APPLICATION OF A REAL-TIME 'UNIVERSAL' PRIMER FOR PCR DETECTION OF *LISTERIA MONOCYTOGENES* FROM MEAT PRODUCTS

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PREFACE

This study was conducted to provide support for the new approaches in developing rapid real-time PCR detection methods for the screening of *Listeria monocytogenes* from both raw and ready-to-eat meat products. Specific objectives of this research was to (a) optimize the fluorescence based Amplifluor^{TM} Universal primer (Uniprimer^{TM}) real-time PCR system for the detection of *Listeria monocytogenes*, (b) Apply the Uniprimer^{TM} real time PCR system for the detection of *Listeria monocytogenes* from artificially inoculated food samples (raw ground meats and hotdogs), (c) testing of retail food samples for *L. monocytogenes* by both Uniprimer^{TM} real-time PCR and traditional method in order to validate the sensitivity of Uniprimer^{TM} PCR.

I sincerely thank my advisor Dr. Peter M. Muriana for the valuable guidance and support in the completion of this project. I also thank my master's committee members – Dr. William McGlynn and Dr. Udaya DeSilva for sharing their expertise in this research.

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LIST OF ABBREVIATIONS

ActA	Actin A
BCM	Baylor College of Medicine, HGSC
BHI	Brain Heart Infusion
CAMP	Christie Atkins Munich Peterson
CDTX	Cholesterol Dependent Toxin
cfu	Colony Forming Unit
CNS	Central Nervous System
CSF	Cerebral Spinal Fluid
Ct	Threshold Cycle
Ctl	Controls
DNA	Deoxyribo Nucleic Acid
dNTP	Deoxyribo Nucleic Acid Triphosphate
ds	Double Stranded
ELISA	Enzyme Linked Immuno Sorbent Assay
FA	Fluorescent Antibody
FB	Fraser Broth
FDA	Food and Drug Administration
FSIS	Food Safety and Inspection Services
g	Gram

hlyA	Hemolysin
hrs	Hours
i.v.	Intravenously
Inl	Internalin
LEB	Listeria Enrichment Broth
LLO	Listeriolysin O
LPM	Lithium Chloride Phenyl Ethanol Plating Media
MDL	Minimum detection level
mg	Milligram
$MgCl_2$	Magnesium Chloride
min	Minutes
ml	Milliliter
mM	Millimolar
MOPS-BLEB	3-(N-Morpholino) Propane Sulfonic Acid- Buffered Listeria Enrichment Broth
MOX	Modified Oxford Agar
MPL	MetalloProtease
NCBI	National Center for Biotechnology Information
nm	Nanometer
PCR	Polymerase Chain Reaction
Plc	Phospholipases C
PrfA	Positive Regulatory Factor
RFU	Relative Fluorescence Unit

RNA	Ribose Nucleic Acid
RTE	Ready-to-eat
Sec	Seconds
ss	Single Stranded
TSA	Tryptic Soy Agar
USDA	United States Department of Agriculture
UVM	University of Vermont Media
VIDAS	Vitek Immuno Diagnostic Assay System
µL	Microliter
µM	Micromolar
1^{o}	Primary
2^{o}	Secondary

CHAPTER I

INTRODUCTION

1.1 Background of *Listeria* species.

Listeria monocytogenes, a small Gram-positive bacillus which caused monocytosis, was first isolated in 1924 (Murray et al. 1924). Hence the organism was named *Bacterium monocytogenes*. Three years after Murray et al. (1924), Pirie isolated it from a gerbille (an African jumping mouse) and named it *Listerella hepatolytica* in honor of Lord Lister (Pirie et al. 1940). However, *B. monocytogenes* and *L. hepatolytica* was the same organism and the name was changed to *Listerella monocytogenes*. In 1939, it was discovered that the name *Listerella* had been applied to a group of slime molds and the name was changed to *Listeria monocytogenes* by Pirie (Pirie et al. 1940).

1.2 Taxonomical classification

The genus *Listeria* initially contained *L. monogytogenes*, *L. ivanovii*, *L. innocua*, *L. welchimeri*, *L. seeligeri*, *L. denitrificans*, *L. murrayi*, and *L. grayi* (Ryser and Marth, 1991).The taxonomic position of *L. grayi* and *L. murrayi* is controversial. The numerical taxonomic studies based on cell wall, cytochrome, menaquinone and fatty acid composition revealed that *L. murrayi* and *L. grayi* is indistinguishable and were placed in a single species *L. grayi* (Fiedler and Sager, 1983). In 1974 based on DNA homology, *L. murrayi* and *L. grayi* was found to be sufficiently distinct from *Listeria monocytogenes* and therefore were placed into separate single genus *Murraya* (Stuart and Welshimer, 1974). In addition, morphological and biochemical characteristics of *L. denitrificans* indicated that *L. denitrificans* was not a member of genus *Listeria*. Because of these

ambiguities, in Bergey's manual of systematic bacteriology, these three species are characterized as "incertae sedis" (species of uncertain position) (Seeliger and Jones, 1993). Three of the eight *Listeria* species, *L. monocytogenes*, *L. ivanovii* and *L. seeligeri* can cause human and/or animal infections. However, the later two are extremely rare and are considered to be far less virulent than *L. monocytogenes* which is currently responsible for all cases of human listeriosis (Ryser and Marth, 1991).

1.3 Characteristics of *L. monocytogenes*

Listeria monocytogenes is a Gram-positive, pleomorphic short rod shaped bacterium with rounded ends that is not spore forming and is not acid fast (Fig. 1). When observed microscopically, fresh isolates are in the smooth pathogenic form and appear as short diptheroid-like rods measuring 1.0-2.0 μm x 0.5 μm. Cells of young *Listeria* isolates may appear as diplococci or cocci. They exhibit an end-over-end tumbling motility when grown at 20-25°C but not at 37°C (Ryser and Marth, 1991).

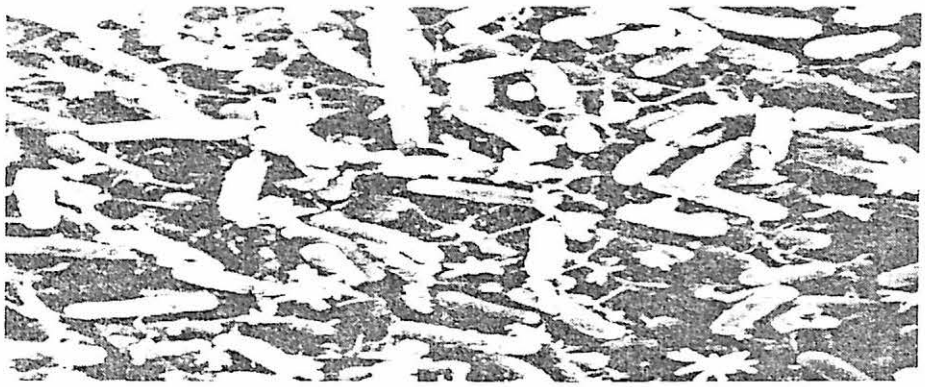

Figure 1. An electron micrograph picture of *L. monocytogenes* (Ryser and Marth, 1991).

Listeria monocytogenes is a psychrotrophic organism that can grow at a wide range of temperature from 1- 45°C with optimum growth at the temperature of 30-37°C (Gray,

1960). It is not fastidious and can reproduce readily in simple bacteriological media. Typical colonies on Tryptic Soy Agar (TSA) are between 0.3 and 1.5 mm in diameter, translucent, slightly raised with a fine textured surface, watery in consistency, bluish gray in color under normal illumination and have entire margins. In blood agar plates after incubation, the colonies of *L. monocytogenes* are surrounded by a narrow zone of β - hemolysis (Ryser and Marth, 1991). *L. monocytogenes* is generally recognized as facultatively anaerobic mesophile organism because of its propensity to grow better with reduced oxygen tension than in air. However, it fails to grow under strict anaerobic conditions (Seeliger, 1961). *Listeria* species hydrolyse esculin and sodium hippurate. They are catalase-positive, oxidase-negative, urease-negative, methyl red-positive and Voges-Proskauer-positive. Acid is produced without any gas production from the fermentation of glucose. The CAMP test (first discovered in streptococci by Christie, Atkins and Munch-Peterson) is useful in differentiating *Listeria* species based on their β-hymolysis on sheep blood agar plates. Cultures of β-hemolytic *Staphylococcus aureus* and *Rhodococcus equi* are streaked vertically on sheep blood agar plate and *Listeria* species. are streaked right angle to these two cultures. After 48 hours (hrs) of incubation at 35°C, β-hemolysis of *L. monocytogenes* and *L. seeligeri* are produced near *Staphylococcus* streak and *L. ivanovii* is enhanced near the *Rhodococcus* streak. However, other species are non hemolytic (Fig. 2). Furthermore, all the eight species of *Listeria* can be differentiated and identified based on their biochemical properties and CAMP test (Table 1).

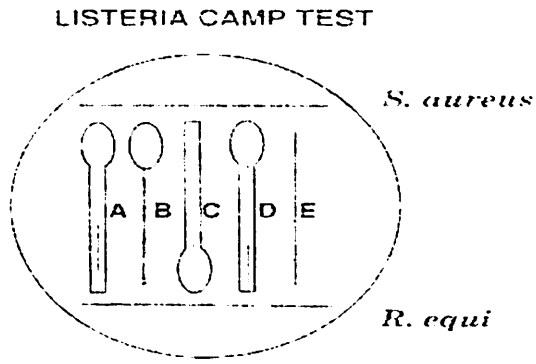

Figure 2. CAMP test for identifying *Listeria* species. The white zone of β-hemolysis indicates a positive reaction. *L. monocytogenes* typical (A), *L. monocytogenes* atypical (B), *L. ivanovii* (C), *L. seeligeri* (D), *L. innocua* negative reaction (E) (Ryser and Marth, 1991).

Table 1. Differentiation of *Listeria* species based on traditional biochemical tests (Ryser and Marth, 1991).

	Listeria sp.							
Biochemial test	*Monocytogenes*	*Ivanovi*	*Innocua*	*Welshimeri*	*Seeligeri*	*Grayi*	*Murrayi*	*denitrificans*[a]
dextrose	+	+	+	+	+	+	+	+
Esculin	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+
Mannitol	-	-	-	-	-	-	+	+
Rhamnose	+	-	V[b]	V	-	-	V	-
Xylose	-	+	-	+	+	-	V	-
Hippurate hydrolysis	+	+	+	+	+	-	-	-
Voges-proskauer	+	+	+	+	+	+	+	-
Methyl red	+	+	+	+	+	+	+	+
Beta-hemolysis	+	+	-	-	V	-	-	-
Urea hydrolysis	-	-	-	-	-	-	-	-
Nitrate reduction	-	-	-	-	-	-	+	+
Catalase	+	+	+	+	+	+	+	+
H_2S on TSI	-	-	-	-	-	-	-	-
H_2S by lead acetate strip	-	-	-	-	-	+	+	-
CAMPpositive/S.aureus	+	-	-	-	+	-	-	-
CAMP positive/R. equi	-	+	-	-	-	-	-	-

[a]Reclassified as *Jonesia denitrificans*
[b]V- Variable

1.4 habitat and Transmission

L. monocytogenes is widely distributed in the environment from a variety of sources including soil, vegetation, fecal material, sewage and water. Improperly fermented silage has been cited as the source of infection in numerous cases of listeriosis (Weishimer 1966). The mode of transmission is highly complex and humans can come into contact with this pathogen through animals, meat, dairy products, sea foods, plants, insects and other humans (Fig. 3).

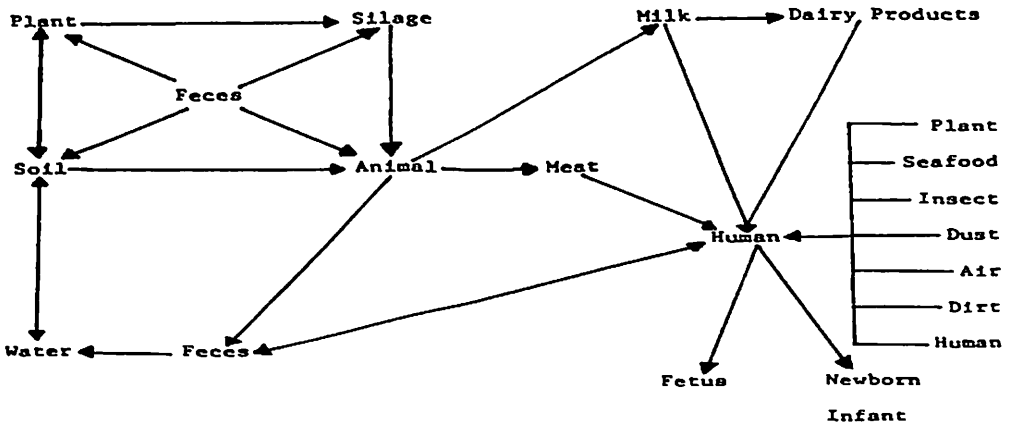

Figure 3. The mode of transmission of *L. monocytogenes* in humans (Ryser and Marth, 1991).

L. monocytogenes also has the ability to survive under adverse environmental conditions for an extended time which makes this organism a particular threat to the food industry (Ryser and Marth, 1991). Survival of *L. monocytogenes* under various environmental conditions has been summarized in Table 2.

Table 2. Survival conditions of *L. monocytogenes* in the environment (Ryser and Marth, 1991)

Sample	Storage temperature (°C)	Survival (days)
Soil		
Sterile soil (I)[a]	Outside—Winter/Spring	154
Clay soil (I)		
sealed tubes	24–26	225
cotton-plugged tubes	24–26	67
Fertile soil (I)		
sealed tubes	24–26	295
cotton-plugged tubes	24–26	67
Top soil (I)		
exposed to sunlight	NG[b]	12
not exposed to sunlight	NG	182
Moist soil	NG	ca. 497
Dry soil	NG	>730
Soil	4–12	240–311
Soil	18–20	201–271
Fecal material		
Cattle feces (NC)[c]	5	182–2190
Moist horse/sheep feces (I)	Outside	347
Dry horse/sheep feces (I)	Outside	730
Sheep feces	Outside	242
Liquid manure	Summer	36
Liquid manure	Winter	106
Sewage		
Sewage sludge cake (NC)		
surface	28–32	35
interior	48–56	49
sprayed on field	Outside	>56
Water		
Sterilized pond water (I)	Outside	7
Unsterilized pond water (I)	Outside	<7–63
Pond water	35–37	346
Pond water	15–20	299
Pond water/ice	2–8	790–928
Pond/river water	37	325
Pond/river water	2–5	750
Water	Outside	140–300
Distilled water (I)	4	<9
Animal feed		
Silage (NC)	4	450
Silage (NC)	5	180–2190
Mixed feed (I)	Outside	188–275
Oats (I)	Outside	150–300
Hay (I)	Outside	145–189
Straw (NC/I)	ca. 22	365
Straw (I)	Outside	47–207
Straw	Outside–Summer	23
Straw	Outside –Winter	135

[a]Inoculated.
[b]Not given.
[c]Naturally contaminated.
Source: Adapted from Refs. 4,5,49.

Chapter II

Review of the Literature

2.1 Pathogenesis of *L. monocytogenes*

2.1.1 Entry and colonization of host tissue

Listeria host cell interactions have been studied extensively. Both macrophage and non-professional phagocytes can serve as host cells for *L. monocytogenes*. Before reaching the intestine, the pathogen must survive the gastric acidity barriers in the stomach which may destroy significant number of organisms ingested with the contaminated food. There are two basic theories proposed regarding the entry and the mechanism of intestinal translocation of *L. monocytogenes*. Early studies by Racz et al. (1972) suggested that the organism penetrates the host by invading the intestinal epithelium and this is consistent with another study where *L. monocytogenes* is able to penetrate the apical surface of polarized, differentiated human enterocytes like Caco-2 cells with an intact brush border (Racz et al. 1972, Karunasagar et al. 1994). In other studies, using mice, no invasion of intestinal villous epithelium was observed; instead there was colonization of Peyer's patches which suggested that *L. monocytogenes* uses M-cell epithelium as a portal of entry (Mac Donald et al. 1980, Marco et al. 1997). An epithelial phase involving bacterial multiplication in the intestinal mucosa is not required by *L. monocytogenes* for systemic infection, and prior to intraepithelial replication the organisms are transported to deeper organs very rapidly (Fig. 4).

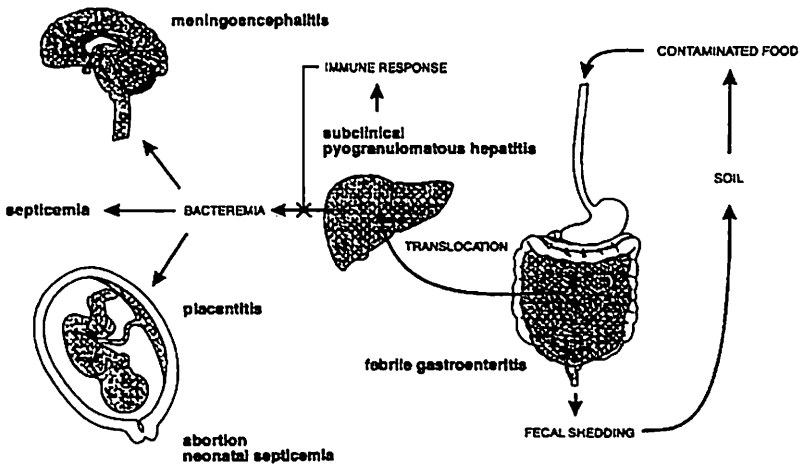

Figure 4. Schematic diagram for the pathophysiology of *Listeria* infection (Vazquez-Boland et al. 2001a).

After crossing the intestinal barrier the *L. monocytogenes* are carried by the lymph or the blood to the mesenteric lymph nodes, the spleen and the liver. If the infection is not controlled by an adequate immune response in the liver, unlimited proliferation of *L. monocytogenes* in the liver parenchyma may result in the release of bacteria into the circulation. *L. monocytogenes* is a multi-systemic pathogen which may cause septicemia involving multiple organs with localized *Listeria* infection and has pathogenic tropism towards the gravid uterus and Central Nervous System (CNS). *L. monocytogenes* gains access to the fetus by hematogenous penetration of the placental barrier. Colonization of the trophoblast layer followed by translocation of the bacteria across the endothelial barrier enables the bacteria to reach fetal bloodstream, resulting in generalized infection and subsequent death of fetus in uterus or to premature birth of a severely infected neonate with miliary pyogranulomatous lesions (small skin lesions that have size and appearance like millet seeds with an infiltration of macrophages, plasma cells and polymorphonucleocytes) (Kathariou 2000, Vazquez-Boland et al. 2001a).

2.1.2 Intracellular infectious cycle

The cycle begins with adhesion to the surface of eukaryotic cells and subsequent penetration of the bacterium into host cells. The invasion of non-pathogenic cells involves a zipper-type mechanism in that the bacterium gradually sinks into dip like structures of the host cell surface until it is finally engulfed in a phagocytic vacuole where *L. monocytogenes* ensures its intravacuolar viability by preventing phagosome maturation to the phagolysosomal stage (Alvarez-Dominguez et al.1997). Internalization is rapidly followed by the lysis of the phagocytic vacuole and liberation of the bacterium into the cytosol where it replicates (Gedde et al. 2000). Intracytoplasmic *L. monocytogenes* are immediately surrounded by fine, fuzzy, fibrillar actin filaments which rearrange to form 40 μm polar tails. These tails propel *L. monocytogenes* in the cytoplasm with a mean speed of 0.3 μm/s and the bacteria eventually reach the cell periphery, come into contact with the cell membrane and pushes it where they induce the formation of pseudopods that penetrates uninfected neighboring cells and are, in turn, engulfed by phagocytosis (Vazquor-Boland et al. 2001a). The bacteria escape rapidly from the newly formed vacuole by dissolving its double membrane, and are released into the cytoplasm where they initiate a new round of intracellular proliferation and spread (Fig. 5).

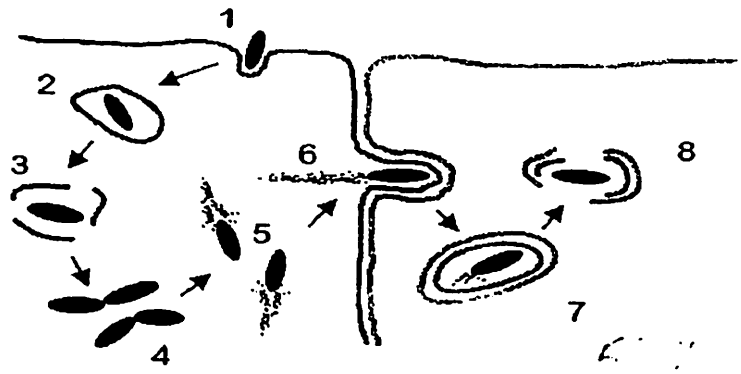

Figure 5. Schema of intracellular life cycle of pathogenic *Listeria* Entry in host cell (1), survival in phagosome (2), escape from phagosomal membrane (3), multiplication inside host cell (4), formation of actin tail and movement (5), cell to cell spread (6), survival inside double membrane of host cell (7), dissolution of host cell double membrane (8) (Vazquez-Boland et al. 2001b).

2.2 Virulence factors in *Listeria monocytogenes*

In *Listeria*, the virulence genes are clustered into so-called "pathogenicity islands" in the chromosome. The genetic structure and transcriptional organization of virulence genes includes three transcriptional units (Fig. 6).

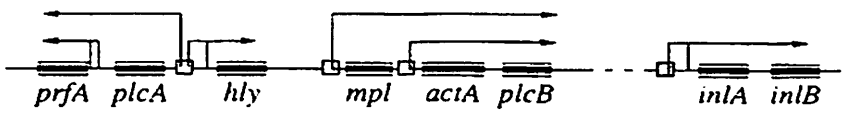

Figure 6: The scheme of the *prfA* regulon. Boxes with arrows in it indicates PrfA binding sites for PrfA dependent transcriptions and arrows without the boxes indicates PrfA independent transcription (Ermolaeva 2001).

The hemolysin gene, *hly*A, was the first virulence determinant to be identified and sequenced in *Listeria* species. The *hly*A gene, which encodes the protein hemolysin (Hly), occupies the central position of the transcriptional unit and is a key virulence

factor essential for pathogenesis. The production of this soluble hemolysin by *L. monocytogenes* was first reported by Harvey and Faber in 1941. Thereafter an extensive effort was made by several researchers to characterize it and finally in 1987 the first unambiguous evidence was provided that hemolysin is a pore-forming toxin protein called listeriolysin O (LLO). It is a streptolysin-O (SLO) related cytolysin that belongs to the family of cholesterol-dependent toxin (CDTX) with characteristic low pH optimum (5.5) and narrow range pH (4.5-6.5) over which it is active (Geoffroy et al. 1987). There is a direct correlation between hemolytic activity and pathogenecity of genus *Listeria* and it was observed that the spontaneous loss of hemolysin production results in avirulence (Hof 1984). The role of LLO in virulence is as a mediator of phagosome membrane disruption after the uptake of extracellular bacteria upon its initial entry into the host cell and it is also required for the efficient escape of *L. monocytogenes* from the double membrane vacuole during cell to cell spread (Gedde et al. 2000) (Fig. 7). *L. monocytogenes* also secretes two phospholipase C (Plc) enzymes, PlcA and PlcB, production of which was first observed as an opacity reaction in egg yolk agar that correlated with the hemolytic activity of the strain (Fuzi and Pillis, 1962). The *plc*B gene, which encodes the PlcB protein is located downstream from *hly*A in a 5.7 kb operon along with two other genes *act*A and *mpl*. The PlcB is secreted as inactive propeptide and needs extracellular processing by the metalloprotease (MPL) enzyme, the product of *mpl* gene of the same operon. Although the primary function of PlcB is to mediate dissolution of double membranes formed during cell to cell spread, PlcB works with Hly in dissolving primary vacuole during phagocytosis of extracellular *Listeria* (Vazquez-Boland et al. 2001a, Ryan et al. 2002). The third gene of same operon *act*A encodes the

surface protein Actin A (Act A) that is attached to the bacterial cell wall. ActA is responsible for listerial intracellular motility and cell to cell spread. ActA is also involved in internalization of *Listeria* by host cells. Thus, the 3 protein products of *mpl-act*A-*plc*B operon are responsible for the direct passage of bacteria from one cell to another. The *plc*A gene which encodes phosphatidyl inositol (PI)-specific phospholipase C (PlcC) is located upstream region from *hly*A and forms the bicistronic operon, *plc*A-*prf*A. PI-specific PlcC synergizes with Hly and PlcB in the destabilization of primary phagosomes. The *prf*A gene encodes the positive regulatory factor (PrfA) protein, a transcriptional factor that is required for the transcriptional activation of all the genes of the cluster including PrfA itself. Besides these proteins, internalins (Inl) are the protein products of a family of virulence-associated genes found in pathogenic *Listeria* species. The first members of this family characterized in *L. monocytogenes* are InlA and InlB proteins encoded by the *inlAB* operon. InlA was shown to function as an invasion protein, mediating bacterial internalization through non-phagocytic epithelial cells like those of the intestinal epithelium. InlA is sufficient for inducing adhesion and uptake of *Listeria* by epithelial cells (Gaillard et al. 1991). PrfA is the only virulence regulator for all the virulence genes. A number of environmental and growth phase dependent signals control the expression of virulence genes via PrfA. Hly and InlAB are partially regulated and can be transcribed by *prf*A independent promoters (Ermolaeva 2001) (Fig.7).

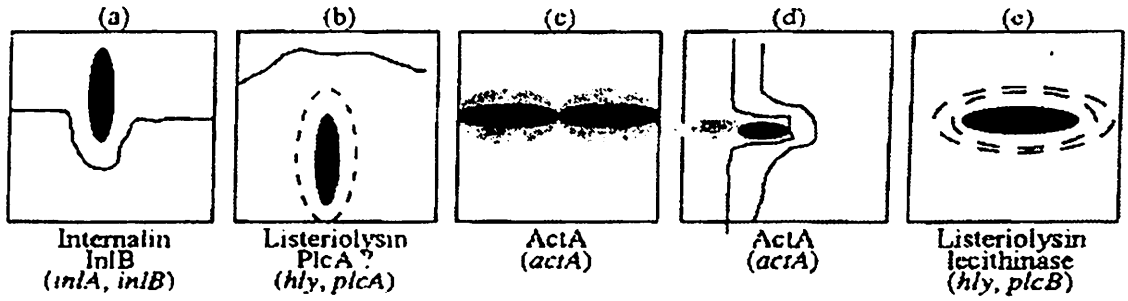

Figure 7. Schematic representation of the virulence factors of *L. monocytogenes*. The encoding genes are presented in the brackets. (A) internalization, (b) lysis of primary vacuole, (c) cell division and formation of actin tail, (d) cell to cell spread, (e) lysis of two plasmatic membranes after entering the neighboring cell and commencement of new cycle (Ermoleaeva 2001)

2.3 Clinical features

The clinical features of *L. monocytogenes* are often confused with other illnesses since these infections are very similar in all susceptible groups. Human listeriosis is generally characterized by puss formation and miliary granulomas (masses of inflamed tissue comprised of many small lesions and focal necroses). Mainly, Human listeriosis is distinguished into two basic forms- Perinatal listeriosis and listeriosis in adults.

In both cases, the predominant clinical forms correspond to disseminated infection or to local infection of the Central Nervous System (CNS). Size, number and severity of the disease depend on the infectious dose, route of infection, and host risk factors. Table 3 describes prominent clinical symptoms although combinations of two or more manifestations may occur simultaneously or in succession. Listeriosis is a very severe disease with a mean mortality of 20-30% or higher despite early antibiotic treatment (Vazquez-Boland et al. 2001a, Ryser and Marth, 1991, and Rocourt et al. 2000).

Table 3. Manifestations of listeriosis in humans (Ryser and Marth, 1991)	
1.	Listeriosis during Pregnancy
2.	Meningitis, meningoenchepalitis, and encephalitis
3.	Cutaneous form
4.	Septicemia with pharyngitis and mononucleosis
5.	Oculoglandular form
6.	Listeriosis of the new born (granulomatosis infantiseptica)
7.	Cervicoglandular form
8.	Granulomatosis septica and typhoid-pneumonic form
9.	Other forms

2.4 Host risk factors and target groups

Host susceptibility plays a major role in establishing listerial infection. *L. monocytogenes* more commonly infects individuals with depressed T-cell mediated immunity (Rocourt 2000). Therefore, *L. monocytogenes* is classified as an opportunistic pathogen. The groups at risk for listeriosis are pregnant women, adults with underlying diseases (including cancer patients, organ transplantation recipients, people with AIDS, hepatitis, chronic disorder, and diabetes) and the elderly (55-60 years and older) (Schuchat et al. 1991). Listeriosis in pregnancy may be asymptomatic and characterized by flu-like illness with fever and headaches. However, serious consequences for fetus include spontaneous abortion, stillbirth, severe neonatal septicemia, and meningitis (Frederickson et al.1992).

In non-pregnant women listeriosis mainly causes CNS and meningeal and/or brain paranchymal infection. Gastroenteritis with vomiting and diarrhea are also observed. In severely immunodepressed people, listeriosis results in bacteremia (Skoberg et al. 1992, Goulet et al.1995). People with human immunodeficiency virus (HIV) infection are also at significant risk of contracting listeriosis. It has been found that prevalence for listeriosis is 300 to 1000 times higher for AIDS patients than for the general population (Vázquez-Boland et al. 2001a). Therefore the health status of an individual is highly responsible for the contraction of listeriosis. Immunocompetent patients usually survive listeriosis, whereas those with underlying weakening diseases have a high chance of succumbing to the infection with a mean mortality rate greater than 30-40% (Vázquez-Boland et al. 2001a, Skoberg et al. 1992).

2.5 Treatment of foodborne listeriosis

Although some individuals may recover spontaneously from listeriosis, early antibiotic therapy is usually required to prevent permanent disabilities and possible death. Untreated invasive listeriosis infection is usually fatal, except in pregnant woman who deliver infants with early onset of listeriosis and clear their infection after delivery. Clinical results regarding susceptibility of *L. monocytogenes* to various antibiotics is conflicting.

Most isolates of *L. monocytogenes* are sensitive *in vitro* to penicillin, ampicillin, tetracycline, erythromycin, chloramphenicol and cephalosporin, but these antibiotics may have side effects when administered *in vivo* (Ryser and Marth, 1991). *In vitro* data and *in vivo* clinical analysis suggest a combination of ampicillin and an aminoglycoside like

gentamicin is the favored treatment for invasive listeriosis (Schlech 2000). Ampicillin is bacteriostatic for *L. monocytogenes* and relapsing infection may occur. All strains of *L. monocytogenes* are resistant to cephalosporin antibiotics which are unsuitable due to poor diffusion through the blood brain barrier during meningitis (Seeliger and Finger, 1976). However, third generation cephalosporins may be combined with ampicillin when listerial meningitis is suspected. Although chloramphenicol, streptomycin and sulphonamides readily penetrate the blood brain barrier, these can be toxic to neonates and are seldom used to treat neonatal listeriosis (Ryser and Marth, 1991). Vancomycin, in combination with an aminoglycoside, can be successfully used as a treatment for penicillin allergic patients with listeriosis. Another regimen in the literature is a combination of trimethoprim-sulphamethoxazole (TMP-SMZ) and rifampin (Schlech 2000). Despite the development of many new antibiotics, the optimal antibiotic therapy for listeriosis is not yet known (Margret and Seeliger, 1988). However, favorable results have most frequently been obtained either by ampicillin alone or in combination with gentamicin (Margret and Seeliger, 1988, Ryser and Marth, 1991).

A relatively high dose of ampicillin is required for effective treatment. Adults with septisemic or neurological listeriosis need 6-12 g of ampicillin intra venously (i.v.) daily in 3-4 doses; whereas infants should receive 200-400 mg ampicillin/kg bodyweight/day. Expectant mothers with minor symptoms of listeriosis should receive 3-6 g of ampicillin daily (i.v. or orally). The generally accepted standard duration of therapy is 3 weeks. (Schlech 2000, Ryser and Marth, 1991). However, for profound and irreparably immunocompromised patients, life long suppressive therapy may be required.

2.6 Detection of *L. monocytogenes* from foods

L. monocytogenes is a non-fastidious organism that can be subcultured in most common bacteriological media. Hence, efforts to isolate *L. monocytogenes* from the blood and the Cerebrospinal fluid (CSF) of infected patients have been successful. However, attempted isolation or re-isolation from inoculated or naturally contaminated food is often unsuccessful mainly because of low numbers of *L. monocytogenes* in combination with large numbers of other background microorganisms. Thus, in spite of current stringent procedures, the food industry faces a big challenge to prevent the occurrence of foodborne listeriosis. An efficient detection method plays a crucial role in preventing foodborne listeriosis. Therefore, a heightened interest in food borne listeriosis has led to intense efforts towards devising one or more optimal detection methods that can be used commercially for screening food products for *Listeria*

2.6.1 Traditional detection system from food

The early attempts for isolation of small numbers of *Listeria* from samples containing large population of indigenous micro flora were based on the direct plating of samples in simple agar media but this often ended in failure (Beumer et al. 2003). Gray et al. (1948) introduced the use of a cold enrichment procedure for the isolation of *L. monocytogenes* from highly contaminated food samples. Better results were obtained with the use of the technique of cold enrichment for several weeks at 4°C. The major disadvantage of this method was the long incubation period that lasted for several weeks (Gray et al. 1948, Beumer and Hazeleger, 2003). After the major listeriosis outbreaks, two federal agencies, USDA / FSIS and FDA, which are responsible for foods including sea food, dairy

products and vegetables, recommended their own methods for the detection of *Listeria* species based on variety of selective and elective isolation enrichment media. The original USDA procedure introduced by McClain in 1986 has undergone several changes and has been revised frequently. This procedure could detect *Listeria* colonies within 3-6 days (Fig. 8) (Ryser and Marth, 1991, USDA/FSIS 2002). The revised method varies from the original protocol in that *Listeria* Enrichment broth (LEB)I has been replaced by Fraser broth (FB) (LEBI to which lithium chloride and ferric ammonium citrate was added) as a secondary enrichment broth and lithium chloride phenylethanol plating (LPM) agar was replaced by modified oxford agar (MOX) as the only plating medium (USDA/FSIS 1998). FB and MOX will both blacken during incubation from the ability that *Listeria* species to hydrolyze esculin with colonies of *Listeria* exhibiting black halos on MOX following 24-48 hrs incubation at 30°C. While LEBI and FB are similar, the darkening of FB during incubation differentiates *Listeria* containing samples within three days thus conserving time. The positive samples are then confirmed for *L. monocytogenes* by streaking on horse blood overlay agar for β-hemolytic colonies (USDA/FSIS 1998).

FDA, like USDA, has also developed frequently revised standard procedures for detection and isolation of *Listeria* from dairy, seafood and vegetable products. Like USDA, the FDA method saved 5-6 days of analysis time but it differed from the USDA method in the selective enrichment and plating media used (Lovett 1988). Although these two standard methods have considerably reduced the time period for detection of the pathogen, the 3-6 day period to determine whether the food sample is free from *L. monocytogenes* creates problems for food industries that handle perishable food products.

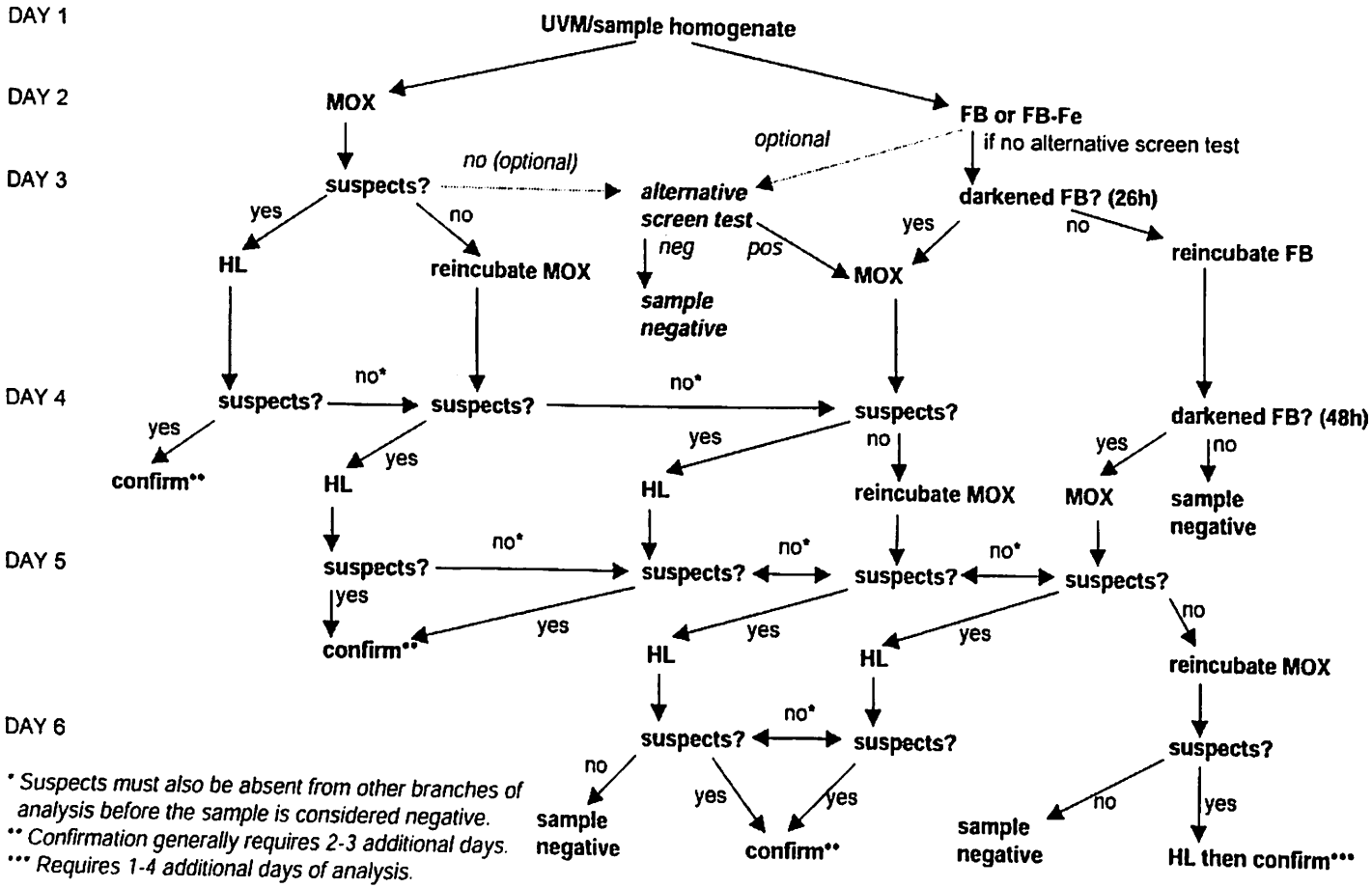

Figure 8. Traditional method of detection of *L. monocytogenes* from meats (USDA/FSIS 1998).

2.6.2 Alternative detection methods

With the traditional agar method, the screening of *L. monocytogenes* requires the formation of visible colonies to obtain isolates, then purified and confirmed. All of these methods are costly, labor intensive, and time consuming. Several automated biochemical kits can decrease the time required for biochemical confirmation to less than 24 hrs but they do not contribute in speeding up the initial detection of *L. monocytogenes*. Thus a faster method for the detection of *L. monocytogenes* is required, especially for foods with a shorter shelf life. The advancement in immunology and genetics has led to the development of methods used to detect pathogen from food within several hours following enrichment. However, enrichment of low levels of *Listeria* to higher detectable levels will always be the bottleneck of any detection method. Some of these methods are briefly covered in this section.

2.6.2.1 Miniatured Biochemical Tests

In miniatured techniques, heavily inoculated small tubes of bacteriological media with pure cultures of organisms are used for biochemical identification (Weaver 1954). Several commercial kits are available for rapid identification of *Listeria* species and can provide biochemical identification of *L. monocytogenes* within 4-24 hours (Ryser and Marth, 1991). One of these kits, the API 20 Strep system, successfully identified 147 known clinical and environmental isolates of *Listeria* species to genus level after only 4 hours of incubation. However, this system could not distinguish *L. monocytogenes* from *L. seeligeri* (Mac Gowan et al. 1989). In 1976, the Micro ID system was introduced by Organon Teknika Corp, in which following 4 hours of incubation, all the *Listeria* cultures

were identified based on their octal codes derived from the number of positive and negative biochemical reactions. However, all these kits require that the organism first be isolated in pure culture. Therefore these techniques are more useful in rapid identification of isolated *Listeria* species rather than reducing the total time for detection of *Listeria monocytogenes* from food. In addition neither could identify *L. monocytogenes* with absolute certainty (Ryser and Marth, 1991, Mac Gowan et al. 1989).

2.6.2.2 Fluorescent antibody (FA) techniques

The FA technique is based on selective fluorescence which results from specific binding of a fluorescently-labeled antibody to antigens on the bacterial cell surface. After treatment, the bacterial cell surface is completely coated with antigen-antibody complex and emits fluorescence which can be detected with a fluorescence microscope. (Fig. 9) (Ryser and Marth, 1991).

Sheridan et al. (1997) used this technique to isolate *L. monocytogenes* and *L. innocua* from minced beef enrichment. The technique demonstrated that it has a detection level of Log_{10} 3.11 cfu/ml and no false positive or negative results were reported. Efficient surface adhesion with the immunofluorescence technique is influenced by temperature, pH and medium of culture (Duffey and Sheridan, 1997). Although this approach is effective, it is heavily reliant on the specificity of monoclonal antibodies that can be too specific or too general. In addition, sourcing and preparation of immunofluorescent antisera can prove to be time consuming also the sophisticated nature and excessive cost of instrumentation may limit its use to the largest food industries (Duffey and Sheridan, 1999).

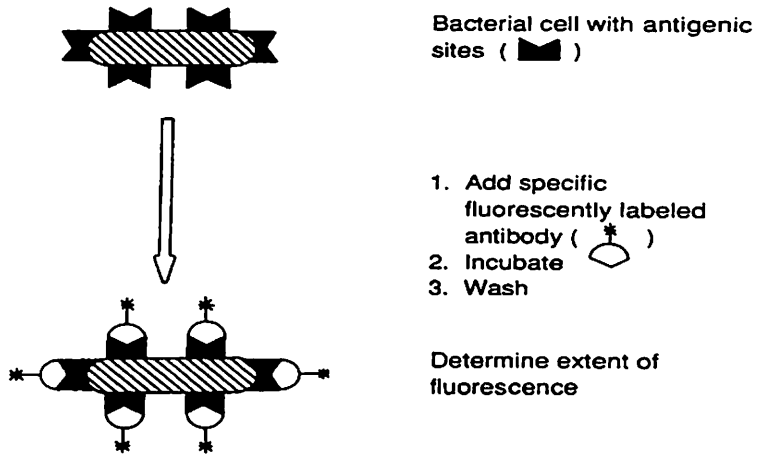

Figure 9. Direct Fluorescent Antibody technique (Ryser and Marth, 1991).

2.6.2.3 Enzyme-linked immunosorbent Assay (ELISA)

The ELISA technique is based on the principle that either an antibody or antigen can be linked to an enzyme with the resulting complex retaining the immunological as well as the enzymatic activity. It is one of the important developments in the labeled reagent assays (Ryser and Marth, 1991). ELISA is relatively objective, far less labor intensive and can be automated with relative ease. Advances in hybridoma/monoclonal antibody technology, coupled with numerous recalls of *Listeria*-contaminated food products have prompted the development of an ELISA for detecting *Listeria species* including *L. monocytogenes* in various food and environmental samples. The 2-hour double antibody sandwich ELISA known as Listeria-Tek assay was commercially introduced by Organon Teknika Corp. in November 1987 (Fig. 10). It is a one step double antibody sandwich procedure in which antigenic material from heat inactivated enrichment cultures is incubated with an enzyme-labeled monoclonal antibody in polystyrene micro-ELISA wells that have been commercially pre-coated with an unlabelled "capture" monoclonal antibody. The enzyme used is a horse-radish peroxidase, which upon adding a substrate

(tetra-methybenzidine) produces an intense blue color that turns yellow by the addition of acid. Results are read with a spectrophotometer and are completed within 2 hrs (Ryser and Marth, 1991). Mattingly applied this technique to 136 food samples which showed no false negative reactions and 10 positive ELISA tests which were identical to results obtained with the cultural procedure (Mattingly et al. 1988).

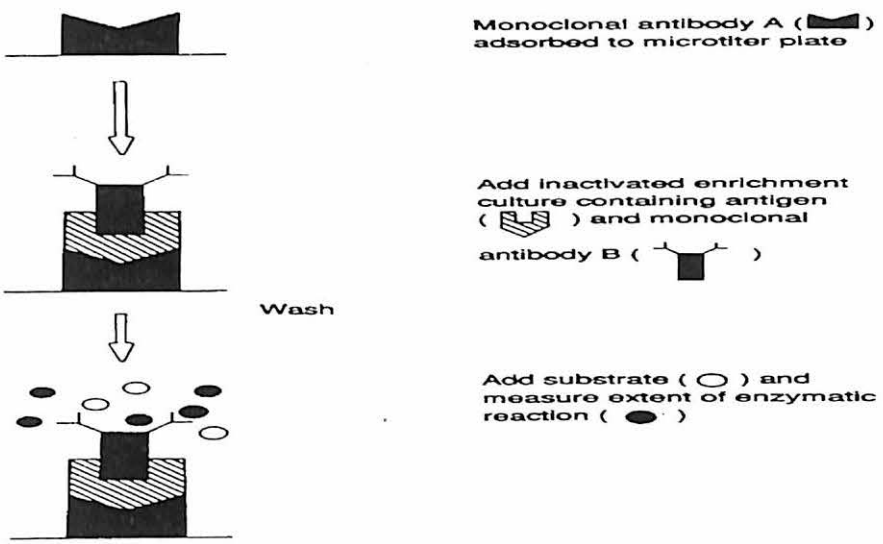

Figure 10. Listeria-Tek assay for detecting *Listeria* species in food (Ryser et al. 1991).

A *Listeria* antibody (B4) was evaluated by ELISA in its ability to bind live vs. dead *Listeria* cells indicated that the antibody was able to detect only viable cells when heat treatment was done. But, when cells were killed by methods other than heat treatment, the results were ambiguous as to whether the antibody detected the organisms or not (Solve et al. 2000). A collaborative study for detection of *Listeria* in foods was done using Vitek Immuno Diagnostic Assay System (VIDAS). The VIDAS test is an enzyme linked fluorescent assay (ELFA) which couples automation with ELISA technology. Here the conjugate enzyme catalyses the hydrolysis of the substrate (4-methyl-umbellferyl phosphate) emitting fluorescence (4-methyl-umbellferon phosphate)

measured at 450nm (Gangar et al. 2000, Sewell 2003). Of 1558 samples tested, 935 were positive by the VIDAS method and 809 by the standard method. The false positive rates were 10.3% in comparison to that of the culture method (13.5%). The agreement between both the methods was 86% (Gangar et al. 2000). In addition the ELFA system was compared with Palcam broth enrichment method for *Listeria* detection. The efficiency of ELFA was 97.5% with a false positive rate of 1.9% and 3.0% (Sewell 2003).

2.6.2.4 DNA hybridization probe

Nucleic acid hybridization technology offers an exciting approach for rapid and definitive detection of foodborne pathogens, including *L. monocytogenes*. A number of DNA probes were constructed to specifically identify *L. monocytogenes* based on the listeriolysin gene (Chenevert et al. 1989, Datta et al. 1990), hymolysinA gene (Datta et al. 1988), 16S rRNA sequence (Bobbit and Betts, 1992) and the internalinA gene (Ingianni et al. 2001). All of the above hybridizations were based on isotopic (Klinger et al. 1988) and colorimetric nucleic acid hybridization techniques (King et al. 1989). The essential principle of nucleic acid hybridization is the specific formation of double stranded complementary nucleic acid molecules from two single stranded molecules; one of the strands is produced in the laboratory in the form of probe molecules, and nucleic acids from the target organism provide the other strand. (Olsen et al. 1995). The target organism's nucleic acid can either be DNA or RNA, and the probe used is either radioactively labeled with phosphorous (^{32}P) or an enzyme label (streptavidin alkaline phosphatase). The degree of radioactive or enzymatic activity emitted above threshold values (negative control) indicates positive reaction that can be detected using a scintillation counter or a spectrophotometer (Ryser and Marth, 1991) (Fig. 11). In 1988,

Gene-Trak systems synthesized a probe complementary to *Listeria* specific 16S rRNA sequence and radioactively labeled it with P^{32}. Inclusivity and exclusivity of the probe were confirmed with 139 *Listeria* isolates representing all known species and 73 non-listerial strains. The end result was genus specific DNA probe that was specific for *Listeria* isolates only (Klinger, 1988). This assay was done with 2 days of cultural enrichment and required a total assay time of less than 2.5 days. The false negative rate was less than the rate of the culture method. However, their method had two major obstacles : a 10-day shelf life for the radioactive probe (^{32}P half life=14.3 days) and the need for special licensing to handle the radioactively-labeled DNA probe and accompanying waste materials (Ryser and Marth, 1991).

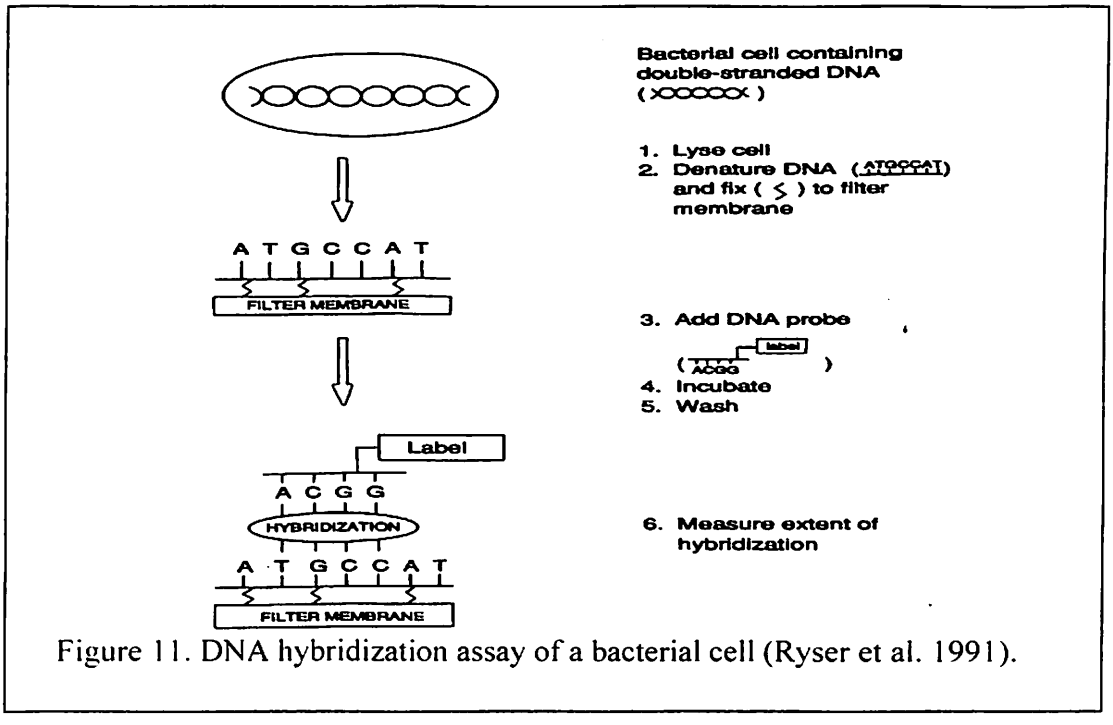

Figure 11. DNA hybridization assay of a bacterial cell (Ryser et al. 1991).

Realizing the limitations of the above assay, in 1989 Gene-Trak came up with a new colorimetric nucleic acid hybridization assay. This non-isotopic detection has been applied in a rapid nucleic acid dip-stick hybridization assay for detection of *Listeria* species in food samples. The assay takes approximately 2.5-3 hrs after a two day broth

and plate enrichment. Hybridization occurs between fluorescent labeled detector probes, polyA (deoxyadenosine) -tailed capture probe, and *Listeria*-specific regions of 16s rRNA. These target probe complexes are captured on polyT (deoxythymidine) coated plastic dip-sticks. Detection is based on binding of horse-radish peroxidase conjugated anti-fluorescent antibody to the hybridization complex and enzyme mediated color development. In this study 306 food samples were tested. When compared with total culture results with the in hybridization method, they had 1.4-2% false positives and 3.8-4.7% false negatives (King et al 1989). In 1992, a *L. monocytogenes* –specific acridium ester labeled DNA probe was evaluated in chemiluminescent homogeneous protection assay (HPA). After the hybridization, the acridium label on the unhybridized probe was inactivated by a chemical differential hydrolysis step and the positive target was detected in a luminometer after the addition of detection reagent (Okwumabua et al. 1992). The assay of simultaneous detection of several samples can be completed in 30-45 min. The probe showed 100% sensitivity and specificity for *L. monocytogenes*. However, the lower detection level of this method was between 10^4 and 10^5 cells (Okwumabua et al. 1992).

ELISA and DNA hybridization have several advantages since they are highly sensitive, can detect stressed or injured cells that may elude conventional methods and further provide species identification. However, most of these methods require either pure cultures or high cell number (10^4-10^5 cfu/ml) before they can be applied. Also these methods can only be performed by trained personnel and are expensive. While the USDA and other regulatory agencies are actively evaluating the DNA probes, ELISA assays and other rapid methods to detect *Listeria* in foods, their approval is still debatable especially when no single method has identified all positive samples. These methods although

included in the USDA microbiology laboratory guidebook (1998), are restricted for use in combination with the standard culture methods particularly for the confirmation of a sample which showed a suspected *Listeria* colony on selective media. The USDA proposed scheme of identification is shown in the following figure (Fig. 12).

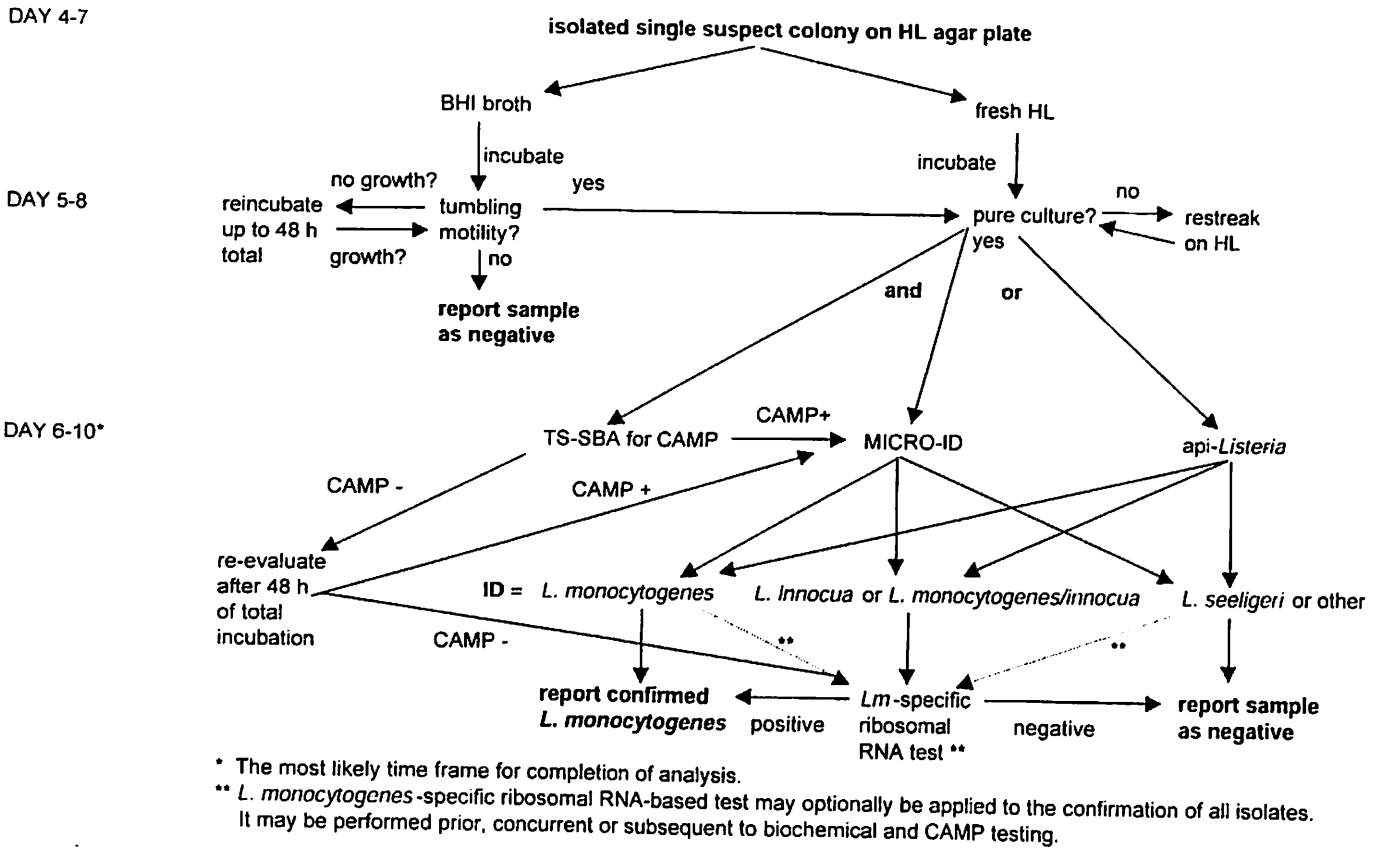

Figure12. Identification of *Listeria* species by combination of traditional method and alternative methods. (USDA/FSIS 1998).

2.6.3. Rapid real-time polymerase chain reaction (PCR) detection of *L. monocytogenes*

Molecular methods of detection have improved upon some aspects of traditional means for detection, and their development continues in earnest. Nucleic acid amplification technologies have been developed in recent years and show tremendous potential for detection and identification of *L. monocytogenes*.

PCR is an *in vitro* enzymatic process for the amplification of a specific DNA sequence by as much as a factor of 10^7 (Saiki et al. 1988). The assay is simple and based upon oligonucleotide primer directed DNA synthesis by a polymerase enzyme. Briefly two oligonucleotide primers, designed to flank the target DNA are present in a reaction mixture containing DNA polymerase, enzyme cofactors, deoxyribonucleotide triphosphates (dNTP) and the target DNA. The reaction proceeds with the cycling rounds of 3 temperature based steps: denaturation, annealing and extension, resulting in an exponential increase in the number of copies of target DNA within 2-3 hrs (Fig.13). Followed by the amplification, the target DNA can be detected by ethidium bromide stained agarose gel electrophoresis (Norton 2002, Saike et al. 1988).

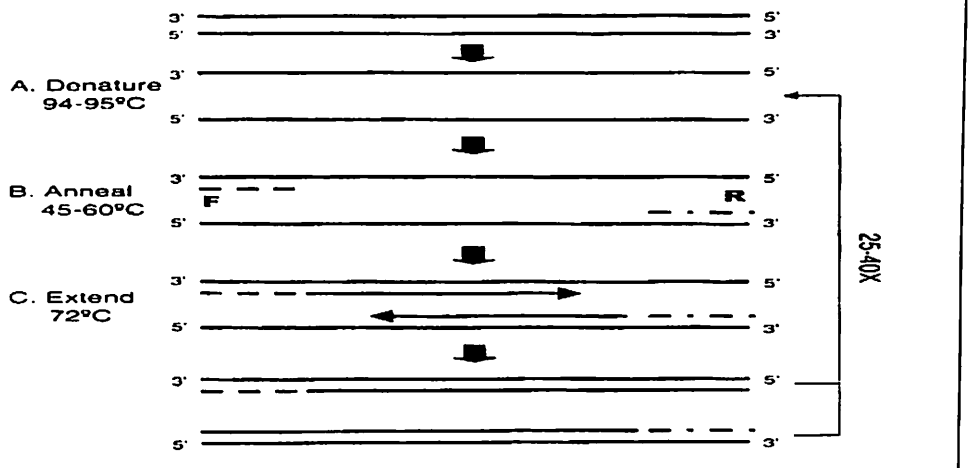

Figure 13. Principle of the polymerase chain reaction (Norton 2002).

In 1990, PCR was performed for specific detection of *L. monocytogenes* targeting the listeriolysin (*llo*) gene. The technique identified 95 of 95 *L. monocytogenes* strains, 0 of 12 *Listeria* strains of other species and 0 of 12 non *Listeria* strains (Bessesen et al. 1990).These results were in agreement in terms of specificity with others when used to detect *L. monocytogenes* from food (Bansal 1996). Other than gel electrophoresis, the detection of *L. monocytogenes* was performed using PCR in combination with ELISA (Scheu et al. 1999, Mckie et al. 2002) and in combination with post PCR DNA hybridization probe capture assays (Blais et al. 1993).The sensitivity of the PCR technology typically approximates to the detection of a single bacterium (Border et al. 1990). However, complex sample preparation and the use of gel electrophoresis end point detection for confirmation is both laborious and time consuming, thus limiting the number of samples to be analyzed and makes it unsuitable for food sample testing. Although ELISA and hybridization probe methods can increase the number of samples to be tested, manipulation of the PCR products after thermal cycling is still required (Sails et al. 2003).

In an effort to reduce the time necessary for detection, methods to simplify or even eliminate the need for post amplification assays are introduced. Recently, a new method for PCR quantification has been invented. This is called "real-time" PCR because it allows the user to actually view the increase in the amount of target DNA as it is amplified. The real-time PCR system is based on detection and quantification of a fluorescent reporter. This signal increases in direct proportion to the amount of PCR product in the reaction (Heid et al. 1996). A thermocycler fitted with a fluorescence detection system measures the fluorescence in specially designed tubes that contain the reaction components. The amplification results in a characteristic sigmoid shaped curve which represents 3 phases of PCR: the lag phase (little product accumulation), the exponential phase (rapid product accumulation) and the plateau phase (no further product is amplified) (Mahoney et al. 2002). By recording the amount of fluorescence emission in each cycle, it is possible to monitor the PCR during the exponential phase where the first significant increase in the fluorescence directly correlates with the initial amount of target template and the cycle where this fluorescence is measured is referred as a threshold cycle (Ct) (Higuchi et al. 1992, Sawyer et al. 2003).

There are generally several methods of fluorescence quantification based on the principle of fluorescent probes or DNA binding agent which includes: 1) TaqMan probes 2) Molecular Beacons 3) Scorpion probes 4) Amplifluor™ 5) SYBR Green I dye (Koo et al. 2003). Since the real-time PCR does not require a post PCR manipulation (closed tube) and also avoids the cross contamination of the PCR amplicons, it becomes highly suitable for food industries where they can be used for routine applications towards rapid pathogen detection with ease of use and high throughput (Zhang et al. 2003).

2.6.3.1 SYBR Green I method

SYBR Green is a fluorescent intercalating agent that is not sequence specific. It is a double stranded (ds) DNA binding dye thought to bind in the minor groove (Zhang et al. 2003). It does not bind to single stranded (ss) DNA whereas the fluorescence of SYBR Green I is greatly enhanced upon binding ds DNA in each cycle (Fig. 14) which makes it ideal for the detection of amplification product (Morrison et al. 1998). The maximum absorbance of SYBR Green I is ~497nm and the emission maximum is 520nm. When SYBR Green I is used as the fluorescent dye, a subsequent melting curve analysis of the PCR product may be used to generate a specific profile. (Mahoney and Hill, 2002).

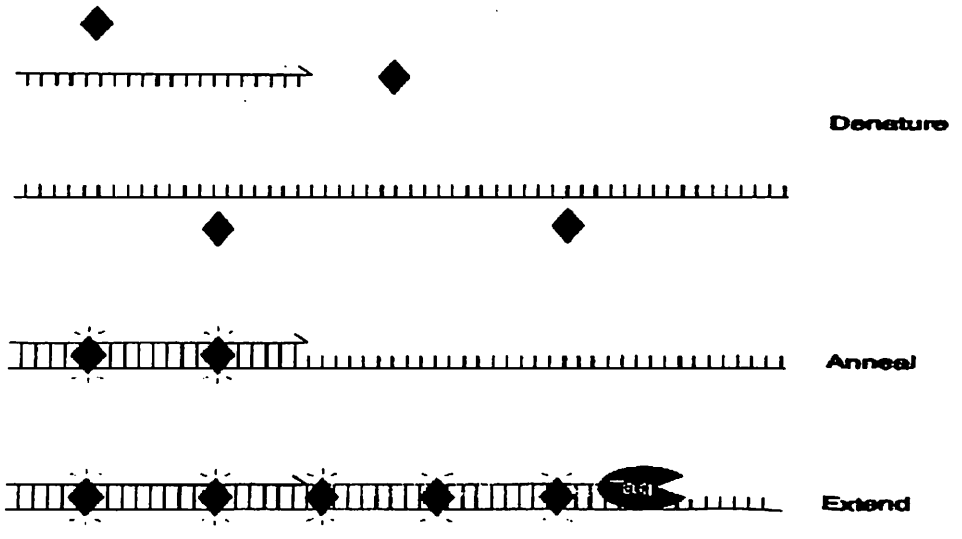

Figure 14. Action of SYBR Green I in the polymerase chain reaction.

SYBR Green I has several advantages over molecular probes because they allow the real-time PCR to be applied without the need for probes linked with fluorescent molecules. Protocols that are already in use for classic PCR can thus be used with only slight

modifications and therefore is cost effective (Medici et al. 2003). Further SYBR Green I is very sensitive because multiple dye molecules bind to a single amplification product. However, it binds to all ds DNA and hence false positive signals from primer-dimers, secondary structures or spurious priming can interfere with accurate quantification. Measuring fluorescence at elevated temperatures may help reduce the detection of non-specific product (Morrison et al.1998). Thus the Lightcycler™'s continuous monitoring capability and subsequent melting curve analysis has been used to overcome this problem (Rasmussen et at. 1998). Though it is simple this might not be suitable for large industrial food applications because of the technical knowledge and skill required for routine operation.

2.6.3.2 TaqMan probe

A fluorogenic PCR which allows homogeneous quantification of the initial template concentration was developed in 1992 (Bassler et al. 1995). They are oligonucleotide probes called TaqMan probes whose fluorescence depends on the amplification of a target sequence. TaqMan probes are designed to anneal the target sequence between the forward and the reverse primers. TaqMan PCR takes the advantage of the 5`→3` nuclease activity of the Taq DNA polymerase to digest the probe which is labeled with both fluorescent reporter dye (6-carboxyflourescein [FAM]) and a quencher dye 6-(6-carboxytetramethylrhodamine). When the intact probe anneals to the target sequence, excitation of the reporter is quenched due to its proximity to the 3`quencher. However as extension proceeds, the 5` exonuclease activity of the polymerase cleaves the probe, separating the reporter from the quencher and generating an increase in the reporter dye`s fluorescence intensity (Fig. 15) (Nogva et al. 2000). Repeated cycles of thermocycling

result in an exponential amplification of the PCR product and are quantitative for the initial amount of template (Bassler et al. 1995). Quantitative detection of *L. monocytogenes* in pure culture, water, skim and unpasteurised whole milk was performed using TaqMan PCR. The detection limit was ~6 to 60 cfu/ml with quantification linear over at least 7 log units, and the method could be completed within 3 hrs (Nogva et al. 2000). TaqMan probe was also applied for real-time detection of *L. monocytogenes* from artificially contaminated cabbage. The result showed a linear response over 7 log cycles from $1.4x10^2$ to $1.4x10^9$ cfu in 25g of cabbage (Hough et al. 2002). One advantage of the TaqMan probe, particularly for quantitation is that the fluorescence is dependent not only on the presence of target, but also on amplification of the target. However TaqMan probes have to be individually designed for each specific target. In addition the 5'→3' hydrolysis performed between the fluorophore and the quencher can be met only when these 2 moieties are not located too close to each other. This creates a serious problem for the assay since the efficiency of the energy transfer decreases with inverse sixth power of the distance between the reporter and the quencher. As a consequence, the background emission from unhybridized probe can be quite high (Nazarenko et al. 1997).

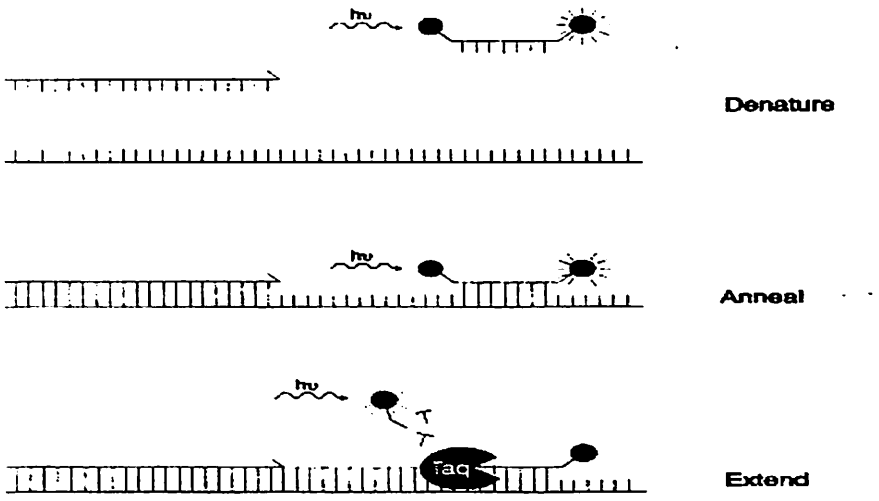

Figure 15. Schematic representation of action of TaqMan probe in fluorescent emission

2.6.3.3 Molecular Beacons

A new principle for the construction of probes that are useful for detecting specific nucleic acids in homogeneous solutions was developed. These probes are called "Molecular Beacons" because they emit a fluorescent signal only when hybridized to target molecules (Tyagi and Kramer, 1996). Molecular beacons are dual-labeled oliginucleotide probes designed to form a stem and loop structure in the absence of target (Fig. 16). In the hairpin configuration, the loop portion of the molecule is a probe sequence that is complementary to a predetermined sequence in a target nucleic acid. In the stem, the fluorophore 5-(2'-aminoethyl)aminonaphthalene-1-Sulfonic acid (EDANS) is at one end of the molecule is brought into close proximity with a quenching moiety 4-(4'-dimethylaminophenylazo) benzoic acid (DABCYL) at the other end of the stem by annealing of two complementary arm sequences that are on either side of the probe sequence. The arm sequences are unrelated to the target sequence (Fig. 16). When the fluorophore is excited in this configuration, it transfers energy as light, in a process known as fluorescence resonance energy transfer (FRET). As a result of the hairpin configuration the fluorophore is unable to fluoresce. When the probe encounters a target molecule it forms a hybrid that is longer and more stable than the hybrid formed by the short hairpin sequence, and thus causes a conformational change of the probe leading fluorophore and quencher to move away from each other. When illuminated in the hybrized configuration, fluorescence can be detected (Fig. 16) (Tyagi and Kramer, 1996). One advantage of molecular beacons is that unlike SYBR Green, this method specifically detects the target of interest. However, each probe must be carefully and uniquely designed for the detection specific target. Similar to TaqMan, the assay it does not detect

the amplified DNA directly and therefore the signal will be affected by the efficiency of the probe hybridization (Nazarenko et al 1997).

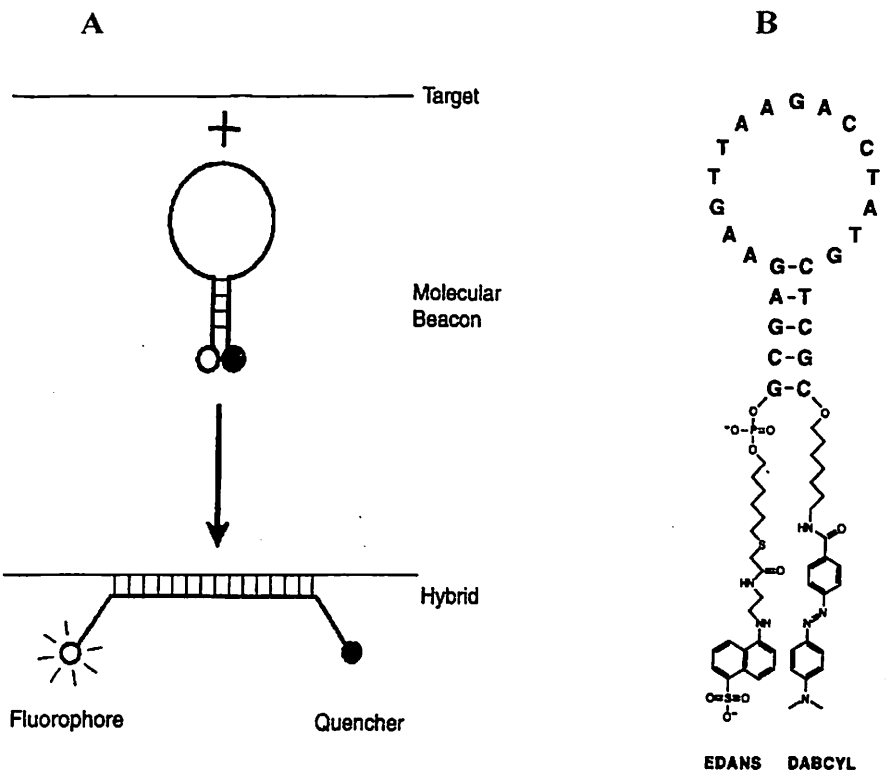

Figure16. The structure and principle of molecular beacons. Fluorescence emission by molecular beacon during amplification of the target DNA (panel A). Basic structure of the molecular beacon (panel B) (Tyagi and Kramer 1996).

2.3.6.4 Hybridization probes

Hybridization probes are for specific detection of the product. These probes also uses the concept of fluorescence resonance energy transfer (FRET). In FRET technology, two fluorescently labeled probes are used where one is labeled with a donor fluorophore at 3' end (fluorescein) and the other is labeled with an acceptor fluorophore at 5' end (LC red 640). The technique relies on the transfer of energy from one fluorescent dye to another, however, unlike the previously mentioned probes that are on the same strand, each probe label is on a separate oligonucleotide strand. The probes are designed to hybridize to target DNA adjacent to each other within the amplified fragment (Fig. 17). During the amplification cycle, once both probes hybridize correctly to the target sequence, energy emitted from the fluorophore of the donor probe can be transferred to the acceptor fluorophore on the adjacent probe which then emitts fluorescent signal at a different wavelength that can be detected by the Light Cycler™. The signal increases with each thermal cycle and is proportional to the amount of specific PCR product available for hybridization. However, this method also requires specific design and optimization of probes (Zhang et al. 2003, Koo and Jaykus, 2003).

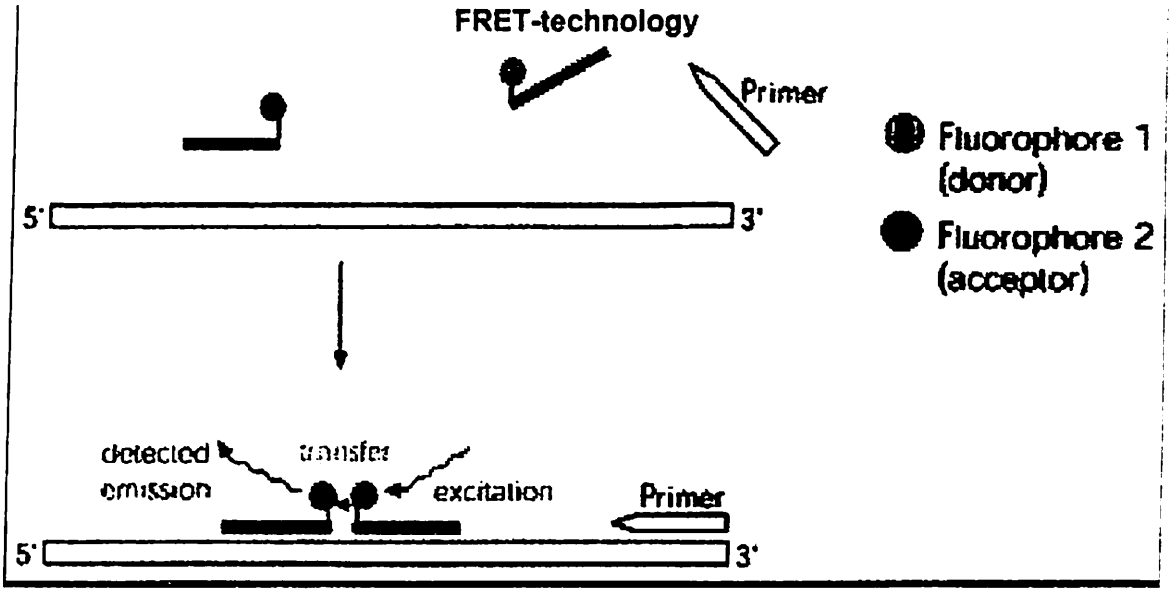

Figure17. FRET technology of hybridization probes for real-time PCR by the Light Cycler™ method.

2.3.6.5 Scorpion probe

A new method based on a primer with a tail attached to its 5' end by a linker that prevents copying of the 5' extension has been devised (Whitcombe et al. 1999). Scorpion probes contain both an amplification primer and a target specific probe separated by an amplification blocker. The probe portion is flanked by complementary sequences favoring formation of a stem structure which brings a fluorophore and quencher into close proximity. The probe element is designed so that it hybridizes to its target only when the target site has been incorporated into the same molecule by extension of the tailed primer (Fig. 18) (Whitcombe et al. 1999). During amplification, extension of the target sequence proceeds from the primer-portion of the Scorpion probe. As the reaction cools following denaturation, a unimolecular rearrangement occurs such that the Scorpion probe loop sequence hybridizes to the amplified target sequence, separating the complementary stem sequences and the fluorophore and quencher (Whitcombe et al. 1999).

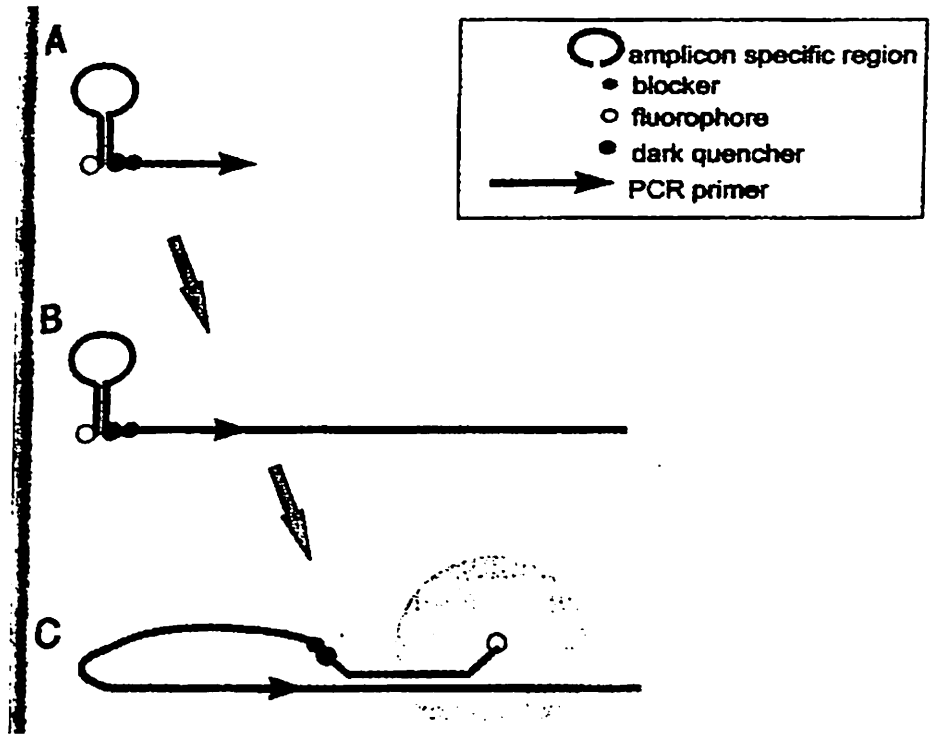

Figure 18: Mechanism of incorporation of Scorpions probes in target sequence during PCR. The molecular structure of Scorpion probe (Panel A), The binding of Scorpion probe with the target DNA (Panel B), Fluorescence emission by the Scorpion probe during the extension of the target (Panel C) (Whitcombe et al. 1999)

2.3.6.6 Amplifluor™ Uniprimer™ real-time PCR

A new method for the direct detection of PCR-amplified DNA in a closed system is now described. This method is designed for direct measurement of the target DNA by incorporation of labeled primers into the reaction product (Nazarenko et al. 1997). It is based on the method of Tyagi and Kramer which utilizes the conformational transition of the oligonucleotide as a switch of energy transfer between labels (Tyagi et al. 1996).

In this method, the donor (fluorescein) and the acceptor/quencher 4-(dimethylamine) azo benzene sulfonic acid (DABSYL) moieties are both attached to a hairpin structure on the 5` end of the amplification primers (Hermendez et al. 2004). The Off-to On transition occurs when the conformation of the Amplifluor™ primer changes from "closed" intramolecular stem-loop structure to an "open" extended structure (Uehara et al. 2000).The Amplifluor™ system makes the use of a universal primer that emits fluorescence signal only following the incorporation of the primers into the amplification product. This oligonucleotide primer is called Uniprimer™ Energy Transfer-labeled Primer because it affords the user a universal format to detect PCR products using fluorescence energy transfer .

The universal primer (Uniprimer™) consists of an 18 base primer tail (Z sequence) coupled to a hairpin sequence labeled with the fluorophore and the quencher. First, the target is amplified using direct target specific primers one which has the Z sequence added to its 5` end. In the following round of amplification, the complementary of the Z sequence is incorporated into the actual product. The Uniprimer™ then anneals to the complement of the Z sequence and the extension proceeds. In the next cycle, extension

proceeds through the Uniprimer™ incorporating it into the amplification product. In the process, the hairpin is unfolded separating the fluorophore and the quencher, thus emitting a fluorescence signal that is proportional to the amplified product (Fig. 19) (Nazarenko et al. 1997).

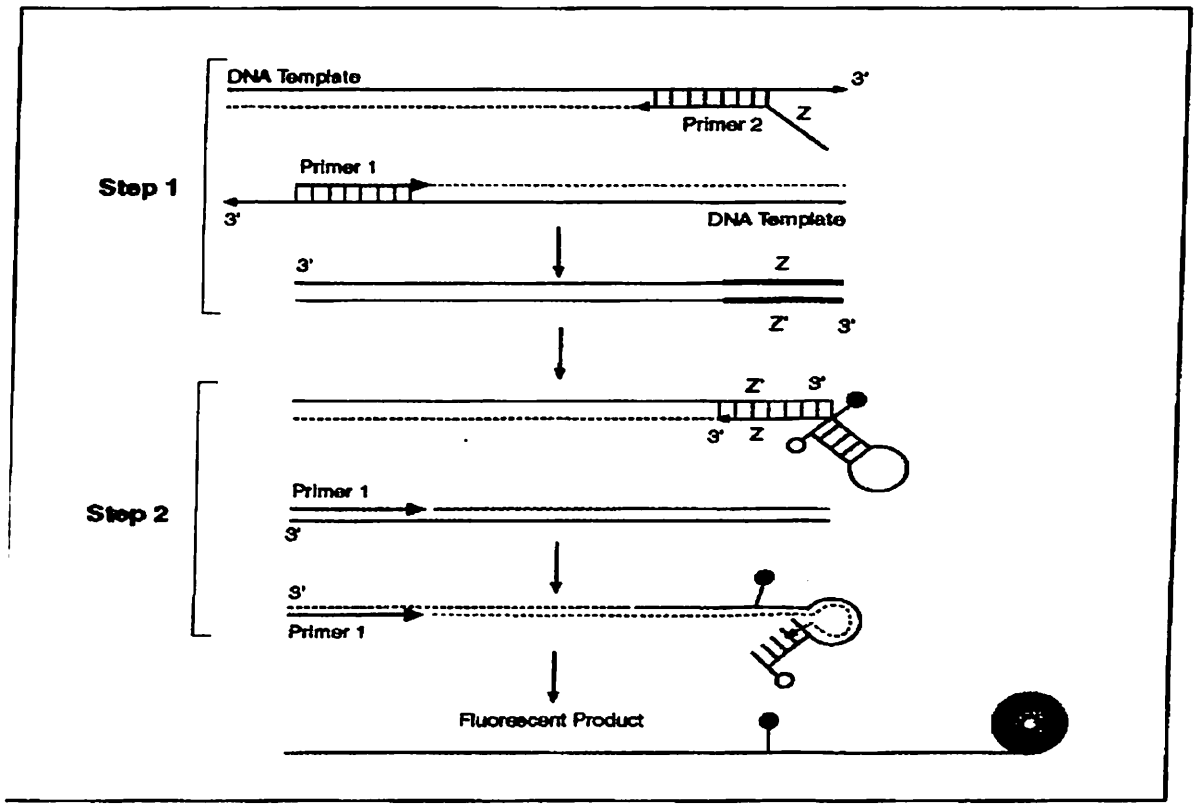

Figure 19. Principle of the Amplifluor™ Uniprimer™ amplification and detection system. The Z sequence is added onto the 5' end of a target specific primer (tailed primer) (Step 1). After the first few cycles of the PCR, a complementary sequence to the Z sequence (Z'sequence) is incorporated into the PCR products. In subsequent cycles of PCR the Uniprimer™ anneals to the template containing the Z'sequence resulting in the incorporation of the fluorescein and the quencher moieties into the PCR product.
This PCR product in turn serves as a template for Primer 1. As the extension takes place the hairpin unfolds causing the fluorescein and the quencher to move away from each other and thus results in the emission of a fluorescence signal (Step 2) (Intergen Co.).

There are several advantages of the Amplifluor™ Uniprimer™ system which are difficult to achieve using other fluorescent detection systems like hybridization probes. Direct incorporation of Uniprimer™ into the amplicon and the high signal-to-noise ratio of the Uniprimer™ due to its unique structure and low fluorescence background with unincorporated primers allow closed-tube quantification of amplicon by multiple instrument platforms in both endpoint and real-time analysis. Elimination of laborious post-PCR sample processing enables high-throughput analysis and greatly reduces the chance of carryover contamination. The Amplifluor™ Uniprimer™ can be universally used for PCR by simply modifying the target specific primers and thus opening up new possibilities of multiplex PCR by allowing detection of different targets in same reaction tube with minimum optimization. This method is simple and makes it well suitable for high throughput PCR assays.

2.7 Typing of *L. monocytogenes* strains.

The main purpose in epidemiology is to correlate outbreak strains with potential sources which often require differentiation. Prevention and control of foodborne diseases very much depends on early outbreak recognition that good surveillance systems can provide. *Listeria* and other bacterial foodborne pathogens are widely distributed in the environment. Some of them can also be a part of the resident flora of animals. In addition, global food trade may spread infected foods worldwide in a few days. All of these factors makes the specific detection difficult to trace the source of contamination. Thus, along with the detection of a pathogen from food, the ability to differentiate pathogenic isolates beyond the species level and their detailed strain characterization

plays a pivotal role in the epidemiology of infectious diseases, generating the information necessary for identifying, tracking, and intervening against foodborne disease outbreaks.

The optimal typing method in a laboratory will depend on several factors. Ideally, the typing method should have high typeability, reproducibility and discriminatory power. It should be simple to perform as should be the interpretation of results. The cost must also be taken into account. Various methods have been employed using traditional phenotypic methods and genotypic methods (molecular typing) to differentiate strains of *L. monocytogenes* and these methods vary in their degree of resolution, cost and labor.

2.6.4.1. Phenotypic methods

Phenotypic methods detect the presence or absence of metabolic or biological activities as expressed by microorganisms. These methods include biotyping, serotyping, phage typing and multilocus enzyme electrophoresis (MLEE). Biotyping includes metabolic activities expressed by an isolate and may include specific biochemical reactions, colonial morphology and environmental tolerances. Biotyping has only a limited ability to differentiate among strains within species and has relatively poor discrimination power. Serotyping is a classical tool for strain differentiation and is based on different variations of antigenic determinants expressed on the cell surface, including lipopolysaccharides, capsular polysaccharides, membrane proteins and extra-cellular organelles (flagella and fimbrie). Serotyping identifies 13 serotypes of *L. monocytogenes* (Seeliger and Hohne, 1979). It is of restricted value because most sporadic human cases and outbreaks have reportedly been caused by *L. monocytogenes* serotype 4b and this indicates that more sensitive strain discrimination method among serotype 4b is required. Phage typing is based on the capability of a standard set of viruses to infect and lyse

bacterial cells. In the WHO study on subtyping isolates of *L. monocytogenes* by phage typing, limited comparability between results was obtained from testing the same cultures using the same phages in six different laboratories (McLauchlin et al. 1996). The MLEE differentiates isolates according to the electrophoretic mobility of a large number of their metabolic enzymes. Electromorph profiles over the enzymes assayed (electrophoretic types, ETs) are equated with multilocus genotypes, indexing the whole chromosomal genome of the isolates (Selander et al. 1986). The discriminatory power of MLEE in an international study for characterizing *L. monocytogenes* isolates ranged from 0.827 to 0.925 (Caugant et al.1996). MLEE has many attractive features as a typing scheme, yet it is laborious, somewhat subjective and produces results that are difficult to compare between laboratories.

2.6.4.2. Genotypic Methods

Genotyping methods account for DNA- based analyses of chromosomal or extra chromosomal genetic elements. These methods include, Plasmid analysis, Pulsed-field gel electrophoresis (PFGE), Ribotyping, PCR based methods such as Random amplified polymorphic DNA (RAPD), amplification fragment length polymorphism (AFLP) and multilocus sequence typing (MLST). The selection of the suitable and most applicable typing technique depends on the purpose of analysis.

A) Plasmid analysis-

Plasmid analysis can include typing of plasmid profiles, plasmid fingerprinting and identification of plasmid mediated virulence genes. The main disadvantage of this typing method is that many pathogens may not possess plasmids which limit the use of plasmid analysis for epidemiological studies.

B) Pulsed field gel electrophoresis

In 1984, Schwartz and Cantor described that pulsed field gel electrophoresis (PFGE) was a technique used to separate especially long strands of DNA by length in order to tell differences among samples. raising the upper size limit of DNA separation in agarose from 30-50 kb to well over 10 Mb (10,000 kb) (Schwartz and Cantor, 1984). During continuous field agarose gel electrophoresis, DNA above 30-50 kb migrates with the same mobility regardless of size. This is seen in a gel as a single large diffuse band. However in PFGE, the electrical field alternately pulses and switches the direction of the current and the DNA is forced to change direction during electrophoresis and different sized fragments within this diffuse band begin to separate from each other. With each reorientation of the electric field relative to the gel, smaller sized DNA will begin moving in the new direction more quickly than the larger DNA. Thus, the larger DNA lags behind, providing a separation from the smaller DNA.

PFGE has been successfully used in epidemiological investigations involving *L. monocytogenes* such as in cold smoked rainbow trout (Miettinen et al. 1999), from cold-smoked salmon processing plants (Dauphin et al. 2001), meat processing plants (Stephan et al. 2000) and in other cases of listeriosis outbreaks. PFGE takes advantage of restriction enzymes that cut genomic DNA infrequently and thus produces simple profiles (10-20 bands); computer based analysis is simplified, enabling rapid, and easy comparison of strains. Currently PFGE seems to be the most widely used subtyping method and is often the standard one to which other methods are compared. However one of the biggest disadvantages of this method is the time needed to finish the assay and its

labour intensive work. DNA degradation is also a problem in PFGE for some strains as in conventional electrophoresis (Graves and Swaminathan, 2001).

C) Ribotyping

Ribotyping utilizes the analysis of restriction fragment length polymorphisms of ribosomal RNA (rRNA) genes and their surrounding sequences. These genes are highly conserved and vary in number and position within the genome of bacteria (Tang et al. 2003). The chromosomal DNA is isolated and digested by restriction enzymes, then the DNA carrying rRNA genes are separated by agarose gel electrophoresis and detected by labeled 16S, 23S or 5S rRNA hybridization probes. The major limitations include a need for skilled technical staff and significant hands-on time required for the performance (De Cesare et al. 2001).An automated Riboprinter (Qualicon) has been introduced that performs a restriction digest of the chromosomal DNA, separates the restriction fragments by gel electrophoresis and simultaneously blots the DNA fragments to a membrane which is used for Southern blot analysis. Restriction digest fragments are hybridized synthetic probe that is based on the conserved regions of the genes for the ribosomal DNA. This often yields a DNA fingerprinting which is strain specific. Each fingerprint is stored in a database so it can be accessed for future comparisons and identifications (Tang et al. 2003). Automated ribotyping improved the discrimination of *L. monocytogenes* isolates, particularly serotype 4b strains with a discriminatory power of >0.900 (De Cesare et al. 2001). This automated system has several benefits that allows increased standardization, lower labor costs, increased speed and better between-run

comparison (Bruce 1996). However, the setup of an automated ribotyping laboratory requires considerable capital investments.

D) PCR based methods-

PCR can offer several advantages over other nucleic acid based typing methods. RAPD methods use PCR for creating genomic fingerprints by the amplification of randomly chosen sequences. AFLP is a recently developed PCR-based typing method. In this method the genomic DNA is digested with a frequently cutting and an infrequently cutting enzyme. The resulting fragments are ligated to specific adaptors and amplified using primers. Finally the labeled products are separated by electrophoresis. Although both RAPD and AFLP have been successfully applied for the genotyping of *L. monocytogenes*, poor reproducibility for comparing DNA fragments in gels within and between laboratories makes it difficult to standardize (Autio et al. 2003, Paillard et al. 2003, Byun et al. 2001, Martinez et al. 2003).

The full genome sequences have opened new opportunities to study bacteria and the diseases they cause. Sequencing allows us to catalogue all genetic variables giving us knowledge of bacterial pathogenicity. Sequencing the entire bacterial genome is likely to differentiate *Listeria* strains extremely well and to yield information about their phylogeny. However, that approach is not yet technically feasible for use in the investigation of listeriosis outbreaks. Based on sequencing of specific genes a method called **multilocus sequence typing** (MLST) was recently developed by Maiden et al. 1998 and Chan et al. 2001. This method makes the use of automated DNA sequencing to characterize the alleles present at different housekeeping and virulence genes of bacteria (Salcedo et al. 2003). MLST targets slowly diversified gene sequences to address global

epidemiology of pathogenic microorganisms. MLST, (i) Because it is based on nucleotide sequence, it is highly discriminatory and provides unambiguous DNA sequence data that can be easily exchanged and compared via worldwide web databases; (ii) combines PCR and automated DNA sequencing to reduce labor and analysis time; and (iii) provides discriminatory power comparable to, or higher than, that provided by fragment –based methods (Zhang et al. 2004).

MLST is based on the well-tested principles of MLEE but assigns the alleles at each locus directly by nucleotide sequencing, rather than indirectly from the electrophoretic mobilities of their gene products on starch gels. The sequences of 450-500 bp internal fragments of some housekeeping genes are determined for each isolate. In most bacterial pathogens, it provides sufficient variation to identify many different alleles within the population (Enright and Spratt, 1999).

Upon gaining an understanding of the sensitivity and versatility of Amplifluor™ Uniprimer™ system, in my thesis I propose to examine the use of this real-time PCR technology for rapid and sensitive detection of *L. monocytogenes* from meat products following primary and secondary enrichment of the food. In addition to the above study, the isolates of *L. monocytogenes* detected from the food products by the Amplifluor™ Uniprimer™ real-time PCR method were used for further strain differentiation by the application of PCR based multilocus sequence typing.

CHAPTER III

MATERIALS AND METHODS

3.1 Bacterial cultures

Listeria species and strains used in this study are listed in Table 4. A 100 ul aliquot of each culture used was transferred twice out of a frozen stock into 10 ml of BHI broth and incubated at 30°C overnight before use. *L. monocytogenes* ScottA-2 (an outbreak strain) was used as the main target organism for optimization of the Amplifluor™ Uniprimer™ real time PCR system and in further studies for detection from food samples.

3.2 Primer designing and selection

Primers for real-time PCR were specifically designed to target a short amplicon (~100 bp) for the *hly*A gene of *L. monocytogenes*. A short amplicon would amplify more efficiently than a longer amplicon since the PCR reagents would not be consumed too early during real-time PCR detection. Primers examined in this study are listed in Table 4. Primer set-I was designed using Vector NTI Suite 9.0 primer analyses program. It targets *hlyA/llo* gene of *Listeria monocytogenes* and amplifies a short 86 base pair (bp) PCR product. These primers were selected from a region of the gene which has homology at the 3' end for *Listeria monocytogenes* but was different for other species of *Listeria* for which homologous sequences have been identified. Primer set-II was designed from within a conserved sequence of 45 *L. monocytogenes* *hly*A gene sequences (~600 bp in length) composed from different serotypes (4b, 1/2a, 4a, 1/2c, 1/2b, 3a, 3b, 4d) present in NCBI GenBank. These sequences were then aligned using

the multiple sequence alignment program of the Vector NTI Suite 9.0. The alignment program aligned all the sequences and provided the consensus sequence of *hly*A gene (Fig. 20). This consensus sequence was then used to design the primers using the Vector NTI Suite 9.0 primer analyses program. The primer set-II amplified a PCR product of 110 bp. Primer set-III is the same as primer set-II except that the reverse primer of Primer set III does not have an additional "Z tail" sequence of 18 bp attached to its 5' end which is present in the reverse primer of primer set-II. The primer set-IV was designed in a manner that could allow it to specifically target the 16S rRNA gene of *L. monocytogenes*. All the primer sets except that of primer set-III has the "Z tail" of Uniprimer™ incorporated in their reverse primer. The primers were synthesized from IDT Inc (Coralville, IA).

Table 4. List of strains of *Listeria* used in this study	
Listeria species	Strain numbers
Listeria monocytogenes	ScottA-2
Listeria monocytogenes	V7-2
Listeria monocytogenes	PMM 39-2
Listeria monocytogenes	PMM 383-2
Listeria innocua	ATCC 33090
Listeria ivanovii	ATCC 19119
Listeria grayi	NRRL B-33023
Listeria seeligeri	NRRL B-33019
Listeria welshimeri	NRRL B-33020

Table 5. List of primers used in this study

Primer	Target Gene	Sequence (5' → 3')	Product Size (bp)
Primer set-I	Hemolysin A (*hly*A)		86
	Forward	AAT CAT CGA CGG CAA CCT C	
	Reverse (+ "Z tail")	ACT GAA CCT GAC CGT ACA AAT GGG AAC TCC TGG TG	
Primer set-II	Hemolysin A (*hly*A)		110
	Forward	CAA AAG CTT ATA CAG ATG GAA	
	Reverse (+ "Z tail")	ACT GAA CCT GAC CGT ACA AAT TTC GTT ACC TTC AGG A	
Primer set-III	Hemolysin A (*hly*A)		110
	Forward	CAA AAG CTT ATA CAG ATG GAA	
	Reverse (w/o "Z tail")	CAA TTT CGT TAC CTT CAG GA	
Primer set-IV	16S rRNA		90
	Forward	TAC ACA CGT GCT ACA ATG GAT A	
	Reverse (+ "Z tail")	ACT GAA CCT GAC CGT ACA CCT ACA ATC CGA ACT GAG AAT A	

```
  1                                                          tcagtgaagggaaaatgcaa
 61    gaagaaGTCATTAGTTTTAAACAAATTTACTATAACGTGAATGTTAATGAACCTACAAGA
121 CCTTCCAGATTTTTCGGCAAAGCTGTTACTAAAGAGCAGTTGCAAGCGCTTGGAGTGAAT
181 GCAGAAAATCCTCCTGCATATATCTCAAGTGTGGCATATGGCCGTCAAGTTTATTTGAAA
241 TTATCAACTAATTCCCATAGTACTAAAGTAAAAGCTGCTTTTGACGCTGCCGTAAGTGGG
301 AAATCTGTCTCAGGTGATGTAGAACTGACAAATATCATCAAAAATTCTTCCTTCAAAGCC
361 GTAATTTACGGTGGCTCCGCAAAAGATGAAGTTCAAATCATCGACGGTAACCTCGGAGAC
421 TTACGAGATATTTTGAAAAAAGGTGCTACTTTTAACCGGGAAACACCAGGAGTTCCCATT
481 GCCTATACAACAAACTTCTTAAAAGACAATGAATTAGCTGTTATTAAAAACAACTCAGAA
541 TATATTGAAACAACTTCAAAAGCTTATACAGATGGAAAAATCAACATCGATCACTCTGGA
                    >>>>>>>>>>>>>>>>>>>>>>>>>>>
601 GGATACGTTGCTCAATTCAACATCTCTTGGGATGAAATAAATTATGATCCTGAAGGTAAC
                                                <<<<<<<<<<<<<<<<
661 GAAATTGTTCAACATAAAAACTGGAGCGAAAACAATAAAAGtaagctagctcatttca
     <<<<<<<
721 catcgtccatctatttg
```

Figure 20. Conserved sequence of *hly*A gene from 45 different *hly*A sequences of *L. monocytogenes* present in NCBI GenBank. The arrows indicate the position of forward (>) and reverse (<) primers of primer set-II/III. Small letters indicate variable region among the individual *hly*A gene sequences obtained from NCBI GenBank).

3.3 Template preparation/DNA extraction

We used two different template preparation for PCR: one where we used extracted and purified DNA of target cells and another where we directly used the crude target cell lysates (as would be required for detection from food samples).

BAX™ Lysis. The BAX™ kit for *L. monocytogenes* (DuPont Qualicon, Wilmington, DE) was used to perform the lysis of the culture in order to obtain the DNA template for PCR. The BAX™ system consists of BAX™ Lysis buffer and protease. Strains of *L. monocytogenes* were grown overnight in 10ml of sterile BHI at 30°C. A 5 ul Aliquot of overnight culture was taken and mixed with 200 ul of BAX™ lysis reagent (1.0 ml of BAX lysis buffer + 12.5 ul of protease) and the lysis was performed by incubating at 55°C for 60 min and then 95°C for 10 min followed by a cooling period of 5 min at 4°C. Of the resulting lysate, 50 ul is generally used for PCR using the BAX™ protocol, but for Uniprimer™ real time PCR, we initially used only 2 ul of this lysate a DNA source.

DNeasy method. The DNeasy tissue kit (Qiagen Inc, Valencia, CA) for DNA extraction was used in order to obtain purified DNA for detection instead of a crude lysate. All the reagents were provided by the kit. A 2 ml of the overnight culture of *L. monocytogenes* in BHI was pelleted at 5000xg (7500 rpm) for 7-8 min. The pellet was resuspended in 180 ul of enzymatic lysis solution [1 ml of lysis buffer (20 mM Tris HCL, pH 8.0, 2 mM EDTA, 1.2% Triton® X-100) + 2.0 mg of lysozyme], incubated for 30 min at 37°C, mixed with 25 ul Proteinase K and 200 ul Buffer AL (Lysis buffer) and incubated at 70°C for 30 min. After incubation, the sample was mixed thoroughly with 96-100% ethanol by vortexing and then added into the DNeasy mini column containing 2 ml

collection tube. The column was then centrifuged at ≥6000x g for 1 min. The mini column was then washed by adding 500 ul of Buffer AW1 (Wash buffer 1) and centrifuging at ≥6000x g for 1 min. After centrifugation. 500 ul of Buffer AW2 (Wash buffer 2) was added and centrifuged again for 3 min at full speed to dry the DNeasy membrane. The DNA was then eluted out of the column in a clean microcentrifuge tube by adding 200 ul of Buffer AE (Elution buffer) and centrifuging at ≥6000 x g for 1 min. The eluted DNA was resuspended in 2 ml of sterile water in order to equalize it with the original volume of cells. The pure DNA obtained was then used as a template source for Amplifluor™ Uniprimer™ real-time PCR detection.

3.4 Amplifluor™ Uniprimer™ PCR reagents and thermocycling program before optimization

For real time PCR we used the fluorescence-based Amplifluor™ Uniprimer™ universal system (Chemicon Intl., Temecula, CA). A "Master Mix" was prepared by mixing the target specific primers (mentioned above) and reagents equilibrated to room temperature outlined in Table 6. A 23 ul aliquot of this master mix was placed in PCR reaction tubes [MJ white low profile tubes (MJ Research Inc, Waltham MA)], mixed with 2 ul of the lysate (DNA source) and then subjected to real time PCR detection. PCR amplification and fluorescence detection was performed simultaneously using the Opticon monitor-2 DNA engine (MJ Research Inc, Waltham MA) with the Amplifluor™ Universal system temperature cycling program (Table 7).

Table 6. Amplifluor™ Uniprimer™ universal System PCR components		
Component	**Volume**	**Final concentration in the reaction tube**
Master Mix		
10X PCR buffer B	2.5 ul	1 X
25 mM $MgCl_2$	1.5 ul	1.50 mM
2.5 mM dNTP mix	2.5 ul	0.25 mM
0.5 uM Reverse primer with "Z tail"	2.5 ul	0.05 uM
5.0 uM Forward primer	2.5 ul	0.50 uM
5.0 uM Amplifluor™ Uniprimer™	2.5 ul	0.50 uM
Taq Polymerase (5 units/ul)	0.25 ul	1.25 units(0.05 units/ul)
dH20	8.75 ul	-
Total volume of master mix	**23.00 ul**	-
DNA sample/ lysate	2.00 ul	-
Total volume of PCR reaction	**25.00 ul**	

Table 7. Amplifluor™ Uniprimer™ System thermocycling Program		
Cycling Parameter	**Time**	**Temperature**
Denaturation	4 minutes	95°C
Denaturation	15seconds	95°C
Annealing	20seconds	55°C
	Plate read for fluorescence detection	
Extension	40 seconds	72°C
	Go to step 2 for 39 times	
Final extension	4 minutes	72°C
Hold		4°C

3.5 Optimization of Amplifluor™ Uniprimer™ real-time PCR conditions

L. monocytogenes ScottA-2 was selected as the target organism for the study. Primer set-1 was used for the optimization of PCR reagents and thermocycling conditions. The Opticon Monitor-2 thermocycling temperature parameters for strand denaturation, strand extension, and primer annealing temperature and cycle time was optimized by performing real-time PCR in triplicate replications with denaturing temperature gradients from 92°C to 95°C, extension temperature gradients from 66°C to 76°C, and annealing temperature gradients from 50°C to 60°C. Although the thermocycler permitted temperature gradients, it could not perform cycle time gradients which had to be done manually. Using optimized temperatures for denaturation, annealing and extension, the optimum anneal time was selected by performing different PCR of anneal time 14 sec, 16 sec, 18 sec, 20 sec (original) and 22 sec.

Optimization of the reagents in the original master mix involved PCR with original (1.25 units/reaction) and double concentration of Taq polymerase (2.5 units/reaction), original (0.25 mM) and double concentration of dNTP mix(0.5 mM/reaction), and a gradient of $MgCl_2$ concentration(final) [1.0 mM, 1.2 mM, 1.5mM (original), 1.8 mM, 2.0 mM, 2.2 mM]. In addition to the master mix, the volume of the lysate used was also optimized by performing multiple PCR replications with different volumes of lysate (0.5 ul, 1 ul, 2 ul {original}, 5 ul and 10 ul).

After optimization statistical analysis was performed. Data are expressed as the means of triplicate replications ± SD. Statistical comparison of Uniprimer™ PCR for all the four strains of L. moncytogenes before and after optimization was performed by one way

pairwise analysis of variance (Sigma Stat 3.0, SPSS, Chicago, IL). Data were considered significant when their computed probabilities were less than 0.05 ($P < 0.05$).

Our own Uniprimer™ Master mix (with optimized volumes and concentration of the reagents) was then compared with other available commercial mixes. In order to reduce the variability of the real-time PCR due to human error during preparation of the fresh master mix between runs, a storable main mix for 500 reactions was prepared with the optimized volumes and concentration of the reagents [1.0 X PCR buffer-B, dNTP mix(0.25 mM), double concentration of Taq polymerase (2.5 units/reaction) and 1.8 mM of $MgCl_2$]. A shelf-life study of this Uniprimer™ main mix at -20°C from week 0 to week 3 was performed and compared with one of the commercially available master mix and the freshly prepared Uniprimer™ master mix. The effect of purified DNA template obtained by DNeasy kit with that of the crude BAX lysate was also studied using optimum conditions

Using our optimized thermocycling conditions (Table 8) and reagent concentration (Table 9) the target specific primer set-II for *L. monocytogenes* was then selected from a pool of different primers by testing them against *Listeria* species, including *L. innocua* ATCC 33090, *L. ivanovii* ATCC 19119, *L. grayi* NRRL B-33023, *L. seeligeri* NRRL B-33019, *L. welshimeri* NRRL B-33020 and *L. monocytogenes* strains ScottA-2, V7-2, PMM39-2 and PMM383-2. Primer set-II with the optimized PCR conditions (Tables 8 & 9) was then used for the application of Uniprimer™ real-time PCR for detection of *L. monocytogenes* from enrichment broths using pure culture conditions as well as from food matrices after primary and secondary enrichment.

Table 8. Optimized Amplifluor™ Uniprimer™ System PCR components			
Component		**Volume**	**Final concentration in the reaction tube (25ul).**
I Master Mix			
i)Main Mix		7.30 ul	
	10 X PCR buffer B	2.50 ul	1 X
	25 mM $MgCl_2$	1.80 ul	1.8 mM
	2.5 mM dNTP mix	2.50 ul	0.25 mM
	Taq Polymerase B (5 units/ul)	0.50 ul	2.5 units (0.1 units/ul)
ii)5.0 uM Forward primer		2.50 ul	0.5 uM
iii)5.0 uM Amplifluor™ Uniprimer™		2.50 ul	0.5 uM
iv)1.0 uM reverse-Z primer		2.50 ul	0.1 uM
v) Sterile dH20		5.20 ul	
Total volume of master mix		**20.00 ul**	
II DNA sample/ lysate		**5.00 ul**	
Total volume of PCR reaction		**25.00ul**	

Table 9. Optimized Amplifluor™ System for Opticon Monitor-2 thermocycling Program		
Cycling Parameter	**Time**	**Temperature**
1)Denaturation	4 minutes	95°C
2)Denaturation	15 seconds	95°C
3)Annealing	18 seconds	51°C
4)Plate read for fluorescence detection		
5)Extension	40 seconds	72°C
	Go to step 2 for 39 times	
6)Final extension	4 minutes	72°C
7)Hold		4°C

3.6 Application of Uniprimer™ in real-time PCR detection using primer set-II

3.6.1 From pure culture detection

3.6.1.1 Washing of the culture:

Effect of washing the culture prior to lysis was also studied in order to improve the lysis and/or the effect of culture media on PCR. For this purpose, 4 laboratory strains (ScottA2, V7-2, 39-2 and 383-2) of *L. monocytogenes* were grown overnight in University of Vermont Media (UVM) at 30°C. A 1.5 ml aliquot of each culture was centrifuged at 8000 rpm for 6 min and resuspended in 1.5 ml of sterile 0.1% buffered peptone water. A 5 ul aliquot of the washed and unwashed cultures were used for the BAX™ lysis and subsequent optimized Uniprimer™ real-time PCR. Both washed and unwashed cultures were simultaneously plated to determine the percent recovery after washing using TSA. Plates were then counted after 48 hrs of incubation at 30°C.

3.6.1.2. Comparison of live cells vs. dead cells

This experiment was performed to evaluate whether the PCR detected live cells as well as the DNA of dead cells from the culture broth inoculated at the same level. Since the DNA of dead cells can equally serve as the template source and would show a false positive result, it may be a concern for the food industry. Therefore PCR of both viable cells and the dead cells were compared before and after 24 hrs of primary enrichment in UVM at 30°C and 20-22 hrs of secondary enrichment in MOPS-BLEB at 37°C. Dead cells were obtained by autoclaving an overnight culture (10^9 cfu/ml). The protocol is described in Figure 21.

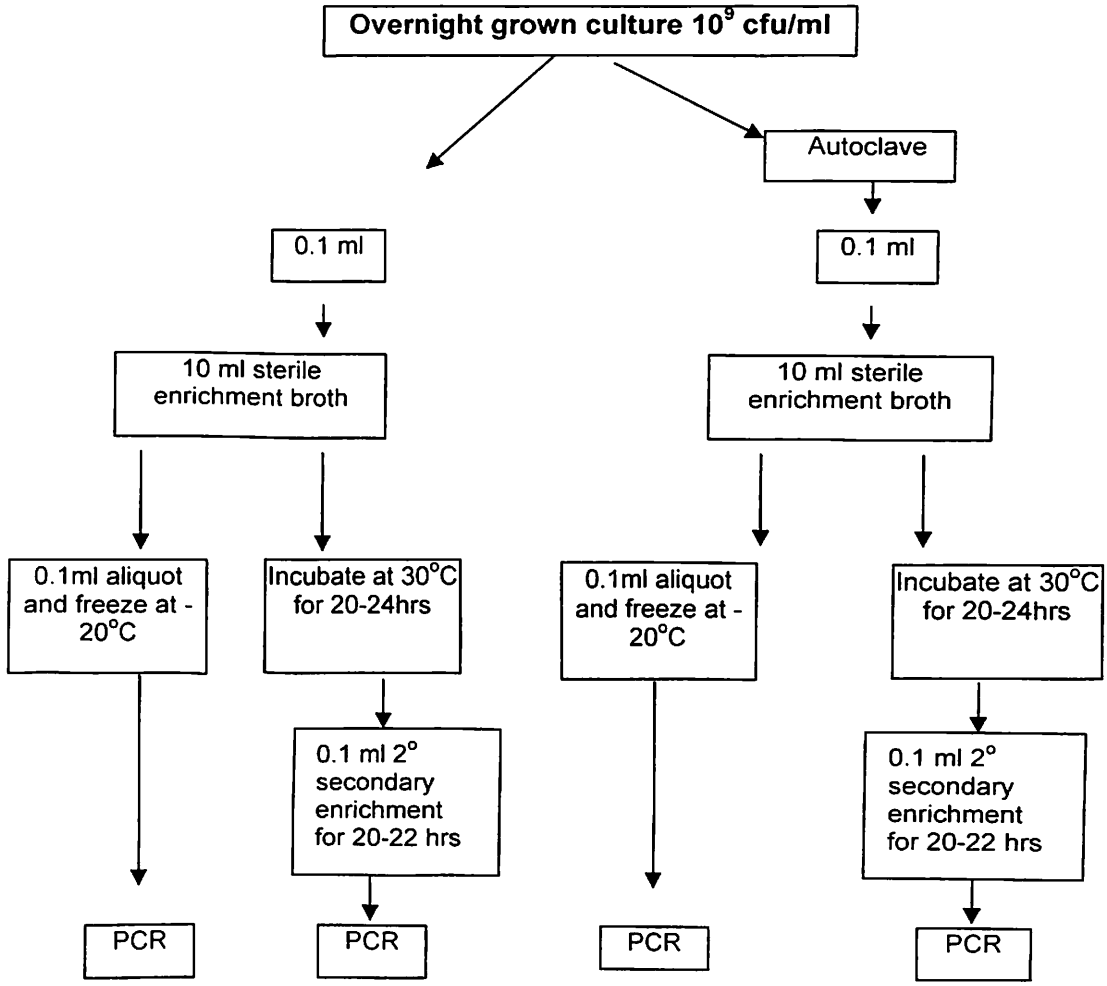

Figure 21. Schematic representation of comparing viable and dead cells

3.6.1.3. Determination of minimum cell level required for efficient PCR detection

In order to check the capability of the Uniprimer™ real-time PCR system for the efficient detection of *L. monocytogenes* within 40 cycles, we had to determine the minimum cell level or cfu/ml of *L. monocytogenes* required in media broth to be used prior to the assay. This is referred to as the minimum detection limit (MDL).

For this purpose, we used five different media including one non-selective media (BHI) and four selective media such as Fraser broth (FB), University of Vermont (UVM) broth, Demi Fraser (DF) broth and 3-(N-Morpholino) Propane Sulfonic Acid-Buffered Listeria Enrichment Broth (MOPS-BLEB). All five medias were inoculated with 100 ul of *L. monocytogenes* ScottA-2 and incubated for 20-22 hrs at 30°C. After incubation, all of them were serially diluted (1:10) in 9 ml of their respective sterile broth down to 10^{-9th} dilution (i. e.,10^{0} cfu/ml). A 5 ul of each dilution from each media was used for BAX™ lysis and for subsequent Amplifluor™ Uniprimer™ real time PCR. The undiluted cultures from all the 5 media were also plated in TSA to determine the initial culture level. The experiment was performed in triplicate replications and then averaged.

3.6.1.4. PCR detection following enrichment

Before examining Uniprimer™ real time PCR within a food matrix, real-time PCR detection of *L. monocytogenes* from primary and secondary enrichment using pure culture was performed to eliminate any interference which might occur due to the food product. The enrichment method used was the same as used by BAX™ PCR system for *L. monocytogenes* (DuPont Qualicon, Wilmington, DE). Overnight grown culture of *L.*

monocytogenes strain in BHI was serially diluted by 10-fold dilution scheme into 9 ml of sterile DF broth and UVM broth down to the 10^0cfu/ml level. Both the inoculated series (UVM and DF broth) had to undergo primary enrichment at 30°C for 22-24hrs. Following primary enrichment of both UVM and DF broth, 100 ul aliquot of initially inoculated 10^6cfu/ml to 10^0cfu/ml cultures were inoculated in 9.9 ml of sterile MOPS-BLEB for secondary enrichment and incubated at 35°C+/- 2°C for 18-20 hrs. After secondary enrichment, 5 ul samples from each tube of secondary enrichment broth were taken for real time PCR. The purpose of this experiment was to determine the ability of even 1 cfu/ml to grow in the enrichment media to levels high enough to allow real-time PCR detection.

3.6.2. From artificially inoculated food samples.

For the testing of inoculated foods, both raw and RTE meats were used. *L. monocytogenes* ScottA-2 was serially-diluted in 0.1% sterile buffered peptone water until 10^0cfu/ml. A 25 g sample of ground meat (i.e., ground beef) or RTE meat (i.e., hotdogs) was added to 225 ml of primary enrichment broth (DF broth for raw meat; UVM broth for processed meat), mixed with a stomacher, and 1 ml of each dilution from 10^6cfu/ml to 10^0cfu/ml was inoculated into identical suspensions and incubated at 30°C for 24 hrs. Samples inoculated with sterile water served as the negative control. Raw ground beef samples were chosen that tested negative for indigenous *Listeria* by enrichment and stored frozen until needed. After incubation primary enrichment, 0.1 ml of the primary enrichment from each sample was inoculated into 9.9 ml of secondary enrichment broth (MOPS-BLEB) and incubated at 35+/-2°C for 22-24 hrs followed by real-time PCR

detection. This helped identify whether the enrichment protocols were capable of providing sufficient enrichment for detection, regardless of the initial level of contaminating *Listeria* in the raw or processed meat. Additionally, the experiment examined whether food particles interfere with the real-time PCR detection. The experiment with both raw and RTE meats was performed in triplicate.

3.6.3 Detection from uninoculated food samples

For the testing of retail foods for *L. monocytogenes*, 25 g of raw ground meat (ground beef, pork, or chicken) from different retailers was incubated in enrichment broths as mentioned above, and tested by both real-time PCR and traditional culture methods mentioned in section 2.6.1 (Fig. 22). By comparing with the traditional method we determined the sensitivity and reliability of the Uniprimer^{TM} real-time PCR method.

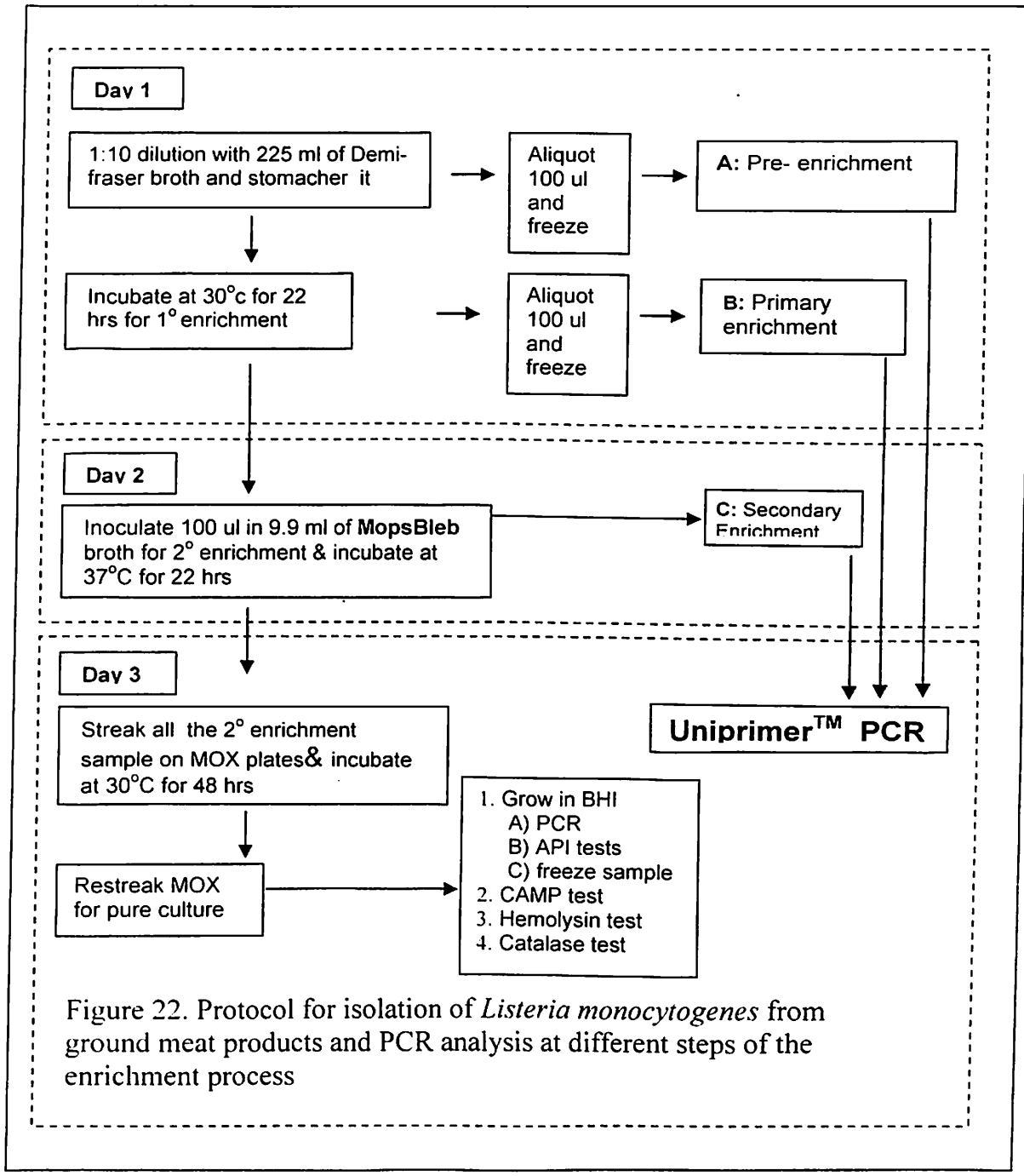

Figure 22. Protocol for isolation of *Listeria monocytogenes* from ground meat products and PCR analysis at different steps of the enrichment process

3.7. Improvement in fluorescence level

Primer set III and IV was used in my study to improve the fluorescence emission at lower cycles in comparison to primer set-II. Primer set-III has the Uniprimer™ directly attached to the 5` end instead of the indirect "Z tail" and it targets the *hly*A gene. Primer set-IV targets 16S rRNA gene which has 6 copies in the chromosome as compared to only 1 copy of *hly*A gene. Unlike primer set-III, primer set-IV has a "`Z tail"' attached to its 5` end of the reverse primer which incorporates the Uniprimer™ indirectly to its target gene. For both the sets, 100 ul of *L. monocytogenes* ScottA-2, V7-2, 39-2 and 383-3 was inoculated in BHI and grown overnight at 30°C. Following BAX™ lysis, real-time PCR was performed using the optimized conditions determined from our study. In the case of primer set-III, we used both the forward and the reverse primer (with attached Uniprimer™) in the same concentration (0.5 uM final concentration).

CHAPTER IV

RESULTS AND DISSCUSSION

In recent years numerous outbreaks of foodborne listeriosis have been caused by raw and ready to eat meat products contaminated with *Listeria monocytogenes*. The heavy economic losses due to product recalls and the high mortality rates associated with listeriosis have raised the need for reliable and rapid detection of the pathogen. Although reliable, there are several disadvantages of conventional bacteriological methods which are time consuming, laborious, and needs additional biochemical tests to confirm the species. These limitations could be overcome by the use of PCR techniques which are highly specific, rapid, and which permit automation. Real-time PCR using the TaqMan system is widely used and has also been applied in real-time PCR detection of *L. monocytogenes* from artificially contaminated cabbage (Hough et al. 2002). But the TaqMan system requires a specific probe to be synthesized for each PCR assay. In our study, we applied a real-time PCR assay using the new Amplifluor™ Uniprimer™ system for the detection of *Listeria monocytogenes* from the food matrices (meat samples). In contrast to the TaqMan system, the Amplifluor™ system uses a universal energy transfer hairpin primer (Uniprimer™) which emits a fluorescence signal when unfolded during its incorporation into an amplification product. Although, in a comparative study, the Uniprimer™ technique was slightly less sensitive than the TaqMan system (Rodriguez-Lazaro et al. 2004), the Uniprimer™ system offers the advantage of being less sensitive to mutations in the target DNA. It does not require a third probe target sequence (i.e., in addition to the primer annealing target sequences). It

is more cost effective when different genes are to be targeted due to its universal primer and it is easy to adapt on previous conventional PCR systems with minimum optimization. These factors justify our selection of the Uniprimer™ system for our study.

4.1 Examining primer set-I

The primer set-I was first designed for the study and tested for its efficiency and specificity using four strains of *L. monocytogenes* (ScottA-2, V7-2, 39-2, 383-2), *L. innocua* ATCC 33090, *L. ivanovii* ATCC 19119 and a non-template control. The PCR conditions and reagents used were the ones described by the original Amplifluor™ protocol prior to optimization. The resulting PCR efficiently amplified all four strains of *L. monocytogenes* with a logarithmic increase release. However, the primers did not show a high degree of specificity towards the target and also other species of *Listeria* (Fig. 23).

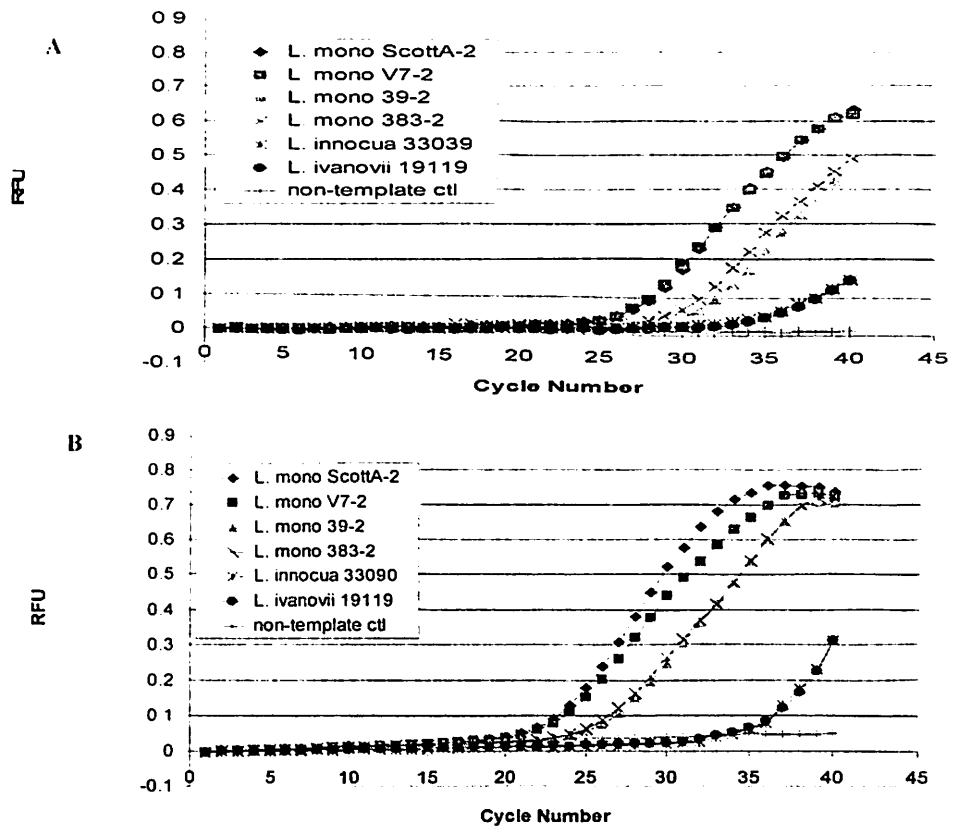

Figure 23. Uniprimer™ PCR amplification of 4 strains of *L. monocytogenes*, *L. innocua* ATCC33090, *L. ivanovii* ATCC 19119 and non-template control using primer set-I. Panel A, PCR before optimization. Panel B, PCR after optimization.

Table 10. Statistical analysis of Uniprimer™ PCR before and after optimization for 4 strains of *L. monocytogenes*

L. monocytogenes strains	Maximum RFU*± S.D. [1]		Ct value*± S.D. [2]	
	Before	After	Before	After
ScottA-2	0.65 ± 0.15 ^{A}	0.76 ± 0.02 ^{A}	27.77 ± 0.64 ^{c}	22.93 ± 0.26 ^{d}
V7-2	0.63 ± 0.17 ^{A}	0.73 ± 0.02 ^{A}	27.97 ± 1.32 ^{c}	23.43 ± 0.40 ^{d}
39-2	0.47 ± 0.04 ^{A}	0.71 ± 0.02 ^{B}	31.80 ± 0.20 ^{c}	26.48 ± 0.19 ^{d}
383-2	0.49 ± 0.15 ^{A}	0.72 ± 0.00 ^{B}	31.00 ± 1.82 ^{c}	26.14 ± 0.17 ^{d}
Non-template control	0.02 ± 0.01 ^{A}	0.05 ± 0.01 ^{A}	0.00 ± 0.00 ^{c}	0.00 ± 0.00 ^{d}

*Note: values are the means of triplicate replications. Ct values are calculated by the Opticon monitor-2 (M J Research Inc. Alameda, CA) at 0.1 RFU.

[1] RFU values in the same row with different upper case letters are significantly different from each other ($P<0.05$)

[2] Ct values in the same row with different lower case letters are significantly different from each other ($P<0.05$)

4.2 Determining the conditions for optimum PCR performance

The thermocycling temperatures such as denaturing, annealing and extension temperatures were examined. After testing various ranges of temperature and time we found that the denaturing temperature of 95°C, the extension temperature of 72°C, the annealing temperature of 51°C and an annealing time of 18 sec worked best in terms of higher fluorescence levels and/or reducing the threshold cycle to lower cycle numbers. (Fig. 24).

We next used our optimized thermal cycling parameters to examine various components to our PCR recipe. Using Different PCR we compared 1.25 units/reaction Taq polymerase with 2.5 units/reaction, 0.25 mM dNTP mix with 0.5 mM dNTPmix and reactions containing both 2.5 units/reaction of Taq polymerase and 0.5 mM dNTP mix with reactions containing 1.25 units/reaction of Taq polymerase and 0.25 mM dNTP mix. The results of the PCR indicated that by increasing the concentration of Taq polymerase (2.5 units/reaction), the fluorescence levels increased, and at 0.097 Relative Fluorescence Unit (RFU), it reduced the threshold cycle (Ct) level by 2 cycles that is cycle 24.2 in comparison to the original Ct cycle 26 (Fig. 25A). We therefore increased the concentration of Taq polymerase from 1.25 units to 2.5 units per reaction for our further optimizations. On the other hand the change in the dNTP concentration did not improve the fluorescence detection in comparison to the original. The non-template control amplification was eliminated (data not shown) when the pipettors used during reagent preparation were separated from the other pipettors used for microbial analysis, washed, cleaned with 70% ethanol before each use. In addition to the cleaning of the pipettors, the barrier pipette tips were used to prevent any contamination from aerosols

and the sterile water and reagents were stored in sterile vials which had not been previously used for any microbiology work in the laboratory. This result indicated that the fluorescence from the non-template control reactions were due to contamination with the target DNA and may not be due to the primer dimer formation. For optimization of the $MgCl_2$ concentration, we performed different PCR with 2.5 units/reaction of Taq polymerase and different concentration (final) of $MgCl_2$ (1.2 mM, 1.5 mM, 1.8 mM, 2.0 mM, and 2.2 mM). The best fluorescence yield was best at 1.8 mM $MgCl_2$ (1.72 RFU) in comparison to our original 1.5 mM $MgCl_2$ (1.60 RFU) (Fig. 26C). We also performed PCR with different lysate volumes (0.5 ul, 1 ul, 2 ul, 5 ul, and 10 ul). The 5 ul lysate volume had the highest RFU (0.41) and lowest Ct at cycle 23 in comparison to the original 2 ul volume suggested by BAX^{Tm} protocol (RFU 0.37 and Ct 27).

We performed statistical analysis of the primer set-I using the conditions that maximize our response (optimized) and the conditions before optimization, The data indicates that, after optimization, the Ct levels for all four strains of *L. monocytogenes* improved and were significantly different ($P<0.05$) from their respective Ct levels before optimization. Although, the maximum RFU levels for all strains tested were higher after optimization, some of the strains were not significantly different due to high standard deviations between the replicates of those strains. However, after optimization the S.D. within the replicates of each strain were smaller and more consistent (Table 10). The specificity of these primers were rechecked using the four strains of *L. monocytogenes* (ScottA-2, V7-2, 39-2 and 383-2), *L. innocua* ATCC 33090, *L. ivanovii* ATCC 19119 and non-template control. The primers still amplified *L. innocua* and *L. ivanovii* (Fig. 23). Therefore these primers were not very target specific and were not suitable for our further application.

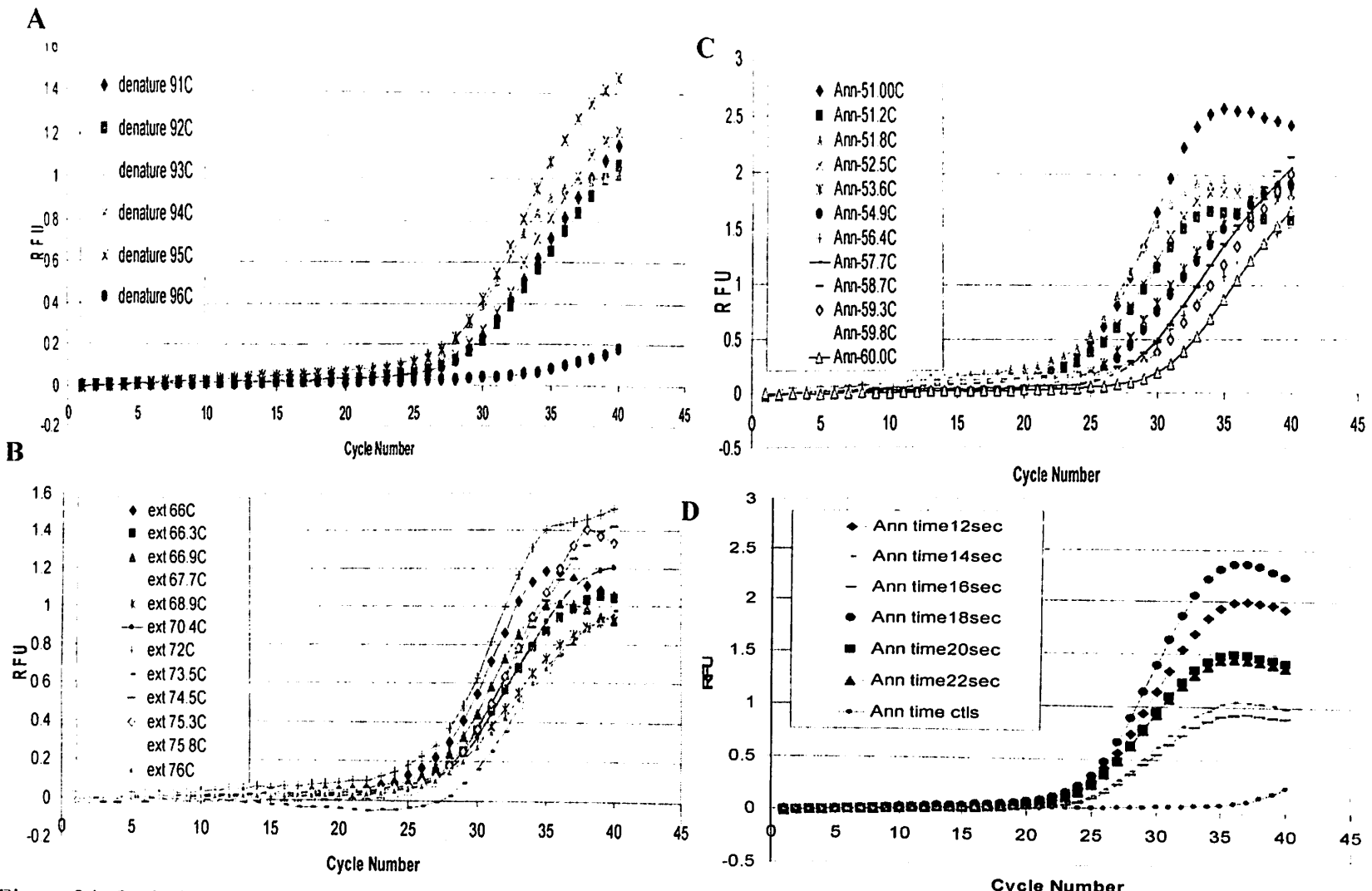

Figure 24. Optimization of thermocycling parameters using *L. monocytogenes* ScottA-2 and primer set-I. Panel A. denaturing temperature gradient of 91°C-96°C. Panel B, extension temperature gradient of 66°C-76°C. Panel C, Annealing temperature gradient of 50°C-60°C. Panel D, PCR with different annealing time for primers from 12 sec-22 sec.

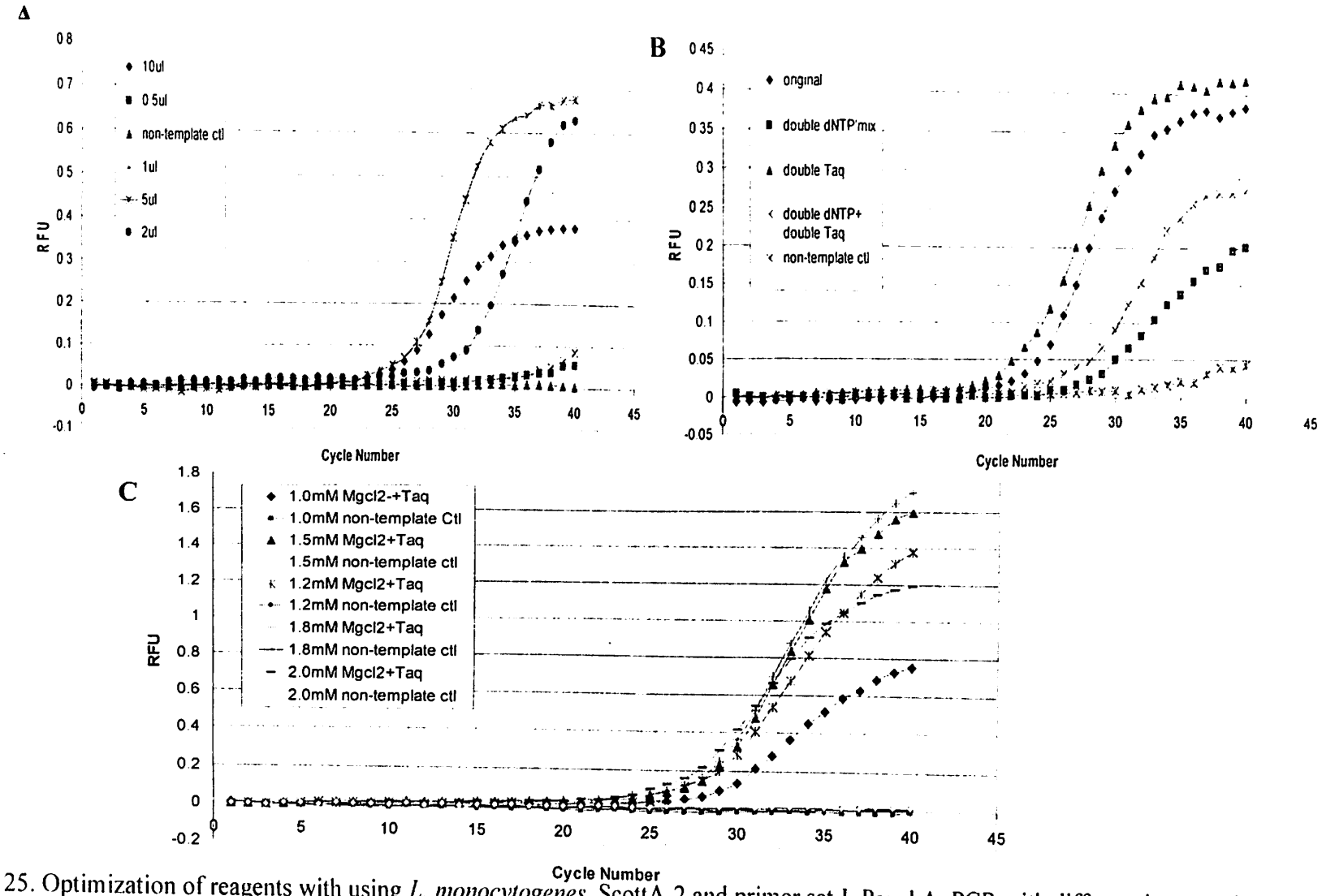

Figure 25. Optimization of reagents with using *L. monocytogenes* ScottA-2 and primer set-I. Panel A, PCR with different lysate volumes from 0.5 ul to10 ul. Panel B, PCR with different concentrations of dNTP mix and Taq polymerase. Panel C, PCR with different $MgCl_2$ concentrations

4.3 Examining the primer set-II

The primer set-II which was designed using the consensus sequence of 45 different *hlyA* gene sequences of different serotypes of *L. monocytogenes* was then checked for its specificity towards the target. These primers were again tested against 4 strains of *L. monocytogenes* (ScottA-2, V7-2, 39-2 and 383-2), *L. innocua* ATCC 33090, *L. ivanovii* ATCC 19119, *L. grayi* NRRL B-33023, *L. seeligeri* NRRL B-33019, *L. welshimeri* NRRL B-33020 and the non-template control reaction. The PCR conditions used was the ones we had optimized before.

The result indicated that these primers were specifically positive for *L. monocytogenes* and did not amplify *L. ivanovii, L. innocua, L. grayi, L. seeligeri, L. welshimeri* and the non-template control (Fig. 26). We then optimized the concentration of the reverse-Z primer of the primer set-II to 0.1 uM from 0.05 uM original concentration. This primer set-II was then used for our further studies and for the detection of *L. monocytogenes* from food samples.

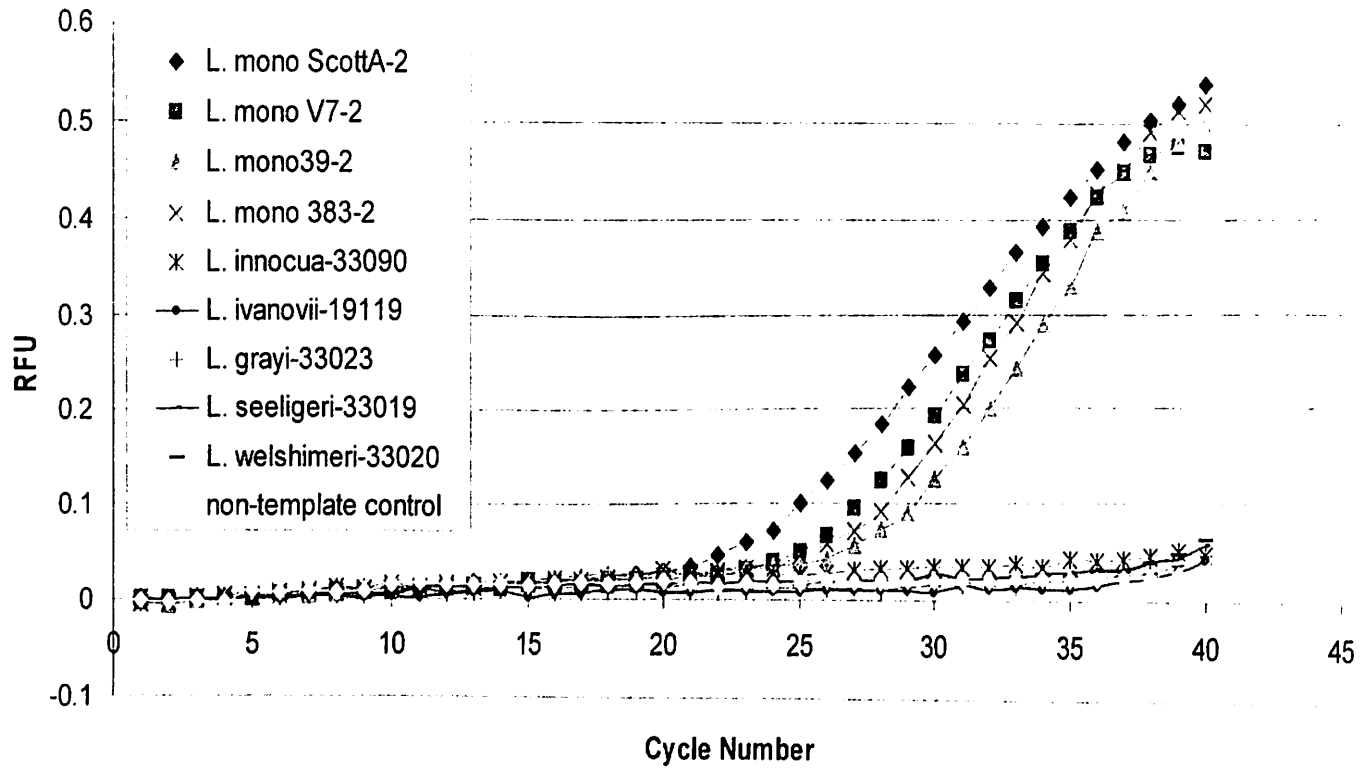

Figure 26. Uniprimer™ PCR of *L. monocytogenes* ScottA-2, V7-2, 39-2, 383-2, *L. innocua* ATCC 33090, *L. ivanovii* ATCC 19119, *L. grayi* NRRL33023, *L. seeligeri* NRRL 33019, *L. welshimeri* NRRL 33020 and non-template control using primer set-II.

4.4 Studies using different master mixes

Master mixes are premixed components of PCR reactions that are useful when performing many PCR reactions. A master mix avoids inaccuracies of measuring out small volumes required of various components for individual reactions. Therefore reagent master mix plays a critical role for successful PCR. There are many commercial master mixes available in the market which may reduce the effort and time required for the preparation when multiple samples are to be tested. Hence the efficiency of the optimized Uniprimer™ master mix was compared to other 6 commercially available master mixes (Qbiogene. Biomix Red, Qiagen, Promega, Eppendorf and Jumpstart mix). Our result show that the master mix we prepared worked better with our optimized Amplifluor™ protocol for real-time PCR detection than when any of the commercial master mixes were used (Fig. 27). The stability of our master mix was tested along with that produced by Qbiogene when stored at -20°C were then compared over a period of 4 weeks along with freshly prepared Uniprimer™ master mix. The results of the entire 4 weeks study indicated that the stored Uniprimer™ master mix maintained a consistency in its performance and was better than Qbiogene's master mix as well as the fresh Uniprimer™ master mix (Fig. 28). The stored Uniprimer™ master mix might have worked better because it was prepared in bulk and any human error was avoided while pipetting small volumes of reagents during preparation of small volumes of fresh master mix. The stability of the master mix at -20°C is important because it shows that it could be easily prepared and stored, which can significantly reduce the time, error and complexity of preparing the master mix and thereby making it suitable for the routine microbiological analysis for food testing applications.

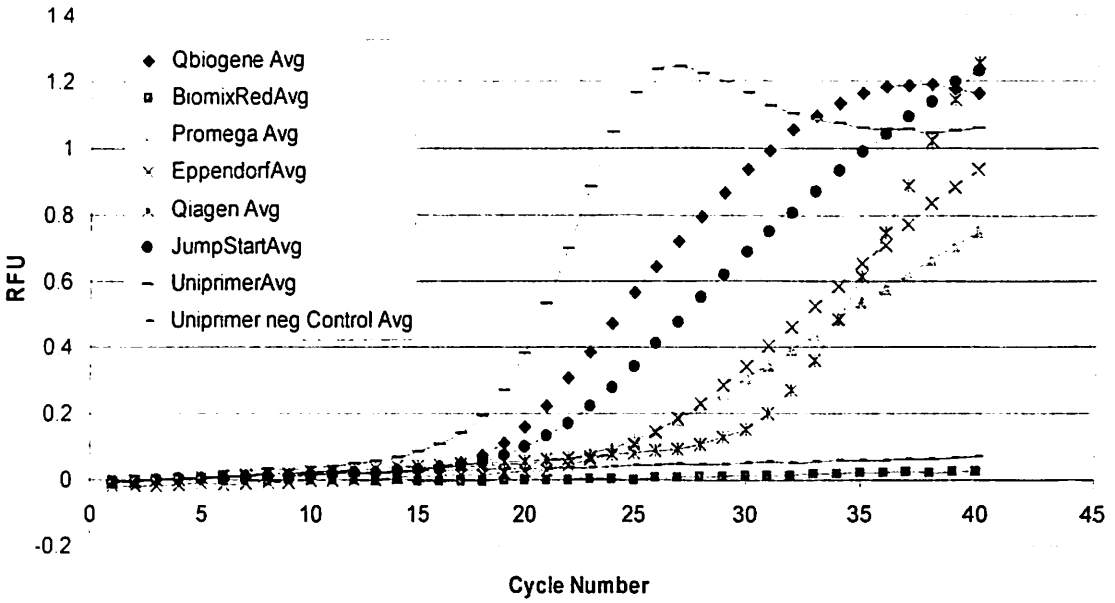

Figure 27.Comparison of the Uniprimer™ master mix with different commercially available master mix.

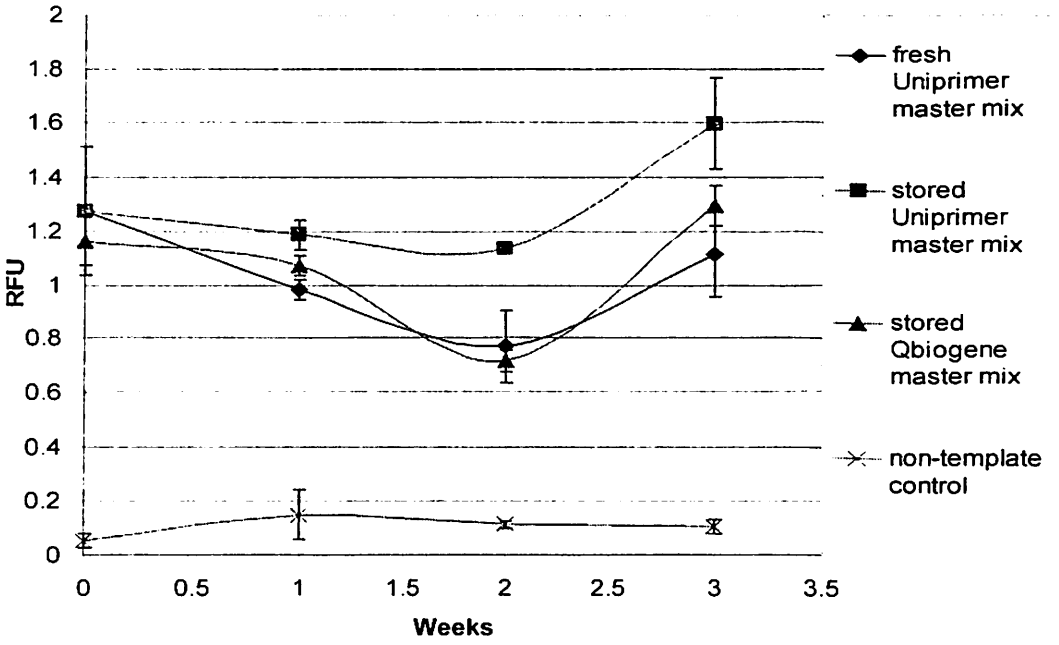

Figure 28. Storage stability of Uniprimer™ master mix at -20°C in comparison to stored Qbiogene master mix and fresh prepared Uniprimer™ master mix.

4.5 Template Preparation and treatments

The BAX™ lysate is a crude lysate which also contains cell debris and other unrequired materials that may interfere with an optimum enzymatic reaction. Thus, in order to improve the fluorescence detection we compared real-time PCR with crude lysate with that of using purified DNA prepared by DNeasy Kit. The purified DNA was resuspended in the volume as was originally taken from the cell culture broth so that the DNA amount in the crude lysate is equal to the DNA amount in the purified DNA suspension. The pure DNA amplified and crossed the threshold fluorescence 0.1RFU much earlier at cycle 17 than the crude lysate at cycle 26 (Fig. 29).

The washing of the culture prior to the BAX™ lysis also improved the fluorescence in 3 out of 4 strains of *L. monocytogenes* (ScottA-2, V7-2 and 39-2) when compared to their respective unwashed culture. However, the fluorescence emission of the washed *L. monocytogenes* strain 383-2 was lower than its unwashed culture (Fig. 30). The total plate count of both the washed and the unwashed culture indicated that with the washing of *L. monocytogenes* strain 383-2 only 38% of the original cells was recovered, resulting in a decreased fluorescence in comparison to the unwashed culture of the same strain (Table 11).

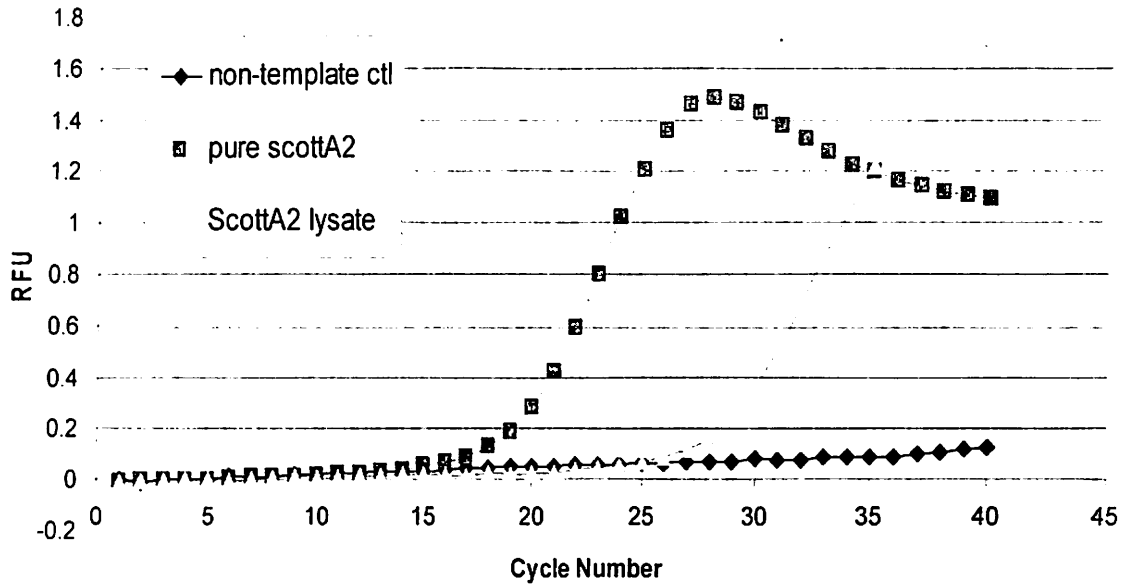

Figure 29. Uniprimer™ PCR comparison of pure DNA with that of the BAX™ crude lysate with *L. monocytogenes* strain ScottA-2.

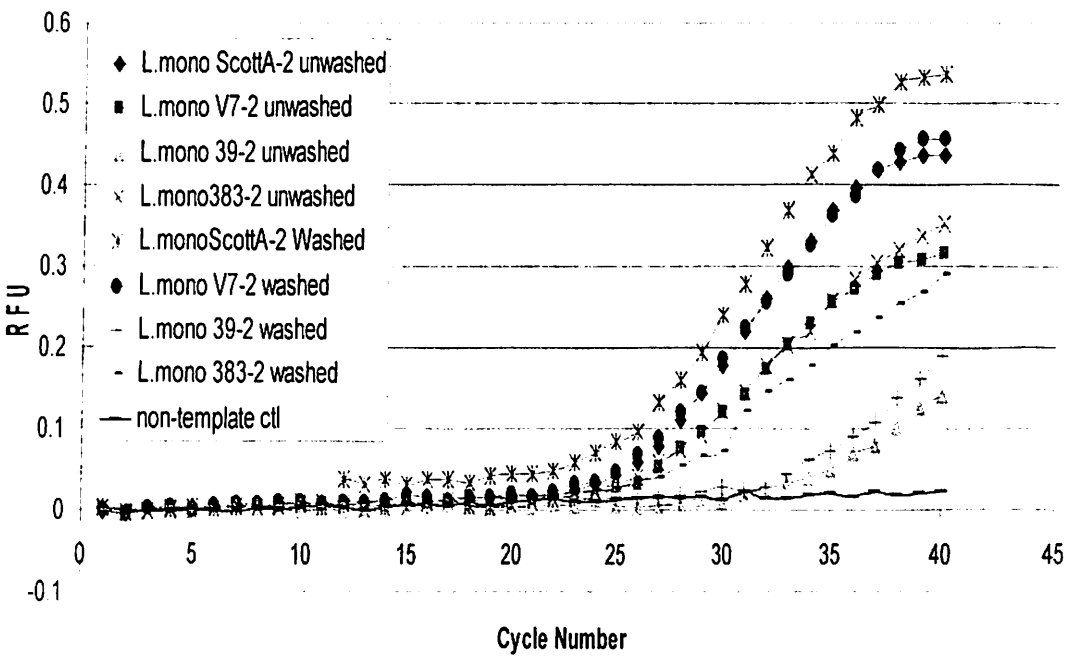

Figure 30. Comparison of washed sample with unwashed samples for *L. monocytogenes* strain ScottA-2, V7-2, 39-2 and 383-2.

Table 11. To determine the % recovery of cells of *L. monocytogenes* from washing			
Strain	Treatment	Cfu/ml	Recovery %
ScottA-2	unwashed	$8.9x10^8$	77.52
	washed	$6.9x10^8$	
V7-2	unwashed	$3.6x10^8$	100
	washed	$3.6x10^8$	
39-2	unwashed	$7.2x10^8$	100
	washed	$7.2x10^8$	
383-2	unwashed	$7.9x10^8$	36.7
	washed	$2.9x10^8$	
Formula for calculating % recovery= washed (cfu/ml)/unwashed (cfu/ml) x100.			

4.6 Determination of minimum detection level (MDL)

The purified DNA and the washed culture samples yielded better fluorescence detection (i.e. higher fluorescence or lower Ct) than with the crude lysate providing potential areas of consideration for additional improvements in our detection protocol. The main objective of this study was to examine the Uniprimer™ system for food analysis, whereby the extraction of purified DNA or washing of the culture would be an additional cumbersome step unsuitable for testing a large number of food samples. In addition, with washing there is also a probability of losing some cells which is undesirable and introduces error.

In order to test the efficiency of the Uniprimer™ system to detect the minimum number of target cells from the crude lysate, real-time PCR was performed with 10-fold dilutions of culture from 10^9 cfu/ml -10^0 cfu/ml grown in selective media (UVM, DF broth, FB and MOPS-BLEB) and non-selective media (BHI). For all 4 selective media, the minimum number of target cells required for the detection by Uniprimer™ system within 40 cycles was 10^5 cfu/ml. However, if grown in a non-selective media like BHI, the Uniprimer™ system can detect *L. monocytogenes* down to 10^4 cfu/ml (Fig. 31). For all the five medias the plot of Ct level vs. cfu/ml shows a negative correlation, and thus with the increase in cfu/ml there is a decrease in the Ct level.

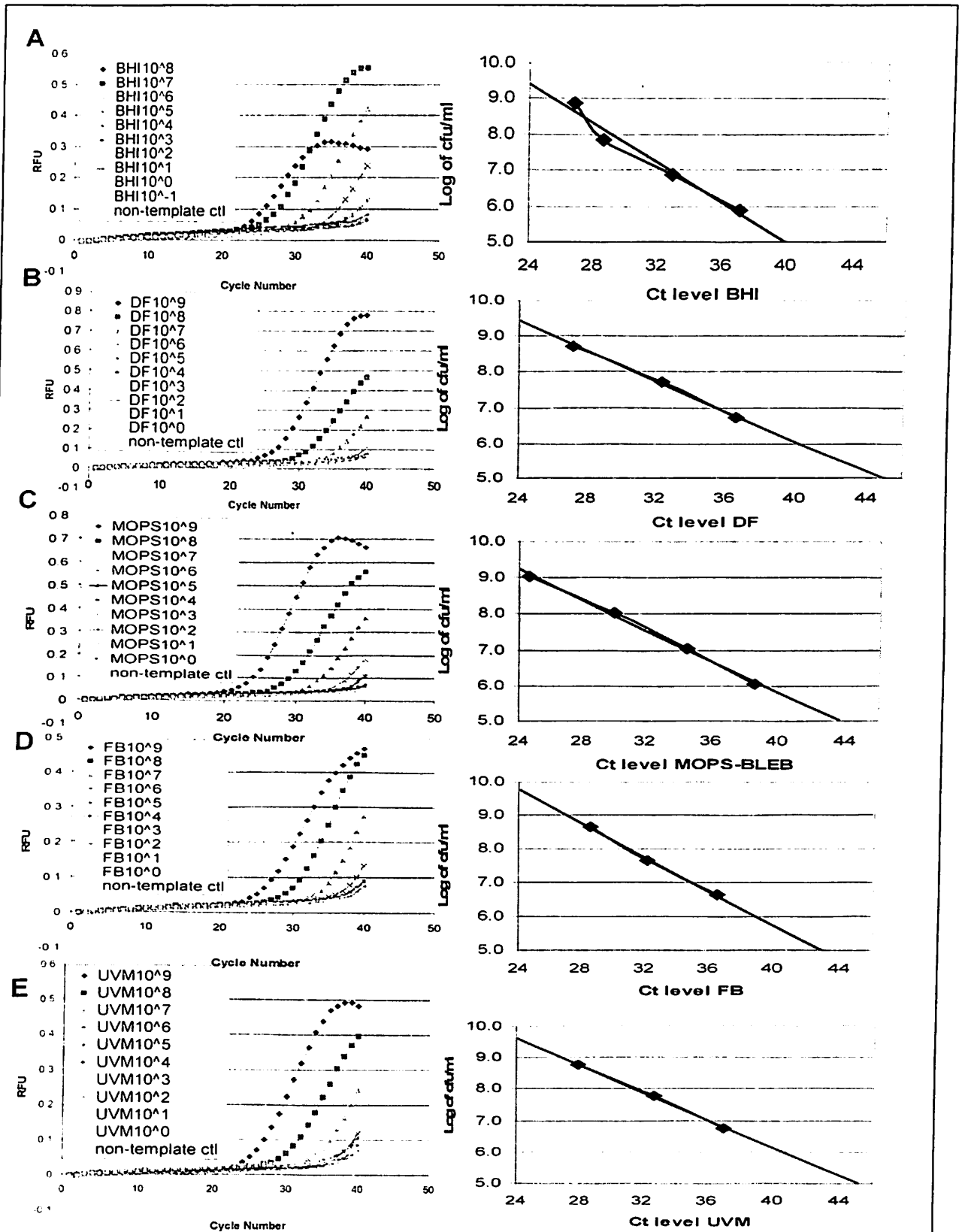

Figure 31. Minimum level of cfu/ml required in different medias for Uniprimer™ PCR and the correlation of threshold cycle (Ct) with the cfu/ml. Panel A, BHI broth. Panel B.DF broth. Panel C. MOPS-BLEB broth. Panel D. FB broth. Panel E. UVM broth.

4.7 Studies using pure culture

We performed additional studies using pure cultures. Since the target of a PCR reaction is the DNA template, there is a possibility that the PCR would equally detect a dead cell as it would detect the DNA from a live cell. Therefore, if the Uniprimer™ (or any PCR) system were to detect the dead cells, it would be a concern for its application in the food industry because it would lead to false positive results.

For this purpose, we inoculated 2 tubes of UVM with the same level (10^9 cfu/ml) of live culture and dead (autoclaved) culture, and pulled samples for PCR from each before and after primary and secondary enrichment. The results showed that on day 1 (before incubation) there was no difference in the detection of dead cells or live cells and the Uniprimer™ system detected both equally. This was because the 2 tubes of UVM were inoculated with the same culture concentration of dead and live cells (i.e., 10^9 cfu/ml). Therefore, even the dead cells had the same level of template/DNA as that of the live cells. After 24 hrs of incubation in UVM, the live cells had increased in number, resulting in a decrease in the Ct level. On the other hand, the dead cells did not increase further after incubation, resulting in comparatively lower fluorescence and an increase in Ct level. The lag in the fluorescence release by the dead cells after incubation might be due to the degradation of the DNA which was held at 30°C for 24hrs and/or dilution of (dead cell) culture when inoculated into the primary enrichment broth and again transferred to the secondary enrichment broth (Fig. 32).

Therefore our results show that although high levels of dead cells may be detected, there is a need for primary and secondary selective enrichment for *L. monocytogenes* before its

detection with the Uniprimer™ (or any PCR) system in order to avoid false positive results from dead cells. The enrichment protocols provide for dilution of dead cells to undetectable levels while live cells increase to higher numbers within the range that can be detected.

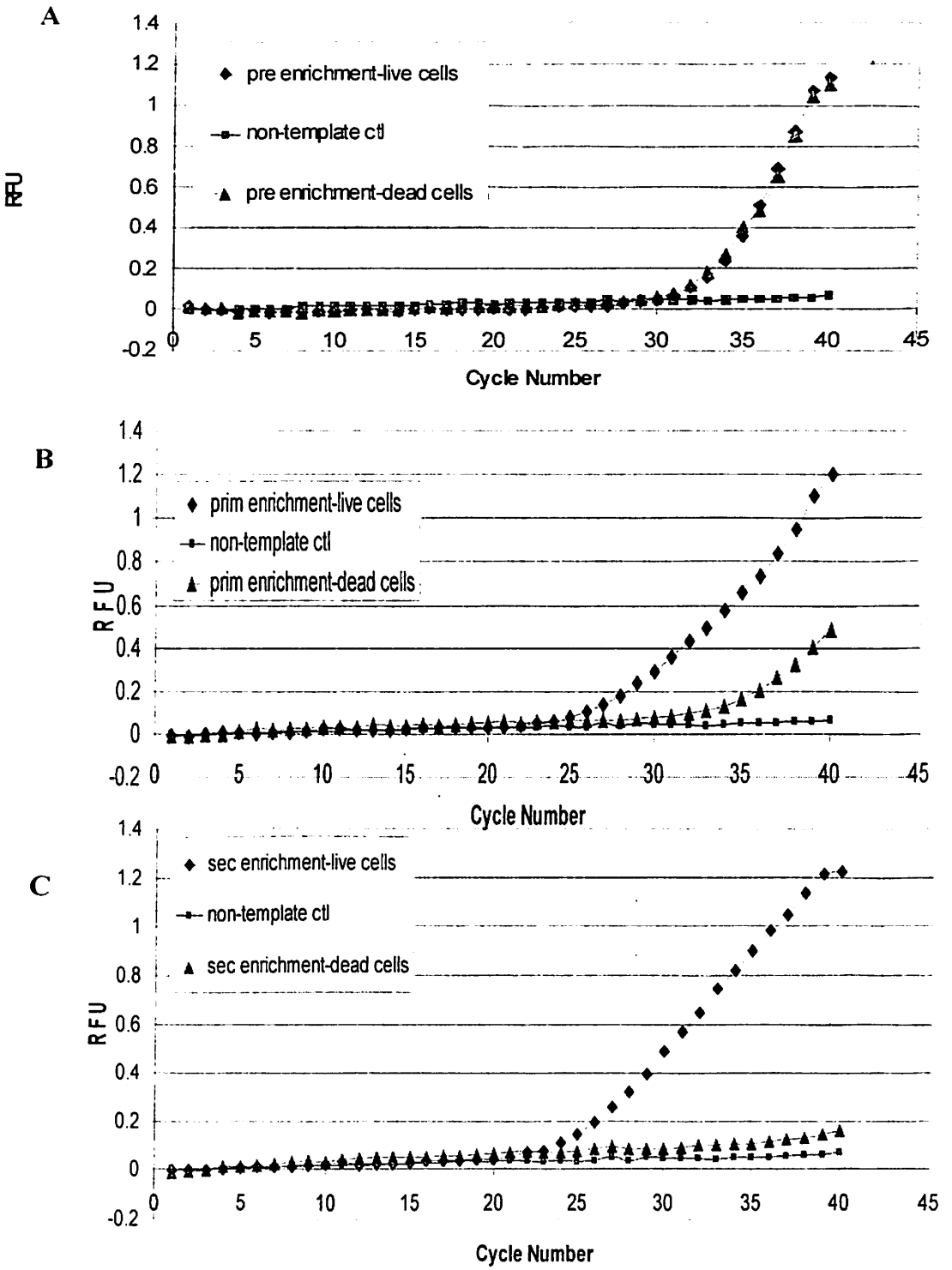

Figure 32. Uniprimer™ PCR for comparing live vs. dead cells of *L. monocytogenes* ScottA-2. Panel A, before primary enrichment. Panel B, after primary enrichment. Panel C, and after secondary enrichment.

4.8 Studies with different enrichment broths

For enrichment we followed the protocol recommended by the BAX^{Tm} PCR system (DuPont Qualicon, Wilmington, DE). The BAX^{Tm} PCR method recommends either UVM (for RTE meats) or DF broth (for raw meats) as primary enrichment and MOPS-BLEB as the secondary enrichment media. On the other hand, the USDA/FSIS traditional method for detection of *L. monocytogenes* includes UVM as primary enrichment (irrespective of the type of the meat used) and FB for secondary enrichment. The BAX^{Tm} method for enrichment for *L. monocytogenes* was selected for our study because this method is approved by the USDA/FSIS and is included in the current version of USDA/FSIS Microbiological Laboratory guidebook for PCR detection of *L. monocytogenes* from meats (USDA/FSIS 2002). This method varies from the traditional method in that the FB is replaced by the MOPS-BLEB as secondary enrichment broth. We examined the suitability of the BAX^{Tm} enrichment media in comparison to FB enrichment media for the Uniprimer^{TM} PCR detection of *L. monocytogenes*. Uniprimer^{TM} real-time PCR was performed on 4 strains of *L. monocytogenes* (ScottA-2, V7-2, 39-2, 383-2), each grown by both the BAX^{Tm} enrichment methods (UVM + MOPS-BLEB and DF + MOPS-BLEB) and the traditional enrichment method (UVM + FB). The result showed that by BAX^{Tm} enrichment methods all the four *L. monocytogenes* strains were almost similar in their fluorescence yield (0.52-0.62 RFU or 0.44-0.50 RFU), well amplified, and detected within 40 cycles. In contrast when FB was used, there was a high inconsistency within the strains in terms of their fluorescence yield (0.09-0.33 RFU) (Fig. 33). In addition, total plate counts after using the different media indicated that of the 5

broths used the FB had the lowest growth level and the MOPS-BLEB had the highest growth levels (Table 12).

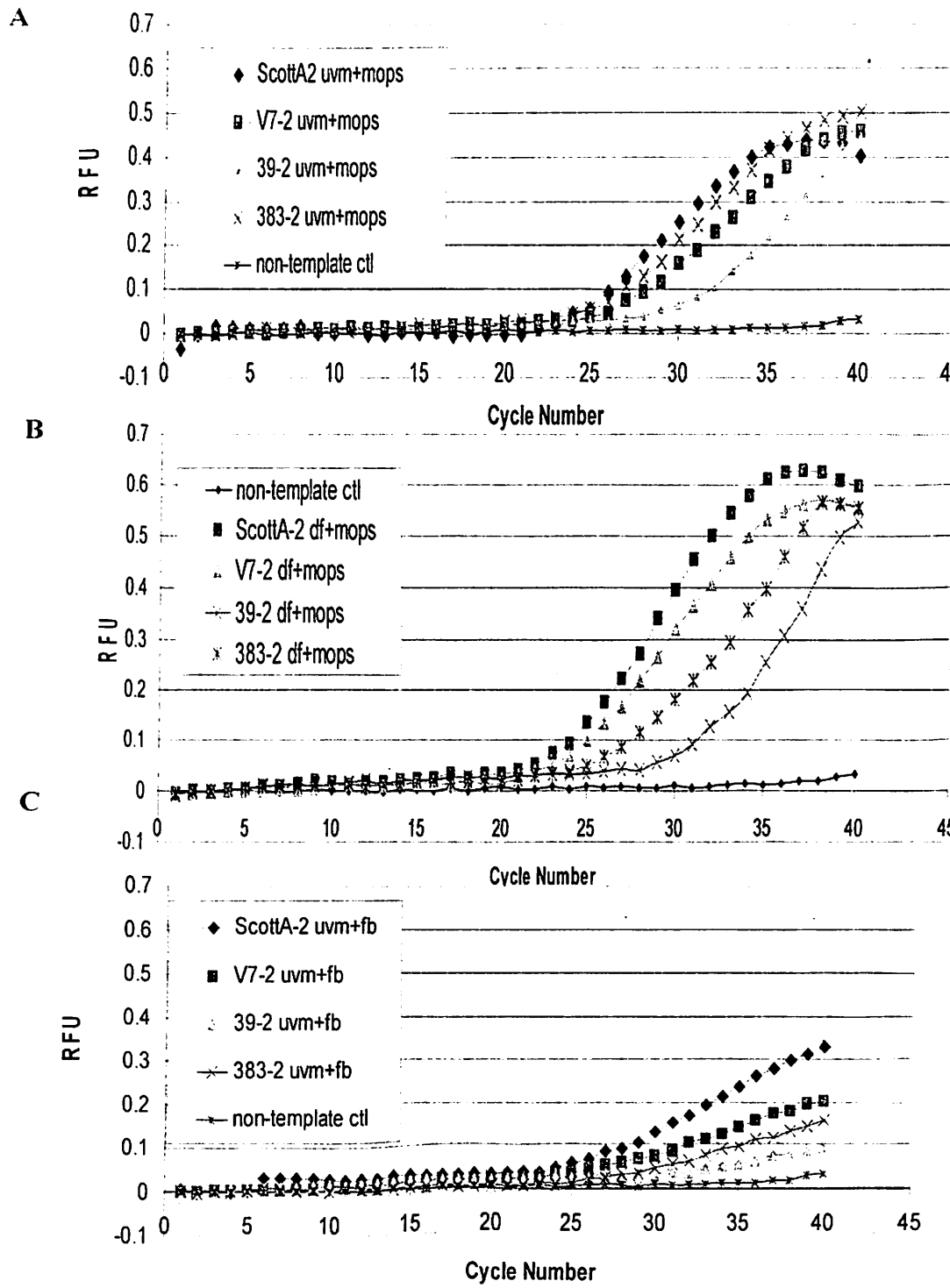

Figure 33. Uniprimer™ PCR of 4 strains of *L. monocytogenes* after enrichment in various media. Panel A. UVM broth (primary enrichment) followed by MOPS-BLEB (secondary enrichment). Panel B DF broth (primary enrichment) followed by MOPS-BLEB (secondary enrichment). Panel C. UVM broth (primary enrichment) followed by FB (secondary enrichment).

Table 12. Cell levels (cfu/ml) of *L. monocytogenes* ScottA-2 in different broth media.

Media	Log $_{10}$ cfu/ml			Mean Log $_{10}$ cfu/ml	Standard deviation
	Run1	Run2	Run3		
BHI broth	8.9	8.8	8.8	8.9	0.07
UVM broth	8.7	8.8	8.8	8.8	0.06
MOPS-BLEB broth	9.0	9.0	9.1	9.0	0.09
DF broth	8.7	8.8	8.8	8.8	0.05
FB broth	8.6	8.7	8.6	8.7	0.08

4.9 Detection of *L. monocytogenes* following enrichment

Our prior data showed that the Uniprimer™ system required at least 10^5 cfu/ml of *L. monocytogenes* in the culture/enrichment broth to be detected well within 40 cycles (Fig. 31) and the primary and secondary enrichment broths of the BAX ™ system work well with the Uniprimer™ PCR (Fig. 33). The minimum detectable level compelled us to arrive at number of initial ($<10^5$ cfu/ml) target cells. In order to verify this assumption, we artificially inoculated serial dilutions of the cultures of *L. monocytogenes* ScottA-2 in the primary enrichment broths (both the UVM and the DF broth) at a level of 10^6 cfu/ml to 10^0 cfu/ml along with a negative control (only sterile water). This was followed by both primary enrichment and secondary enrichment of each and then we performed the real-time PCR and were conducted in triplicate replications. This primary enrichment dilution series was conducted with pure culture (tubes) and with raw sausage emulsion and RTE meats (hotdogs). The results showed that in both sets of experiments broth and meat enrichments were successful in allowing PCR detection even when initially inoculated at a level of 1 cfu/ml (pure culture tubes) (Fig. 34) or 1 cfu/25 grams of meat (Fig. 35). Even at the lowest inoculation levels (1 cfu/25 gm meat) exceeded our minimum detection limit of 10^5 cfu/ml and all were efficiently detected by the Uniprimer™ real time PCR. Moreover, we also observed that the efficiency of the detection directly from the meat samples was equally good as from the pure culture. Therefore the presence of the food particles did not interfere with the Uniprimer™ PCR reaction.

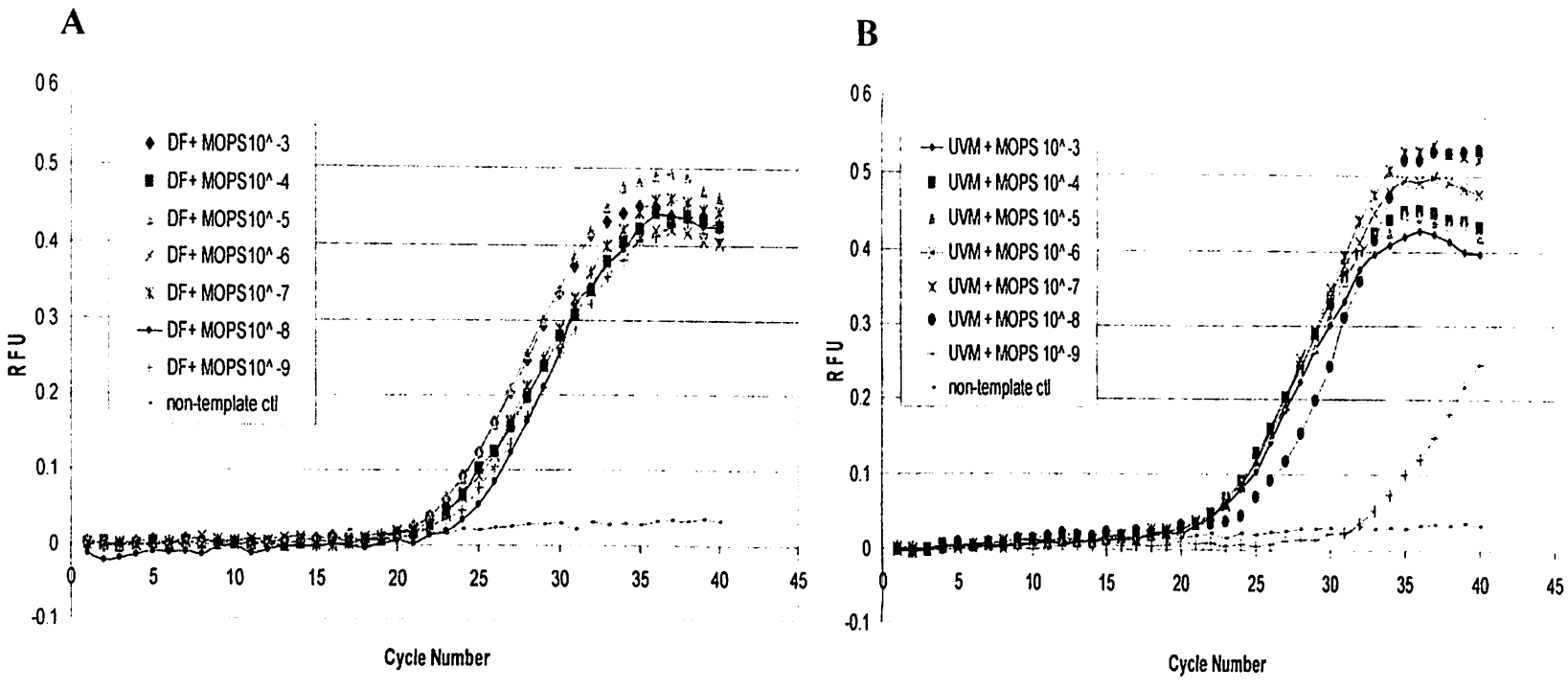

Figure 34.Uniprimer™ real-time PCR detection of *L. monocytogenes* strain ScottA-2 from pure culture tube enrichment starting with primary enrichments inocluated at 10^6 cfu/ml to 10^0cfu/ml followed by transfer to secondary enrichment and Uniprimer™ PCR detection. Panel A, primary enrichment in DF broth followed by secondary enrichment in MOPS-BLEB. Panel B, primary enrichment in UVM broth followed by secondary enrichment in MOPS-BLEB. primary enrichment UVM (10^{-3} to 10^{-9} dilution) (panel B).

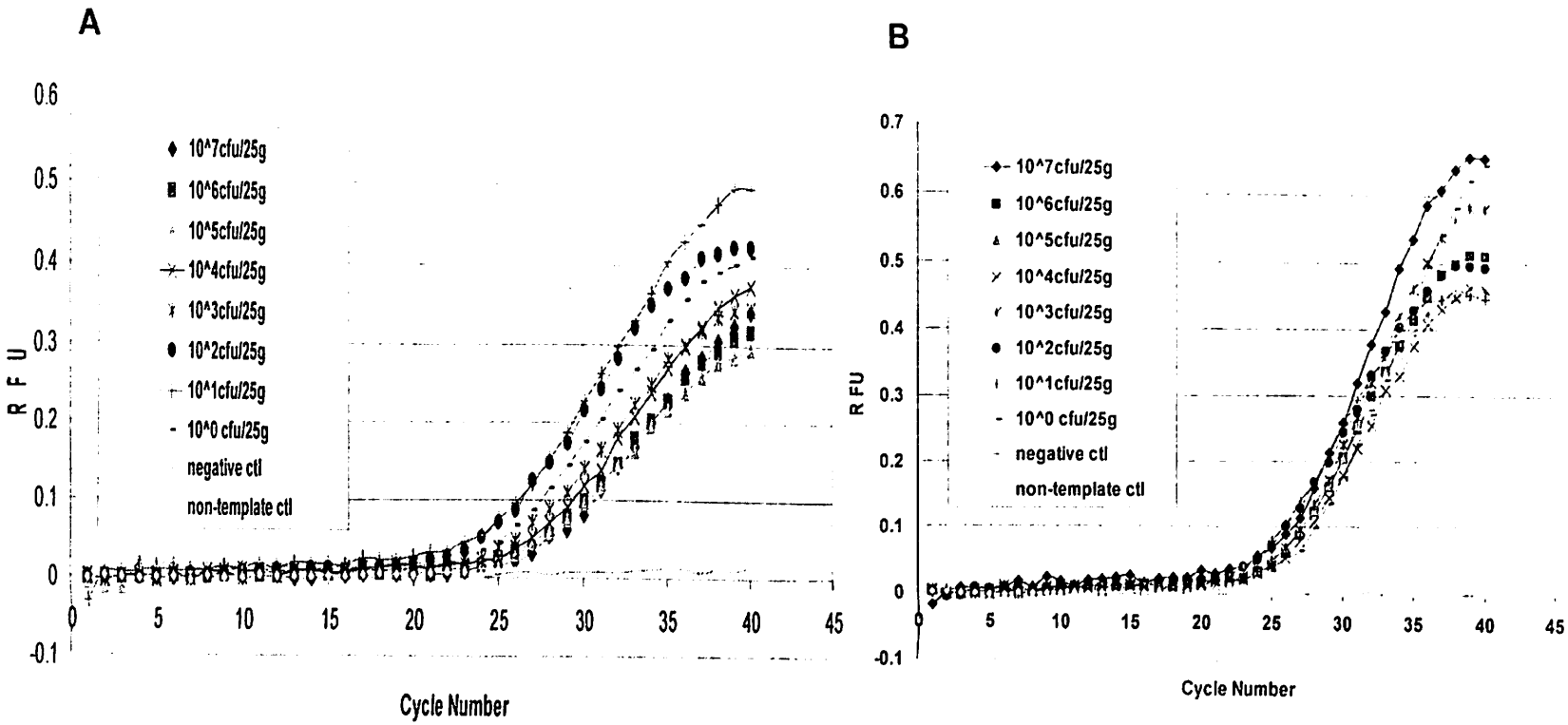

Figure 35. Uniprimer™ PCR detection of *L. monocytogenes* ScottA-2 in sausage emulsion (panel A) or hotdogs (panel B) inocluated at various levels (10^7cfu/25g- 10^0 cfu/25g) followed by primary and secondary enrichment. Panel A, detection from sausage emulsion after enrichment in DF broth and MOPS-BLEB. Panel B, detection from hotdogs after enrichment in UVM broth and MOPS-BLEB.

4.10 For the testing of retail foods for *L. monocytogenes*

We tested the Amplifluor™ Uniprimer™ real-time PCR technique on a variety of retail foods including raw ground meats, RTE cold cuts, RTE hotdogs, cheese and salads for *L. monocytogenes* using both the Uniprimer™ PCR assay and the traditional USDA/FSIS method .The types of retail foods tested in this study and the number positive samples detected by both the methods are listed in Table 13. The Uniprimer™ PCR was performed on sample enrichment (pre-enrichment), after 20-22 hrs of primary enrichment, and after 18-20hrs of secondary enrichment. In our limited screening of food samples, only detected and isolated *L. monocytogenes* from raw ground meats and did not detect positive samples from any of the RTE products. The positive results from the PCR were also positive for typical *Listeria* colonies in MOX agar plates and were identified and confirmed by biochemical tests as *L. monocytogenes*. Other species of *Listeria* were positive on MOX agar and negative by PCR assay. These other species were not found to be *L. monocytogenes* after various biochemical and confirmatory tests that are not necessary with the real-time PCR assay. This is attributed to the species specificity of the primers used in this study for real-time PCR detection. So far our food sampling studies indicate that PCR results after ok were in accordance with the traditional culture method and did not show any false positive or false negative results. However, some pre-enrichment samples tested positive with the PCR reaction which later tested negative after primary and secondary enrichment. These samples also tested negative with the traditional method. The pre-enrichment samples may be showing a false positive result which might be due to the presence of DNA from the dead cells of *L. monocytogenes* in food and the DNA was degraded or lost by subsequent enrichment procedures and were

therefore also negative by the traditional plating method. This result were in agreement with our prior study of dead vs. live cells and supports our hypothesis that in order to avoid false positive result from dead cells, a primary and secondary selective enrichment of *L. monocytogenes* from the food is required prior to its detection using Uniprimer™ real-time PCR for routine microbiological analysis of food before its actual application in food industry.

Table 13. Detection of *L. monocytogenes* from raw and RTE meats by Uniprimer™ PCR.

Detection Method	Type of Sample (# of samples)	Manufacturer										Total Positive Samples	
												PCR	USDA
	Raw Meats(34)											5	5
	Ground beef(9)	A	B	C	D	E	F	G	H	I		3	3
Uniprimer™													
Pre-Enrichment		+	+	+	-	-	-	-	-	-			
1° Enrichment		-	+	+	-	-	-	-	-	-			
2°Enrichment		-	+	-	-	-	-	-	+	+			
USDA Method		-	+	-	-	-	-	-	+	+			
	Pork Sausages(10)	A	B	C	D	E	F	G	H	I	J	1	1
Uniprimer™													
Pre-Enrichment		-	+	-	-	-	-	-	-	-	-		
1° Enrichment		-	-	-	-	-	-	-	-	-	-		
2° Enrichment		-	-	-	-	-	+	-	-	-	-		
USDA Method		-	-	-	-	-	+	-	-	-	-		
	Ground Turkey (9)	A	B	C	D	E	F	G	H	I		1	1
Uniprimer™													
Pre-Enrichment		-	+	-	-	-	-	-	-	-			
1° Enrichment		-	+	-	-	-	-	-	-	-			
2° Enrichment		-	-	-	-	+	-	-	-	-			
USDA Method		-	-	-	-	+	-	-	-	-			
	Ground Pork (3)	A	B	C								0	0
Uniprimer™													
Pre-Enrichment		+	-	-									
1° Enrichment		+	-	-									
2° Enrichment		-	-	-									
USDA Method		-	-	-									
	Ground Chicken (3)	A	B	C								0	0
Uniprimer™													
Pre-Enrichment		+	-	-									
1° Enrichment		+	-	-									
2° Enrichment		-	-	-									
USDA Method		-	-	-									
	RTE Meats (10)											0	0
	Hotdogs	A	B	C	D	E	F	G	H	I	J		
Uniprimer™													
Pre-Enrichment		-	-	-	-	-	-	-	-	-	-		
1° Enrichment		-	-	-	-	-	-	-	-	-	-		
2° Enrichment		-	-	-	-	-	-	-	-	-	-		
USDA Method		-	-	-	-	-	-	-	-	-	-		
	RTE Salads (10)	A	B	C	D	E	F	G	H	I	J	0	0
Uniprimer™													
Pre-Enrichment		+	+	+	+	+	+	+	+	+	+		
1° Enrichment		-	-	-	-	-	-	-	-	-	-		
2° Enrichment		-	-	-	-	-	-	-	-	-	-		
USDA Method		-	-	-	-	-	-	-	-	-	-		

4.11 Improvement in fluorescence yield

Although the Uniprimer™ PCR assay was successful in its application for the detection *L. monocytogenes* strains from food, it has a limitation in its MDL of 10^5 cfu/ml. The MDL could be improved by the decreasing the current Ct (cycle 24) for fluorescence detection. This would help in detecting the amplification of the target earlier than cycle 24, whereby a larger number of samples with target cell load levels below 10^5 cfu/ml may be detected. We took the advantage of the universal applicability of the Uniprimer™ and adapted it for real-time amplification of 16S rRNA gene of *L. monocytogenes*. The PCR resulted in a 3-fold increase in the fluorescence yield over that of the *hly*A gene target (Fig. 36). The Ct was also reduced by 4 cycles at 0.1 RFU. The reason for the enhancement is likely the presence of 6 copies of 16S rRNA gene per chromosome in *L. monocytogenes* in comparison to a single copy of the *hly*A gene. We also attached the reverse primer for the *hly*A gene target onto the Uniprimer™ (Direct Amplifluor). This was compared to the indirect incorporation of the Uniprimer™ using a "Z tail" on both the reverse primer and the Uniprimer ™. With the direct attachment of the Uniprimer™, the Direct Amplifluor primer might be incorporating more efficiently and initiating exponential amplification sooner. The fluorescence yield was approximately double than the indirect Uniprimer™ (Fig. 36).However, unlike the 16S rRNA primers the *hly*A primers with attached Uniprimer™ did not decrease the threshold cycle significantly and therefore may need further optimization.

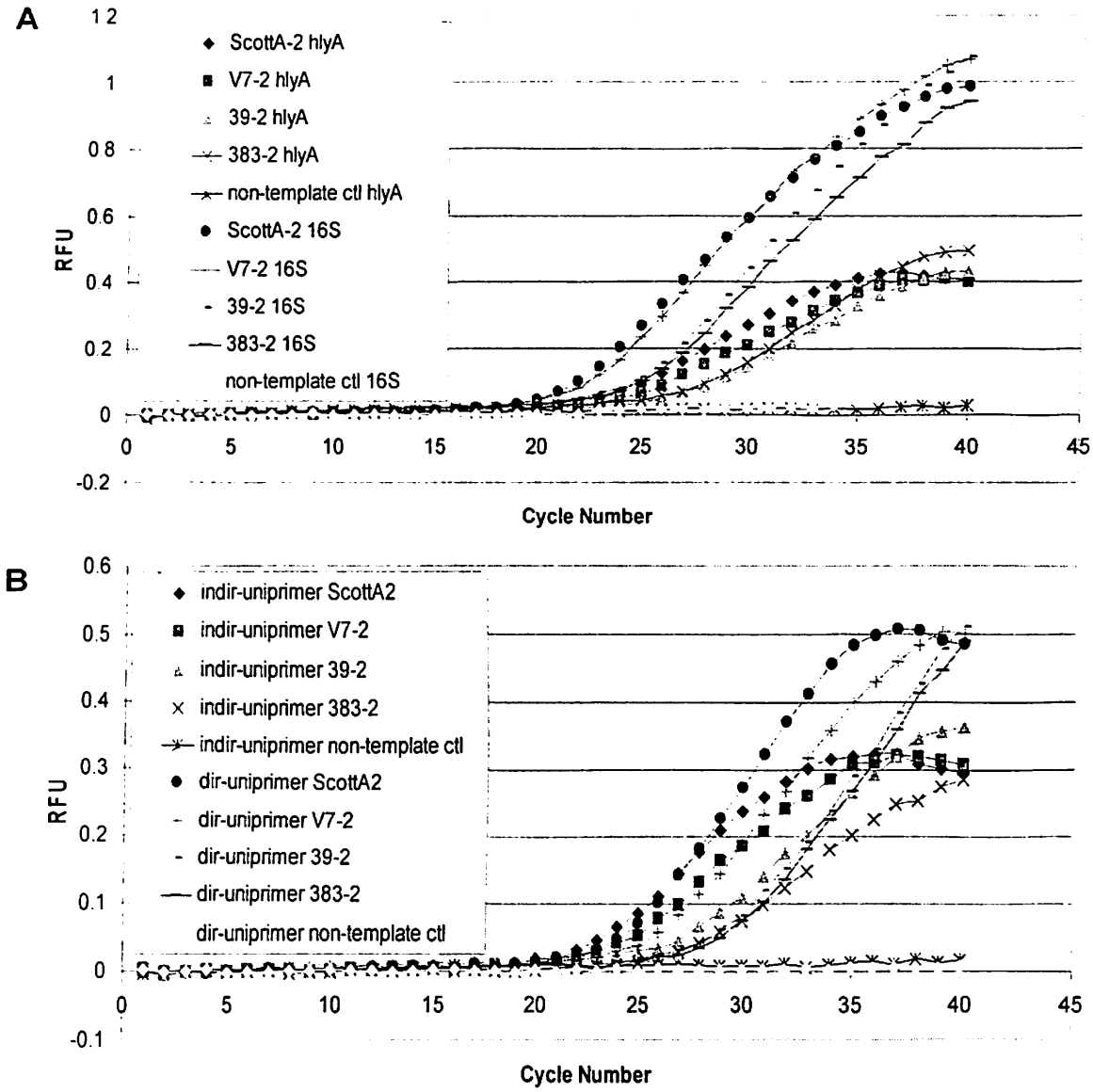

Figure 36. Primer modifications attempting to improve detection sensitivity. Panel A, primer sequences were made to target the 16S rRNA gene of *L. monocytogenes* in comparison to that targeting the *hly*A gene. Panel B, *hly*A reverse primer sequence was placed directly on the Uniprimer™ (direct Amplifluor) in comparison with the indirect method.

CHAPTER V

CONCLUSIONS AND FUTURE RESEARCH

In our study, we examined and applied the Amplifluor™ Uniprimer™ real-time PCR system for the detection of *L. monocytogenes* in raw and RTE meat products. In comparison to the traditional method of detection recommended by USDA/FSIS which is cumbersome and time consuming, our method was simple in operation and rapid which confirms the presence of *L. monocytogenes* in meats within 2 days, including enrichment, making it well suited for application in the food industry.

The Amplifluor™ Uniprimer™ real-time PCR detection enables the user to actually view the amplification of positive targets if present in the reaction by the exponential increase of fluorescence with increasing cycles of PCR, thereby gives an immediate positive or negative result and eliminates the need for post amplification manipulation like agarose gel electrophoresis or the end point fluorescence detection. Furthermore, the real-time PCR also opens an opportunity to quantify the initial number of target molecules if required rather than a qualitative analysis by end point PCR detection. In our study, we did not require the quantification of initial target cells since *L. monocytogenes* has a zero tolerance in RTE meats and the presence of a single cell in 25 g of meat product is a matter of concern.

One of major observations was the formation of non-specific products from the *Listeria* species other than the *L. monocytogenes* strains when the primer set-I was used for the study. We tested several different primers targeting *hly*A gene and finally the primer set-

II gave us a species-specific 110 bp *hly*A gene product for strains of *L. monocytogenes* used in our study. It is therefore highly essential to meticulously design and select target-specific primers for the Uniprimer™ PCR in order to avoid any false positive results during food sampling. Therefore before its application to food industries, the primers to be used should be validated for their specificity with various species of *Listeria* as well as detection of *L. monocytogenes* from a variety of food stuffs in comparison with current standard method.

The Amplifluor™ Uniprimer™ PCR system was optimized using crude lysates of *L. monocytogenes* strain ScottA-2. After optimization the Amplifluor™ Uniprimer™ PCR was applied for the detection of *L. monocytogenes* from the pure culture as well as the raw and RTE meat samples. We included a non-template control reaction so that the background fluorescence units can be subtracted from the positive samples; it also helps in identifying the undesirable fluorescence resulting from any contamination of the reagents by the target DNA or due to any primer-dimer formation.

The minimum detection level (MDL) for the Amplifluor™ real-time PCR was 10^5 cfu/ml. Primary and secondary selective enrichment of *L. monocytogenes* is required for Uniprimer™ PCR detection to be comparable to the detection limit specified by USDA/FSIS (10^0 cfu/25g of meat). The enrichment steps also eliminate any false positive results due to DNA from dead cells of the target organism that might be present in the food samples.

When artificially inoculated, in both raw and RTE meats we were able to detect *L. monocytogenes* (after enrichment) with inoculum levels as low as 1 cfu/25g of meat with

a maximum detection time of 2 days, including primary and secondary enrichments. When detecting *L. monocytogenes* from a non-sterile food matrix, neither the food matrix nor the indigenous bacteria present in the food interfered with Uniprimer™ PCR detection.

No false positive or false negative results were obtained using the Uniprimer™ PCR system to screen various retail foods for *L. monocytogenes*. A positive control as pure culture of *L. monocytogenes* and a non-template reaction control should always be included for such validations. Additionally, any suspected positive sample reported by the Uniprimer™ system should be cross-checked by traditional method for a final conclusive report.

Even though the Uniprimer™ real-time PCR system was efficient for its application in detection of *L. monocytogenes* from food samples, its MDL is 10^5 cfu/ml. The MDL can be improved/decreased by decreasing the threshold cycle (Ct) at a particular RFU. We observed that the Ct could be reduced by using purified DNA or by washing culture prior to PCR. But both of these steps would be cumbersome in actual food testing applications. Uniprimer™ PCR targeting the 16S rRNA gene reduced the Ct by 4 cycles and yielded a greater fluorescence at a lower Ct due to six copies of the 16S rRNA gene per *Listeria* genome. In addition, primers targeting the *hly*A gene with Uniprimer™ directly attached to the 5` end of one of the primers also increased the fluorescence at a lower Ct. Therefore; future studies remain for optimizing these primers in order to improve the MDL of the Uniprimer™ system for detection of pathogens from food stuffs.

Future studies should focus on reducing the time of detection by minimizing or eliminating the secondary enrichment steps and perhaps making the use of immunomagnetic beads which can specifically pull live *Listeria monocytogenes* cells out of primary enrichment culture. The implementation of this technique would reduce the detection time by 20-22 hrs.

The Amplifluor™ Uniprimer™ can be universally used for PCR amplification of different nucleic acid targets by simply modifying the target-specific primers and, therefore, if different fluorophores were used, the Amplifluor™ Uniprimer™ system could be used for multiplex PCR reactions for the detection of different foodborne pathogens.

REFERENCES:

Alvarez-Dominguez, C., C. R. Roberts, and P.D. Stahl. 1997. Internalized *Listeria monocytogenes* modulates intracellular trafficking and delays maturation of the phagosome. *J. Cell Sci.* 110: 731-743.

Autio, T., R. Keto-Timonen, J. Lunden, J. Bjorkroth, H. Korkeala. 2003. Characterization of persistent and sporadic *Listeria monocytogenes* strains by pulsed-field gel electrophoresis (PFGE) and amplified fragment length polymorphism (AFLP). *Syst Appl Microbiol.* 26(4): 539-45.

Bansal, N. S. 1996. Development of a polymerase chain reaction assay for the detection of *Listeria monocytogenes* in foods. *Lett Appl Microbiol.* 22(5): 353-356.

Bassler, H. A., S. J. A. Flood, K. J. Livak, J. Marmaro, R. Knorr, and C. A. Batt. 1995. Use of a fluorogenic probe in a PCR-based assay for the detection of *Listeria monocytogenes*. *Appl. Environ. Microbiol.* 61(10): 3724-3728.

Bessesen, M.T., Q. Luo, H. A. Rotbart, M.J. Blaser, and R.T. Ellison. 1990. Detection of *Listeria monocytogenes* by using the polymerase chain reaction. *Appl. Environ. Microbiol.* 56: 2930–2932.

Beumer, R.R., and W. C. Hazeleger. 2003. *Listeria monocytogenes* : diagnostic problems. *FEMS Immunol. Med Microbiol.* 35: 191-197.

Blais, B. W., and L. M. Phillippe. 1993. A simple RNA probe system for analysis of *Listeria monocytogenes* polymerase chain reaction products. *Appl. Environ. Microbiol.* 59(9): 2795-2800.

Bobbitt, J. A., and R. P. Betts. 1992. Confirmation of *Listeria monocytogenes* using a commercially available nucleic acid probe. *Food Microbiol.* 9: 311–317.

Border, P.M., J. J. Howard, G. S. Plastow, and K.W. Siggens. 1990. Detection of *Listeria* species and *Listeria monocytogenes* using polymerase chain reaction. *Lett. Appl. Microbiol.* 11: 158–162.

Bruce, J. 1996. Automated system rapidly identifies and characterizes microorganisms in food. *Food Technol.* 50: 77-81.

Byun, S. K., S. C. Jung and H. S. Yoo. 2001. Random amplification of polymorphic DNA typing of *Listeria monocytogenes* isolated from meat. *Int. J Food Microbiol.* 28(69): 227-35.

Caplin, B. E., R. P. Rasmussen, P. S. Bernara, and C.T. Wittwer. 1999. LightCycler™ hybridization probes, the most direct way to monitor PCR amplification for quantification and mutation detection. *Biochemica.* 1: 5-8.

Caugant, D. A., F. E. Ashton, W. F. Bibb, P. Boerlin, W. Donachie, C. Low, A. Gilmour, J. Harvey, and B. Norrung. 1996. Multilocus enzyme electrophoresis for characterization of *Listeria monocytogenes* isolates: results of an international comparative study. *Int. J. Food Microbiol.* 32: 301-311.

Chan, M., M. C. J. Maiden, and B. G. Spratt. 2001. Database-driven multilocus sequence typing (MLST) of bacterial pathogens. *Bioinformatics*. 17: 1077-1083.

Chenevert, J., J. Mengaud, E. Gormley, and P. Cossart, 1989. A DNA probe specific for *Listeria monocytogenes* in the genus *Listeria*. *Int. J. Food Microbiol.* 8: 317–319.

Datta, A. R., B. A. Wentz, D. Shook, and M. W. Trucksess. 1988. Synthetic oligodeoxyribonucleotide probes for detection of *Listeria monocytogenes*. *Appl. Environ. Microbiol.* 54: 2933–2937.

Datta, A.R., B. A. Wentz, and J. Russell, 1990. Cloning of the listeriolysin O gene and development of specific gene probes for *Listeria monocytogenes*. *Appl. Environ. Microbiol.* 56: 3874–3877.

Dauplin, G., C. Ragimbeau, and P. Malle. 2001. Use of PFGE typing for tracing contamination with *Listeria monocytogenes* in three cold-smoked salmon processing plants. *Int. J. Food Microbiol.* 64: 51-61.

De Cesare, A., J. L. Bruce, T. R. Dambaugh, M. E. Guerzoni, and M. Weidmann. 2001. Automated ribotyping using different enzymes to improve discrimination of *Listeria monocytogenes* isolates, with a particular focus on serotype 4b strains. *J. Clin. Microbiol.* 39(8): 3002-3005.

De Silva, D., M. Hermann, A. Reiser, K. Tabiti, and C. Wittwer. 1998. Rapid genotyping and quantification on the lightCycler™ with hybridization probes. *Biochemica*. 2: 12-15.

Duffy, G. and J. J. Sheridan 1997. The effect of temperature, pH and medium in a surface adhesion immunoflourescent technique for detection of *Listeria monocytogenes*. *J. Appl. Microbiol.* 83(1): 95-101.

Duffy, G., O. M. Cloak, and J. J. Sheridan. 1999. The development of a combined surface adhesion and PCR technique in the rapid detection of *Listeria monocytogenes* in meat and poultry. *Int. J. Food Microbiol.* 49(3): 151-159.

Enright, M. C., and B. G. Spratt, 1999. Multilocus sequence typing. *Trends Microbiol.* 7(12): 482-487.

Ermolaeva, S.A. 2001. Genetics mechanisms of virulence in *Listeria monocytogenes*. *Russ. J. Genet.* 37(3): 213-219.

Franciosa, G., M. Pourshaban, M. Gianfranceschi, and P. Aureli, 1998. Genetic typing of human and food isolates of *Listeria monocytogenes*. *Eur. J. Epidemiol.* 14: 205-210.

Frederiksen, B. and S. Samuelsson. 1992. Foeto-maternal listeriosis in Denmark 1981–1988. *J. Infect.* 24: 277–287

Feidler, F. and J. Sager. 1983. The murein types of *Listeria grayi*, *Listeria murrayi* and *Listeria denitrificans*. *Syst. Appl. Microbiol.* 4: 444-450.

Füzi, M., and I. Pillis, 1962. Production of opacity in egg-yolk medium by *Listeria monocytogenes*. *Nature* 13:195.

Gaillard, J.-L., P. Berche, C. Frehel, E. Gouin, and P. Cossart. 1991. Entry of *Listeria monocytogenes* into cells is mediated by internalin, a repeat protein reminiscent of surface antigens from Gram-positive cocci. *Cell* 65: 1127-1141.

Gangar, V., M. S. Curiale, A. D'Onorio, A. Schultz, R. L. Johnson, and V. Atrache. 2000. VIDAS enzyme-linked immunoflourescent assay for detection of *Listeria* in foods: collaborative study. *J AOAC Int.* 83(4): 903-18.

Gedde, M. M., D. E. Higgins, L. G. Tilney, and D. A. Portnoy. 2000. Role of listeriolysin O in cell-to-cell spread of *Listeria monocytogenes*. *Infect. Immun.* 68: 999-1003

Geoffroy, C., J.-L. Gaillard, J. E. Alouf, and P. Berche, 1987. Purification, characterization, and toxicity of the sulfhydryl-activated hemolysin listeriolysin O from *Listeria monocytogenes*. *Infect. Immun.* 55: 1641-1646

Giovannacci, I., C. Ragimbeau, S. Queguiner, G. Salvat, J.-L.Vendeuvre, V. Carlier, and G. Ermel. 1999. *Listeria monocytogenes* in pork slaughtering and cutting plants use of RAPD, PFGE and PCR-REA for tracing and molecular epidemiology. *Int. J. Food Microbiol.* 53 :127–140.

Goulet, V., C. Jacquet, V. Vaillant, I. Rebiere, E. Mouret, C. Lorente, E. Maillot, F. Stainer, and J. Rocourt. 1995. Listeriosis from consumption of raw-milk cheese. *Lancet* 345: 1581–1582.

Graves, L. M., and B. Swaminathan. 2001. PulseNet standardized protocol for subtyping *Listeria monocytogenes* by macrorestriction and pulsed-field gel electrophoresis. *Int. J. Food Microbiol.* 65: 55-62.

Gray, M. L., H. J. Stafseth, F. Thorp Jr., L. B. Sholl, and W. F. Riley, Jr. 1948. A new technique for isolating listerellae from the bovine brain. *J. Bacteriol.* 55: 471-476.

Gray, M.L., 1960. Isolation of *Listeria monocytogenes* from oat silage. *Science.* 132: 1767-1768.

Heid, C. A., J. Stevens, K. J. Livak, and P. M. Williams, 1996. Real-time quantitative PCR. *Genome Res.* 6: 986-994.

Hernandez, M., T. Esteve, S. Prat, and M. Pla. 2004. Development of real-time PCR systems based on SYBR® Green I, Amplifluor™ and TaqMan® technologies for specific quantitative detection of the transgenic maize event GA21. *J. Cereal. Sci.* 39(1): 99-107.

Higuchi, R., G. Dollinger, S. Walsh, and R. Griffith, 1992. Simultaneous amplification and detection of specific DNA sequences. *BioTechnology.* 10: 413-417.

Hof, H. 1984. Virulence of different strains of *Listeria monocytogenes* serovar 1/2a. *Med. Microbiol. Immunol.* 173:207-218.

Hough, A. J., S. A. Harbison, M. G. Savill, L. D. Melton, and G. Fletcher, 2002. Rapid enumeration of *Listeria monocytogenes* in artificially contaminated cabbage using real-time polymerase chain reaction. *J Food Prot.* 65(8): 1329-32

Ingianni, A., M. Floris, P. Palomba, M. A. Madeddu, M. Quartuccio, and R. Pompei. 2001. Rapid detection of *Listeria monocytogenes* in foods, by a combination of PCR and DNA probe. *Molecular and Cellular probes.* 15: 275-280.

Jacquet, C., B. Catimel, R. Brosch, C. Buchrieser, P.Dehaumont, V.Goulet, A. Lepoutre, P. Veit, and J. Rocourt, 1995. Investigations related to the epidemic strain involved in the French listeriosis outbreak in 1992. *Appl. Environ. Microbiol.* 61: 2242–2246.

Johansson, T., L. Rantala, L. Palmu, and T. Honkanen-Bulzalski. 1999. Occurrence and typing of *Listeria monocytogenes* strains in retail vacuum-packed fish products and in a production plant. *Int. J. Food Microbiol.* 47: 111–119.

Karunasagar, I., G. Krohne, and W. Goebel. 1993. *Listeria ivanovii* is capable of cell-to-cell spread involving actin polymerization. *Infect. Immun.* 61:162-169

Kathariou, S. 2000. Pathogenesis determinants of *Listeria monocytogenes*, p 295-314. In W. J. Cary (ed.), Microbial Foodborne diseases, mechanisms of pathogenesis and toxin synthesis. Tachnamic publishing, Lancester.

King, W., S. Raposa, J. Warshaw, A. Johnson, D. Halbert, and J. D. Klinger, 1989. A new colorimetric nucleic acid hybridization assay for *Listeria* in foods. *Int. J. Food Microbiol.* 8(3): 225-232.

Klinger, J. D., A. Johnson, D. Croan, P. Flynn, K. Whippie, M. Kimball, J. Lawrie, and M. Curiale. 1988. Comparative studies of nucleic acid hybridization assay for *Listeria* in foods. *J. Assoc. Off. Anal. Chem.* 71(3): 669-673.

Koo, K., and L-A. Jaykus. 2003. Detection of *Listeria monocytogenes* from a model food by fluorescence resonance energy transfer-based PCR with an asymmetric fluorogenic probe set. *Appl. Environ. Microbiol.* 69(2): 1082-1088.

Lovett. J. 1988. Isolation and identification of *Listeria monocytogenes* in dairy products. *J. Assoc. Off. Anal. Chem.* 71: 658-660.

M. J. Research Inc. 2002. Compatible chemistry, sample vessel and sealing options. In Operations Manual version 1.0, DNA Engine Opticon ™ 2 System for continuous fluorescence detection.

Mac Gowan, A. P.R., Marshall, R. J. and Reeves, D. S. 1989. Evaluation of API 20 STREP system for identifying *Listeria* species. *J. Clin. Pathol.* 42: 548-550.

MacDonald, T. T., and P. B. Carter 1980. Cell-mediated immunity to intestinal infection. *Infect. Immun.* 28:516-522.

Mafu, A. A., D. Roy, J. Goulet, and P. Magney. 1990. Attachment of *Listeria monocytogenes* to stainless steel, glass, polypropylene, and rubber surfaces after short contact time. *J. Food Prot.* 53:742–746.

Mahoney, J. O., and C. Hill. 2002. A real time PCR assay for the detection and quantification of *Mycobacterium avium* subsp. Paratuberculosis using SYBR Green and the light cycler™. *J. Microbiol. Methods.* 51: 282-293.

Maiden, M. C. J., J. A. Bygraves, E. Feil, G. Morelli, , J. E. Russell, R. Urwin, , Q. Zhang, J. Zhou, K. Zurth, D. A. Caugant, I. M. Feavers, , M. Achtman, and B.G Spratt. 1998. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. *Proc. Natl. Acad. Sci.* USA. 95: 3140-3145.

Marco, A. J., J. Altimira, N. Prats, S. Lopez, L. Dominguez, M. Domingo, and V. Briones. 1997. Penetration of *Listeria monocytogenes* in mice infected by the oral route. *Microb. Pathog.* 23: 255-263.

Margret, W., and H. P. R. Seeliger. 1988. *Listeria monocytogenes* infections-therauptic possibilities and problems. *Infection.* 16(2): 175-177.

Martinez, I., L-M. Rorvik, V. Brox, J. Lassen, M. Seppola, L. Gram, and B. Fonnesbech-Vogel. 2003. Genetic variability among isolates of *Listeria monocytogenes* from food products, clinical samples and processing environments, estimated by RAPD typing. *Int. J. Food Microbiol.* 84(3): 285-297.

Mattingly, J. A., B. T. Butman, M. C. Plank, R. J. Durham, and B. J. Robinson. 1988. Rapid monoclonal antibody-based enzyme-linked immunosorbent assay for detection of *Listeria* in food products. *J. Assoc. Off. Anal. Chem.* 71(3): 679-681.

Mckie, A., D. Samuel, B. Cohen, and N. A. Saunders. 2002. Development of a quantitative immuno-PCR assay and its use to detect mumps-specific IgG in serum. *J. Immunol. Methods.* 261: 167-175.

McKillip, J. L., and M. Drake. 2000. Molecular beacon polymerase chain reaction detection of *Escherichia coli* O157:H7 in milk. *J. Food Prot.* 63: 855-859.

Medici, D. D., L. Croci, E. Delibato, S. D. Pasquale, E. Filetici, and L. Toti. 2003. Evaluation of DNA extraction methods for use in combination with SYBR Green I Real-time PCR to detect *Salmonella enterica* serotype Enteritidis in poultry. *Appl. Environ. Microbiol.* 69(6): 3456-3461.

Mienttinen, M.K., and P. A. D. Grimont. 1993. Factors affecting reproducibility of random amplified polymorphic DNA fingerprinting. *Res. Microbiol.* 144: 377-379.

Miettinen M. K., A. Siitonen, P. Heiskanen, H. Haajanen, K. J. Bjorkroth, H. J. Korkeala. 1999 Molecular epidemiology of an outbreak of febrile gastroenteritis caused by *Listeria monocytogenes* in cold-smoked rainbow trout. J Clin Microbiol. 37(7): 2358-60.

Morrison, T. B., J. J. Weis, and C.T. Wittwer. 1998. Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification. *Biotechniques.* 24(6): 954-962.

Murray, E.G.D., R. A. Webb, and M. B. R. Swann. 1924. A disease of rabbits characterized by a large mononuclear leucocytosis caused by a hitherto undescribed bacillus *Bacterium monocytogenes* (n.sp.). *J. Pathol. Bacteriol.* 29: 407-439.

Nazarenko, I. A., S. K. Bhatnagar, and R. J. Hohman. 1997. A closed tube format for amplification and detection of DNA based on energy transfer. *Nucl. Acid. Res.* 25:2516-2521.

Nogva, H. K., K. Rudi, K. Naterstad, A. Holck, and D. Lillehaug. 2000. Application of a 5'-nuclease PCR for quantitative detection of *Listeria monocytogenes* in pure cultures, water, skim milk, and unpasteurized whole milk. *Appl. Environ. Microbiol.* 66:4266-4271.

Norton, D. M. 2002. Polymerase chain reaction –based methods for detection of *Listeria monocytogenes*: toward real-time Screening for food and environmental samples. *J. Assoc. Off. Anal. Chem.* 85(2): 505-515.

Norton, D., and C. A. Batt. 1999. Detection of viable *Listeria monocytogenes* with a 5' nuclease PCR assay. *Appl. Environ. Microbiol.* 65: 2122-2127.

Okwumabua, O., B. Swaminathan, P. Edmonds, J. Wenger, J. Hogan, and M. Alden, 1992. Evaluation of a chemiluminiscent DNA probe assay for the rapid confirmation of *Listeria monocytogenes*. *Res Microbiol.* 143(2): 183-189.

Olsen, J. E., S. Aabo, W. Hill, S. Notermans, K. Wernars, P. E. Granum, T. Popovic, H. N. Rasmussen, and O. Olsvik. 1995. Probes and polymerase chain reaction for detection of foodborne bacterial pathogens. *Int. J. Food Microbiol.* 28: 1-78.

Pirie, J.H.H. 1940. *Listeria*: Change of a name for a genus of a bacterium. *Nature*. 145: 264.

Racz,P., K. Tenner, and E. Mero. 1972. Experimental *Listeria* enteritis I. An electron microscopic study of the epithelial phase in experimental *Listeria* infection. *Lab. Investig.* .26: 694-700.

Rasmussen, R., T. Morisson, M. Hermann, and C. Wittwer. 1998. Quantitative PCR by continuous fluorescence monitoring of a double strand DNA specific binding dye. *Biochemica*. 2: 8-11.

Revazishvili, T., M. Kotetishvili, O. Colin Stine, A. S. Kreger, , J. Glenn Morris Jr., and A. Sulakvelidze. 2004. Comparative analysis of Multilocus Sequence typing and Pulsed-field gel electrophoresis for characterizing *Listeria monocytogenes* strains isolated from environmental and clinical sources. *J. Clin. Microbol.* 42(1): 276-285.

Rocourt, J., Ch. Jacquet, and A. Reilly. 2000. Epidemiology of human listeriosis and sea foods. *Int. J. Food Microbiol.* 62(3): 197-209.

Rodríguez-Lázaro, D ., M. Hernández, M. Scortti, T. Esteve, J. A. Vázquez-Boland, and M. Pla. 2004. Quantitative Detection of *Listeria monocytogenes* and *Listeria innocua* by Real-Time PCR: Assessment of *hly*, *iap*, and *lin02483* Targets and Amplifluor Technology. *Appl. Environ. Microbiol.* 70 (3): 1366-1377.

Ryan, M., T. O. Zaikova, J. F. W. Keana, H. Goldfine, and O. H. Griffith. 2002. *Listeria monocytogenes* phosphatidylinositol-specific phospholipase C: activation and allostery. *Biophysical Chemistry*. 101-102: 347-348.

Ryser, E. T., and E. H. Marth. 1991. *Listeria*, listeriosis and food safety, Marcel Dekker Inc, NY.

Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science*. 238 (4839): 487-91.

Sails, A. D., A. J. Fox, F. J. Bolton, D. R. A. Wareing and D. L. A. Greenway. 2003. A real-time PCR assay for the detection of *Campylobacter jejuni* in foods after enrichment culture. *Appl. Environ. Microbiol.* 69(3): 1383-1390.

Salcedo, C., L. Arreaza, B. Alcaca, L. De la Fuente, and J. A. Vazquez. 2003. Development of a multilocus sequence typing method for analysis of *Listeria monocytogenes* clones. *J. Clin. Microbiol.* 41(2): 757-762.

Sawyer, J., C. Wood, D. Shanahan, S. Gout, and D. McDowell. 2003. Real-time PCR for quantitative meat species testing. *Food Control.* 14: 579-583.

Scheu, P., A. Gasch, and K. Berghof. 1999. Rapid detection of *Listeria monocytogenes* by PCR-ELISA. *Lett. Appl. Microbiol* . 29: 416-420.

Schlech, W. F. III. 2000. Food borne listeriosis. *CID.* 31: 770-775.

Schuchat, A., B. Swaminathan, and C. V. Broome. 1991. Epidemiology of human listeriosis. *Clin. Microbiol. Rev.* 4:169-183

Schwartz, D. C., and R. C. Cantor, 1984. Seperation of yeast chromosome-sized DNAs by pulsed field gel electrophoresis. *Cell.* 37: 67-75.

Seeliger, H. P. R., and H. Finger, 1976. Listeriosis, p. 333- 365. In J.S.Remington and J.O.Klein (eds.), Infectious diseases of the fetus and newborn infant. W. B. Saunders Co., Philadalphia.

Seeliger, H. P. R., and D. Jones. 1993. *Listeria*, p. 1235-1245. In J.G.Holt (ed.), Bergey's Manual of Systematic Bacteriology, 9th ed. Williams and Wilkins, Baltimore.

Seeliger, H. P. R. 1961. Listeriosis in human, Listeriosis. Hafner Publishing Co., NY.

Seeliger, H. P. R., and K. Hohne. 1979. Serotyping of *Listeria monocytogenes* and related species. *Methods Microbiol.* 13:31-49.

Selander, R.K., A.D.Caugant, H. Ochman, M. J. Musser, N. M. Gilmour, and T. S. Whittam. 1986. Methods of multilocus. Enzyme electrophoresis for bacterial population's genetics and systamatics. *Appl. Environ. Microbiol.* 51: 31-49.

Senczek, D., R. Stephan, and F. Utermann. 2000. Pulsed-field gel electrophoresis (PFGE) typing of *Listeria* strains isolated from a meat processing plant over a 2-year period. *Int. J. Food Microbiol.* 62: 155-159.

Sewell, A. M., W. D. Warburton, A. Boville, F. E. Daley, and K. Mullen. 2003. The development of an efficient and rapid enzyme linked fluorescent assay method for the detection of *Listeria* spp. from foods. *Int. J. Food Microbiol.* 81(2): 123-129.

Sheridan, J. J., G. Duffy, D. A. McDowell, I. S. Blair. 1997. Development of a surface adhesion immunofluorescent technique for rapid isolation of *Listeria monocytogenes* and *Listeria innocua* from meat. J. Appl. Bacteriol. 82: 225- 232.

Skogberg, K., J. Syrjanen, M. Jahkola, O.V. Renkonen, J. Paavonen, J. Ahonen, S. Kontiainen, P. Ruutu, and V. Valtonen. 1992. Clinical presentation and outcome of listeriosis in patients with and without immunosuppressive therapy. *Clin. Infect. Dis.* 14: 815–821.

Solve, M., J. Boel, and B. Norrung. 2000. Evaluation of a monoclonal antibody able to detect live *Listeria monocytogenes* and *Listeria innocua*. *Int. J. Food Microbiol.* 57: 219-224.

Stuart, M. R., and H. J. Welshimer. 1974. Taxonomy reexamination of *Listeria* and transfer *Listeria grayi* and *Listeria murrayi* to a new genus, *Murraya*. *Int. J. System. Bacteriol.* 24: 177-185

Tang, J., D. Cleland, and D. Emerson. 2003. Characterization of bacteria by ribotyping. *ATCC connection*. 23(1): 1-11.

Tyagi, S., and F. R. Kramer. 1996. Molecular beacons: probes that fluorescence upon hybridization. *Nat. Biotechnol.* 14: 303-308.

Uehara, H., G. Nardone, S. Randall, Y. Khirpin, and D. St.Louis, 2000. Rapid and sensitive closed-tube quantification of human interferon-γ mRNA by reverse transcription-PCR utilizing energy-transfer labeled primers. *Clin Chem.* 46(9): 1500-1501.

USDA/FSIS. 1998. Isolation and identification of *Listeria monocytogenes* from Red Meat, Poultry, Egg and Environmental Samples. Microbiology laboratory guidebook. Chapter 8.00. Available at: http://www.fsis.usda.gov/ophs/microlab/mlgbook.htm.

USDA/FSIS. 2002. Isolation and identification of *Listeria monocytogenes* from Red Meat, Poultry, Egg and Environmental Samples. Microbiology laboratory guidebook. Chapter 8.00. Available at: http://www.fsis.usda.gov/ophs/microlab/mlg8.03.pdf

Vazquez-Boland, J. A., G. Dominguez-Bernal, B. Gonzalez-Zorn, J. Kreft, and W. Goebel. 2001b. Pathogenicity islands and virulence evolution in *Listeria. Microb. Infect.* 3: 571-584.

Vázquez-Boland, J. A., M. Kuhn, P. Berche, T. Chakraborty, G. Domínguez-Bernal, W. Goebel, B. González-Zorn, , J. Wehland, and J. Kreft. 2001a. *Listeria* Pathogenesis and Molecular Virulence Determinants. *Clin. Microbiol. Rev.*14 (3): 584-640.

Vogel, B. F., L.V Jorgensen, B. Ojeniyi, H. H. Huss, and L. Gram. 2001. Diversity of *Listeria monocytogenes* isolates from cold-smoked salmon produced in different

smokehouses as assessed by random amplified polymorphic DNA analyses. *Int. J. Food Microbiol.* 65: 83-92.

Wagner, M., and F. Allerberger. 2003. Characterization of *Listeria monocytogenes* recovered from 41 cases of sporadic listeriosis in Austria by serotyping and pulsed-field gel electrophoresis. *FEMS Immun. Med Microbiol.* 35: 227-234.

Weaver, R. H. 1954. Quicker bacteriological results. *Am. J. Med. Technol.* 20: 14-26.

Weishimer, H. J. 1966. Isolation of *Listeria monocytogenes* from vegetation. *J. Bacteriol.* 95: 300.

Whitcombe, D., J. Theaker, S. P. Guy, T. Brown, and S. Little. 1999. Detection of PCR products using self-probing amplicons and fluorescence. *Nat. Biotechnol.* 17: 804-807.

Zhang, W., B. M. Jayarao, and S. J. Knabel. 2004. Multi-virulence-locus sequence typing of *Listeria monocytogenes*. *Appl. Environ. Microbiol.* 70(2): 913-920.

Zhang, Y., D. Zhang, W. Li, J. Chen, Y. Peng, and W. Cao. 2003. A novel real-time quantitative PCR method using attached universal template probe. *Nucl. Acid. Res.* 31(20): 1-8.

APPENDIX

Differentiation of various food isolates of *L. monocytogenes* by Multilocus Sequence Typing.

Differentiation of various food isolates of *L. monocytogenes* by Multilocus Sequence Typing

Introduction

Bacteria of the genus *Listeria* are ubiquitous in the environment. Food borne strains of *Listeria monocytogenes* have been the causative agents of several outbreaks of human listeriosis. Amongst others, the main products associated with listeriosis are ready-to-eat meat products, typically those with a long shelf life at refrigerated temperatures. (Jacquet et al. 1995). Cross contaminations which can occur within the environment or on equipment of processing facilities are considered to be possible sources. *L. monocytogenes* is able to attach itself and survive on various working contact surfaces (Mafu et al. 1990). In spite of cleaning and disinfection procedures, the persistence of *Listeria* species over a long period in the environment of food processing plants has been reported (Giovannacci et al. 1999, Johansson et al. 1999). Because of these characteristics of *L. monocytogenes*, and despite the "zero-tolerance" policy by the USDA, several major food borne listeriosis outbreaks and multimillion dollars food recalls have occurred due to contaminated food, especially with RTE meats. In addition, global food trade may spread infected foods worldwide within a few days making it more difficult to trace the source of infection. Thus improving the ability to identify outbreak-causing strains rapidly, and tracing them back to pinpoint the source of contamination, is crucial for preventing recurrent outbreaks and addressing many of the epidemiological, clinical and legal issues associated with listeriosis outbreaks. Several genotypic and phenotypic typing methods have been used to subtype *L. monocytogenes*. These methods differ in their discriminatory power and reproducibility. In general, molecular typing methods

such as ribotyping and pulsed-field gel electrophoresis (PFGE) are considered to have better discriminatory power than phenotypic methods and are well suited for investigating *L. monocytogenes* outbreaks. Although these methods provide better strain differentiation, their discriminatory abilities are optimal and sometimes they cannot differentiate epidemiologically unrelated strains. In comparison, the Multilocus Sequence Typing (MLST) is an automated DNA sequencing method that discriminates strains based on the nucleotide sequence of their slowly diversified genes (Salcedo et al. 2003). This method is highly discriminatory, and provides results that can be easily shared and compared by different laboratories via the internet.

Our objective is to develop a MLST method based on virulence genes of *L. monocytogenes* for the subtyping of various food isolates of *L. monocytogenes.*

Materials and methods

Bacterial cultures:

Food isolates of *L. monocytogenes* obtained by our Uniprimer™ real-time PCR detection method, as well as other food isolates, were used for multilocus sequence typing (MLST). A total of 16 isolates were examined in this study. Aliqouts (100 ul) of the frozen concentrated culture of these isolates were inoculated into 10 ml of sterile BHI broth and incubated overnight at 30°C. These overnight grown cultures of *L. monocytogenes* were used to obtain a crude lysate for PCR amplification of specific genetic loci.

Multilocus Sequence Typing (MLST):

Three genetic loci were selected for MLST analysis, including a region encoding the hemolysin/listeriolysin O protein (*hly*A), another encoding the surface protein internalinA (*inl*A), and a third loci encoding for the positive regulatory factor for virulence (*prf*A).

Primers for these 3 genetic loci (Table 14) were designed by the Vector NTI Suite 9.0 primer analysis program. The primers were then used for PCR amplification and sequencing.

Table 14. List of primers used for the MLST.

Primer	Target Gene	Sequence (5' → 3')	Product Size (bp)
Primer I	Hemolysin A (*hly*A)		
	Forward	TGA ACC TAC AAG ACC TTC CA	560
	Reverse	CAA TTT CGT TAC CTT CAG GA	
Primer II	Internalin A (*inl*A)		
	Forward	GCT TCA GGC GGA TAG ATT AG	575
	Reverse	AAC TCG CCA ATG TGC C	
Primer III	Positive Regulatory Factor (*prf*A)		
	Forward	ATT TTT AAC CAA TGG GAT CC	590
	Reverse	CAT TCA TCT AAT TTA GGG GC	

The bacterial template for PCR was obtained by using a crude lysate (containing the chromosomal DNA) of *L. monocytogenes* isolates. The Protease and lysis solution for the BAX^{Tm} kit for *L. monocytogenes* (DuPont Qualicon, Wilmington, DE) was used to prepare the crude lysate. A 5 ul volume of the overnight grown culture of *L. monocytogenes* isolates were taken and mixed with 200 ul of BAX ^{TM} lysis reagent (1.0 ml of BAX^{Tm} lysis buffer + 12.5 ul of BAX ^{Tm} protease) and the lysis was performed by incubating at 55°C for 60 min and 95°C for 10 min followed by a cooling period of 5 min at 4°C. A 5ul aliquot of the lysate was then used separately for PCR amplification of the three genetic loci mentioned above. A 25 ul PCR reaction system was composed as follows: 7.7 ul of sterile water, 2.5 ul of dNTP mix (final concentration 0.25 mM each), 2.5ul of 5 uM forward primers (0.5 uM final concentration), 2.5 ul of 5 uM reverse

primer (final concentration 0.5 uM), 1.8 ul of 25 mM $MgCl_2$ (final concentration 1.8 mM), 2.5 ul of 10 X PCR buffer B, 0.5 ul of 5 units/ul Taq Polymerase B (final concentration 2.5 units/ rxn) and 5 ul of the *L. monocytogenes* lysate solution.

A single PCR program for the amplification of the three virulence genes was used as follows: Initial denaturation at 95°C for 4 min followed by 40 cycles of 95°C for 15 sec, 51°C for 18 sec and 72°C for 40 sec, a final extension at 72°C for 4 min and then continuous holding at 4°C. The amplicons were then purified with the Montage PCR cleaning kit (Millipore, Billerica, MA), examined by gel electrophoresis for quantitative analysis, and then sequenced in both forward and reverse direction by the Department of Biochemistry core facility (Oklahoma State University, Stillwater, OK).

Data Analysis

The forward and reverse sequence of each gene amplicon were compared using the NCBI BLAST 2 sequence program to determine the correct base sequence incase of a discripency between them. The amplicons with ≤ 97% match between forward and reverse sequence were rejected and re-sequenced.

For each strain of *L. monocytogenes*, the PCR amplified sequences of the three genes were artificially joined by the "Construct DNA/RNA Molecule" tool of Vector NTI Suite 9.0 software to form an artificial composite gene. These composite genes for different strains were then compared by multiple sequence alignment and finally clustal analysis was performed. The AlignX tool of Vector NTI Suite 9.0 software was used to construct the neighbor-joining phylogenic tree of *L. monocytogenes* isolates which group these isolates based on their degree of divergence.

The MLST involves simultaneous sequence-based analysis of several genes and differentiation based on their genetic homology (Enright and Spratt, 1999). It can effectively distinguish strains with high degrees of homology within the compared gene sequences (Fig. 37). In our study, the method could differentiate the 16 food isolates into 9 groups (Fig. 38). We also observed that with the increase in the number of genes for comparison, the diversification among the strains also increased. Although some of the strains within each group were indistinguishable by MLST in terms of their degree of divergence, this does not necessarily indicate that these are the same isolates. It might be possible that a greater number of genetic loci or perhaps loci with a greater degree of sequence heterogeneity between different strains can differentiate between them. Based on these observations, future work would include the addition of more virulence genes such as *inl*B encoding for inlB surface protein and *Act*A gene encoding for ActA filamentous protein since these virulence genes may provide more sensitive discrimination for clustal analysis.

This technique is user friendly and not as laborious as pulsed-field gel electrophoresis (PFGE), or as expensive as ribotyping, it provides an ideal balance between sequence-based resolution and technical feasibility. Furthermore, future research may also include the application of this technique in distinguishing strains collected from different meat processing plants in order to identify sources of contamination and comparison the sensitivity and discriminatory power of this technique with ribotyping and PFGE.

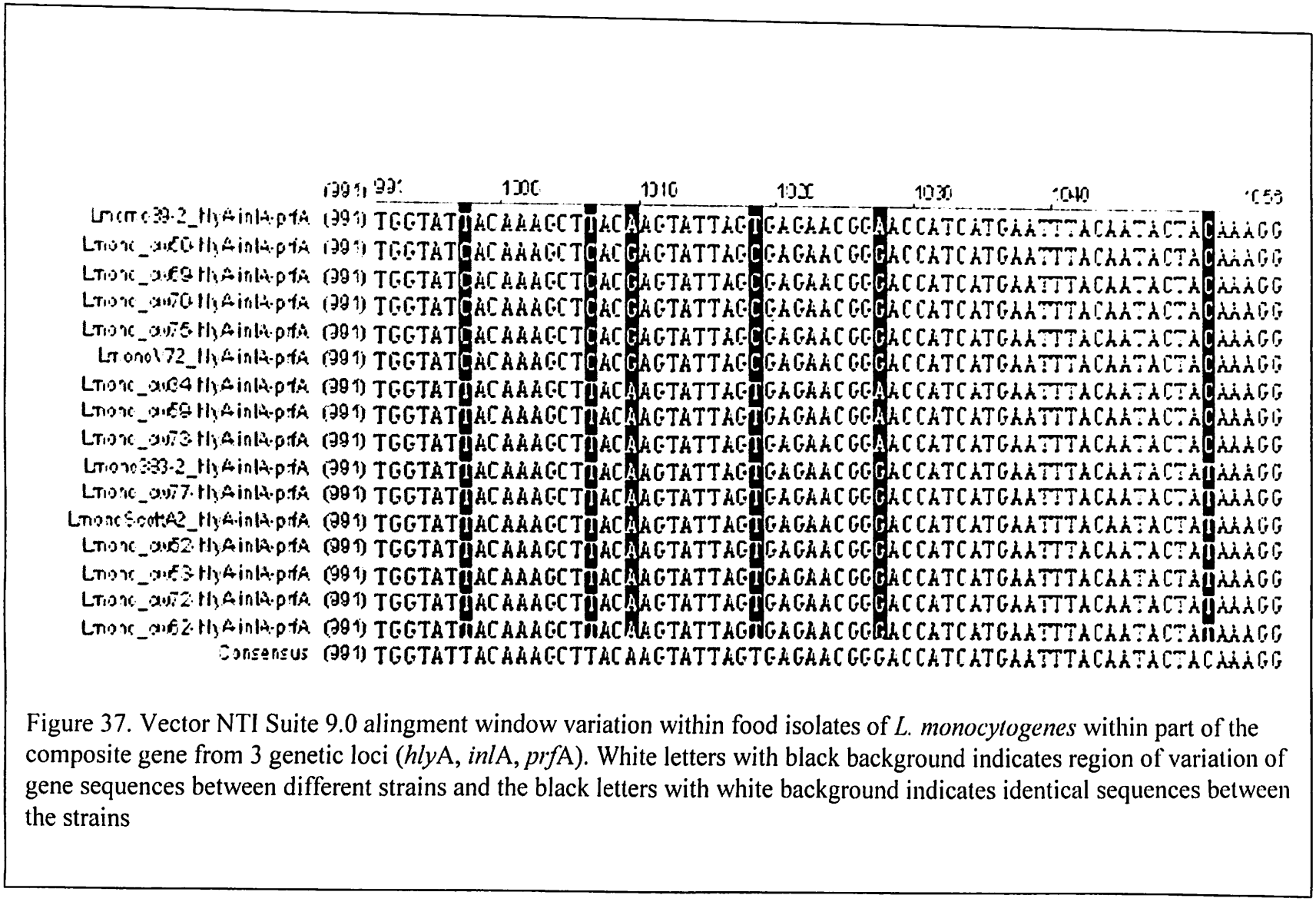

Figure 37. Vector NTI Suite 9.0 alingment window variation within food isolates of *L. monocytogenes* within part of the composite gene from 3 genetic loci (*hly*A, *inl*A, *prf*A). White letters with black background indicates region of variation of gene sequences between different strains and the black letters with white background indicates identical sequences between the strains

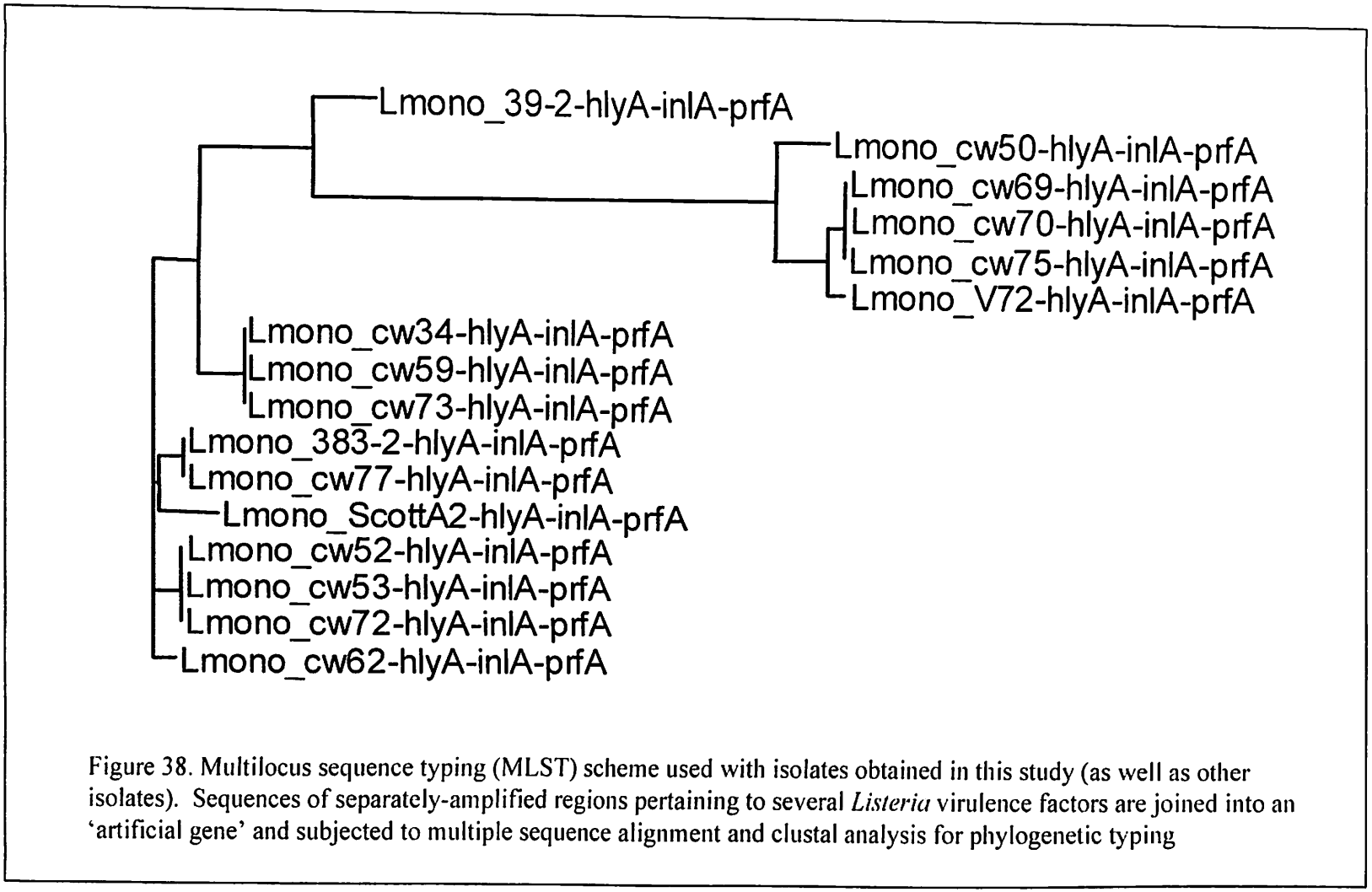

Figure 38. Multilocus sequence typing (MLST) scheme used with isolates obtained in this study (as well as other isolates). Sequences of separately-amplified regions pertaining to several *Listeria* virulence factors are joined into an 'artificial gene' and subjected to multiple sequence alignment and clustal analysis for phylogenetic typing

Reference:

Enright, M. C., and B. G Spratt.1999. Multilocus sequence typing. *Trends Microbiol.* 7(12): 482-487.

Giovannacci, I., C. Ragimbeau, S. Queguiner, G. Salvat, J.-L. Vendeuvre, V. Carlier, and G. Ermel. 1999. *Listeria monocytogenes* in pork slaughtering and cutting plants use of RAPD, PFGE and PCR-REA for tracing and molecular epidemiology. *Int. J. Food Microbiol.* 53: 127-131.

Jacquet, C., B. Catimel, R. Brosch, C. Buchrieser, P. Dehaumont, V. Goulet, A. Lepoutre, P. Veit, and J. Rocourt. 1995. Investigations related to the epidemic strain involved in the French listeriosis outbreak in 1992. *Appl. Environ. Microbiol.* 61: 2242–2246.

Johansson, T., L. Rantala, L. Palmu, and T. Honkanen-Bulzalski. 1999. Occurrence and typing of *Listeria monocytogenes* strains in retail vacuum-packed fish products and in a production plant. *Int. J. Food Microbiol.* 47: 111–119.

Mafu, A. A., D. Roy, J. Goulet, and P. Magney. 1990. Attachment of *Listeria monocytogenes* to stainless steel, glass, polypropylene, and rubber surfaces after short contact time. *J. Food Prot.* 53: 742-746.

Salcedo, C., L. Arreaza, B. Alcaca, L. De la. Fuente, and J. A. Vazquez. 2003. Development of a multilocus sequence typing method for analysis of *Listeria monocytogenes* clones. *J. Clin. Microbiol.* 41(2): 757-762.

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