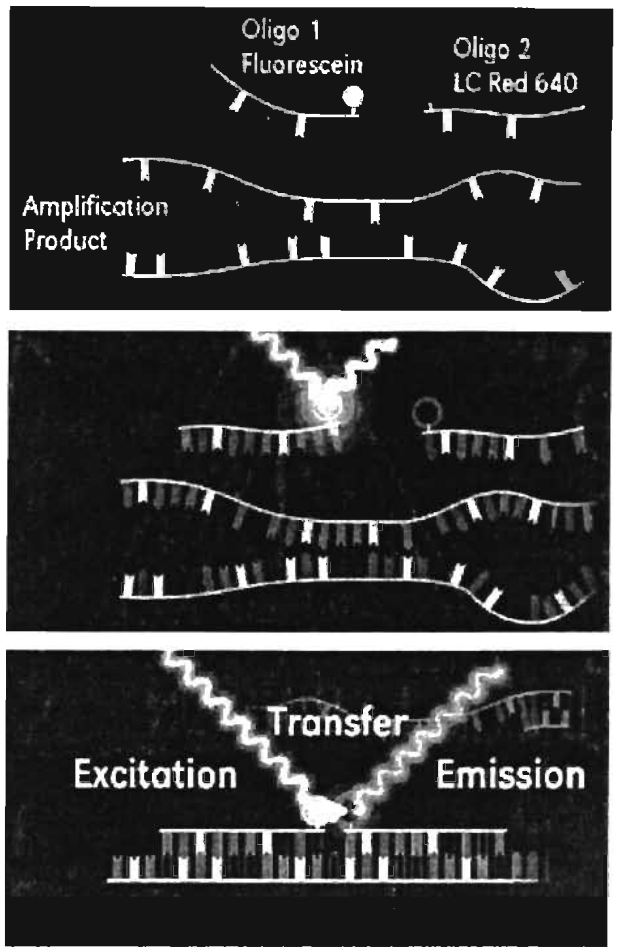


Figure 7. Hybridization probes used for PCR monitoring by the Light Cycler™

(A) The top panel shows the four essential components for this technique, two different fluorescence-labeled oligonucleotides, and the amplification product. As shown in the figure, oligo 1 has fluorescein at its 3 end whereas oligo 2 has LC Red 640 at its 5' end.

(B) The oligonucleotides are selected and synthesized such that they hybridize to the amplification product in a head to tail manner

(C) When the hybridization takes place the two-labeled oligonucleotides are in close proximity resulting in FRET (which is highly dependent on the distance between the two fluorescent moieties). The light emitted from the light cycler's LED (Light Emitting Diode) excites the first dye (fluorescein) causing it to emit a green fluorescence. This emitted energy excites LC Red 640 attached to the second oligonucleotide. As a result LC Red 640 emits red fluorescence of even longer wavelength (From Boehringer Mannheim , Switzerland)

3.3 Amplifluor Uniprimer™ system

The system used in the work reported here is known as the Amplifluor Uniprimer™. This system has been designed for the direct detection and measurement of the amplified DNA using primers that are labeled with fluorescent moieties (Bhatnagar et. al 1997) The system was developed based on Tyagi and Kramer's molecular beacon technology (Kramer and Tyagi, 1996). As described above the molecular beacons are hairpin-shaped oligonucleotides that possess a fluorophore on one arm of the beacon and a quencher on the other arm. When the molecular beacon is in its closed state, the fluorophore and the quencher are present in close proximity thus reducing any fluorescence emission. However in the presence of a complementary sequence the beacon undergoes a conformational change forcing the two fluorescent moieties away from each other thus resulting in emission of fluorescence (Kramer and Tyagi. 1996). In comparison the UniPrimer™ consists of a similar 5' hairpin-structure and an additional 3' oligonucleotide sequence (Bhatnagar et al. 1997). Fluorescein is used as the fluorophore while 4-(dimethylamine) azo benzene sulfonic acid (DABSYL) is used as the quencher (Wilkinson, 1999). The UniPrimer™ or the energy transfer-labeled primer is named so because of its universal application for the detection of amplified DNA.

The actual PCR reaction involves three primers, two target-specific primers synthesized by the user and the UniPrimer™. (Wilkinson. 1999) The Amplifluor system can be optimized for use in any PCR reaction just by modifying one of the target specific primers such that it has the tail (Z-) sequence on its 5' end (Wilkinson. 1999). The Z-sequence has been specifically designed to reduce any background fluorescence caused by heterodimer formation (Wilkinson, 1999). During PCR, the two target-specific primers (5'-tailed primer and the untailed primer) anneal to the target sequence and extend After the first few cycles the Z-sequence of the tailed primer is incorporated into the target DNA Thus the target DNA now contains the Z-sequence on one strand and its complementary Z'-sequence on the other strand (Fig 8) As the PCR proceeds into the third cycle the UniPrimer™ anneals to the Z-sequence of the amplicon and extends and is thus incorporated into the target sequence In the next cycle, as the primer

extends through the UniPrimer™ template, its hairpin- structure is unfolded increasing the distance between the fluorescein and the DABSYL moieties (Fig. 8). Quenching is therefore no longer possible. The fluorescent signal generated increases as the number of cycles increases and is proportional to the amount of amplified DNA formed. The background signal generated is low (Wilkinson, 1999). Based on its mechanism of action the UniPrimer™ has been described as a molecular switch that detects amplification of DNA by energy transfer between fluorophore and quencher (Khripin et al. 2000). This switch undergoes a "off" to "on" transition when the Amplifluor primer changes its conformation from a closed hairpin-loop to an open extended structure (Khripin et al. 2000).

When compared to the other fluorescent detection techniques available, the Amplifluor UniPrimer system has the following advantages:

1. The technique involves a specific single-step PCR and so eliminates the need for any hybridization probes
2. The reaction and the analysis of the product are performed in a single closed tube, thus eliminating any chance of carry-over contamination that could lead to false-positive results
3. The amplified DNA is detected directly by measuring the fluorescence emission
4. The Amplfluor UniPrimer system can be used in any PCR by simply modifying one of the target-specific primers.
5. The technique eliminates the laborious post-PCR sample processing and thus enables high-throughput sample analysis
6. The technique can be used for both end-point and real-time analysis.

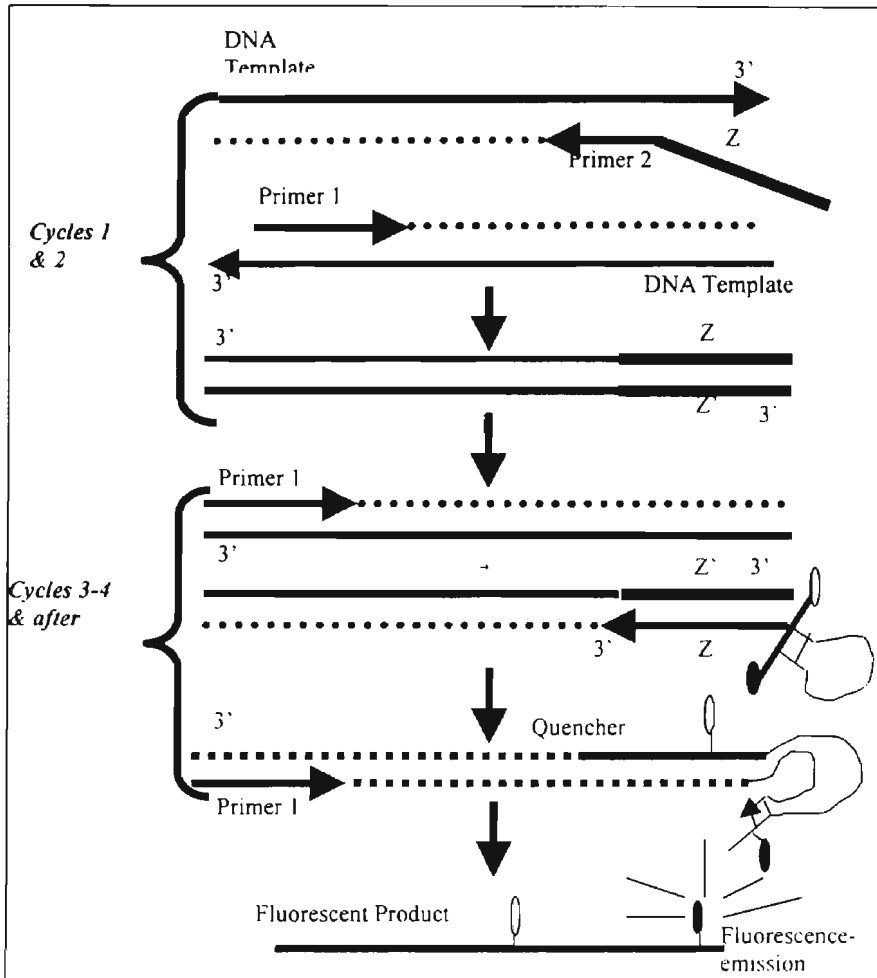


Figure 8. Principle of the Amplifluor UniPrimer™

Cycles 1 & 2: During the initial cycles of the PCR, the target-specific primers (5'-tailed primer 2 and the Untailed primer 1) are extended This yields a product that has the Z-sequence on one strand and its complementary Z'-sequence on the other strand

Cycles 3, 4 & after: The UniPrimer™ anneals to the Z'-sequence of the template The subsequent polymerization results in the reporter and the quencher molecules being incorporated into the product The product now acts as a template for primer 1 As this primer is extended, the hairpin-structure of the UniPrimer™ is unfolded causing the reporter and the quencher dye to move away from each other. Quenching is therefore no-longer possible and the fluorescence emitted by the reporter dye is detected directly The detected fluorescence is proportional to the amount of amplified product present.

The Amplfluor UniPrimer™ system has been used successfully for various purposes including, SNP genotyping (Hamer et al. 2001), detection of prostate-specific antigen cDNA (Bhatnagar et al. 1997), identification of point mutations (Winn-Deen, 1998), closed-tube telomeric repeat amplification protocol (Hohman et al. 1999), and in situ PCR and RT-PCR (Bhatnagar et al. 1997). In my thesis, I propose to use the Amplifluor UniPrimer™ technique for the detection of the food-borne pathogen *Listeria monocytogenes*.

1.4 Phenol chloroform extraction

According to this procedure, 200 ml of fresh culture was centrifuged at 7,438 Xg for 10 min at 4 ^{0}C and the pellet obtained was resuspended in 50 ml of TES buffer (50 mM NaCl, 30 mM Tris pH 8.0, 5 mM EDTA) This resuspension was again centrifuged and the supernatant discarded. To the pellet 25 ml of cell suspension buffer (8% sucrose, 50 mM Tris pH 8.0, 1 mM EDTA) was added. To this 5 ml of lysozyme (15 mg / ml) was added and the mixture was incubated at 37 ^{0}C for 1 hr. After incubation, 4 ml of EDTA solution (250 mM EDTA, 50 mM Tris pH 8.0), 3 ml of SDS solution (20% SDS, 50 mM Tris pH 8.0), 10 μL of RNAse A (1 mg / ml) and 20 μL of proteinase K (20 mg /ml) were added and were mixed together mixed. The mixture was then incubated at 50 ^{0}C for 15 min. Then, 4 ml of 2.0 M Tris HCl and 6 ml of 5.0 M NaCl were added by mixing gently. Then 55 ml of phenol was added and the mixture was stirred gently for approximately 10 min. This was followed by the addition of 55 ml of chloroform The contents of the tube were mixed and centrifuged at 12,296 Xg for 15 min at 4 ^{0}C. The aqueous phase was removed to a separate bottle and to it 55 ml of chloroform–isoamyl alcohol (24: 1) was added. The bottle was stirred gently to mix its contents The aqueous phase was removed again and treated with an equal volume of isopropanol. The resulting pellet was then resuspended in TE

1.5 DNA extraction using the Wizard® genomic DNA purification kit (Promega)

The kit is designed for isolation and purification of genomic DNA from white blood cells, tissue culture cells and animal tissue, plant tissue, yeast, gram-positive and gram-negative bacteria. The reagents provided in the kit include the nuclei lysis solution, RNase, protein precipitation solution and DNA rehydration solution

A fresh culture (1.0 ml) was centrifuged and cells pelleted The pellet was mixed with 600 μL of the nuclei lysis solution and the cells were lysed by incubation at 80.0 ^{0}C for 5 min The incubated mixture was then cooled at room temperature and 3 μL of RNAse was added to the lysed cells and incubated at 37.0 ^{0}C for 15 min-30 min The RNAse treated cell lysate is then treated with 200 μL of protein precipitation solution and incubated on ice for 5 min The cell debris

was pelleted by centrifuging at 13,000 Xg for 3 min. The supernatant containing the DNA was then mixed with 600 μL of isopropanol in a separate tube and centrifuged at 13,000 Xg for 2 min The supernatant was discarded and the pellet containing the DNA was cleaned by ethanol precipitation. The resulting purified DNA is resuspended in 100 μL of DNA rehydration solution.

1.6 Plasmid extraction

Cells (1.0 ml) from a fresh culture were centrifuged as above and the pellet was resuspended in 200 μL of solution I (50 mM glucose, 10 mM EDTA, and 25 mM Tris-HCl pH 8.0) To this 400 μL of solution II (0.2 mM NaOH and 1% SDS) and 300 μL of solution III (3.0 M Na-acetate pH 4.8) was added and mixed by vortexing. The mixture was then centrifuged at 12,570 Xg for 10 min. The supernatant containing the plasmid was recovered and an equal volume of isopropanol was added and mixed. The tube was then centrifuged at 14.000 Xg for 5 min The supernatant was discarded and the pellet containing the plasmid was air-dried. The dry pellet was resuspended in 50 μL of TE (10 mM Tris, 1 mM EDTA pH 8.0) and washed with ethanol.

2. Extraction of PCR product from the agarose gel

The Ultrafree® - DA kit (Millipore) was used to extract the PCR product from agarose gel The system can extract DNA of 100 bp to 10,000 bp It consists of pre-assembled Gel Nebulizer™, ultrafree-MC (0.45 μm durapore) and microcentrifuge filtrate vials. The agarose containing the band of interest was cut and placed into the gel nebulizer and firmly capped The device was then centrifuged at 5,000 Xg for 10 min Centrifugal forces cause the agarose to pass through the gel nebulizer where it was converted into fine slurry and captured by ultrafree-MC DNA with the electrophoresis buffer passes through the membrane in ultrafree-MC and is collected in the filtrate vial The ultrafree-MC and the gel nebulizer were discarded while the DNA in the filtrate was used for cloning

3. Procedure for inoculation of the food samples tested

The food sample was first mashed and mixed thoroughly without the addition of any diluent. From this a 10 gm portion was weighed into a netted stomacher bag (Nasco). To this 90 ml of UVM broth was added. The food sample in the bag was then inoculated with 1.0 ml of diluted cultures (approximately, 10^3 CFU / ml, 10^2 CFU / ml or 10^1 CFU / ml) and the contents of the bag were mixed again. The bags were then incubated at 30 ^{0}C for 24 hr. From this enrichment, 1.0 ml was transferred into 9.0 ml of FB and incubated at 30 ^{0}C for 24 hr. After 24 hr 200 µL of the culture was lysed and the DNA was recovered using isopropanol before using it for PCR.

4. Procedures involved in cloning and sequencing the target genome

4.1 BAX PCR

PCR is performed using the BAX Kit for *L. monocytogenes* (Qualicon) The BAX system consists of 0.2 ml PCR tubes. Each PCR tube contains a pellet that consists of all the ingredients for PCR including the primers. The system has a built-in control that gives a product of approximately 200 bp. The template for the PCR was prepared using the BAX lysis procedure. The cycle used for PCR was 90.0 ^{0}C for 1 sec, 93.0 ^{0}C for 2 min and 10 sec followed by 37 cycles of 94.0 ^{0}C for 25 sec, and 69.0 ^{0}C for 3 min and 10 sec.

4.2 Cloning and transformation

The PCR product was extracted from the agarose gel using the Ultrafree® - DA kit (Millipore). TOPO TA cloning® kit (Invitrogen) was used for cloning. The kit is used to clone and sequence PCR products amplified by Taq-polymerase. It consists of pCR®4-TOPO cloning vector that has covalently bound topoisomerase I enzyme The vector has 3'-T overhangs, multiple cloning sites with flanking EcoR I sites, kanamycin & ampicillin resistant genes, LacZ gene and multiple primer sites (T7, T3, M13 forward & M13 reverse) The kit contains reagents for both cloning and transformation The reagents for cloning include pCR®4-TOPO vector,

6. PCR using set II primers (Table 3)

6.1 PCR with the target-specific primers and the modified primers

The template for the PCR was obtained using the phenol-chloroform extraction The PCR cycle used was 95.0 ^{0}C for 2 min, followed by 35 cycles of 95.0 ^{0}C for 30 sec, 55.0 ^{0}C to 63.5 ^{0}C for 1 min and 72.0 ^{0}C for 2 min. The reaction mix contained 0.2 μM of each primer, 2 5 mM $MgCl_2$ and 0.2 mM dNTP mix. Taq polymerase used was 1.25 U. The total volume of the PCR reaction mixture was 100 μL. The concentration of reagents and the PCR cycle used for the modified primers were similar to the PCR with the target-specific primers except that an annealing temperature of 60.0 ^{0}C was used.

6.2 PCR with the UniPrimer™

The Template for the PCR with the UniPrimer™ was obtained using the BAX lysis procedure. The PCR cycle used was 94 ^{0}C for 4min, followed by 35 cycles of 94 0 ^{0}C for 15 sec, 60.0 ^{0}C for 20 sec and 72.0 ^{0}C for 40 sec with a final extension of 72.0 ^{0}C for 4 min. The total volume of the reaction mix was 25 μL. Concentrations of the UniPrimer™ and the non-tailed primer were 0.5 μM each whereas the tailed primer concentration was varied. Concentrations of the tailed primer used for the PCR were 0.05 μM, 0.06 μM, 0.07 μM, 0 08 μM and 0.1 μM $MgCl_2$ concentration was 1.5 mM and 0.2 mM nucleotide mix was added to the PCR

7. PCR using set III primers (Table 3)

7.1 PCR with the target-specific primers

The template for the PCR using the 3rd set of primers was extracted using the Wizard® Genomic DNA purification kit (Promega) The cycle used for the PCR with the target-specific primers was, 94 ^{0}C for 4 min followed by 35 cycles of 94 0 ^{0}C for 15 sec, 60 0 ^{0}C for 20 sec and 72.0 ^{0}C for 40 sec with a final extension of 72.0 ^{0}C for 4 min DNA of *L monocytogenes* Scott A-2 DNA was used as the template The total volume of the reaction mixture was 25 μL

containing 0.2 mM dNTP mix and 0.5 μM of each primer. The $MgCl_2$ concentration was varied from 1.5 mM to 2.0 mM. The amount of Taq polymerase used was 1.25 U.

7.2 PCR with the UniPrimer™

The Wizard kit (Promega) extracted the chromosomal DNA used, while the lysate used was obtained using the BAX kit (Qualicon). The PCR cycle used was 94 ^{0}C for 4 min, followed by 35 cycles of 94.0 ^{0}C for 15 sec, 60.0 ^{0}C for 20 sec and 72.0 ^{0}C for 40 sec with a final extension of 72.0 ^{0}C for 4 min. The concentration of the UniPrimer™ and the non-tailed primer was 0.5 μM and that of the tailed primer was 0.05 μM. The $MgCl_2$ concentration was 1.5 mM and dNTP concentration was 0.2 mM.

8. PCR using set IV primers (Table 3)

8 1 PCR with modified (tailed and non-tailed) primers and the UniPrimer™

The template was extracted using the Wizard® Genomic DNA purification kit PCR with the modified primers included 0.5 μM of each primer and 1.5 mM $MgCl_2$ The PCR cycle was 94 ^{0}C for 4 min, followed by 35 cycles of 94.0 ^{0}C for 15 sec, 60.0 ^{0}C for 20 sec and 72 0 ^{0}C for 40 sec with a final extension of 72.0 ^{0}C for 4 min

The PCR with the UniPrimer™ included 0.5 μM of the UniPrimer™ and the non-tailed primer and 0.05 μM of the tailed primer The other reagents were used at similar concentrations as with the modified primers.

9. Optimization of PCR conditions

The annealing temperature was optimized by doing a PCR with an annealing temperature gradient of 57.0 ^{0}C to 63.0 ^{0}C. The positive control was performed using only the modified primers at a concentration of 0.5 μM each. The annealing temperature used for the positive control was 62.0 ^{0}C. In order to optimize the $MgCl_2$ concentration, different PCRs were performed with 0.75mM, 1.00 mM, 1.25 mM, 1.50 mM, 1.80 mM, 2.00 mM, 2.25 mM and 2.50 mM $MgCl_2$. An annealing temperature of 60.0 ^{0}C was used. The positive control reaction for the $MgCl_2$ optimization was performed at an annealing temperature of 62.0 ^{0}C with 1.50 mM $MgCl_2$

Optimization of the template concentration involved PCRs with various amounts of templates (36 ng, 66 ng, 99 ng 198 ng, 264 ng, 330 ng and 396 ng). The concentration of the template was determined using the DNA fluorometer TKO 100 (Hoefer Scientific Instruments, SanFransisco). The positive control reaction was performed with 132 ng of template. The annealing temperature used for all the PCRs including the positive control was 60.0 ^{0}C. The concentration of the UniPrimer™ and the non-tailed primers was 0.5 μM each whereas the tailed primer concentration was 0.05 μM.

In order to optimize the primer concentration, PCR was performed with six master mixes. The first master mix contained only the target specific primers (primers 1 & 2. set IV Table 3) at a concentration of 0.5 μM each. The second master mix contained the non-tailed and the tailed primers (primers 1 & 4. Set IV Table 3) at a concentration of 0.5 μM each The third master mix contained the non-tailed and UniPrimer™ (primer 1 Set IV Table 3) at a concentration of 0.5 μM each and the tailed primer (primer 4 Set IV Table 3) at a concentration of 0.05 μM. In the fourth, fifth and the sixth master mixes the concentration of the tailed primer was increased gradually. The fourth mix had 1/9th tailed primer (0.055 μM) while the fifth and the sixth mix had 1/8th and 1/7th the concentration of the non-tailed primer (0.0625 μM and 0.0714 μM respectively). The $MgCl_2$ concentration was 1.5 mM 35 cycles of PCR were performed with annealing at 60.0 ^{0}C.

10. Template preparation and treatment

10.1 Culture media effects

L. monocytogenes 383-2, *L. innocua* ATCC 33090, and *E. feacalis* were grown overnight in BHI (Bovine Heart Infusion, Difco) and FB (Frasier Broth, Difco). The cells were lysed using BAX lysis and 10.0 μL of the resulting lysate was used as template in the PCR. A positive control reaction was performed with purified *L. monocytogenes* DNA extracted with the Wizard® Genomic DNA purification kit. A non-template reaction was used as the negative control. The optimized PCR conditions and primer concentrations were used.

10.2 Cell lysis and DNA extraction

In order to use the lysate for PCR, the DNA in the lysate must be recovered before it can be used as template for the UniPrimer™ This resulted in the development of a new protocol for DNA extraction that would be simpler and less time consuming than the DNA extraction and purification methods used earlier. This procedure was a modification of the BAX lysis procedure.

Primers

Set	Primer Description	Primer Sequence (5'-3')	Size (bp)
I	1. *L. monocytogenes* P3 O- For 2. *L. monocytogenes P3 O-* Rev	GGTAGAATAGGTTAACTGTCC CATGCTAGAAGCCGTCAAGG	21 20
II	1. *L. monocytogenes* Patent- For 2. *L. monocytogenes* Patent -Rev 3. *L. monocytogenes* Patent-For+Z 4. *L. monocytogenes* Patent-Rev+Z	GGTAGAATAGGTTAACTGTCCAGTTCC TTTGTTGTTCTGCTGTACGATCTTCGG ACTGAACCTGACCGTACA GGTAGAATAGGTTAACTGTCCAGTTCC ACTGAACCTGACCGTACA TTTGTTGTTCTGCTGTACGATCTTCGG	27 27 45 45
III	1. *L. monocytogenes* GS- For 2. *L. monocytogenes* GS -Rev 3. *L. monocytogenes* GS-For+Z 4. *L. monocytogenes* GS-Rev+Z	AGCGGTTTATACAGCCTTGC GGGTTCCCTGTGTTTCTTCC ACTGAACCTGACCGTACA AGCGGTTTATACAGCCTTGC ACTGAACCTGACCGTACA GGGTTCCCTGTGTTTCTTCC	20 20 38 38
IV	1. *L. monocytogenes* Patent- For 2. *L. monocytogenes* Patent -Rev 3. *L. monocytogenes* Patent-For+Z 4. *L monocytogenes* Patent-Rev+Z	GCGATACGACAAATCTGTTAGGCACC AAATCCCTGAGGAACAAATCATCGTC ACTGAACCTGACCGTACA GCGATACGACAAATCTGTTAGGCACC ACTGAACCTGACCGTACA AAATCCCTGAGGAACAAATCATCGTC	26 26 44 44

Table 3. List of primers tested for the optimization of the UniPrimer™

11. Machines

11.1 PCR machine

PCR was performed using a PTC-200, gradient thermal cycler from MJ Research The machine allows the use of a temperature gradient of over 24 ^{0}C across the 96 well sample block. Both 96 well PCR plates and 0.2 μL PCR tubes can be used in the machine.

11.2 Fluorescence plate reader

A fluorescence plate reader known as Genios from Tecan and distributed by Phenix research products was used for fluorescence detection.

Results and Discussion:

I chose to examine the Amplifluor UniPrimer™ system because of its ability to work with different primer sets. In order to optimize the system target-specific primers have to be synthesized and the PCR conditions should be optimized. The optimized system would then be tested with different ready-to-eat food samples

1. Synthesis of target-specific primers

1.1 Cloning and sequencing the target genome

Since the genomic sequence of *L. monocytogenes* was not known, the best alternative strategy for primer synthesis was to clone and sequence a PCR product of *L. monocytogenes* obtained by using the "Qualicon BAX PCR system" specific for *L. monocytogenes*. This system detects *L. monocytogenes* in enrichment cultures derived from food and environmental samples using PCR and gel electrophoresis (Gendel et al. 1988) The BAX-system is highly specific for *L. monocytogenes* and is not affected by the presence of other *Listeria* spp. or microbes of other genera (Gendel et al. 1988). The built-in control of the system aids in accurate detection by providing proof for proper functioning of the PCR. The BAX kit was used to perform 8 identical PCRs. Lysate of *L. monocytogenes* Scott A-2 was used as the template.

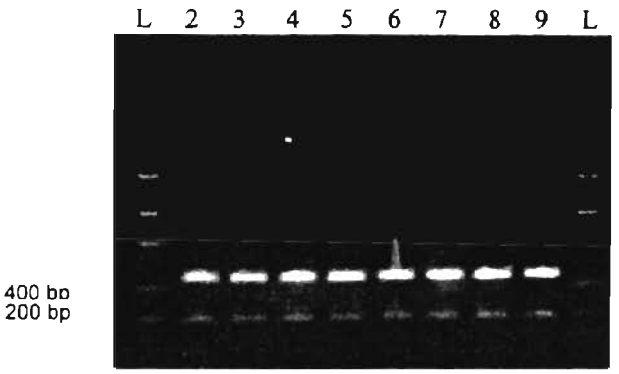


Figure 9. Gel electrophoresis analysis of PCR product obtained by using the BAX PCR system specific for *L. monocytogenes*.

L. Low molecular mass ladder

Lanes 2 through 9: PCR product of *L. monocytogenes* Scott A-2 (approximately 450bp) and BAX control reaction product (200 bp)

The PCR product was separated from the agarose gel and cloned into TOPO-TA cloning vector. The recombinant vectors were transformed into competent *E. coli* cells and positive transformants were selected using kanamycin and ampicillin. The plasmid was extracted and analyzed by gel electrophoresis (Fig. 10).

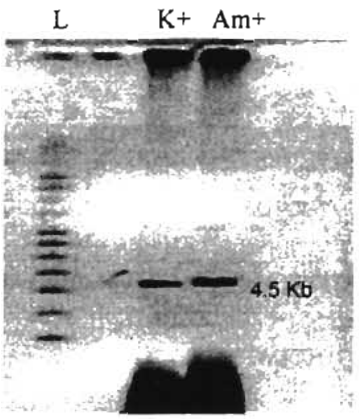


Figure 10. Gel electrophoresis analysis of the extracted recombinant plasmid

L. Supercoiled DNA ladder (2-10 Kb)

The gel shows plasmid extracted from transformants selected using kanamycin and ampicillin respectively

The extracted plasmid was submitted to the Recombinant DNA/Protein Resource Facility (Department of Biochemistry and Molecular Biology) for sequencing. The T3 and the T7 primers were used for sequencing. Primers were designed from the sequence obtained (Fig. 11) using the Primer3 Output program (NCBI) (Primers 1 & 2. set I. Table 3).

Primer 1

1 GGTAGAATAGGTTAACTGTCCAGTTCCAGTTCCATTTTCAACTATTGAAGGCGTTAT

59 CACATCAGAGGTTTTTAATTAAGCGGAATTAATTTTTTTACTGGTGTTTGTGAAAATA

119 TATCAAAGTTTCTTGTAATTTTTATTATGGAGAGGAGATAATTTTTATGACAAATGCA

178 AATGCAAATGGCAACCTAAAAAAATGCCCCATCACGATTAGCTCTTATACGCTTGGA

238 ACAGAGGTATCTTTTCCTAAACGAGTAAAAGTCGCTGCGGAAAAGCGTTTTGACGG

297 ATTGGCTTGCGTGCAGAAAACTATGTAGATGCACTAGCTGCCGGATTAACGGATGA

356 AGACATGTTGCGGATTTTAGACGAGCATAACATGAAAGTAACAGAAGTGGAGTACA

415 TAACTCAGTGGGGAACTGCCGAAGATCGTACAGCAGAACAACAAAAAGGGCGAAT

473 TCGTTTAAACCTGCAGGACTAGTCCC

Primer 2

Figure 11. Sequence of the amplified and cloned target genome and the primers designed from the sequence using the Primer 3 output program (Whitehead - Institute and Howard Huges Medical Institute)

1.1.1 PCR with *L. monocytogenes* DNA and recombinant plasmid

The newly synthesized primers were first used in PCR with the chromosomal DNA of *L. monocytogenes* Scott A-2 and also with the recombinant plasmid. A band of approximately 0.45 Kb was detected by gel electrophoresis (Fig. 12).

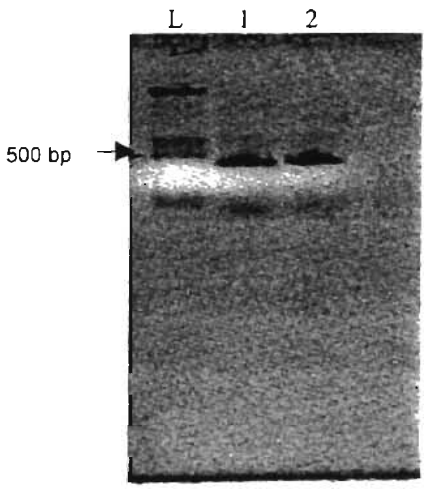


Figure 12. Gel electrophoresis analysis of the PCR product formed by set I primers (Table 3)

L. 50 bp DNA marker

Lane 1: PCR product obtained by using chromosomal DNA of *L. monocytogenes* strain ScottA-2

1.1.2 PCR with other *Listeria* spp.

PCR was then performed with 4 different *Listeria* spp. in order to test the specificity of the primers synthesized. *L. monocytogenes* Scott A-2, *L. innocua* ATCC 33090, *L. ivanovii* ATCC 33091, and *L. welshimeri* were tested. The agarose gel electrophoresis analysis (Fig. 13) showed that the primers reacted with all the *Listeria* spp. tested giving a product band of approximately 450 bp. It was also observed that the intensity of the product band for *L monocytogenes* was higher than the product bands for the other *Listeria* spp. This suggested that by varying the PCR conditions the primers could be made specific for *L. monocytogenes*.

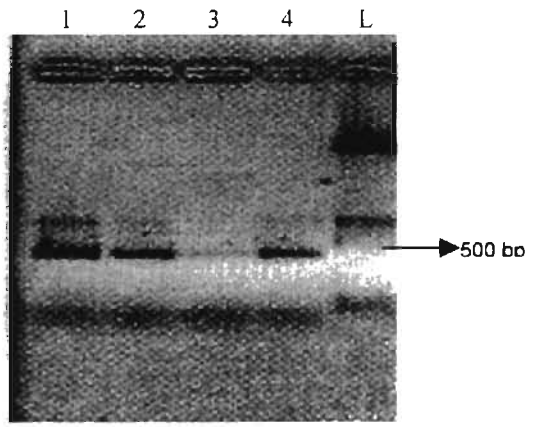


Figure 13. Gel electrophoresis analysis to test the specificity of primer (Set 1. Table 3) to *L. monocytogenes*

Lane 1: *L. monocytogenes* Scott A-2

Lane 2: *L. innocua* ATCC 33090

Lane 3: *L. ivanovii* ATCC 33091

Lane 4: *L. welshimeri*

L. 50 bp ladder

1.1.3 PCR with annealing temperature gradient

A PCR was designed with an annealing temperature gradient of 60.8 ^{0}C to 70.0 ^{0}C The effect of annealing temperatures on the specificity of the primers was compared using *L. monocytogenes* Scott A-2 and *L. innocua* ATCC 33090 as templates. On gel electrophoresis of the product (Fig. 14), it was observed that at a temperature of 66.0 ^{0}C, the primers were specific to *L. monocytogenes* since a product of approximately 450 bp was formed with *L monocytogenes* whereas no product was formed with *L. innocua*. However in order to be used with the UniPrimer™. the primers should function specifically within an annealing temperature range of 55.0 ^{0}C to 60.0 ^{0}C. Hence though the primers were specific to *L. monocytogenes* at a higher annealing temperature, they could not be used.

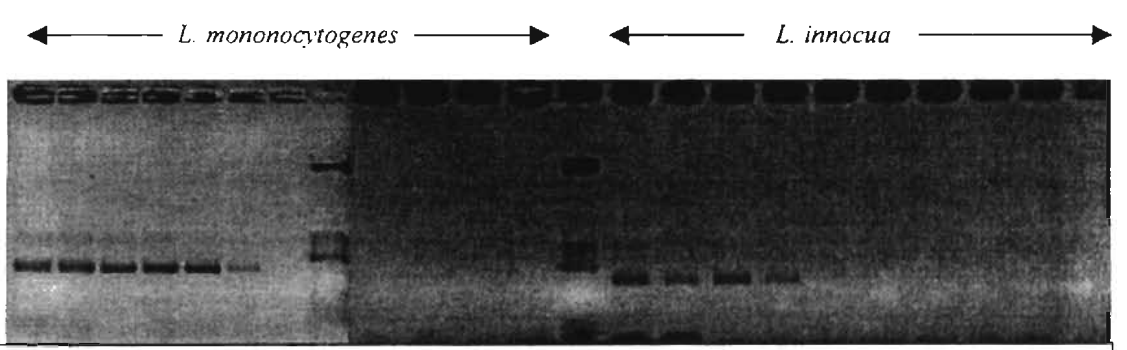


Figure 14. Gel electrophoresis analysis to test the primer (Set I. Table 3) at various annealing temperatures

Annealing temperatures (given below the lanes) used in the gradient were measured in ^{0}C

L: 50 bp DNA marker

1.2 Analysis of various primers:

1.2.1 Primer set II

Oligonucleotides specific for *L. monocytogenes* are listed in U. S. patent 5,922,538 (Hazel and Anton, 1999). A set of primers from this patent (Primers 1 & 2. Set II. Table 3) were synthesized for PCR before conducting any further experiments to make the previous set of primers (Primers 1 & 2. set I. Table 3) specific for *L. monocytogenes*. DNA of *L. monocytogenes* and *L. innocua* were used as templates. PCR was performed with an annealing temperature gradient of 55.0 ^{0}C to 63.5 ^{0}C. Gel electrophoresis of the products obtained (Fig. 15) showed that the primers were specific to *L. monocytogenes* within an annealing temperature range of 55.0 ^{0}C to 63.5 ^{0}C.

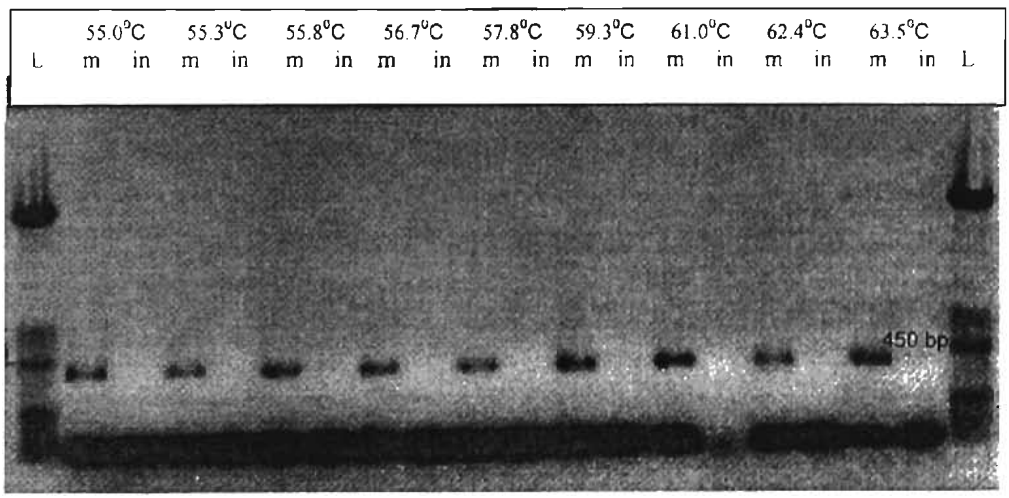


Figure 15: PCR product with the 2nd set of primers (Primers 1 & 2. Set II. Table 3) over an annealing gradient of 55.0 ^{0}C to 63.5 ^{0}C

L: 50 bp DNA marker

Since the primers were specific to *L. monocytogenes* within an annealing temperature range that is required for the UniPrimer ™, these primers were chosen for further research. The Z-sequence (5' ACTGAACCTGACCGTACA 3') was added to the 5' end of reverse primer (primer 4. Set II. Table 3). Before using the UniPrimer™, PCR was conducted using the tailed and the non-tailed primers with *L. monocytogenes* Scott A-2 and *L. innocua* ATCC 33090. An annealing temperature of 60.0 ^{0}C was chosen for the PCR. The gel electrophoresis analysis of the product (Fig. 16) showed that a product of approximately 450 bp was formed with *L. monocytogenes* whereas *L. innocua* did not give a product.

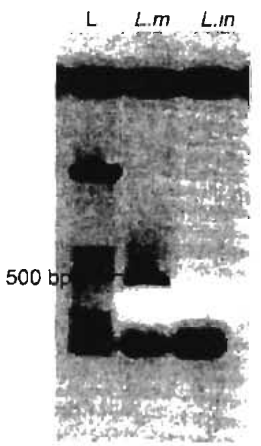


Figure 16. Gel electrophoresis analysis of the PCR product obtained using the modified set II primers (Primers 1 & 4. Set II Table 3)

L: 50 bp DNA marker

L. m: L. monocytogenes, L. in: L. innocua

PCR with the UniPrimer™ and primer from set II

Primers were then tested with the UniPrimer™ PCR was performed using *L. monocytogenes* Scott A-2 and *L. innocua* ATCC 33090. Also in an attempt to increase the product yield, PCRs were performed with increasing concentration of the tailed primer. The gel analysis showed that the yield of the product (intensity of the product band) is highest when 0.06 μM tailed primer was used. But, in addition to the product band the gel picture (Fig. 17) also showed the presence of primer artifacts or primer dimers of approximately 100 bp. The presence of such non-specific products result in high background fluorescence that interferes with accuracy of detection.

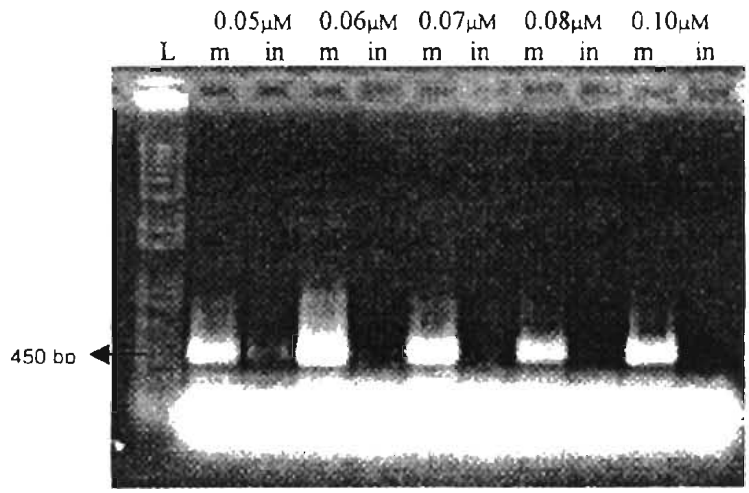


Figure 17. Gel electrophoresis analysis of the PCR product obtained using the modified primers of set II and the UniPrimer™

The varying concentrations of the tailed primer are listed above the gel picture.

In order to avoid formation of primer artifacts, PCR conditions including the primer concentration, dNTP concentration and Mg^{++} concentration were varied but the dimerization of primers could not be prevented. Further, primers were synthesized such that the Z-sequence was present on the 5' end of the forward primer (Primer 3. Set II. Table3). When used in PCR these primers also formed dimers. Variation in annealing temperature, $MgCl_2$ concentration, primer concentration and template concentration did not eliminate the formation of primer artifacts.

1.2.2. Primer set III

A third set of primers was chosen for testing (Primers 1 & 2. Set III. Table 3). These primers were designed based on genome subtraction analysis of *L. monocytogenes* (Muriana and Wu, 1995). Initially PCR was performed with only the forward and the reverse primer at a concentration of 0.5 μM each. *L. monocytogenes* Scott A-2 DNA was used as the template. In order to test the optimum $MgCl_2$ for these primers, PCR was performed with varying $MgCl_2$ concentrations. Gel analysis of the PCR product (Fig. 18) showed that there were no primer artifacts or primer dimers formed at the different $MgCl_2$ concentrations.

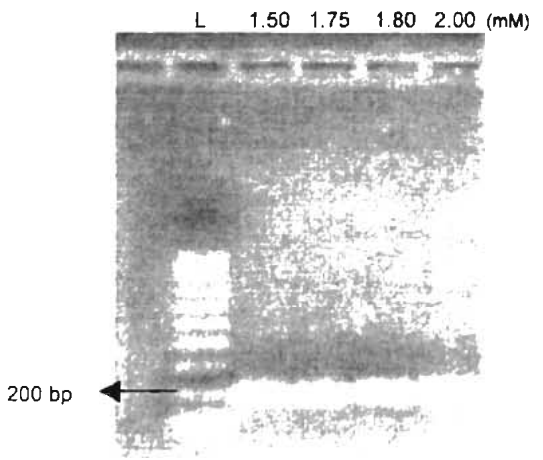


Figure 18. Gel electrophoresis analysis of the of the PCR product obtained using the set III primers (Primers 1 & 2. Set III. Table 3) at different concentrations of $MgCl_2$.

L: EZ load 100 bp DNA marker

The different $MgCl_2$ concentrations tested are given above the lanes in the gel picture.

PCR with UniPrimer™

PCR was then performed using the UniPrimer™. The Z-sequence (tail sequence) was added to the 5' end of the forward primer (Primer 3. Set III. Table 3) In order to compare the efficiency of the PCR, two reaction mixes were made, one with purified *L. monocytogenes* Scott A-2 DNA and the other with a crude lysate of *L. monocytogenes* Scott A-2 (prepared by BAX lysis). As a negative control a reaction mix was prepared without any template. The gel electrophoresis analysis of the 3 reactions (Fig. 19) shows that a product of 250 bp was formed with both the purified DNA and the lysate of *L. monocytogenes*. However,

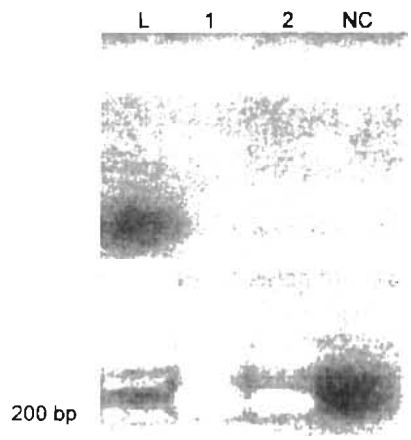


Figure 19. Gel electrophoresis analysis of the of the PCR product obtained using the 3rd set of primers (Primers 1 & 3. Set III. Table 3) and the UniPrimer™

L. EZ load 100 bp DNA marker

1 Product obtained from *L. monocytogenes* DNA (250 bp)

2 Product obtained from *L. monocytogenes* lysate (250 bp)

NC: Non-template control reaction

the non-template control reaction resulted in the formation of primer artifacts at approximately 100 bp. This suggested that when used in the presence of other *Listeria* spp. (i. e non-*monocytogenes*), these primers could form dimers. In order to verify this assumption PCR was now conducted with different *L. monocytogenes* strains and other *Listeria* spp. As a positive control PCR was performed with *L. monocytogenes* using only the target specific primers (Primers 1 & 2. Set III. Table 3).

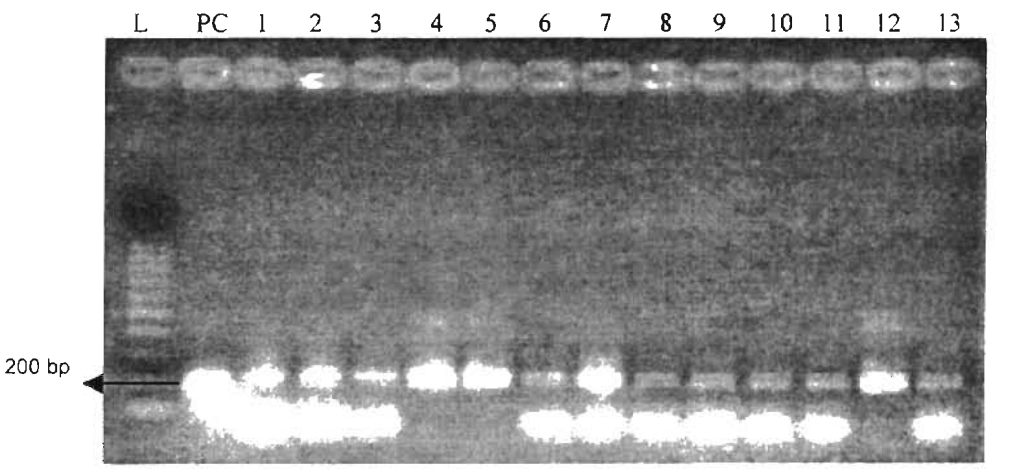


Figure 20. Gel electrophoresis analysis showing the reactivity of the 3rd set of primers and the UniPrimer™ for different *L. monocytogenes* strains and other *Listeria* spp.

L. EZ load 100 bp marker

PC: Positive control (target-specific primers with *L. monocytogenes* SA-2)

Lane 1. *L. monocytogenes* 383-2

Lane 2: *L.monocytogenes* 39-2

Lane 3: *L. monocytogenes* V7-2

Lane 4 *L. monocytogenes* SA-2

Lane 5: *L. monocytogenes* 82-2

Lane 6 *L. monocytogenes* 383-2

Lane 7: *L. monocytogenes* H224

Lane 8. *L. innocua* CW-36

Lane 9: *L. welshemen*

Lane 10. *L. ivanovii* 33090*L*

Lane 11 *L. ivanovii* 19119

Lane 12. *L. innocua* ATCC

Lane 13 *L. innocua* 33090

From the gel picture (Fig. 20) it can be seen that the primers reacted with the other *Listeria* spp. and also formed primer artifacts with many of the *L. monocytogenes* strains.

1.2.3 Primer set IV

PCR with the modified primers

A new set of primers specific for *L. monocytogenes* (Primers 1 & 2. Set IV. Table 3) were chosen from the US patent 5,922,538 (Hazel and Anton, 1999). These primers were found to be 99.5% accurate when evaluated using 323 strains of *L. monocytogenes* and 30 non-*L. monocytogenes* strains (Hazel and Anton, 1999). The Z-sequence was added to the 5' end of the reverse primer (Primer 4. Set IV. Table 3). PCR was first performed with the modified primers (tailed and the non-tailed primers) without the UniPrimer™ DNA from four different strains of *L. monocytogenes* (*L. monocytogenes* Scott A-2, *L. monocytogenes* 39-2, *L. monocytogenes* 383-2 and *L. monocytogenes* V7-2) was used as template in four identical reactions. The gel electrophoresis analysis of the product (Fig. 21) revealed that the addition of the Z-sequence did not result in the formation of primer artifacts.

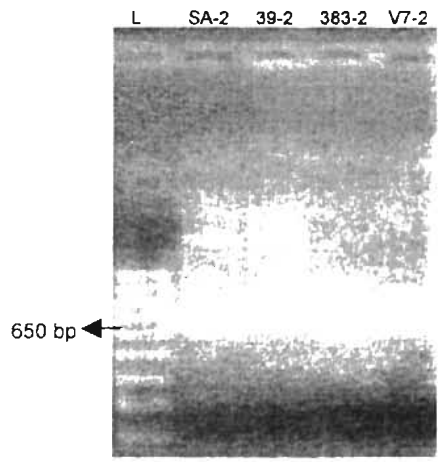


Figure 21 Gel electrophoresis analysis of the PCR products of different *L. monocytogenes* strains formed by the modified set IV Primers (Primers 1 & 4 Set IV Table 3)

L: EZ load 100 bp DNA marker

PCR with the UniPrimer™

PCR was then performed using the UniPrimer™ along with the tailed and the non-tailed primers (Primers 4 & 1. Set IV. Table 3). Four identical PCRs were performed as in the previous experiment. Gel electrophoresis analysis (Fig. 22) revealed that along with a product of approximately 650 bp, the PCR also resulted in the formation of primer artifacts that appeared as faint bands at about 100 bp.

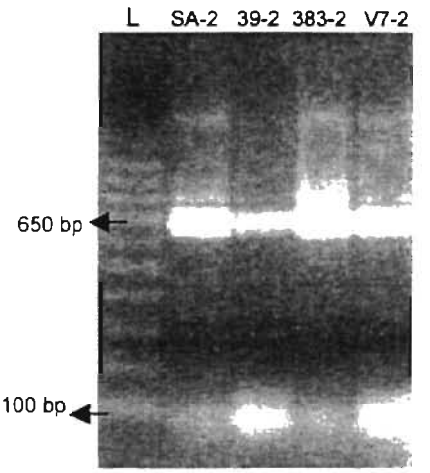


Figure 22. Gel electrophoresis analysis of the PCR products of different *L. monocytogenes* strains formed by the UniPrimer™ and the 4th set of primers (Primers 1 & 4. Set IV. Table 3)

L: EZ load 100 bp DNA marker

PCR with UniPrimer™ using different species of *Listeria*

Before optimizing various PCR conditions to eliminate the formation of primer dimers, the specificity of the primers (to *L. monocytogenes*) in the presence of the UniPrimer™ was tested. PCR was performed using four different *Listeria* spp. These included *L monocytogenes* Scott A-2, *L. innocua* ATCC- 33090, *L. ivanovii* ATCC- 33091 and *L. welshimeri* As a negative control, PCR was performed without any template. The gel electrophoresis analysis of the PCR product (Fig. 23) showed that a product of approximately 650 bp was formed with *L. monocytogenes* whereas no product was formed with the other *Listeria* species tested. Thus on confirming the primer specificity to *L. monocytogenes*, further research was focused on optimizing the conditions for PCR such that the primer dimer formation could be eliminated.

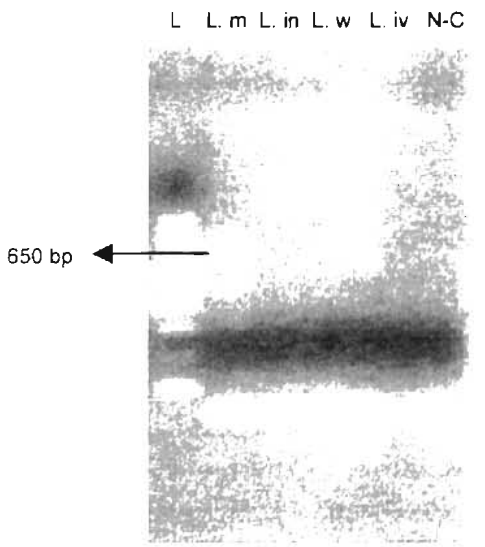


Figure 23. Gel electrophoresis to analyze the specificity of the 4th set of primers (Primers 1 & 4. Set IV Table 3) to *L. monocytogenes* in comparison to other *Listeria* spp.

L: EZ load 100 bp DNA marker

L. m L. monocytogenes *L. iv: L. ivanovii*

L. in: L. innocua *L. w: L. welshimeri*

2. Optimization of PCR conditions

2.1 Annealing temperature:

The recommended annealing temperature range for the UniPrimer™ was 55°C to 60°C and as stated, the previous PCRs were performed at an annealing temperature of 60°C. In an attempt to avoid primer-dimer formation, PCR was performed with an annealing temperature gradient of 57 °C to 63 °C. A PCR with only the tailed and the non-tailed primers at an annealing temperature of 62.0 °C was used as the positive control. From gel electrophoresis (Fig 24) it was concluded that the increase in annealing temperature did not eliminate primer dimer formation but decreased the product yield.

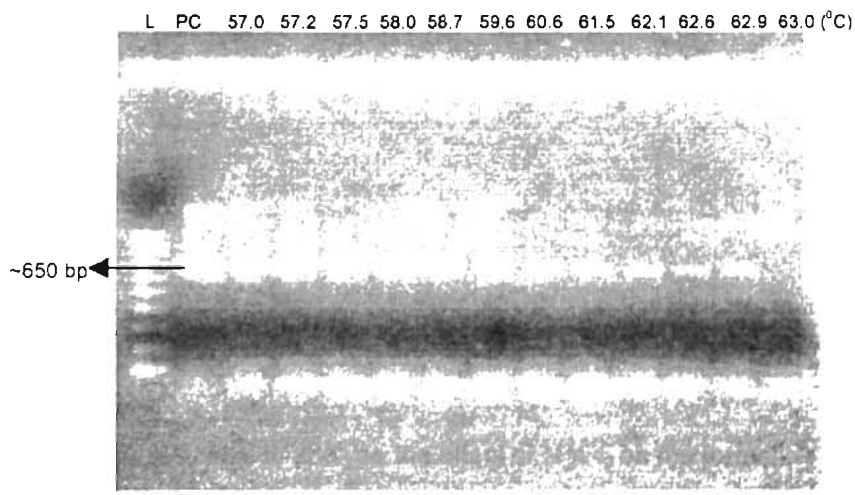


Figure 24. Gel electrophoresis performed to analyze the effect of annealing temperature on the formation of primer artifacts using the set IV primers (Primers 1 & 4 Set IV Table 3) and the UniPrimer™

PC: Positive Control performed using only the tailed and the non-tailed primers of set IV

Template *L monocytogenes* 383-2 DNA

L. EZ load 100 bp DNA marker

2.2 $MgCl_2$ concentration:

As the next step in the optimization of PCR, the concentration of $MgCl_2$ was varied and its effect on the formation of the primer artifacts was analyzed. Two sets of reactions were performed, one at an annealing temperature of 57.2 ^{0}C and the other at an annealing temperature of 60.6 ^{0}C. DNA of *L. monocytogenes* Scott A-2 was used as the template. The gel analysis (Fig. 25) showed that at both annealing temperatures the product was formed with only 1.5 mM and 1.8 mM $MgCl_2$. The product yield was greater at 1.8 mM than at 1.5 mM $MgCl_2$ However, no significant effect on the formation of primer artifacts was observed.

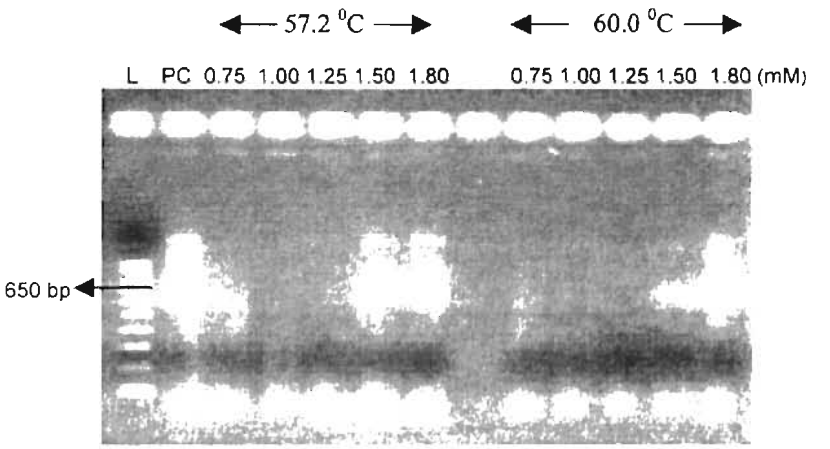


Figure 25. Gel electrophoresis to analyze the effect of $MgCl_2$ on the formation of primer artifacts using set IV primers (Primers 1 & 4. Set IV Table 3) and the UniPrimer™

PC: Positive Control Template: *L. monocytogenes* 383-2 DNA

L: EZ load 100 bp DNA marker

PCR was then performed with higher $MgCl_2$ concentrations, equal to and greater than 1.8 mM. The gel electrophoresis (Fig. 26) performed to analyze the product showed that increased $MgCl_2$ concentration did not have any significant effect on either the product yield or the formation of primer dimers.

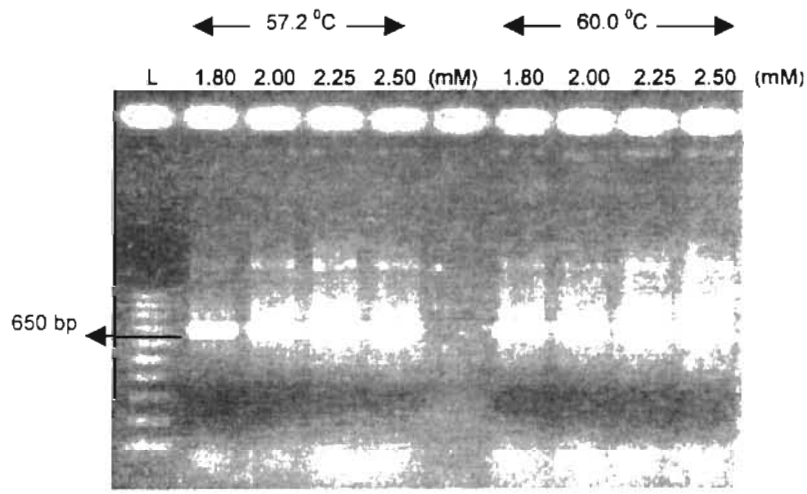


Figure 26: Gel electrophoresis to analyze the effect of increased $MgCl_2$ concentration on the formation of primer artifacts using set IV primers (Primers 1& 4. Set IV. Table 3) and the UniPrimer™.

L. EZ load 100 bp DNA marker Template: *L. monocytogenes* 383-2 DNA

2.3 Template concentration:

The concentration of purified DNA used as template during optimization of the PCR conditions was 0.066 μg / μL. The volume of the DNA used for PCR was 2.0 μL making the total amount of template in the PCR equal to 132ng. PCR was performed with varying amounts of template (lower than 132 ng). A positive control reaction was performed with the UniPrimer™ and 132 ng of DNA. On analyzing the products, it was seen that at lower template concentrations the PCR resulted in low product yield which appeared as faint bands on the gel (Fig. 27). The positive control reaction (132 ng of DNA) however, resulted in good product yield.

The effect of the lower template concentrations on the primer dimer formation could not be established, as the primer dimers are not visible on the gel picture. The reason may be that the primer dimers have run off the gel.

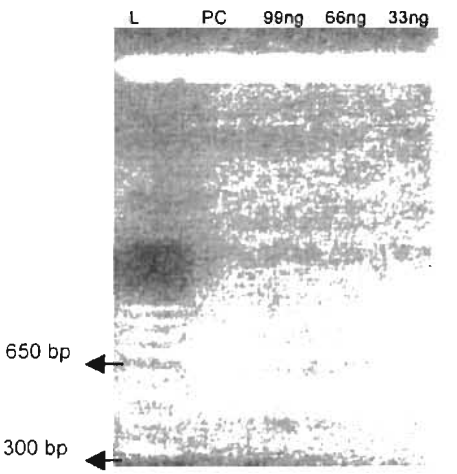


Figure 27. Gel electrophoresis to analyze the effect of decreased template concentration on the formation of primer artifacts in PCR

L: EZ load 100 bp DNA marker

PC: Positive control with 132 ng of template using the UniPrimer™

Primers used: Primers 1 & 4, Set IV Table 3 and UniPrimer™

Template: *L. monocytogenes* 383-2 DNA

PCR was then performed by increasing the amount of template added into the PCR. Analysis of the product by gel electrophoresis showed that only the controls resulted in positive reactions giving a product 650 bp (Fig. 28) whereas the reactions with higher template concentrations did not result in any product. Primer dimers still appeared on the gel.

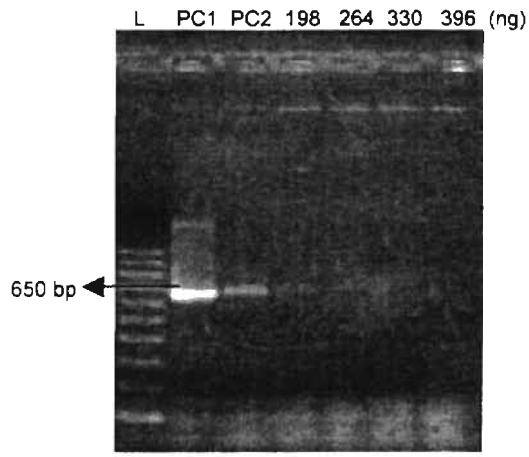


Figure 28. Gel electrophoresis to analyze the effect of increased template concentration on the formation of primer artifacts in PCR

L. EZ load 100 bp DNA marker

PC1: Control PCR with 132 ng of template using only the tailed and the non-tailed Primers

PC2: Control PCR with 132 ng of template using the UniPrimer™

Primers used: Primers 1 & 4. Set IV. Table 3 and the UniPrimer™

Template: *L. monocytogenes* 383-2 DNA

2.4 Primer concentration:

A PCR was devised and performed such that the primers involved in the formation of dimers could be identified. Six master mixes containing different primer combinations were prepared and used for PCR. The products were then analyzed using gel electrophoresis (Fig. 29).

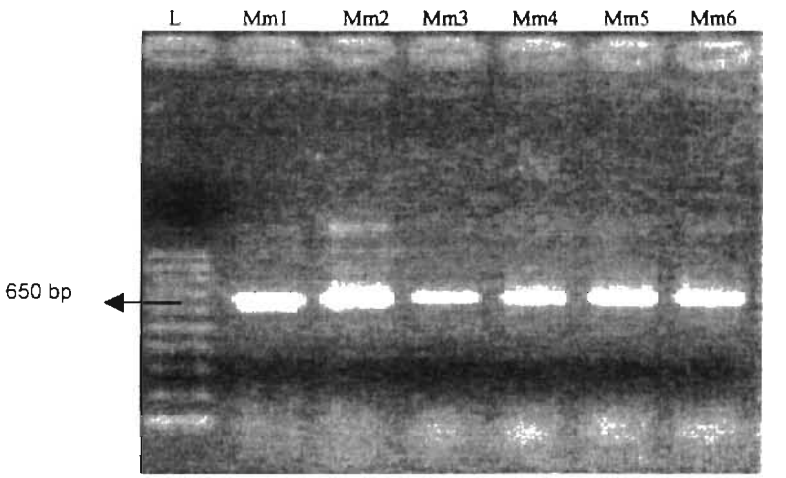


Figure 29: Gel electrophoresis of PCR products performed to identify primers involved in dimerization

L EZ load 100 bp DNA marker

Mm1. Target specific primers at 0.5 μM each

Mm2: Modified (tailed and non-tailed) primers at 0.5 μM each

Mm3: UniPrimer™ and non-tailed primer at 0.5 μM each and 0.05 μM tailed primer

Mm4: UniPrimer™ and non-tailed primer at 0.5 μM each and 0.055 μM tailed primer

Mm5. UniPrimer™ and non-tailed primer at 0.5 μM each and 0 0625 μM tailed primer

Mm6: UniPrimer™ and non-tailed primer at 0.5 μM each and 0.0714 μM tailed primer

Primers used: Primers 1, 2 & 4. Set IV Table 3

Template: *L. monocytogenes* 383-2 DNA

Gel analysis showed that on addition of the UniPrimer™ in the PCR, the band intensity of the primer dimers increased on the gel (Mm3, Fig. 29). This led to an assumption that the presence of the UniPrimer™ initiated or enhanced the formation of primer dimers. Hence in order to verify my assumption, the concentration of the UniPrimer™ used in the in the PCR was lowered while the concentrations of the non-tailed and the tailed primers were maintained at 0.5 μM and 0.05 μM respectively. The product obtained was analyzed using gel electrophoresis and the fluorescent plate reader. On analysis (Fig. 30) it was observed that even at low UniPrimer™ concentration, the PCR resulted in a good product yield for *L. monocytogenes*. This could be observed both from the gel picture and the relative fluorescence units (RFU) obtained (Fig. 30). *L. innocua* gave faint bands on the gel but its fluorescent reading was equal to that of the non-template control (Fig. 30). Considering the lower negative RFU values obtained with 0.05 μM UniPrimer™ and also assuming that the RFU values of the positive controls (i. e; *L monocytogenes*) can be improved, 0.05 μM UniPrimer™ was chosen for further studies.

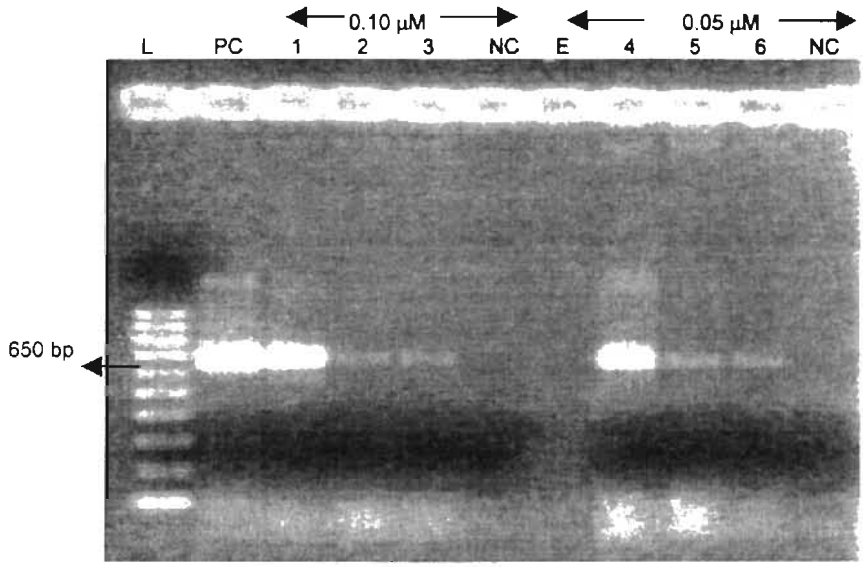


Figure 30. Gel electrophoresis analysis of PCR products testing the effect of low UniPrimer™ concentration when used with primers of set IV (Primers 1 & 4 Set IV. Table 3)

Listed below are the corresponding relative fluorescent units measured at an optimal gain of 111.

Description of samples run on the gel	RFU at optimal gain of 111
L. 100bp molecular mass ladder	
PC PCR product of L. monocytogenes 383-2 with no UniPrimer™	-
Lane 1 *L monocytogenes* 383-2 DNA (36µg/ml) as template	45,442
Lane 2: *L innocua* ATCC 33090 DNA (88µg/ml) as template	18,029
Lane 3. *L. innocua* ATCC 33090 DNA (44µg/ml) as template	17,466
NC. Non-template control	17,613
E: Empty	-
Lane 4: *L monocytogenes* 383-2 DNA (36µg/ml) as template	36,935
Lane 5 *L. innocua* ATCC 33090 DNA (88µg/ml) as template	11,010
Lane 6 *L. innocua* ATCC 33090 DNA (44µg/ml) as template	11,494
NC Non-template control	11,546

In an attempt to optimize the concentrations of the non-tailed and the tailed primers, a series of PCRs were performed with various primer concentrations. Upon fluorescence and gel analysis it was found that these variations did not improve either the fluorescent readings (RFU values) or the product yield. Hence it was determined that 0.5 μM non-tailed primer, 0.05 μM tailed primer and 0.05 μM UniPrimer™ were optimum for the PCR.

The tailed primer with the Z-sequence was 44 bases long. A primer of this length may result in non-specific binding or annealing, which would in turn result in high background fluorescence. Therefore, in order to further optimize the PCR the tailed primer was shortened on its 5' end and 2 modified tail primers were synthesized. One of these primers was 3 nucleotides shorter on its 5' end while the other was 6 nucleotides shorter.

PCR reaction mixes were prepared with *L. monocytogenes*, *L. innocua* and no template. Three sets of PCRs were performed with each reaction mix using the normal, -3 and the –6 tailed primers. On comparing the RFU values and the gel analysis (Fig 31), it was observed that the best product yield on the gel and the highest RFU values were obtained using the 41 nucleotides-long modified tailed primer (-3 nucleotides at 5' end). It was also observed that the fluorescence readings of the negative controls (non-*monocytogenes* DNA) obtained using the different tailed primers were approximately the same as their corresponding non-template controls. Hence it was determined that 0.05 μM of the –3 modified tailed primer (41 nucleotides) will be used for further studies.

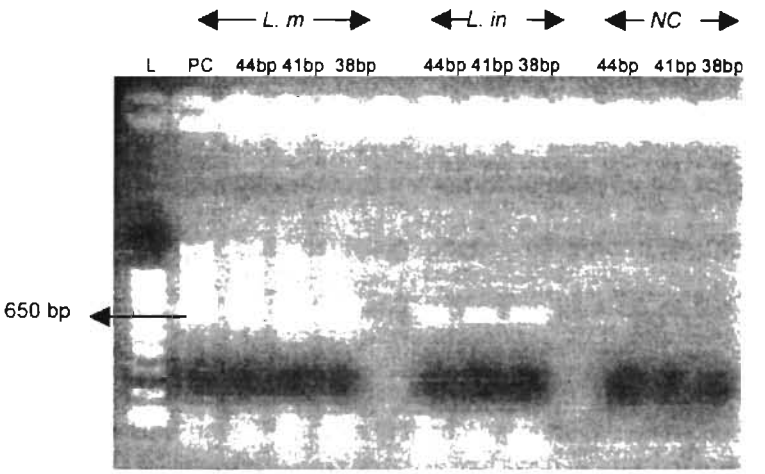


Figure 31. Gel electrophoresis performed to analyze the effect of the Z-sequence on the specificity of the tailed-primer and the product yield using set IV primers (Primers 1 & 4. Set IV. Table 3) and the UniPrimer™.

Given below are the corresponding fluorescent readings at an optimal gain of 110.

Description of the samples run on the gel	RFU at an optimal gain of 110
L. EZ load 100bp DNA marker	-
PC: Positive Control using only the tailed and the non-tailed primer	-
L. monocytogenes 383-2 using the UniPrimer™, the non-tailed primer and the following tailed primers	
44 bp tailed primer	44,887
41 bp tailed primer	45,003
38 bp tailed primer	33,179
L. innocua ATCC-33090 using the UniPrimer™ the non-tailed primer and the following tailed primers	
44 bp tailed primer	15,825
41 bp tailed primer	16,003
38 bp tailed primer	15,682
Non template Control using UniPrimer™ the non-tailed primer and the following tailed primers	
44 bp tailed primer	14 759
41 bp tailed primer	14,028
38 bp tailed primer	15,576

3. Template preparation and treatment

3.1 Culture media effects:

In the previous experiments the template used for the PCR was purified DNA, which was extracted from fresh cultures using phenol chloroform extraction. In some cases a commercially available DNA extraction and purification kit such as the "Promega Wizard kit" was used to obtain the purified template required. Considering that the main objective of this project was to optimize the UniPrimer™ for use in food analyses, the extraction of purified DNA would be an additional cumbersome step that would not be suitable for industrial application or routine food testing. Hence in order to test the efficiency of the UniPrimer™ in the presence of crude lysate, PCR was performed with the fresh lysate of cells grown in BHI (Bovine Heart Infusion) and FB (Frazier broth).

The product obtained was analyzed using both gel electrophoresis and the fluorescence plate reader (Fig. 32). It was inferred from the fluorescence readings of the BHI lysates that PCR product was formed only with *L. monocytogenes*. The gel picture (Fig. 32) also confirmed this. But the fluorescence readings of the FB lysates showed that high fluorescence was present with the positive; the negative; and the non-template controls also, whereas, the gel picture (Fig. 32) did not show any products for the negative and the non-template controls This discrepancy between the results on the gel and the fluorescence analysis led to the assumption that some of the ingredients of FB were fluorescing at the same wavelength as the fluorescein on the UniPrimer™. FB contains acriflavine (0.012 g / 1.00 L) which is a fluorochrome having an excitation wavelength of 436 nm and an emission wavelength of 535 nm. This when compared to the excitation (~ 490 nm) and the emission wavelength (~ 520 nm) of fluorescein suggested that acriflavine was interfering with the fluorescence detection thus resulting in high RFU values for the non-*L. monocytogenes* lysates. From the figure (Fig. 32) it was also observed that the fluorescence reading obtained with the lysate were greater than those obtained with purified DNA (Positive control). This observation added to the assumption that the crude lysate was interfering

with the fluorescence emission. These assumptions were confirmed by several assays in which acriflavin was not added to the FB.

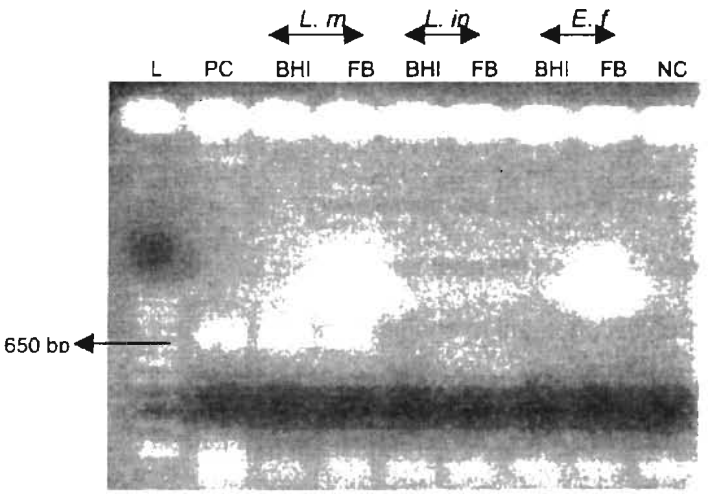


Figure 32. Gel electrophoresis and fluorescence analysis verifying the effect of acriflavine (in FB) on fluorescence detection

Primers used: Primers 1 & 4. Set IV. Table 3

Description of the samples run on the gel	RFU at a manual gain of 135
L EZ load 100 bp DNA marker	
PC positive Control using purified *L monocytogenes* DNA	27,558
L. monocytogenes 383-2 BHI - lysate	30,647
L. monocytogenes 383-2 FB – lysate	48,946
L. innocua ATCC 33090 BHI – lysate	19,239
L. innocua ATCC 33090 FB –lysate	43,622
E feacalis BHI –lysate	18,919
E. feacalis FB –lysate	42,700
Non-template control	7,346

3.2 Cell lysis and DNA extraction

Thus, as observed from the previous experiments, the crude lysate cannot be used as template for PCR. Therefore DNA in the lysate was precipitated using isopropanol and then used for PCR. In order to compare the efficiency of the isopropanol precipitation, PCR was also performed with the crude lysate. The product obtained was analyzed by gel electrophoresis and the fluorescence plate reader (Fig. 33). It was observed that the fluorescence readings of the negative controls of the crude lysates were greater than the negative controls of the isopropanol treated lysates. In addition the fluorescence readings of the negative controls were almost equal to the fluorescence readings of the positive control (*L. monocytogenes*) when the crude lysate was used. Thus, it was concluded that the isopropanol treatment eliminates the background fluorescence caused by cell debris or media ingredients.

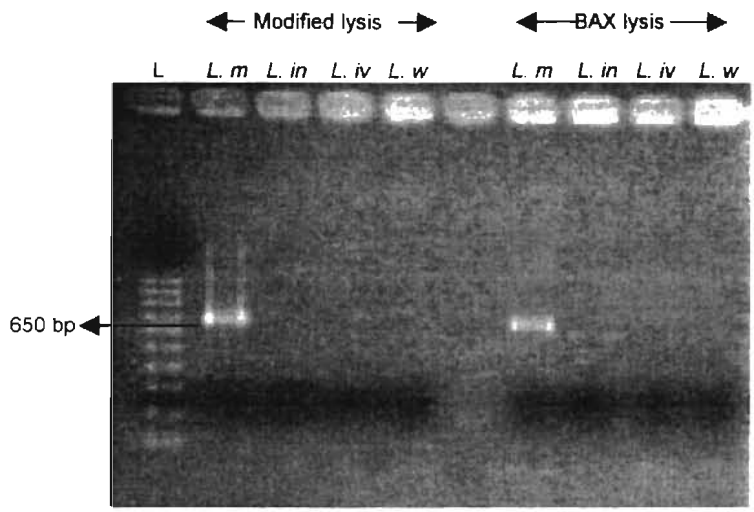


Figure 33. Gel electrophoresis and fluorescence analysis performed to test the effectiveness of the modified lysis procedure using isopropanol to simultaneously recover DNA and remove acriflavine

Description of the samples run on the agarose gel	RFU at a manual gain of 114
L EZ load 100bp DNA marker	
Modified Lysis (with isopropanol)	
L monocytogenes 383-2	35,109
L. innocua ATCC-33090	13,977
L ivanovii ATCC-33091	15,194
L. welshimeri	16,110
BAX lysis (without isopropanol)	
L. monocytogenes 383-2	>50,000
L innocua ATCC-33090	33,467
L. ivanovii ATCC-33091	24,704
L. welshimeri	44,232

4.2 Formed turkey

Since the previous experiment showed that the system can detect the presence of 1 CFU / gm, the food samples to be tested were inoculated with culture containing only 1 CFU / gm. Nine *L. monocytogenes* strains and one non-*L. monocytogenes* strain were tested. The products obtained were analyzed using both gel electrophoresis and fluorescence detection (Fig. 35) and it was observed that all the *L. monocytogenes* strains tested positive while the non-*monocytogenes* strain tested negative with both gel electrophoresis and fluorescence

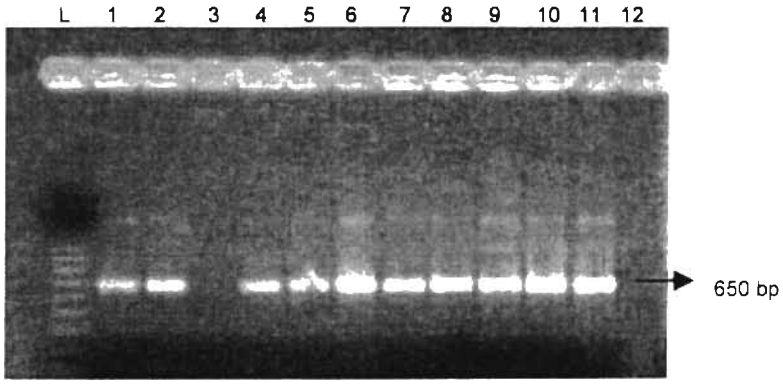


Figure 35. Gel electrophoresis and fluorescence detection of *L. monocytogenes* from inoculated turkey using the Amplifluor UniPrimer™ system

Description of the samples run on the gel	RFU at manual gain of 114
L. EZ load 100 bp DNA marker	
1 *L. monocytogenes* BQ-34	42,252
2 *L. monocytogenes* 99-60	33,270
3. N-m: Non-*monocytogenes* strain	23,297
4 *L. monocytogenes* 99-52	33,580
5 *L. monocytogenes* 99-49	41,817
6 *L. monocytogenes* 99-15	45,049
7 *L. monocytogenes* 99-38	37,504
8 *L. monocytogenes* 99-5	36,674
9 *L. monocytogenes* 98-71	46,430
10 *L. monocytogenes* 98-10	40,544
11 *L. monocytogenes* SA-2	43,690
12 NC: non-template control	24,105

4.3 Roast beef

Nine *L. monocytogenes* strains, one non-*monocytogenes* strain and a non-template control were inoculated into the roast beef product. From the results obtained (Fig. 36), it was seen that *L. monocytogenes* strain 99-60, even though it gave a low RFU value (19,174), was still higher than the non-monocytogenes (12,866) and the non-template control (16,275). All the other strains of *L. monocytogenes* resulted in significantly higher fluorescence values (RFU) than the negative and non-template controls

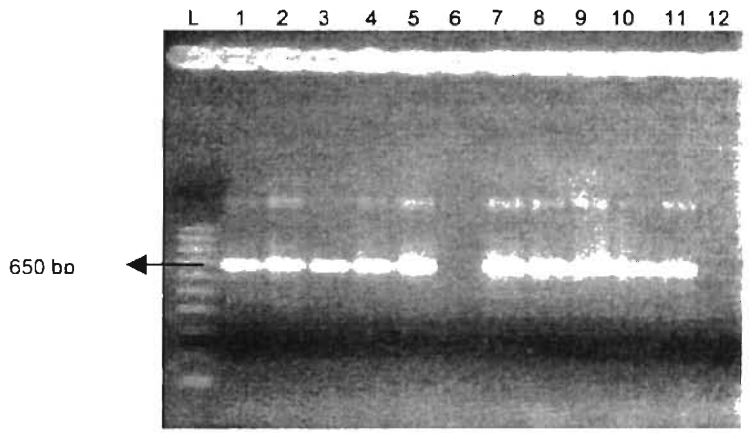


Figure 36: Gel electrophoresis and fluorescence detection of *L. monocytogenes* from Inoculated roast beef using the Amplifluor UniPrimer™ system

Description of the samples run on the gel	RFU at a manual gain of 108
L. EZ load 100 bp DNA marker	
1 *L. monocytogenes* 99-52	36,155
2 *L. monocytogenes* 99-38	37,286
3 *L. monocytogenes* 99-60	19,174
4 *L. monocytogenes* BQ-34	26,659
5 *L. monocytogenes* 99-49	36,192
6 Nm: Non-*monocytogenes* strain	12,866
7 *L. monocytogenes* 99-15	35,496
8 *L. monocytogenes* 98-10	32,515
9. *L. monocytogenes* SA-2	28,142
10. *L. monocytogenes* 99-5	21,111
11 *L. monocytogenes* 98-71	29,655
12 NC Non-template Control	16,275

4.4 Formed ham

Results obtained from the gel and the fluorescence analysis are given (Fig. 37) Even though the *L. monocytogenes* strain 99-60 was negative when compared to the non-*monocytogenes* strain by both gel analysis and fluorescence detection. The other strains of *L. monocytogenes* resulted in fluorescence readings (RFU) higher than the controls.

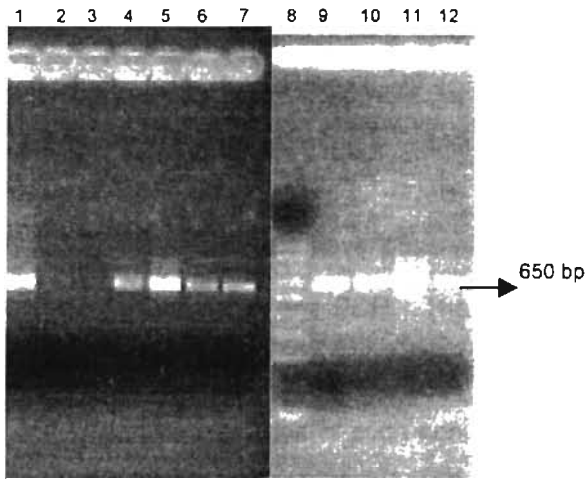


Figure 37: Gel electrophoresis and fluorescence detection of *L. monocytogenes* from Inoculated formed-ham using the Amplifluor UniPrimer™ system

Description of the samples run on the gel	RFU at a manual gain of 114
1. *L.monocytogenes* BQ-34	36,592
2. *L. monocytogenes* 99-60	16,621
3 Nm Non-*monocytogenes* strain	19,450
4. *L. monocytogenes* 99-52	22,364
5. *L. monocytogenes* 99-49	29,482
6. *L. monocytogenes* 99-15	26,050
7 *L. monocytogenes* 99-38	31,312
8. L. EZ load 100 bp DNA marker	
9. *L. monocytogenes* 99-5	25,229
10. *L. monocytogenes* 98-71	36,546
11 *L. monocytogenes* 99-10	49,439
12 *L. monocytogenes* SA-2	28,094

CONCLUSIONS AND FUTURE RESEARCH

The Amplifluor UniPrimer™ system was applied to the detection of *L. monocytogenes* in ready-to-eat food products. When compared to the current detection procedures recommended by the FSIS (Food Safety and Inspection Service), this system enables simple and rapid detection of the pathogen making it applicable for the food industry. The fluorescent detection helps to eliminate the use of agarose gels and gives an instant positive or negative result.

During the course of my research I have observed that the formation of primer dimers or artifacts is a common occurrence in any PCR. The formation of such non-specific products would result in high background fluorescence and thus interfere with the fluorescence detection. In an attempt to eliminate the formation of such non-specific products, I have tested four sets of primers under different PCR conditions. In the first three primer sets tested, none of the variations in PCR conditions eliminated the primer artifacts In the fourth primer set, however, the reduction of primer concentration (UniPrimer™ concentration) played a major role in the elimination of primer artifacts. It is therefore important to carefully select or design target-specific primers that could be used with the UniPrimer™.

One of the major observations during my research was that different tubes of the same reagent purchased from the same company, when used in duplicate reactions could give varying results. This observation was made based on the results obtained from duplicate PCRs where the only difference was that tube-1 of 10X PCR buffer (Promega) was used in the first PCR and tube-2 of 10X PCR buffer (Promega) was used in the second PCR. The first PCR resulted in the formation of a product while the second PCR did not give a product. When the first reaction was repeated with the tube-1 of 10X PCR buffer, the PCR again gave a product. This confirmed that the contents of the two tubes of buffer varied though they were from the same company and had the same lot number.

I optimized the Amplifluor UniPrimer™ system using pure DNA of different *Listeria* spp. which was extracted using various purification and extraction procedures and commercially available DNA purification kits. For food industrial application, the extraction of DNA may be considered as an additional step. Therefore the system was then optimized with the crude lysate. When crude cell lysate was used as template, the ingredients in the enrichment media and the cell debris not only prevented the PCR but also resulted in high background fluorescence. In order to overcome this problem, isopropanol was used to simultaneously recover the DNA and remove the acriflavin in the DNA. Isopropanol precipitation enabled a quick and easy procedure to obtain DNA from the crude lysate and to use it as template for the UniPrimer™.

The optimized system was used to detect different strains of *L. monocytogenes* inoculated into food samples. I observed that one strain of *L. monocytogenes* (99-60) gave low fluorescence values and in some cases resulted in fluorescence values as low as the negative and the non-template controls. Future research could involve optimization of the Amplifluor UniPrimer™ system using this strain of *L. monocytogenes*. The system should also be tested with various other strains of *L. monocytogenes* that were not used in my research. The background fluorescence obtained for each food sample was different, the relative fluorescence units (RFU) of a particular strain of *L. monocytogenes* in one food sample cannot be compared to its RFU in another food sample. Therefore it is essential to perform a negative control reaction (or a non-template control reaction) so that positive and the negative fluorescence units can be distinguished

Future research should be focused towards the improvement of the Amplifluor UniPrimer™ system. Research could be done to improve the signal to background ratio such that there would be a significant difference between the positive and the negative results. The Z-sequence could be added to the 5' end of both the target-specific primers such that the UniPrimer™ would bind to both the strands of the initially formed amplicon and thus result in greater fluorescence emission.

Since the ingredients or the contents of the enrichment media and the food sample being tested are interfering with the PCR and also the fluorescence detection, It would be

beneficial to perhaps use immuno-magnetic beads for separation of *L. monocytogenes* from the enrichment cultures. This will make the Amplifluor UniPrimer™ applicable for raw meats and ready-to-eat foods.

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VITA

Hannah Nayakanti

Candidate for the Degree of

Master of Science

Thesis: APPLICATION OF FLUORESCENT-TAGGED PRIMERS FOR PCR-BASED NON-GEL DETECTION OF *LISTERIA MONOCYTOGENES*

Major Field: Biochemistry and Molecular Biology

Biographical:

Personal Data: Born in Secunderabad, India, on April 23rd 1976. Daughter of James and Geeta Nayakanti.

Education: Graduated from St. Francis Junior College, Secunderabad, India in March 1993: received Bachelor of Science degree in Genetics, Microbiology and Chemistry from Osmania University, Hyderabad, India in April 1996, received a Masters degree in General Biochemistry from Madras University, Madras India in April1998. Completed the requirements for the Master of Science degree with a major in Biochemistry and Molecular Biology at Oklahoma State University, December 2001.

Professional Experience: Teaching Assistant, Department of Biochemistry and Molecular Biology, Oklahoma State University, August 1999 to December 1999. Research Assistant, Department of Biochemistry and Molecular Biology, Oklahoma State University, January 2000 to present.