CHAPTER 2 THEORY AND LITERATURE REVIEW

This chapter offers the theory and literature review related to this study including morphology, taxonomy of the genus *Listeria*, listeriosis outbreaks, symptoms of listeriosis, detection methods, selective enrichment media and selective agents for the growth and selectivity of *Listeria* spp. in this research.

2.1 *Listeria* spp.

Listeria is small, non–acid-fast, noncapsulated, nonsporulating, β-hemolytic, aerobic, and facultative anaerobic short Gram-positive rod bacteria. They are catalase positive, oxidase negative and ferment carbohydrates. *Listeria* species are motile by peritrichous flagella when grown at temperature less than 30°C and displayed a characteristic "tumbling" motility. They are present worldwide in the environment such as soil, decaying vegetable, food and feed and in the gut of humans, nonhuman mammals, birds, arachnids, and crustaceans (Bush and Perez, 2009; Holt et al., 1994).

2.2 History

Listeria monocytogenes was first described in 1923. Murray (1926) isolated an organism from a disease in his stock rabbits in Cambridge. He called the organism *Bacterium monocytogenes.* Pirie (1927) isolated an organism similar to Murray's. He proposed the name *Listerella (Listeria) hepatolytica,* but in view of the blood response to the organism, which was not observed until after Murray's description appeared, he considers *"monocytogenes"* more suitable as a specific name. *L. monocytogenes* has been recognized as a human pathogen since 1929 (Nyfeldt, 1929), but the route of transmission was unclear until the 1980s when a series of outbreaks indicated that *L. monocytogenes* was transmitted by food (Bille, 1990; Fleming et al., 1985, Goulet et al., 1995; Ho et al., 1986; Linnan et al., 1988; Riedo et al., 1994; Schlech et al., 1983). And

also, this food borne pathogen was widely accepted as food borne association in 1981 (Harris, 2006).

2.3 Listeriosis Outbreaks

Listeriosis is an infection caused by *L. monocytogenes* bacteria. People get infected with *Listeria* by eating food that has been contaminated with the bacteria. Foods that are more likely to be contaminated with *Listeria* are unpasteurized (raw) milk or foods made with unpasteurized milk, hot dogs and deli meats, refrigerated paté, and refrigerated smoked seafood. Human disease caused by *L. monocytogenes* occurs most frequently in women of childbearing age, infants, and the elderly (Figure 2.1). The risk of listeriosis is greatest among certain well defined high-risk groups, including pregnant women, neonates, and immunocompromised adults but may occasionally occur in persons who have no predisposing underlying condition. Persons with cancer, diabetes, AIDS, and liver or kidney disease are often predisposed to severe infection and death after infection with *L. monocytogenes* (Gasanov et al., 2005).

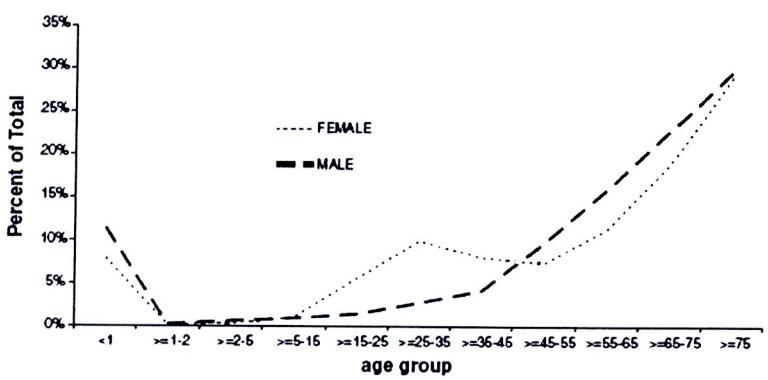


Figure 2.1 Percent of reported laboratory-confirmed listeriosis within age groups for males and females, United States, 2000 to 2004. Isolation of *L. monocytogenes* reported to CDC through the Public Health Laboratory Information System (PHLIS).
(Source: Gasanov et al., 2005)

Scallan et al. (2011) estimated that each year roughly 1 in 6 Americans (or 48 million people) gets sick, 128,000 were hospitalized and 3,000 dies of food borne diseases. All of these numbers, estimated hospitalization caused from listeriosis was about 1,520 cases. And 16 % of hospitalizes cases resulted in death.

In England and Wales the epidemiology of listeriosis had increased from 2.1 cases/million population during 1990–2000 to 3.6 cases/million population during 2001–2009, and more cases have been found in persons more than 60 years of age who had bacteremia (but not meningitis) (Gillespie et al., 2006).

The annual incidence of listeriosis in France in 2004 increased from3.5 cases/ million persons (in 2001-2005) to 4.7 cases/ million persons. In 2007, 159 cases were reported from January through June, which corresponded to an estimated incidence of 5.6 cases/million persons. For the 6-month period from January through June, the incidence of listeriosis increased by 46% in 2006 and 2007 compared with incidence during 2001–2005. The increased incidence of listeriosis in 2006–2007 over that of 2001–2005 was mainly due to a rise in cases in persons more than 60 years of age and was most pronounced in those more than 75 years of age (Report of the French sanitary agencies, 2011).

CBC News (2008) reported that the 2008 Canadian listeriosis outbreak was a widespread outbreak of listeriosis in Canada linked to a Maple Leaf Foods plant in Toronto, Ontario owing to inspectors failed to detect *Listeria*. There were 57 total confirmed cases and 23comfirmed died. Maple Leaf Foods had instated a voluntary recall before the outbreak was linked to their plant. The recall reportedly cost the company 20 million dollars.

2.4 Symptoms of Listeriosis

Symptoms of literiosis are including high fever, severe headache, neck stiffness, tiredness and nausea. The incubation period (time between ingestion and the onset of symptoms) for *Listeria* ranges from 3 to 70 days and averages 21 days (Bryan, 1999). If infection spreads to the nervous system, symptoms such as headache, stiff neck, confusion, loss of balance, or convulsions can occur. In immune-deficient individuals, *Listeria* can invade the central nervous system, causing meningitis and/or encephalitis (brain infection). Infected pregnant women ordinarily experience only a mild, flu-like illness; however, infection during pregnancy can lead to miscarriage, infection of the newborn or even stillbirth.

Newborns may present clinically with early-onset (less than 7 days) or late-onset forms of infection (7 or more days) (Bortolussi, 2008). Those with the early-onset form are often diagnosed in the first 24 h. of life with sepsis (infection in the blood). Early-onset listeriosis is most often acquired from the mother through transplacental transmission. Late-onset neonatal listeriosis is less common than the early-onset form. Clinical symptoms may be subtle and include irritability, fever and poor feeding. The mode of acquisition of late-onset listeriosis is poorly understood.

2.5 Taxonomy of the Genus *Listeria*

On the basis of morphological resemblances, *Listeria* was classified with *Lactobacillus*, *Erysipelothrix*, *Brochothrix*, *Renibacterium*, *Kurthia* and *Caryophanon* in the section of "regular, nonsporing, Gram-positive rods" in *Bergey's Manual of Systematic Bacteriology* (Bergey's manual of systematic bacteriology, 1986). However, with the successive introduction and development of numerical taxonomy, chemotaxonomy, DNA/DNA hybridization, and more recently, rRNA (ribosomal RNA) and DNA

sequencing, the phylogenetic position of *Listeria* has been more clearly determined which demonstrate that *Listeria* is a well defined taxon that possesses a number of features identifying it from neighboring taxa. It is not a coryneform bacterium as evidenced by numerical phenetic studies, chemotaxonomic properties, and various rRNA and DNA sequencing analyses. However, the exact phylogenetic position of this genus still remains debated. Although it is generally agreed that its nearest neighbor is *Brochothrix*, its relationships with other members of the low G + C percent DNA content Gram-positive bacteria, especially with *Lactobacillus*, need further clarification (Rocourt and Buchrieser, 2007).

For many years after its discovery, the genus *Listeria* was monospecific, containing only the *L. monocytogenes* species. Because of its ability to reduce nitrates, *L. denitrificans* was added in 1948 (Sohier et al., 1948); *L. grayi* was included in 1966 in honor of M. L. Gray, an American microbiologist (Larsen and Seeliger, 1966). *L. murrayi* was added in 1971 to honor E. G. D. Murray, a Canadian microbiologist (Welshimer and Meredith, 1971) and *L. innocua* in 1981 that named according to the harmlessness of its (Seeliger, 1981). *L. ivanovii* was added in 1985 to honor I. Ivanov, a Bulgarian microbiologist (Seeliger et al., 1984); *L. welshimeri* was added in 1983 to honor H. J. Welshimer, who is American microbiologist and *L. seeligeri* (in honor of H. P. R. Seeliger, a German microbiologist) in 1983 (Rocourt and Grimont, 1983). As for the phylogenetic analysis of *Listeria*, the introduction of molecular biology methods allowed a better appreciation of the diversity within the genus *Listeria*, which now contains six species: *L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, and *L. grayi*.

2.6 Detection Methods

Since it became obvious that *L. monocytogenes* was pathogenic in man and animals, reliable detection methods were needed. No single procedure can be credited with being sensitive enough to detect *L. monocytogenes* from all types of food (Donnelly, 1999). The detection methods are consists of isolation methods, identification or confirmation methods that shown in Figure 2.2.

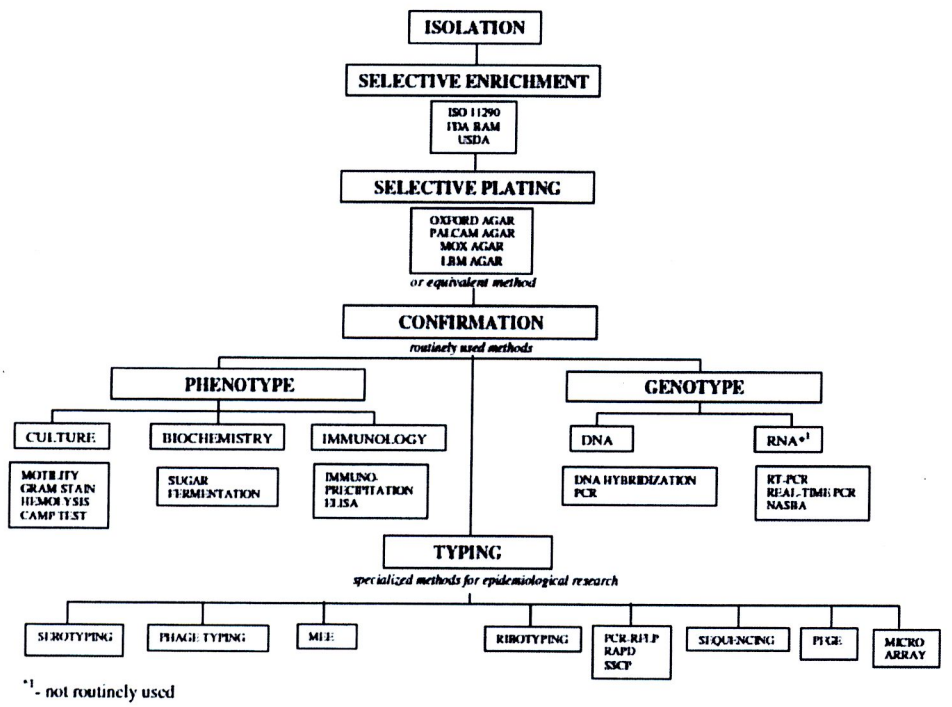


Figure 2.2 Overview of isolation, identification and typing methods for *Listeria* and *L. monocytogenes* in foods and environmental samples.

(Source: Gasanov et al., 2005)

In addition, sublethally injured *L. monocytogenes* cells were able to find in processed food from freezing, heating, acidification and other types of chemical or physical treatments. These injured bacteria require special culture conditions for damage recovery, before being able to be detected in culture. So, the detection of *L.*

monocytogenes from foods and environmental samples require the use of enrichment and selective procedures that can allow the levels of contaminating microorganisms reach to reasonable levels that are enough for detection of the organism (OIE Terrestrial Manual, 2008).

2.6.1 Isolation Methods

Historically, from the studied, it was noted that *Listeria* is able to grow at low temperatures and this feature has been used to isolate these bacteria from clinical samples by incubation for prolonged periods at 4 °C on agar plates until the formation of visible colonies (Gray et al., 1948). This method of isolation carries on up to several weeks and usually does not allow for the isolation of injured *Listeria* cells (Gasanov et al., 2005). Newer techniques were based on the use of a variety of selective in isolation and enrichment media. Results were available much sooner compared with the cold enrichment and for that reason these techniques were more suitable for routine work (Beumer and Hazeleger, 2003). In the food industry, such standard culture procedures are used as reference methods for regulatory purposes and for validation of new technology (Gasanov et al., 2005).

2.6.1.1 Netherlands Government Food Inspection Service Method (NGFIS)

Netherlands Government Food Inspection Service method (NGFIS) is a detection method widely used in Europe. This method was developed by Netten et al. (1989), who reported increased the effectiveness of detection method over that of the USDA-FSIS procedure in foods containing *L. monocytogenes* at lesser than 10 CFU/g. Food samples were enriched in Palcam broth for 48 h at 30°C. After 48 h, 0.1 mL in Palcam broth was plated onto Palcam agar. Then the samples were incubated at 30°C for 48 h under microaerophilic conditions (5% oxygen, 7.5% carbon dioxide, 7.5% hydrogen, and 80% nitrogen).

2.6.1.2 International Dairy Federation (IDF)

International Dairy Federation method (IDF) is recommended for milk products. This method was performed according to the International Dairy Federation Revised Provisional IDF Standard 143:1990 (1990) (IDF method, 1990). Samples were enriched in *Listeria* enrichment broth for 48 h. After that plates were streaked after 48 h on Palcam agar.

2.6.1.3 The United State Food and Drug Administration Bacteriological and Analytical Method (BAM)

For the FDA BAM, the samples were pre-enriched for *Listeria* species at 30°C for 4 h in buffered Listeria enrichment broth (BLEB) then adding selective agents including acriflavin 10 mg/L, Nalidixic acid 40 mg/L and cycloheximide 50 mg/L and incubation at 30°C for a total of 48 h. The enrichment cultures were streaked at 24 and 48 h on one of the prescribed differential selective-agars such as Oxford, Palcam, MOX or LPM in order to isolate *Listeria* species (Hitchins, 2001).

2.6.1.4 Nordic Committee on Food Analysis (NMKL)

Nordic Committee on Food Analysis (NMKL) is Nordic organization from Denmark, Finland, Iceland, Norway and Sweden. The method uses two-stage enrichment procedures. The samples were incubated for 24 h in Half-Fraser broth at 30°C. After 24 h, aliquots were transferred into Fraser broth and incubated at 37°C for 48 h. The cultures obtained from both the enrichment steps were plated out on *L.monocytogenes* specific isolation medium (ALOA or LMBA or Chromogenic Listeria Agar) and incubated at 37°C for 48 h (Johansson and Loncarevic, 2007).

2.6.1.5 International Standard Organization (ISO): ISO 11290 Method

The ISO 11290 method has a two-stage enrichment process starting with enrichment the food samples in half Fraser broth at 30°C for 24 h, then an aliquot was transferred to full

strength Fraser broth at 35°C or 37°C for 48 h. Fraser broth contained the selective agents acriflavin and naladixic acid. And also included esculin, which allows detection of b-D-glucosidase activity by *Listeria*, causing a blackening of the medium (Gasanov et al., 2005). Both the primary and secondary enriched broths were plated on Listeria agar according to Ottaviani and Agosti (ALOA) and on another solid selective isolation medium (Oxford and PALCAM), which is optional.

2.6.1.6 AOAC/IDF Official Method 993.12 *Listeria monocytogenes* in Milk and Dairy Products

AOAC/IDF method is often the method of choice for dairy products and provides specific instructions for sample preparation of specific dairy foods (Gasanov et al., 2005). The samples were enriched in selective enrichment, included selective agents acriflavin, nalidixic acid and cycloheximide, at 30°C for 48 h followed by plating onto Oxford agar. Then inverted plates were incubated 48 h at 37°C.

2.6.1.7 The United States Department of Agriculture (USDA)-Food Safety and Inspection Service (FSIS) Method

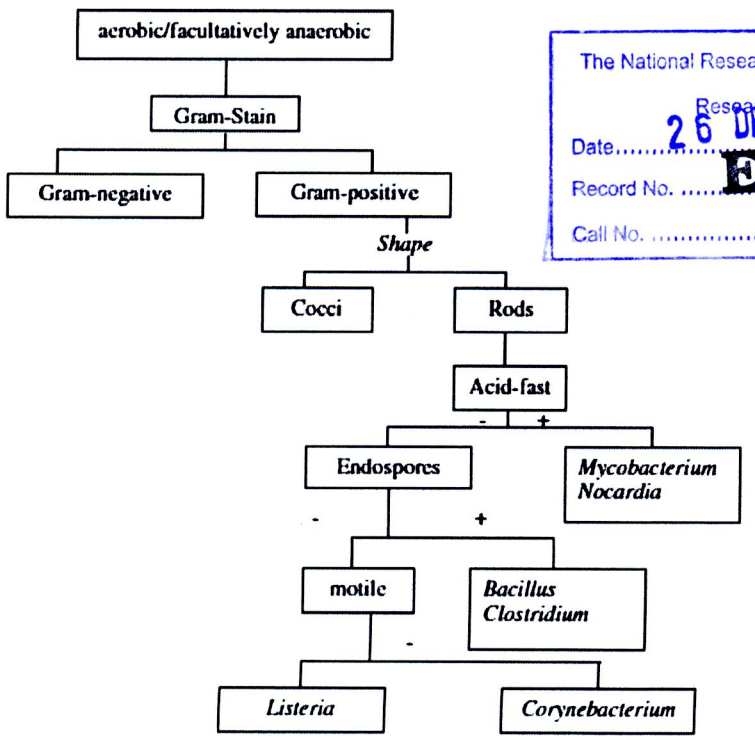
The USDA protocol is often the method of choice for red meat and poultry (raw or cooked ready-to-eat), eggs, egg products and environmental samples. Samples were dispensed to UVM broth (inhibitors: acriflavine and nalidixic acid). Then the samples were incubated at 30°C for 22 h. The UVM enrichment was transferred to FB or MOPS-BLEB and incubated at 35°C for 26 h or for 18-24 h for MOPS-BLEB. The inoculated was plated onto Modified Oxford (MOX) agar containing the selective agents including moxalactam and colistin sulphate (USDA/FSIS, 2002).

2.6.2 Identification or Confirmation Methods

Typical *Listeria* spp. colonies on selective/differential agar plates from the isolation methods are then selected for further identification to the species level, using a battery

of tests that can divided into three groups: phenotypic identification, genotypic identification and Epidemiological identification.

Phenotypic identification is the traditional methods of bacterial identification for example Gram-staining reaction, catalase test, motility test, haemolysis test, carbohydrate use. *Listeria* is Gram-positive rods, aerobic and facultative anaerobic, non-spore forming, catalase-positive, oxidase-negative, fermentative in sugars and producing acid without gas bacteria. Most strains are motile at 28 °C and non-motile at 37 °C (Welshimer, 1981).



		L. monocytogenes	*L. innocua*	*L. ivanovii*	*L. seeligeri*	*L. welshimeri*	*L. grayi*
Hemolysin		+	-	+	+	-	-
Catalase		+	+	+	+	+	+
Oxidase		-	-	-	-	-	-
Fermentation of:	L-Rhamnose	+	+/-	-	-	+/-	+/-
	D-Mannitol	-	-	-	-	-	+
	D-Xylose	-	-	+	+	+	-
	α-Methyl D-Mannoside	+	+	-	-	+	+

Figure 2.3 A methodological system for the phenotypic identification of *Listeria*.

(Source: Gasanov et al., 2005)

Genotypic identification performed by using molecular method such as DNA hybridization that is the simplest method for the detection of *Listeria* and *L. monocytogenes*. Previously, the presence of a target sequence was detected using an oligonucleotide probe of complementary sequence to the target DNA sequence which contained radioactive isotopes as label for detection. More recently, using biotinylated probes, probes incorporating digoxygenin (Boehringer–Mannheim), or fluorescent markers allow detection of target sequences with equivalent sensitivities to radioactive probes, without the biohazards associated with radioactivity (Gasanov et al., 2005). PCR combined with DNA hybridization in a microtitre plate is a convenient and highly sensitive and specific approach for detection of *Listeria* in a high-throughput 96-well format (Cocolin et al., 1997).

Epidemiological identification of *Listeria* is based on species-specific proteins or genes that are relatively stable over time and are passed on from generation to generation (Gasanov et al., 2005) for example phenotypic typing method (base on phenotypic marker such as somatic O), serological typing method (base on antibodies that specifically react with somatic or ''O''), phage typing method (base on phage receptor of *Listeria*) and Multilocus enzyme electrophoresis (MEE) (base on variations of amino acid sequence of enzyme). Due to the variety of epidemiological method, using should considering an appropriate technique such as the ease of use of the method and interpretation of results, labor and material costs, sample throughput ability, the time taken to perform the test, as well as the stability and reproducibility of the test itself (Gasanov et al., 2005).

2.6.3 Inhibitors

The key components which added to selective broths for *Listeria* are selective agents that used to inhibit irrelevant microbial by taking advantage of the resistance of

L. monocytogenes to various selective agents, including chemicals, antimicrobials, and dyes.

2.6.3.1 Acriflavine

Acriflavine is a mixture of proflavine and euflavine. Only euflavine has effective antimicrobial properties (Sridhar Rao P.N, 2008). It is a topical antiseptic. It has the form of an orange or brown powder. Neutral acriflavine (euflavine) was defined as 2, 8-diamino-10-methylacridiniumchloride, but is now considered to be a mixture of the hydrochlorides of 3, 6-diamino-10-methylacridine and 3, 6-diaminoacridine. Acriflavine inhibits RNA synthesis and mitochondriogenesis (De Vries and Kroon, 1970; Meyer et al., 1972). The proposed concentration of acriflavine in the media varies from 2.25 -25 mg/L. The use of arcriflavine was described by Ralovich et al, (1971). They concluded that *L.monocytogenes* grew well when acriflavine (40 mg/L) was added to agar plates whereas growth of Gram-positive cocci was suppressed.

2.6.3.2 Polymyxin B

Polymyxin B is an antibiotic generally used for against Gram-negative bacteria except *Proteas* spp. It was discovered in 1947 and introduced to the medical community in the 1950s. It is derived from the bacterium *Bacillus polymyxa*. Polymyxin B is a polypeptide that has the positively-charged amino groups in the form of cyclic peptides and fatty acid attachment. It acts specifically on anionic cell envelope component like phospholipids and lipopolysaccharides of Gram-negative bacteria (Chen and Feingold, 1973; Newton, 1956). Polymyxins B bind to the cell membrane and alter its structure, making it more permeable, resulting water uptake leads to cell death.

2.6.3.3 Nalidixic Acid

Nalidixic acid is the first of the synthetic quinolone antibiotics (Jones, 2003). It is effective against both Gram-positive such as *Bacillus subtilis* (Cook et al., 1966) and Gram-negative bacteria such as *E. coli* (Goss et al., 1964). In lower concentrations, it acts in a bacteriostatic manner; that is inhibiting growth and reproduction of microorganisms. In higher concentrations, it is bactericidal, meaning that it kills bacteria instead of merely inhibiting their growth. It is especially used in treating urinary tract infections, caused, for example, by *E. coli*, *Proteus*, *Shigella*, *Enterobacter*, and *Klebsiella* (Fraser and Harrower, 1977). Nalidixic acid was shown to inhibit specifically the synthesis of deoxyribonucleic acid (DNA) in Gram negative and slightly Gram-positive by blocking activity of bacterial DNA gyrase and topoisomerase enzyme system (Bhanot et al., 2001; Andriole, 1998; Bryskier and Chantot, 1995). Slight effects on protein and ribonucleic acid (RNA) synthesis were observed only at higher levels of drug or after prolonged incubation (Goss et al, 1964a).

2.6.3.4 Lithium Chloride

Lithium chloride is a chemical compound with the formula LiCl. The salt is a typical ionic compound. It is used as a desiccant for drying air streams. Selectivity is provided by the presence of lithium chloride in the formula of media. The high salt tolerance of *Listeria* is utilized as a means to markedly inhibit growth of enterococci (Ludlam, 1949). Using the combination of phenylethanol and lithium chloride helps to amplify numbers of *L. monocytogenes* in the presence of Gram-negative bacteria (McBride and Girard, 1960). Ryser and Marth (1988) and Yousef and Marth (1988) reported that increasing the lithium chloride concentration to 0.5% increased selectivity of the medium without appreciably decreasing recovery of healthy *Listeria* (Ryser and Marth, 1989; Werner and Lim, 1990).