ABSTRACT

Listeria monocytogenes (*L. monocytogenes*) is an important cause of listeriosis, a common foodborne disease of public health importance. Ready-to-eat (RTE) foods are known as a potential source of listeriosis. There are limited studies in the characterisation of *L. monocytogenes* in Malaysia. The objectives of the study were to determinate the prevalence of *Listeria* species and *L. monocytogenes* in RTE foods in Malaysia and to characterise these isolates by using molecular methods.

Two hundred fifty RTE food samples, including cooked beef and beef products, beverages, cooked chicken and chicken products, fried egg and egg products, packed lunch, salad and vegetables, and cooked seafood and seafood products were purchased from hawkers and hypermarkets in Kuala Lumpur and Petaling Jaya. Conventional and molecular methods were used for the isolation of *Listeria* spp. and *L. monocytogenes.*

Overall, out of 250 RTE food samples, *Listeria* species were found in 52 (20.8%) samples, of which 32 (61.5%) contained *L. monocytogenes.* Twenty-one (65.6%), 7 (21.9%) and 4 (12.5%) of *L. monocytogenes* isolates were grouped into serogroups "1/2a, 3a", "1/2c, 3c" and "4b, 4d, 4e", respectively. All the *L. monocytogenes* harbored virulence genes (*inlA, inlB, inlC* and *inlJ*) as confirmed by PCR and DNA sequence analysis. PCR-RFLP of the *inlA, inlB* and *inlC* had limited variation. Multi-drug resistance was observed in one isolate (3.1%) which belonged to serogroup "4b, 4d, 4e". REP-PCR, BOX-PCR, RAPD and PFGE were performed to genetically characterise the *L. monocytogenes* isolates. Twenty-eight REP profiles, 31 BOX profiles, 32 RAPD profiles and 20 pulsotypes (PFGE profiles) were observed. These *L. monocytogenes* isolates were classified into 8, 9, 4 and 7 distinct clusters at 80% similarity by REP-PCR, BOX-PCR, RAPD and PFGE, respectively. The

discriminatory power was 0.992, 0.998, 1.000 and 0.916 for REP-PCR, BOX-PCR, RAPD and PFGE, respectively.

In summary, the prevalence rate of serogroup “4b, 4d, 4e” indicated that RTE foods are potential sources of listeriosis in humans and the presence of the internalin genes and multi-drug resistant *L. monocytogenes* indicated that contamination of RTE foods could be a public health concern. REP-PCR, BOX-PCR and PFGE could distinguish the *L. monocytogenes* isolates with different flagella antigen groups or serogroups.

ABSTRAK

Listeria monocytogenes (*L. monocytogenes*) adalah punca utama listeriosis, penyakit bawaan makanan kepentingan kesihatan awam. Makanan sedia-untuk-makan (ready-to-eat: RTE) dikenali sebagai sumber yang berpotensi untuk listeriosis. Terdapat kajian yang terhad dalam pencirian *L. monocytogenes* di Malaysia. Objektif utama kajian ini ialah menentukan kelaziman spesies *Listeria* dan *L. monocytogenes* dalam makanan RTE di Malaysia dan mencirikan isolat dengan menggunakan kaedah molekul.

Dua ratus lima puluh sampel makanan RTE, termasuk produk daging lembu dan daging lembu masak, minuman, ayam masak dan produk ayam, telur goreng dan produk telur, makanan bungkusan, salad dan sayur-sayuran, dan makanan laut masak dan produk makanan laut yang dibeli daripada penjaja dan pasar raya besar di Kuala Lumpur dan Petaling Jaya. Kaedah-kaedah konvensional dan molekular telah digunakan untuk pengasingan dan pengenalpastian *Listeria* spp. dan *L. monocytogenes.*

Secara keseluruhannya, daripada 250 sampel produk makanan RTE, spesies *Listeria* telah ditemui di 52 (20.8%) sampel, 32 (61.5%) yang terkandung *L.monocytogenes*. Dua puluh satu (65.6%), 7 (21.9%) dan 4 (12.5%) *L. monocytogenes* isolat telah masing-masing dikumpulkan ke "serogroup" "1/2a, 3a", "1/2c, 3c" dan "4b, 4d, 4e". Semua *L. monocytogenes* mengandungi gen *inlA, inlB, inlC* dan *inlJ* sebagaimana yang disahkan oleh PCR dan analisis DNA urutan. PCR-RFLP daripada *inlA, inlB* dan *inlC* mempunyai variasi terhad. "Multi-drug resistant" telah diperhatikan dalam satu isolat (3.1%) *L. monocytogenes* dari "serogroup" "4b, 4d, 4e". REP-PCR, BOX-PCR, RAPD dan PFGE telah dilakukan untuk mencirikan *L. monocytogenes*. Dua puluh lapan corak REP , 31 corak BOX, 32 corak RAPD dan 20 pulsotypes (corak PFGE) telah diperhatikan. *L. monocytogenes* telah masing-masing dikelaskan kepada 8, 9, 4 dan 7 kelompok yang berbeza berdasarkan 80% persamaan dengan menggunakan

REP-PCR, BOX-PCR, RAPD dan PFGE. Kuasa diskriminasi masing-masing adalah 0.992, 0.998, 1.000 dan 0.916 untuk REP-PCR, BOX-PCR, RAPD dan PFGE.

Secara ringkasnya, kadar kelaziman "serogroup" "4b, 4d, 4e" menunjukkan bahawa makanan RTE adalah sumber potensi listeriosis pada manusia dan kehadiran gen internalin dan "multi-drug resistant" *L. monocytogenes* menunjukkan bahawa pencemaran makanan RTE boleh menjadi satu kebimbangan kesihatan awam. REP-PCR, BOX-PCR dan PFGE dapat membezakan *L. monocytogenes* dengan kumpulan antigen flagella berbeza atau "serogroup".

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ABBREVIATION

>	Greater than
=	Equals to
˚C	Degree Celsius
µl	Microliter
µg	Microgram
%	Percent
AMC	Amoxicillin-clavulanic acid
Aw	Water activity
bp	Basepair
C	Chloramphenicol
CFU	Colony forming unit
CN	Gentamicin
D	Discriminatory Power
DA	Clindamycin
dH_2O	Distilled water
ddH_2O	Double distilled water
DNA	Deoxyribonucleic acid
E	Erythromycin

EC	European Commission
EDTA	Ethylenediaminetetraacetic
ERIC	Enterobacterial Repetitive Intergenic Consensus
EtBr	Ethidium Bromide
EtOH	Ethanol
FDA	Food and Drug Administration
Fig.	Figure
g	Gram
H_2S	Hydrogen sulphite
IND	Indole
Inl	Internalin
K	Kanamycin
Kb	Kilo base pair
L. monocytogenes	*Listeria monocytogenes*
LSA	*Listeria* Selective Agar
M	Molar
MHA	Mueller-Hinton Agar
$MgCl_2$	Magnesium chloride
mM	Millimolar
mg	Milligram

ml	Milliliter
mm	Millimeter
MR	Methyl Red
NaCl	Sodium Chloride
No.	Number
OD	Optical density
P	Penicillin G
PCR	Polymerase Chain Reaction
PFGE	Pulsed field gel electrophoresis
RD	Rifampicin
Ref.	Reference
REP	Repetitive element sequence-based
rRNA	Ribosomal ribonucleic acid
S	Streptomycin
SDS	Sodium dodecyl sulphate
SIM	Motility test
Spp.	Species
SXT	Trimethoprim-sulfamethoxazole
Te	Tetracycline
TBE	Tris-borate-EDTA

TE	Tris-EDTA
Tris	Tris (Hydroxymethyl) methylamine
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
TSAYE	Tryptic Soy Agar with Yeast Extract
TSI	Glucose
UV	Ultraviolet
Va	Vancomycin
VP	Voges-proskaur
w/v	Weight per unit volume
5’-CS 5	conserved segment
3’-CS 3	conserved segment

CHAPTER 1

INTRODUCTION

The genus *Listeria* comprises ten species: *Listeria monocytogenes, Listeria ivanovii, Listeria seeligeri, Listeria innocua, Listeria welshimeri, Listeria grayi, Listeria marthii, Listeria rocourtiae, Listeria fleischmannii,* and *Listeria weihenstephanensis* (Halter *et al.*, 2012). These are Gram-positive, short rods, non-spore, able to grow from pH 4.3 to 9 (Groski, 2008), ~ 0°C to 45°C (Lou & Yousef, 1999), salt content 10% and 200 ppm $NaNO_2$, (Wanger & McLauchlin, 2008) and motile species are widespread in nature (Fenlon, 1985). Member of the genus *Listeria* is known to have the ability to tolerate environmental stresses. Of particular concern is that *Listeria* spp. is osmotically tolerant and grow at refrigerated temperatures (Endang *et al.*, 1998).

L. monocytogenes has emerged as a foodborne pathogen with the first confirmed case in 1981 linked to contaminated coleslaw. It is responsible for the disease listeriosis, which can cause severe malaise in pregnant women, new-born babies, the advanced age and persons suffering from a weakened immune system. Without treatment, listeriosis can develop into septicaemia, encephalitis, meningitis and meningoencephalitis as well as stillbirths and abortion (Montville & Matthews, 2008). With a mortality rate fast approaching 30%, it is considered to exceed those of common foodborne pathogens such as S*almonella enteritidis, Campylobacter* species and *Vibrio* species (Altekruse *et al.*, 1997; Mead *et al.*, 1999; Montville & Matthews, 2008). *L. monocytogenes* is most commonly found in vegetables, meat, dairy products, seafood products, ready to eat (RTE) foods and especially products that are kept at refrigeration temperature as *L. monocytogenes* can grow at these low temperatures (Meng & Doyle, 1997; Gugnani, 1999).

The conventional methods are the most common methods applied to detect *L. monocytogenes* that rely on the use of microbiological media to selectively grow and enumerate this pathogen. These methods are inexpensive, sensitive and provide results

that are both qualitative and quantitative. These methods are, however, time consuming and labour-intensive (De Boer & Beumer, 1999). The approved methods that are currently been using for the detection of *L. monocytogenes* in foods include the Federal Drug Administration (FDA) protocol, ISO-11290-1, Netherlands Government Food Inspection Service (NGFIS) and US Department of Agriculture-Food Safety and Inspection Service (USDA-FSIS) methods (Churchill *et al.*, 2006).

Recent advances in molecular genetics have led to methods targeting unique genes for amplification and subsequent differentiation. Therefore, using a unique gene, *L. monocytogenes* can be distinguished from other *Listeria* spp. These methods are intrinsically more accurate and less affected by natural variation than conventional methods and offers high specificity, sensitivity and a rapid turnover that is essential for producers of minimally processed food products (Liu, 2006).

Various *Listeria* determinants have been reported to mediate bacterial adherence into target cells and invasion. *inlA, inlB, inlC*, and *inlJ* can increase the invasion or virulence of the pathogen in animal models or tissue cultures. (Bierne & Cossart, 2002; Orsi *et al.*, 2007; Sant'Ana *et al.*, 2012). Following ingestion of contaminated food, *L. monocytogenes* can be cause of infection by invading intestinal epithelial cells. The internalin is associated with a receptor on the surface of the host for invasive process (Dramsi *et al.*, 1997; Gaillard *et al.*, 1996). Intestinal epithelial cells need *inlA* for internalization. For entry of *L. monocytogenes* to a wide range of the cell line, such as non-epithelial cells and hepatocytes, *inlB* is involved (Hamon *et al.*, 2006). On the other hand, the presence of *inlC* and *inlJ* genes increase the pathogenicity of *L. monocytogenes* and could be a rapid method to differentiate non-virulent strains of the pathogen from virulent strains (Liu *et al.*, 2007).

Studies on the genomic variation and molecular epidemiology of *L. monocytogenes* often carried out to track sources and spread of the pathogen. Repetitive Extragenic Palindromic (REP-PCR) (Liu, 2006), BOX-PCR (Miteva *et al.*, 1998), Random Amplification of Polymorphic DNA (RAPD) (Gravesen *et al.*, 2000), and Pulsed Field Gel Electrophoresis (PFGE) (Liu, 2006) are appropriate typing methods for *L. monocytogenes* strains because of their reproducibility and high discriminatory ability. They have been well utilized in order to compare strains for epidemiological surveillance (Houhoula *et al.*, 2012; Lukinmaa *et al.*, 2004; Miteva *et al.*, 1998).

Furthermore, the incidence of listeriosis cases in Malaysia is relatively unknown as no system for reporting cases are in place. Information regarding the prevalence and genetic diversity of this pathogen in Malaysia is also limited. Hence, to improve the basic knowledge of the incidence and characteristics of *Listeria monocytogenes*, the present study was undertaken.

The objectives of this research were:

1. To isolate and identify *Listeria* spp. and *L. monocytogenes* by conventional and molecular methods and to determine the prevalence of *L. monocytogenes* in ready to eat (RTE) foods.
2. To determine the prevalence of virulence genes, *inlA, inlB, inlC,* and *inlJ* genes in *L. monocytogenes* and to determine the polymorphism of *inlA* and *inlC* genes.
3. To determine the antimicrobial susceptibility statues of *Listeria monocytogenes*
4. To investigate the genetic diversity of the *L. monocytogenes* from different food matrices.

CHAPTER 2

LITERATURE REVIEW

2.1 *Listeria* species

The history of *Listeria* and *L. monocytogenes* is relatively recent with the first published description of the bacteria by Murray in 1926. Up to the 1970s, the relationship of *Listeria* to other bacteria remained unclear. These bacteria were included in the fourth edition of Bergey's Manual of Determinative Bacteriology and were included in the Corynebacteriaceae family under the genus Kurthia. Based on morphological resemblances, *Listeria* was grouped in the coryneform group of bacteria, being Gram-positive and a non-endospore-forming rod. However, with the development and introduction of new methods and technologies such as numerical taxonomy, chemotaxonomy, DNA/DNA hybridation and ribosomal RNA gene sequencing, the phylogenetic position of *Listeria* has become clearer. During the last three decades, information and data was accumulated and *Listeria* is recognized with distinctive characteristics. It is not a coryneform bacteria, as previously thought, having a low G + C % content, the presence of lipoteichoic acid and absence of mycolic acids (Rocourt, 1999).

The *Listeria* genus was for many years mono-specific containing only *L. monocytogenes*, the type species. With the introduction of molecular methods, the diversity within the genus *Listeria* is better understood and now only contains *L. monocytogenes, L. ivanovii, L. seeligeri, L. innocua, L. welshimeri, L. grayi, L. marthii, L. rocourtiae, L. fleischmannii,* and *L. weihenstephanensis* (Halter *et al.*, 2013; Zhang et al, 2007). *L. monocytogenes* is known as a pathogenic species to animals and human while *L. ivanovii* is considered as a pathogenic species to animals (Bhunia, 2008).

The morphology of *Listeria* can be described as a small (1 - 2 μm in length and 0.5 μm in diameter), regular, Gram-positive rod with rounded ends. The cells can be found alone or in groups, including short chains or V and Y arranged forms depending on the growth conditions and temperature. They do not produce spores and do not form

capsules. The cells might sometimes be coccoid and when older, lose the ability to retain Gram stain. The bacterial cells can then be mistaken for streptococci or *Haemophilus* spp. (Jorgensen *et al.*, 1995; Bhunia, 2008). When cultured at 20°C - 25°C, *Listeria* is motile due to the formation of a few eritrichous flagella. These flagella are very weak or absent when *Listeria* is cultured at 37 °C (Galsworthy *et al.*, 1990).

Listeria can be classified as facultatively anaerobic, microaerophilic, aerobic (Seeliger, 1961). *Listeria* species are psychrophilic and could grow at various temperatures (0°C - 45°C), however, grow below 0°C has been reported (Junttila *et al.*, 1988; Walker *et al.*, 1990). Although, at pH7 *Listeria* genus shows an optimal growth, they could growth at various pH ranges of 4.4 - 9.6 (George & Lund, 1992). At pH values below 4.3, cells do not grow but may survive (Montville & Matthews, 2008). *Listeria* cells can also tolerate a 10 % (w/v) NaCl solution and survive at even higher concentrations (Seeliger & Jones, 1986). The survival at high salt concentrations and low pH is strongly temperature dependent (Cole *et al.*, 1990). *Listeria* species grow best at water activity (Aw) values = 0.97, but some of isolates also grow at an Aw value below 0.93, which is rare for foodborne pathogens. *Listeria* species may even survive at Aw values 0.83 for long periods of time. It has also been shown that the heat resistance of *Listeria* species increases as the Aw decreases (Farber *et al.*, 1992; Montville & Matthews, 2008). Having these extreme tolerances regarding pH, temperature, water activity and salt conditions (Sleator *et al.*, 2003; Liu *et al.*, 2005) makes the survival of this genus in environments. Surface water, soil, sewage, and foods have been identified as suitable environments for the survival and subsequent isolation of *Listeria* species (Liu, 2006).

2.2 *Listeria monocytogenes*

L. monocytogenes is a ubiquitous Gram-positive, psychrotrophic, non-capsule, non-spore, (De Oliveira *et al.*, 2010), foodborne pathogen (Aureli *et al.*, 2000; Salamina *et al.*, 1996; Sim *et al.*, 2002) that is the known cause of listeriosis (De Vasconcelos *et al.*, 2008). *L. monocytogenes* is ß-hemolytic, catalase-positive, oxidase-negative and possesses glucose oxidase and NADH oxidase activities (Farber & Peterkin, 1991). It is highly motile at low temperatures (up to 28°C) and less motile or non-motile at 37°C (Kamp & Higgins, 2011). The resistant nature of *L. monocytogenes* is associated with genetic determinants that encode various components of potential stress response systems (Gandhi and Chikindas, 2007). The bacteria have been reported from environments, soils, surface water, animal faeces, vegetation, sewage, silage (Cocolin *et al.*, 2005; Liu, 2008). It can also be found in the human body and in healthy humans. One to five percent of the world's population is reported to serve as carriers of these bacteria (Bhunia, 2008). *L. monocytogenes* has been isolated from a variety of raw and processed foods (Gugnani, 1999; Meng & Doyle, 1997). Due to the lack of good agricultural practices, vegetables are especially prone to microbiological contamination by the use of untreated water or contaminated organic fertilisers (Francis *et al.*, 1999).

L. monocytogenes thrives as a saprophyte in decomposed organic material and as an intracellular pathogen in macrophages (Liu, 2008). The transmission route for *L. monocytogenes* in humans is primarily via foods, however other routes of transmission have also been identified, including nosocomial and occupational (animal handlers can become infected primarily by open skin wounds) (Bell and Kyriakides, 2005). It was not until 1980s that *L. monocytogenes* was known as a major food-borne pathogen. Since then numerous Listeriosis outbreaks have been documented. Outbreak investigative efforts have led to increased understanding of this pathogen. Nonetheless more than three decades since the first major outbreak, *L. monocytogenes* remains a key

food-safety threat due to the high case fatality rate associated with listeriosis (Scallan *et al.*, 2011).

Based on the somatic (O) and flagellar (H) antigens, *Listeria* has been divided into number of serovars which are ascertained on the basis of unique combinations of O and H antigens. *L. monocytogenes* includes thirteen distinct serovars (1/2a, 3a, 1/2b, 3b, 1/2c, 3c, 4a, 4b, 4ab, 4c, 4d, 4e, and 7). With the use of various subtyping techniques, including serotyping, twelve serovars of the pathogen have been distributed into three genetic lineages and or five phylogenetic groups. Lineage I separate into two phylogenetic groups I.1 and I.2. Phylogenetic group I.1 contains the serovars "1/2a, 3a" and phylogenetic group I.2 comprises two serovars "1/2c, 3c". Lineage II also divide into two phylogenetic groups which phylogenetic group II.1 contains the serovars "4b, 4d, and 4e" and phylogenetic group II.2 includes the serovars "1/2b, 3b, 7". Lineage III contains two serovars 4a and 4c (Doumith *et al.*, 2004a). The lineage II is responsible for most of human listeriosis outbreaks and has the highest pathogenic potential. Those of lineage I have intermediate pathogenic potential and are usually isolated from environments and food categories and those from lineage III have a low pathogenic risk, usually are animal pathogens (Doumith *et al.*, 2004b).

Serovars 1/2a, 1/2b and 4b have been identified as the serovars responsible for the majority of listeriosis cases (De Vasconcelos *et al.*, 2008; Doumith *et al.*, 2004b; Vines & Swaminathan, 1998). Doumith *et al.* (2004b) interestingly found that while serovar 4b, from lineage II, which is considered the most virulent and responsible for the majority of epidemic listeriosis cases, it was in fact serovar 1/2a, from lineage I that was most frequently isolated from contaminated food. This result was in agreement with reports by Kathariou (2002), Gray *et al.* (2004), Jacquet *et al.* (2004) and Ramaswamy *et al.* (2007) and may indicate that not all food contaminated with *L. monocytogenes* will evoke a listeriosis epidemic.

L. monocytogenes is pathogenic at the species level, but various strains display varied virulence and pathogenic potential. There are strains that may be very virulent and cause disease and others that are non-pathogenic and produce no apparent malaise (Erdenlig *et al.*, 2000; Gracieux *et al.*, 2003; Doumith *et al.*, 2004a). The difference between these virulent and non-virulent strains is minimal as they possess the same virulence gene cluster LIPI-1 (consisting of *prfA, hlyA, plcA, mpI, actA*, and *plcB*), and all of the major virulence proteins involved in *L. monocytogenes* pathogenesis, but may differ in the expression of these genes (Liu *et al.*, 2003).

The cellular mechanism of *L. monocytogenes* pathogenesis can be divided into four major steps, namely adhesion and invasion, lysis of vacuoles, intracellular growth and cell-to-cell spread (Figure 2.1) (Bhunia, 2008). In each of these steps there are various virulence proteins that are involved and necessary for *Listeria* pathogenesis. A number of adhesion factors have been identified to be involved in the adhesion of the bacterium to the host cell, namely internalin A (*InlA*), internalin B (*InlB*), virulence-associated invasion protein (Vip), *Listeria* adhesion protein (LAP), fibronectin-binding protein (Fbp), autolysin amidase (Ami), cell wall hydrolase (p60), lipoprotein promoting entry (LpeA) and lipoteichoic acid (LTA).

After the bacterium has attached and entered the host cell, it is trapped inside a vacuole (phagosome). Two virulence proteins have been identified that destroys the phagosome and allows the bacterium to escape, namely Listeriolysin O (LLO) and phosphatidylinositol-specific phospholipase C (PI-PLC) (Bhunia, 2008). LLO, encoded by the *hlyA* gene (part of LIPI-1), is responsible for the haemolysis of blood cells and the disruption of eukaryote membranes (Kingdon & Sword, 1970). The function of LLO, a bacterial pore-forming hemolysin, is to lyse the membranes of phagosomal vesicles and to facilitate the escape of *L. monocytogenes* into the cytoplasm of the cell (Decatur & Portnoy, 2000). The maximum cytolytic activity of LLO coincides with the

phagosomal pH value of 5.5 (Bhunia, 2008). LLO is essential for virulence by *L. monocytogenes* and the presence of LLO is used to detection of the pathogen (Vazquez-Boland *et al.*, 2001).

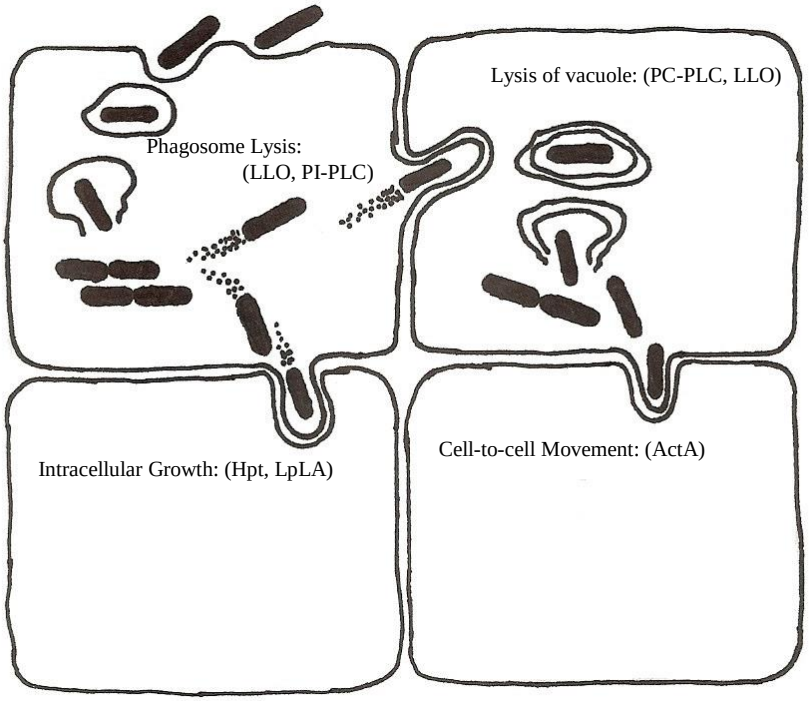


Figure 2.1: Cellular mechanism of *L. monocytogenes* pathogenesis (adapted from Bhunia, 2008).

PI-PLC, encoded by the *plcA* gene (part of LIPI-1), acts synergistically with LLO to destroy the lipid bilayer of the phagosome. Both of the genes that encode for LLO and PI-PLC, *hly* and *plcA*, respectively are regulated by *prfA* (also part of LIPI-1). A mutation in the *prfA* gene will result in the inactivation of all the genes located downstream from it, including *hly* and *plcA*. These two genes are necessary for the maintenance of the virulent status of *L. monocytogenes* (Bhunia, 2008) and this mutation might be the reason for the existence of non-virulent *L. monocytogenes* strains.

After the bacterium has escaped from the phagosome, it first multiplies before moving into a new cell. Although several protein factors are involved during this step, they are not regarded as major virulence proteins. Hexose phosphate translocase (Hpt) and lipoate protein ligase (LpLA1) are expressed to utilise host-derived glucose and lipoic acid for multiplication (Bhunia, 2008). After multiplication, the bacteria spreads to new cells. Three virulence proteins have been identified which aids in this spread, namely actin polymerisation protein (ActA), PC-PLC and zinc metalloprotease (MpI). All three of the genes that encode for ActA, PC-PLC and MpI, namely *actA, plcB* and *mpI*, are part of LIPI-1 and regulated by *prfA* (also part of LIPI-1). A mutation in the *prfA* gene will result in the inactivation of all the genes located downstream from it, including *actA, plcB* and *mpI*. A mutation in the *actA* gene itself will lead to mutant strains that are unable to accumulate actin and, therefore, unable to infect adjacent cells. A mutation in either the *plcB* or *mpI* genes will also lead to reduced virulence (Bhunia, 2008).

2.2.1 Internalin A, internalin B and the internalin Family

InlA and *inlB* are expressed from adjacent genes transcribed both independently and biciscronically from the inlAB locus (Gaillard *et al.*, 1991). They were identified in a genetic screen of *L. monocytogenes* transposon-insertion mutants unable to invade the enterocyte-like colon carcinoma cell line Caco-2 (Gaillard *et al.*, 1991). In the study, *inlA* was found to be necessary for attachment and invasion, and *inlA* was sufficient to reconstitute invasion when expressed in the non-invasive species *L. innocua*. Southern Blot analysis with an *inlA*-based probe suggested that *inlA* and *inlB* were members of a larger highly homologous family (Gaillard *et al.*, 1991). The family now includes at least nine additional members: *inlC, inlC2, inlD, inlE, inlF, inlG, inlH, inlI*, and *inlJ*. In addition, there are also at least 15 Internalin-like genes identified through genomic

analyses (Engelbrecht *et al.*, 1996; Lingnau *et al.*, 1996; Dramsi *et al.*, 1997; Domann *et al.*, 1997; Raffelsbauer *et al.*, 1998; Cabanes *et al.*, 2002; Bierne *et al.*, 2007; Bierne & Cossart, 2007; McGann *et al.*, 2008; Sabet *et al.*, 2008). Only *inlA* and *inlB* are well understood. The defining characteristic of internalins is a leucine rich repeat (LRR) domain of 3 to 28 repeats of 22 amino acids each. Each repeat contains a short ß-strand and a spatially larger 310-helix and each LRR wraps in a right-handed direction to stack upon one another. The entire LRR domain takes a solenoid 'sickle' shape with parallel stacked ß-strands forming the concave face and stacked 310-helix forming the convex face (Marino *et al.*, 1999, 2000; Schubert & Heinz, 2003; Bierne *et al.*, 2007). In addition, each repeat is rotated ~5 degrees with respect to its predecessor giving the sickle-shaped solenoid a superhelical twist (Marino *et al.*, 1999, 2000; Schubert *et al.*, 2001; Schubert *et al.*, 2002; Schubert and Heinz, 2003; Bierne *et al.*, 2007).

A N-terminal cap and an Ig-Like IR domain always flank the LRR domain and it is thought that these domains stabilize the LRR domain by shielding the hydrophobic core from an aqueous environment (Schubert and Heinz, 2003). Internalin and internalin-like proteins all have an N-terminal signal sequence suggesting that these proteins are processed to the bacterial surface by the general secretory pathway (Rafelski & Theriot, 2006; Bierne *et al.*, 2007). All but *inlC*, a secreted internalin, are attached to the bacterial surface, generally through a Cterminal peptidoglycan-anchoring sequence (e.g. LPXTG) or C-terminal domains that associate non-covalently with the bacterial cell wall (e.g. GW domains that bind lipoteichoic acid) (Engelbrecht *et al.*, 1996).

2.3 *Listeria monocytogenes* in foods

In recent years, ready-to-eat (RTE) foods have been implicated in outbreaks of listeriosis. Ready-to-eat foods, smoked fish, cooked marinated products, meat products, and vegetables were found to be contaminated with *L. monocytogenes* (Meloni *et al.*, 2009). Numerous food surveys conducted in Malaysia had reported on the detection of *L. monocytogenes* in various types of foods, including raw and RTE foods (Marian *et al.*, 2012), raw salad vegetables (Ponniah *et al.*, 2010), burger patties (Wong *et al.*, 2012) and vegetarian burger patties (Wong *et al.*, 2012). However, the actual incidence of foodborne listeriosis cases in Malaysia is not known. There is no official data on food poisoning/infection caused by *L. monocytogenes* in Malaysia because *L. monocytogenes* is rarely tested in the food poisoning/infection cases.

2.4 Listeriosis

The first confirmed case of human listeriosis was diagnosed at the end of World War I from a soldier suffering from meningitis. The most susceptible/highest risk group includes pregnant women, neonates, the elderly and immuno-suppressed individuals such as those with HIV, cancer patients receiving chemotherapy or patients receiving treatment for organ transplantation (Vazquez-Boland *et al.*, 2001). The initial symptoms of human listeriosis include fatigue, chills, headache, and also gastroenteritis. Without treatment, the disease can develop into septicaemia, abortion, meningitis, encephalitis and finally death (Vazquez-Boland *et al.*, 2001).

L. monocytogenes has a range of virulence factors that enables it to cross the cerebrospinal, intestinal and placental barriers. The organism escapes the human immune surveillance system by internalisation (moving into the cells) and then multiplying in the cytosol of infected cells (Wiedmann, *et al.*, 1997). Pregnant women

infected with *L. monocytogenes* may transfer the disease to their fetuses, which could lead to abortion, stillbirth or the premature birth of an infected child (Seeliger & Jones, 1986; Spencer, 1987).

The mortality rate of infection with *L. monocytogenes* is approaching 30%, which indicates a high fatality: case ratio and exceeds those of common foodborne pathogens such as *Salmonella Enteritidis, Campylobacter* spp. and *Vibrio* spp. (Altekruse *et al.*, 1997; Mead *et al.*, 1999; Montville and Matthews, 2008). The infectious dose of *L. monocytogenes* is still unknown, but depends on the virulence of the microbe, the immunological status of the human and the contaminated food. Studies with test animals indicated that by reducing the exposure levels reduces the incidence of clinical disease. Foods responsible for serious outbreaks all had >100 cfu g^{-1} *L. monocytogenes*, however, more epidemiologic data is needed to accurately determine the infectious dose (McLauchlin *et al.*, 2004; Montville & Matthews, 2008).

As the infectious dose is still unknown, the official regulations regarding *L. monocytogenes* vary among food products, as well as countries. Regulations range from a "zero tolerance" level in the United States issued by the Food and Drug Administration (FDA) for RTE foods (Chen *et al.*, 2003), to an absence in 1 g in Europe, and to less than 100 cfu g^{-1} in RTE foods that do not support the growth of the pathogen at the time of consumption, also in Europe (EC, 1999; Anonymous 2005).

Listeriosis epidemics have been reported in the past years, but usually occur sporadically with only 2 to 15 cases per million people per year (Farber & Peterkin, 1991; Jacquet *et al.*, 1995). However, 2500 cases of human listeriosis are still reported annually in the United States and of these cases 500 deaths have been reported (Mead *et al.*, 2006; Montville and Matthews, 2008). Although regulations regarding *L. monocytogenes* contamination in food are implemented and strictly adhered to, the

major cause of listeriosis is considered to be the ingestion of contaminated food (Farber & Peterkin, 1991). Six major outbreaks of listeriosis were reported between 1979 and 1999 in North America. The cause was the ingestion of contaminated food sources as diverse as commercially prepared coleslaw, lettuce, carrots, pork tongue in jelly, paté, milk that was contaminated after pasteurisation, chocolate milk, soft cheese made from unpasteurised milk and hot dogs (Donnelly, 2001). The listeriosis outbreak due to contaminated hot dogs was attributed to hot dog meat contamination levels of <0.3 cfu g^{-1} (Donnelly, 2001). This further provides evidence for the very low, but still unknown, infectious dose. These relatively recent listeriosis outbreaks due to contaminated food products have highlighted the importance and necessity of continued surveillance of *L. monocytogenes* in RTE foods (Farber & Peterkin, 1991; Jacquet *et al.*, 1995; Liu *et al.*, 2003).

2.5 Detection methods of *L. monocytogenes* and *Listeria* spp.

To be able to distinguish *L. monocytogenes* from other *Listeria* species, a rapid, specific and sensitive test is essential. The quick and accurate detection of *L. monocytogenes* will lead to the control of the spread of this organism. Two types of detection methods will be discussed, conventional and molecular methods.

2.5.1 Conventional methods of detection of *Listeria*

Listeria spp. and *L. monocytogenes* in food samples often grow in competition with other non-target micro-organisms and are often injured as a result of freezing, heating, drying, irradiation or exposure to chemicals (Bunduki *et al.*, 1994). To be able to detect *Listeria* spp. a primary and secondary enrichment procedure (half and full strength Fraser broth) is essential to recover the sublethally injured *L. monocytogenes*.

After the food samples have been enriched various identification methods, which generally cannot recover the sublethally injured *Listeria*, are used. These methods include cultural and biochemical confirmation, ß hemolysis, the Christie Atkins Munch-Petersen (CAMP) test and more recently chromogenic substrates.

2.5.1.1 Cultural and biochemical confirmation of *Listeria* spp. and *L. monocytogenes*

The earliest identification methods for *Listeria* species relied on biochemical and phenotypic characteristics and are still widely used. However, attempted isolation of *Listeria* from non-selective media had little success as most food specimens are naturally contaminated with large numbers of other micro-organisms and only contain a small number of *Listeria* spp. (Donnelly, 1999). Inhibition of these indigenous micro-organisms, as well as the resistance of *Listeria* to various antibiotics, have led to the formulation of selective agars. Selective agents such as glycine anhydride, phenylethanol, lithium chloride and antibiotics have been added to the media to enable the isolation of *Listeria* in the presence of Gram-negative bacteria and to inhibit Gram-positive contaminants, such as *Staphylococcus* and *Pseudomonas*. McBride *Listeria* agar, LPM agar, Oxford agar, MOX agar, *Listeria* selective agar, and PALCAM agar are used for the isolation of *Listeria* spp. (Donnelly, 1999; Adam & Moss, 2008).

The Henry technique, an oblique illumination technique was developed to facilitate the recognition of *Listeria* colonies on blood-free media. Using the Henry technique, plates are tested under obliquely transmitted white light at an angle of 45° with a binocular scanning microscope. *Listeria* colonies would appear small and round and have a blue-gray to blue-green appearance (Adams & Moss, 2008). Some media, such as Oxford agar, incorporates aesculin and ferric ammonium citrate to produce a

visible colour change which would eliminate the use of the Henry technique (Adams & Moss, 2008). Oxford agar is prepared from Columbia agar base, selective agents and aesculin and ferric ammonium citrate, with the latter two as differential agents. The aesculinase reaction is used to differentiate *Listeria* from other bacteria (Curtis *et al.*, 1989). The ß-D-glucosidase activity of *Listeria* hydrolyses aesculin, producing a black zone surrounding typical colonies due to the formation of black iron phenolic compounds derived from the aglucon after 48 hours of incubation (Greenwood *et al.*, 2005). The selective agents added to Oxford agar, namely lithium chloride, cycloheximide, colistin sulphate, acriflavine, cefotetan and fosfomycin inhibit the growth of other micro-organisms. Unfortunately, *Enterococcus* and *Bacillus* spp. will also grow on these selective plates and also utilise aesculin. Further tests, therefore, are required to conclusively identify colonies of *Listeria*.

These second step tests, used to determine if suspect colonies are *L. monocytogenes*, rely on the unique biochemical and *ß*-hemolysis characteristics of each of the *Listeria* species (Table 2.1) as they all exert the same phenotype (morphology) (Montville & Matthews, 2008). These tests include acid production from xylose, rhamnose, mannitol and alpha-methyl-D-mannoside, ß-hemolysis and the Christie Atkins Munch-Petersen (CAMP) test.

Table 2.1: Biochemical properties of *Listeria* species (Adapted from Swaminathan *et al.*, 1995; Bhunia, 2008).

Characteristics	*L. monocytogenes*	*L. innocua*	*L. ivanovii*	*L. welshimeri*	*L. seeligeri*	*L. grayi*
β-hemolysin	+	-	+	-	+	-
CAMP (*S. aureus*)	+	-	-	-	+	-
CAMP (*R. equi*)	-	-	+	-	-	-
L-Rhamnose	+	v	-	-	v	-
D-Xylose	-	-	-	+	+	-
α-Methyl-D-mannoside	+	+	-	+	-	+
Mannitol	-	-	-	-	-	+
Cytotoxicity	+	-	+	-	±	-
Invasion assay	+	-	+	-	-	-
Mouse virulence	+	-	+	-	-	-

2.5.2 Chromogenic media for detection of *L. monocytogenes*

The recent commercial availability of chromogenic media allows direct identification of colonies by their characteristic colour however biochemical tests or PCR are used for confirmation of the presumptive colonies. The bacteria are differentiated based on their enzymes on chromogenic media. The chromogenic media have many benefits over other tests in being simple, easy to interpret, highly sensitive and specific, cost effective, and allowing a large sample throughput. The activity of the enzyme Phosphatidylinositol-specific phospholipase C (PI-PLC) is measured by the opaque white halo colour reaction produced by the hydrolysis of phosphotidylinositol or lethicin for *L. monocytogenes* identification (Coffey *et al.*, 1996). A commercially available chromogenic agar, CHROMagar^{TM} *Listeria* is applied for the detection of *L. monocytogenes*, producing blue colonies with white halo (Aragon-Alegro *et al.*, 2008; El Marrakchi *et al.*, 2005).

2.5.3 Molecular detection of *Listeria* spp. and *L. monocytogenes*

The low numbers of the *Listeria* spp. is one of the most important problems encountered during detection of the pathogen in contaminated food samples (Hoffman & Weidmann, 2001). Molecular methods have proven to be useful in detecting low numbers of *Listeria* spp., due to the fact that low concentrations of DNA can be specifically detected or amplified (Churchill *et al.*, 2006). Molecular methods detect differences at the DNA level and can differentiate between micro-organisms at the genus, species and even sub-species level (Liu *et al.*, 2003).

PCR has many advantages including high throughput processing, a level of automation, and a relatively short reaction time. It also shows an increase in sensitivity over culturing methods. The inability of some cells to grow in selective media due to low numbers is also excluded as PCR amplifies the specific genetic signals from as little as only a few cells (Shearer *et al.*, 2001). The targets of amplification in *L. monocytogenes* are listeriolysin O gene (Bessesen *et al.*, 1990; Border *et al.*, 1990; Deneer and Boychuk, 1991; Furrer *et al.*, 1991; Thomas *et al.*, 1991; Niederhauser *et al.*, 1992; Wong *et al.*, 1992), *iap* gene (Niederhauser *et al.*, 1992), *Dth18*-gene (Wernars *et al.*, 1991), and 16S RNA (Border *et al.*, 1990). Enrichment cultures were usually obtained and cell lysed for the PCR analysis (Thomas *et al.*, 1991; Niederhauser *et al.*, 1992).

2.6 Molecular subtyping methods

2.6.1 PCR-based subtyping techniques for *L. monocytogenes*

2.6.1.1 Randomly amplified polymorphic DNA (RAPD) and arbitrarily primed PCR (AP-PCR)

Both randomly amplified polymorphic DNA (RAPD) and arbitrarily primed PCR (AP-PCR) use low-stringency PCR amplification to generate anonymous DNA fragments that are strain-specific. The primers applied for RAPD and AP-PCR are single, short and of arbitrary sequence. These primers are used at relatively low temperatures (around 36°C) during PCR, thus effectively lowering the stringency of the primer-annealing temperature. This in turn allowing the annealing of a random primer that shows a perfect match of two or three nucleotides between the template strand and the 3' end of the primer. When annealing and priming occur within a certain distance from one another, the sequence that lies between these two sites can be amplified (Farber & Addison, 1994; O'Donoghue *et al.*, 1995). A comparison between strains or isolates can only be made if the same primer is applied for all the test samples. Otherwise, the fragments yielded would be of no importance as the amount and sizes of the fragments would conflict because of the different primers used. The test samples also have to be pure cultures to avoid contamination and subsequent deceiving or misleading results (Lawrence & Gilmour, 1995). This technique has been applied to trace the source of *L. monocytogenes* contamination in vegetable (Aguado *et al.*, 2004) and poultry (Lawrence & Gilmour, 1995) processing plants. In the year-long study by Lawrence and Gilmour (1995), samples were taken throughout the year, cultured, and RAPD analysis was performed on the isolates. The results indicated that there were two strains of *L. monocytogenes* that remained and were persistent in the processing plant throughout the year. They were able to determine the source of the contamination as the

incoming birds and also demonstrated that there are transitory strains that were isolated which probably came from a variety of contamination sources.

In a study by Ertas and Seker (2005) the presence and genetic variation of *L. monocytogenes* in fresh fish was investigated. Of the 150 fish (Capoeta capoeta umbla) caught, only 10 fish were positive for *L. monocytogenes*. The genetic variation of these 10 isolates were analyzed by RAPD and two distinctive and reproducible RAPD profiles were generated. The different band profiles indicate the feasibility that the *L. monocytogenes* isolates are from different sources. However, the genetic relationship between the isolates needed more discriminative typing techniques (Ertas & Seker, 2005).

Franciosa *et al.* (2001) investigated 32 *L. monocytogenes* strains from listeriosis outbreaks in Italy using AP-PCR, PCR-ribotyping and infrequent-restriction-site PCR (IRS-PCR). The discriminatory ability of the three techniques was evaluated and was found to be 0.714, 0.690 and 0.919 for PCR-ribotyping, AP-PCR and IRS-PCR, respectively. IRS-PCR identified three clusters among the strains of the invasive listeriosis outbreak compared to only two clusters by PCR-ribotyping and AP-PCR each. Within each of the two non-invasive listeriosis outbreaks, the patterns obtained were practically identical, confirmed by all three techniques. Only IRS-PCR could clearly discriminate between the strains of the non-invasive and the invasive listeriosis outbreaks.

AP-PCR and RAPD are faster and cheaper than other subtyping techniques and, therefore, especially appropriate when testing less than fifty strains (Farber & Addison, 1994; O'Donoghue *et al.*, 1995; Louie *et al.*, 1996). AP-PCR can also be used effectively for microbial source tracking and the results obtained can give an indication of contamination sites within a food processing plant (Lawrence & Gilmour, 1995;

Churchill *et al.*, 2006). However, the inconsistency in the discriminatory ability is the disadvantage (Farber & Addison, 1994; O'Donoghue *et al.*, 1995). Franciosa *et al.* (2001) reported that AP-PCR gave less discriminatory results than ribotyping for the subtyping of *L. monocytogenes* isolates involved in listeriosis outbreaks.

2.6.1.2 PCR-Restriction fragment length polymorphism (PCR-RFLP) of *L. monocytogenes*

This method uses restriction endonucleases to cut DNA into fragments of different lengths to obtain different band patterns. These band patterns can then be used to define diversities in the genetic profiles of the organism (Smith & Nelson, 1999). When genomic DNA is digested using restriction endonucleases, separated using electrophoresis and analysed, different electrophoretic patterns of DNA bands are visible, with a very high number of bands. Using genomic DNA would result in a gel containing an abundant amount of DNA bands without adequate distinction between them. This makes the interpretation of the results very difficult (Churchill *et al.*, 2006). It is, therefore, preferred to use an individual gene to minimize the amount of DNA bands visible on the gel, which will result in an easier interpretation of the different patterns. The method is, however, time consuming due to the need for using pure cultures (Paillard *et al.*, 2003).

There are variations in the pathogenic *L. monocytogenes* isolates obtained from food samples and this could be due to deletions of one or more genes encoding for virulence factors (Doumith *et al.*, 2004b). However, there are also studies which indicated that such virulence genes were regarded as a stable part of the *L. monocytogenes* genome (Jaradat *et al.*, 2003; Doumith *et al.*, 2004b). The point mutations of the internalin genes could be responsible to the reduced virulence in *L.*

monocytogenes and the production of a truncated internalin gene. PCR-RFLP can be applied to recognize the internalin genes polymorphism (Rousseaux *et al.*, 2004).

2.6.1.3 REP-PCR

L. monocytogenes, like other prokaryotic organisms, contains a genome with repetitive sequence elements, which are randomly dispersed throughout the genome. They include enterobacterial repetitive intergenic consensus sequences (ERICs) which contains 124-147 base pairs (bp) and have a highly conserved central inverted repeat and repetitive extragenic palindromes (REPs) which contain 35-40 bp and which also have an inverted repeat. These ERIC and REP sequences are both ideal primer binding sites for PCR amplification and could be used for both species and strain discrimination. REP-PCR is known as an alternative subtyping method for *L. monocytogenes*. It is rapid, less expensive and has a similar level of discrimination to ribotyping and PFGE methods (Chou & Wang, 2006; Liu, 2006).

2.6.2 Ribotyping

Polymorphisms associated with ribosomal RNA operons are detected in DNA-DNA hybridizations employing Southern hybridization. An rRNA gene probe is appropriately labeled and is allowed to hybridize on a nylon membrane. For the most part EcoRI has been the preferred restriction enzyme to digest the genomic DNA of *L. monocytogenes* (Graves *et al.*, 1999). Ribotyping has been extensively employed for subtyping *L. monocytogenes* (Graves *et al.*, 1994; Bruce *et al.*, 1995; Weidmann *et al.*, 1997). The Riboprinter (DuPont-Qualicon, Wilmington, DE) is an automated ribotyping system that produces and analyzes ribotyping patterns of bacteria. Therefore, this method has the advantage of automation and standardization. However, resolution is

relatively limited, and ribotyping using the Riboprinter is typically limited to reference laboratories due to the high cost of the equipment.

2.6.3 Pulsed-field gel electrophoresis (PFGE)

PFGE is based on the RFLP method and allows the differentiation of large DNA fragments (10 kbp – 2000 kbp). The size limit of a normal agarose gel is around 20 to 40 kilo base pairs (kb). If larger fragments are separated, they would migrate at the same rate and be visual on the gel as a single band (Churchill *et al.*, 2006). PFGE uses this characteristic as a basis for further separation. Large DNA fragments takes longer than smaller fragments to change into their elongated shapes for movement. By changing the direction of the electric field, it allows the smaller fragments to alter their shape faster and thus commence migration at the limiting mobility rate. By changing the angles and times of the electrophoretic field, it allows the resolution of larger DNA fragments (Moore & Datta, 1994; Finney, 2000).

PFGE is divided into three steps, namely the preparation of agarose plugs with unbroken genomic DNA incorporated into it, the digestion of these plugs with infrequently cutting restriction nucleases to produce large digested fragments (Yde & Genicot, 2004) and the electrophoresis of these fragments using PFGE, allowing separation of fragments ranging in size from 10,000 base pairs (10 kb) to 2 million base pairs (2 Mb) (Smith *et al.*, 1987; Finney, 2000). The preparation of agarose plugs minimizes the shearing and loss of DNA, which are commonly associated with the liquid phase phenol extraction method. The DNA is, therefore, more intact and can be stored for longer periods. The preparation of these plugs also decreases the risk of exposure of virulent human pathogens to laboratory workers (Nair *et al.*, 1999).

Several authors have applied this technique successfully for the epidemiological investigations of listeriosis (Brosch *et al.*, 1991, 1994; Buchrieser *et al.*, 1993; Nguyen *et al.*, 1994; Jacquet *et al.*, 1995; Proctor *et al.*, 1995; Louie *et al.*, 1996). Subtypes within isolates which is indistinguishable by other subtyping methods are identifiable by PFGE (Buchrieser *et al.*, 1993; Brosch *et al.*, 1991, 1994; Jacquet *et al.*, 1995; Louie *et al.*, 1996).

L. monocytogenes isolates from two listeriosis outbreaks in the United States were subtyped by PFGE (Moore & Datta, 1994). Moore and Datta (1994) digested the DNA with *Sma*I and analyzed the band patterns. They found that the two listeriosis outbreaks were not as closely related as previous data from serotyping suggested. In terms of banding patterns, the isolates of each individual outbreak was more closely related to one another, than between outbreaks. However, the isolates of *L. monocytogenes* had the same serotype thus, the serotyping suggested that the two outbreaks might be clonal (Moore & Datta, 1994).

PFGE is currently regarded as the gold standard subtyping method, due to the high discrimination of this technique, reproducibility and the fact that the method is standardized (Gerner-Smidt *et al.*, 2006). The advantage of PFGE being highly reproducible permits the construction of a database based on the different band patterns obtained. This database can then be used for surveillance and also aid in determining the possible vehicle of transmission or infection in listeriosis outbreaks or contaminations in processing plants (Lyytikainen *et al.*, 2006). While PFGE has the advantage of providing a lot of information regarding strains and their differences, it is time consuming, requiring at least three days to obtain results (Finney, 2000).

2.7 Antimicrobial susceptibility test

Many antimicrobial drugs inhibit *Listeria* in vitro. Some literature reported that, *L. monocytogenes* is susceptible to wide range of antimicrobials like ampicillin, erythromycin, kanamycin, streptomycin, nalidixic acid and others except for cephalosporin and fosomycin/ fluoroquinolones (Abelardo *et al.*, 2001; Hansen *et al.*, 2005; Aarestrup *et al.*, 2007). However, it has been reported that resistant *L. monocytogenes* strains were found frequently from time to time, even to common conventional drugs like clindamycin, sulfomethoxazole (Shen *et al.*, 2006) and also to enrofloxacin (Antunes *et al.*, 2002). Plasmid-borne resistance to chloramphenicol, macrolides, and tetracycline has also been identified (FDA/CFSAN, 2003).

2.8 Treatment and prevention

L. monocytogenes is a poor competitor (Desse & Taye, 2001) that does not grow in the presence of high competitor organisms like Lactic Acid Bacteria. Some scientists use this principle for inhibiting its growth from different food samples. Listeriosis can be treated by using conventional antimicrobials like ampicillin and penicillin following diagnosis. However, the antimicrobial profile of the organism at particular place, like within the country, and at the individual level has to be known to be effective in treating patients and in reducing blind antimicrobial treatment, which may lead to the emergence of antimicrobial drug resistant strains of *L. monocytogenes*.

Antibiotic therapy is the treatment of choice in most of the complications and the dose and duration of the treatment differ accordingly. For instance, bacteriamia should be treated for 14 days, if the patient is immuno-competent. Similarly, meningitis should be treated for 21 days; while endocarditis for 4-6 weeks; and brain abscess for a minimum of 6 weeks. Ampicillin without or with gentamycin is generally considered as

the preferred agent but other effective agents like co-trimaxazole can be used for empirical antimicrobial treatment (Beek *et al.*, 2002; Hansen *et al.*, 2005).

Preventive measures have to target the organism's nature (conditions for its normal growth) in addition to good sanitation and adequate heat treatment of food before consuming. Moreover, its growth (doubling time) is much related on the pH, temperature, type of the food sample and background of the microflora present (Morrow *et al.*, 2004). The ability of growth on widely temperatures (1°C to 45°C) is one of the important characters in *L. monocytogenes* strains. There are large strain-to-strain variations but some strains seem to be able to grow down to about –1.5°C. The relationship between temperature and *L. monocytogenes* rate of multiplication was studied and reported that the slower rate of multiplication was observed at colder temperatures (Johan *et al.*, 2004). *L. monocytogenes* is perished by pasteurization (72 to 75°C) such as non-sporing Gram-positive pathogens (Doyle, 1999).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Collection of samples

A total of 250 RTE samples were purchased from Carrefour (Midvalley Mega Mall), Jusco (Midvalley Mega Mall), Pasar Chow Kit at PWTC, Pasar malam PJ-17, food stalls around University of Malaya and University LRT station. A variety of RTE food samples were purchased, including beverages (n=5; orange-flavoured drinks), cooked chicken and chicken products (n=60; non-spicing fried chicken heart, gizzard, leg and breast meat, satay, sausage, *etc*), cooked beef and beef products (n=13; cooked beef meat, sausage, *etc*), cooked egg and egg products (n=28; fried eggs and hard-boiled eggs without the shells, *etc*), packed lunch (n=6), salads and vegetables (n=85; salad, potato salad, fruit salad, cucumber, tomatoes, been sprout, lettuce, *etc*), cooked seafood and seafood products (n=40; fried fish, sushi, fish role, *etc*) and other type of RTE foods (n=13). Isolated *Listeria* spp., samples and locations of sampling are listed in APPENDIX I.

3.1.2 Chemicals, reagents, media, buffers and solutions

Chemicals and reagents used in this study are listed in APPENDIX II. All the media used for isolation, identification and culturing *Listeria* spp. and *L. monocytogenes* are listed in APPENDIX III. Buffers and solutions used in this study are listed in APPENDIX IV.

3.2 Methods

3.2.1 Isolation and identification of *Listeria* spp. and *L. monocytogenes*

3.2.1.1 Conventional methods

In conventional method, different enrichment and selective media could be used for isolation and detection of *Listeria* spp. and *L. monocytogenes* and presumptive colonies are identified by biochemical tests. For isolation of *Listeria* spp. and *L. monocytogenes* from RTE foods three types of media (pre-enrichment medium, enrichment medium, and selective media) were used.

3.2.1.2 Pre-enrichment media

Pre-enrichment media are non-selective and are used for growing bacteria such as half Fraser broth (O'Grady *et al.*, 2009) and *Listeria* enrichment broth (Bang *et al.*, 2013). Half Fraser Broth was used for the first enrichment of the samples in order to provide a suitable situation for *Listeria* spp. to grow and reach to a detectable level for the presumptive identification. A dilution of 10^{-1} was prepared with the samples by combining 25 g of each RTE food 225 ml of half Fraser broth in a sterile stomacher bag. The samples were stomached for 2 min in order to homogenize the mixture. The solution was then incubated at 30±1°C for 24 hours.

3.2.1.3 Enrichment media

Fraser broth was used for detection of *Listeria* spp. A dilution of 10^{-1} was prepared by adding 0.1 ml of half Fraser broth in 10 ml of Fraser broth. The broth was then incubated for 48 hours at 37°C.

3.2.1.4 Selective media

Three types of selective media including CHROMagar™ *Listeria*, *Listeria* selective agar (LSA) and Palcam agar were used for isolation of *Listeria* spp. and *L. monocytogenes*.

3.2.2 Procedure for isolation of *L. monocytogenes*

Listeria spp. were analysed according to the ISO 11290 method (Becker *et al.*, 2006) for detection with a two-step enrichment with half-Fraser (Oxoid, Basingstoke, UK) and Fraser (Oxoid, Basingstoke, UK) enrichment broths. Briefly, 25 g of samples were added to 225 ml of half Fraser broth as the first enrichment culture in stomacher bag and were homogenized in a stomacher (Lab blender 400, Seward Medical, London, UK) and incubated for 24 h at 30 ±1°C. A loopful of first enriched broth culture was streaked on CHROMagar™ *Listeria* and incubated for another 24-48 h at 37 °C. On the other hand, 0.1ml of half Fraser broth was added to 10 ml of Fraser broth as a second enrichment culture and incubated at 37 °C for 48 h. Then, a loopful of enriched Fraser broth-culture was streaked onto LSA and PALCAM agar and incubated for 24-48 h at 37 °C. Then, presumptive colonies were re-streaked on tryptic soy agar (Oxoid, Basingstoke, UK) with 0.6% yeast extract (Oxoid, Basingstoke, UK) (TSAYE) as a non-selective medium and incubated at 37 °C for 24 h. The colonies from TSAYE were confirmed using biochemical tests (Gram determination, catalase, oxidase, SIM, TSI, indole and MR-VP) and Polymerase Chain Reaction (PCR).

3.3 Confirmation *L. monocytogenes* by Polymerase Chain Reaction

3.3.1 Preparation of DNA template for PCR

Bacterial cultures on TSAYE agar plates were used to extract crude DNA, by the use of boiling cell extraction method. Three to 5 single pure colonies were suspended in 100 µl of double distilled water and vortexed shortly. The suspension was heated at 99°C for 10 minutes and then was chilled in ice for 10 minutes. The cell debris was pelleted by centrifugation at 13,400 g for 2 minutes and 5 µl of clear supernatant was used as the DNA template in a PCR.

3.3.2 Specific oligonucleotide primers for identification *Listeria* spp. and *L. monocytogenes*

The primers used in duplex PCR targeting 16S rRNA and *LLO* genes were applied to confirmation of *Listeria* spp. and *L. monocytogenes* (Rossmanith *et al.*, 2006). Primer sequences used in the duplex PCR and the size of the expected amplicons are presented in Table 3.1.

Table 3.1: The primer sequences used for confirmation of *Listeria* spp. and *L. monocytogenes* and the size of the expected amplicons.

Primers	Sequence (5'-3')	Target gene	Specificity	Size (bp)
U1	AGCMGCCGCGGTAATWC	16S rRNA	*Listeria* spp.	938
LI1	CTCCATAAAGTTGACCT			
LM1	CCTAAGACGCCAATCGAA	*LLO*	*L. monocytogenes*	701
LM2	AAGCGCTTGCAACTGCTC			

3.3.3 Reaction mixture and cycling condition for amplification 16S rRNA and *LLO* gene

The total volume of the duplex PCR reaction was 25.0 µl that contained, 1× GoTaq™ Green buffer, 1.5 mM $MgCl_2$, 200 µM each dNTPs, 0.5µM each primers (U1/LI1, LM1/LM2), 1 Unit of Taq Polymerase, and 50 ng DNA template (Table 3.2). The PCR reaction was carried out using the following cycling conditions: pre-denaturation at 95°C for 4 min, 30 cycles of denaturation at 95 °C for 1.5 min, primer annealing at 52 °C for 45 s, primer extension at 72 °C for 2 min, and a final extension at 72 °C for 8 min. The PCR products were then separated using electrophoresis on 1.5% agarose gel. After gel electrophoresis, the gels were stain by ethidium bromide (0.5 µg/ml) and destain three times by H_2O and then visualized under UV (Gel Doc™ XR System, BIO-RAD, CA, USA).

Table 3.2: The volumes of the components used for the duplex PCR targeting 16S rRNA and *LLO* gene.

Component	Stock concentration	Reaction concentration	Volume (µl)
DNA template	--	--	5
Buffer	5 X	1X	5
$MgCl_2$	25 mM	1.5 mM	1.5
dNTPs	10 mM	200 µM	0.5
Primers (U1/LI1)	100 µM each	0.5 µM each	0.125 µl each
Primers (LM1/LM2)	100 µM each	0.5 µM each	0.125 µl each
Taq DNA polymerase	5 U	1 U	0.2
ddH_2O	--	--	Make to 25
Total volume	--	--	25

3.4 Rapid detection assay for *Listeria* spp. and *L. monocytogenes* by Polymerase Chain Reaction

Direct PCR was performed with the crude DNA extracted from the enriched broth cultures. Briefly, after 24 hours of incubation in first enrichment (half Fraser broth), 1ml of first enriched broth was transferred into another test tube containing 9 ml of second enrichment broth culture (Fraser broth). The tube was incubated at 37°C for 4 hours. DNA extraction was done by boiling method. An aliquot of 500µl of enriched broth culture was transferred into microcentrifuge tube. The sample was centrifuged at 10,000 ×g for 5 minutes. The pellet was resuspended in 500µl of sterile distilled water. Then, the sample was boiled for 10 minutes and after that cooled at -20°C for 10 minutes. Therefore, it was centrifuged at 10,000 ×g for 10 minutes and the supernatant was used as DNA template in the PCR.

The nucleotide sequences of the primers used in the PCR which specifically amplify the 938 bp region in the 16S rRNA which shows all *Listeria* spp. and the 701 bp region in the listeriolysin O (*LLO*) gene that shows *L. monocytogenes* (Rossmanith *et al.*, 2006).

The total volume of the duplex PCR reaction was 25.0 µl that contained, 1× GoTaq™ Green buffer, 1.5 mM $MgCl_2$, 200 µM each dNTPs, 0.5µM each primers (U1/LI1, LM1/LM2), 1 Unit of Taq Polymerase, and 50 ng DNA template (Table 3.2). The PCR reaction was carried out using the following cycling conditions: pre-denaturation at 95°C for 4 min, 30 cycles of denaturation at 95 °C for 1.5 min, primer annealing at 52 °C for 45 s, primer extension at 72 °C for 2 min, and a final extension at 72 °C for 8 min. The PCR products were then separated using electrophoresis on 1.5% agarose gel. After gel electrophoresis, the gels were stain by ethidium bromide (0.5 µg/ml) and destain three times by H_2O and then visualized under UV (Gel Doc™ XR System, BIO-RAD, CA, USA).

3.5 Multiplex PCR Differentiation of *L. monocytogenes* Serogroups

The multiplex PCR serogrouping assay developed by Doumith *et al.* (2004a) was used to determine serogroups of *L. monocytogenes* isolates. The multiplex PCR was performed in a total volume of 25 µl using 1× GoTaq™ Green buffer, 2 mM $MgCl_2$, 200µM dNTPs, 1µM each for lmo0737 and ORF2110 primer, 1.2µM each for ORF2819 primer, 1.5µM each for lmo118, 0.2µM each for prs, 1.25 Unit Taq Polymerase and 50 ng DNA template (Tables 3.3 and 3.4). The PCR program consisted of an initial denaturation of 3 min at 94°C, followed by 35 cycles of 94°C for 25 s, 53°C for 70s, 72°C for 70 s and a final extension of 7 min at 72°C. The volumes of the components used for the PCR have been shown in Tables 3.3. Electrophoresis on 2% agarose gel was done to separate the PCR products. After gel electrophoresis, the gels were stain by ethidium bromide (0.5 µg/ml) and destain three times by H_2O and then visualized under UV (Gel Doc^{TM} XR System, BIO-RAD, CA, USA).

Table 3.3: The primer sequences used for the multiplex PCR serogrouping.

Primers	Sequence (5' to 3')	Target gene	Specificity	Amplicon size (bp)	Reference
lmo0737-F	AGGGCTTCAAGGACTTACCC	*lmo0737*	1/2a, 1/2c, 3a, 3c	691	Doumith *et al.*, 2004a
lmo0737-R	ACGATTTCTGCTTGCCATTC				
lmo1118-F	AGGGGTCTTAAATCCTGGAA	*lmo1118*	1/2c, 3c	906	
lmo1118-R	CGGCTTGTTCGGCATACTTA				
ORF2819-F	AGCAAAATGCCAAAACTCGT	ORF2819	1/2b, 3b, 4b, 4d, 4e	471	
ORF2819-R	CATCACTAAAGCCTCCCATTG				
ORF2110-F	AGTGGACAATTGATTGGTGAA	ORF2110	4b, 4d, 4e	597	
ORF2110-R	CATCCATCCCTTACTTTGGAC				
prs-F	GCTGAAGAGATTGCGAAAGAAG	*prs*	*Listeria* species	370	
Prs-R	CAAAGAAACCTTGGATTTGCGG				

Table 3.4: The volumes of the components used for the multiplex PCR serogrouping

Component	Stock concentration	Reaction concentration	Volume (µl)
DNA template	--	--	5
Buffer	5 X	1X	5
$MgCl_2$	25 mM	2 mM	2
dNTPs	10 mM	200 µM	0.5
Primers (ORF2110-F, R)	100 µM each	1 µM each	0. 25 µl each
Primers (ORF2819-F, R)	100 µM each	1.2 µM each	0.3 µl each
Primers (lmo0737-F, R)	100 µM each	1 µM each	0. 25 µl each
Primers (lmo1118-F, R)	100 µM each	1.5 µM each	0.375 µl each
Primers (prs-F, R)	100 µM each	0.2 µM each	0.05 µl each
Taq DNA polymerase	5 U	1.25 U	0.25
ddH_2O	--	--	Make to 25
Total volume	--	--	25

3.6 Multiplex PCR to determine virulotypes of *L. monocytogenes*

A multiplex-PCR comprising of primers that target the three internalin genes (*inlA, inlC* and *inlJ*) from Liu *et al.* (2007) and an addition primer targeting the *inlB* (designed in this study) was used to determine the virulotypes. The cycling conditions were as described by Liu *et al.* (2007). The *inlB* primers designed in this study target the 361 bp region in the *inlB* gene. Representative amplicons of *inlA, inlB, inlC* and *inlJ* were purified by using MEGA quick-spin PCR and agarose Gel DNA extraction kits (Intron Biotechnology, Korea) and sequenced to validate their identities by comparing against reference strains in NCBI database. All experiments were repeated once to confirm their reproducibility.

Detection of the internalin genes was performed in a total volume of 25 µl using 1× GoTaq™ Green buffer, 2.5 mM $MgCl_2$, 400 µM dNTPs, 5.2µM each for inlA primer, 1µM each for inlB-361 primer, 2.6µM each for inlC primer, 0.28µM each for inlJ primer, 1.5 Unit Taq Polymerase, and 50 ng DNA template. The PCR program consisted of an initial denaturation of 2 min at 94°C, followed by 30 cycles of 94°C for 20 s, 55°C for 20 s, 72°C for 50 s and a final extension of 2 min at 72°C. The primers sequencing and the volumes of the components used for the PCR have been shown in Tables 3.5 and 3.6, respectively. Electrophoresis on 1.5% agarose gel was done to separate the PCR products. After gel electrophoresis, the gels were stain by ethidium bromide (0.5 µg/ml) and destain three times by H_2O and then visualized under UV (Gel Doc™ XR System, BIO-RAD, CA, USA). The primers sequencings are listed in APPENDIX VIII.

Table 3.5: The primer sequences used for virulotyping.

Primers	Sequence	Target gene	Amplicon size (bp)	References
InlA-F	5'- ACGAGTAACGGGACAAATGC-3'	*inlA*	800	Liu *et al.*, 2007
inlA	5'- CCCGACAGTGGTGCTAGATT -3'			
InlB-361-F	5'- AGGGGTCTTAAATCCTGGAA -3'	*inlB*	361	This study
InlB-361-R	5'- GGGCTTGTTCGGCATACTTA -3'			
inlC-F	5'- AATTCCCACAGGACACAACC -3'	*inlC*	517	Liu *et al.*, 2007
inlC-R	5'- CGGGAATGCAATTTTTCACTA- 3'			
inlJ-F	5'- TGTAACCCCGCTTACACAGTT -3'	*inlJ*	238	
inlJ-R	5'- AGCGGCTTGGCAGTCTAATA -3'			

Table 3.6: The volumes of the components used for virulotyping

Component	Stock concentration	Reaction concentration	Volume (µl)
DNA template	--	--	5
Buffer	5 X	1X	5
$MgCl_2$	25 mM	2.5 mM	2.5
dNTPs	10 mM	400 µM	1
Primers (inlA-F, R)	100 µM each	5.2 µM each	1.3 µl each
Primers (inlB-361-F, R)	100 µM each	1 µM each	0.25 µl each
Primers (inlC-F, R)	100 µM each	2.6 µM each	0.65 µl each
Primers (inlJ-F, R)	100 µM each	0.28 µM each	0.07 µl each
Taq DNA polymerase	5 U	1.5 U	0.3
ddH_2O	--	--	Make to 25
Total volume	--	--	25

3.7 PCR-Restriction fragment length polymorphism (-RFLP)

3.7.1 PCR-RFLP of *inlA*

For the PCR assay the primer pair consisting of inlA-F (5'-ACG AGT AAC GGG ACA AAT GC-3') and inlA-R (5'-CCC GAC AGT GGT GCT AGA TT-3') was used to amplify an 800 bp fragment of the *inlA* gene (Liu *et al.*, 2007). Each PCR reaction was performed in a total reaction volume of 25 µl containing 1× PCR buffer, 2.5 mM $MgCl_2$, 400 µM dNTPs, 2.4 µM each for inlA primer and 5 µl of DNA template (Table 3.7). The cycling conditions were as follows: initial denaturation 2 min at 94°C, followed by 30 cycles of 94°C for 20 s, 55°C for 20 s, 72°C for 50 s and a final extension of 2 min at 72°C. Each restriction digestion was performed in a total reaction volume of 20 µl containing either 5 U *Xba*I, 5 µl amplicon, 2 µl RE 10× buffer, 0.2 µl BSA and 12.3 µl ddH_2O. The *Xba*I samples were incubated at 37°C for 4 hours. Digestion products were then separated on a 1.5 % agarose gel. After gel electrophoresis, the gels were stain by ethidium bromide (0.5 µg/ml) and destain three times by H_2O and then visualized under UV (Gel Doc^{TM} XR System, BIO-RAD, CA, USA).

Table 3.7: The volumes of the components used for the monoplex PCR amplifications targeting *inlA* gene

Component	Stock concentration	Reaction concentration	Volume (µl)
DNA template	--	--	5
Buffer	5 X	1X	5
$MgCl_2$	25 mM	2.5 mM	2.5
dNTPs	10 mM	400 µM	1
Primers (inlA-F, R)	100 µM each	2.4 µM each	0.6 µl each
Taq DNA polymerase	5 U/µl	1 U	0.2
ddH_2O	--	--	Make to 25
Total volume	--	--	25

3.7.2 PCR-RFLP of *inlC*

For the PCR assay the primer pair consisting of inlC-F (5'-AAT TCC CAC AGG ACA CAA CC-3') and inlC-R (5'-CGG GAA TGC AAT TTT TCA CTA -3') was used to amplify a 517 bp fragment of the *inlC* gene (Liu *et al.*, 2007). Each PCR reaction was performed in a total reaction volume of 25 µl containing 1× PCR buffer, 2.5 mM $MgCl_2$, 400 µM dNTPs, 2.4 µM each for inlC primer and 5 µl of DNA template (Table 3.8). The cycling conditions were as follows: initial denaturation 2 min at 94°C, followed by 30 cycles of 94°C for 20 s, 55°C for 20 s, 72°C for 50 s and a final extension of 2 min at 72°C. Each restriction digestion was performed in a total reaction volume of 20 µl containing either 5 U *Alu*I, 5 µl amplicon, 2 µl RE 10 × buffer, 0.2 µl BSA and 12.3 µl ddH_2O. The *Alu*I samples were incubated at 37°C for 4 hours. Digestion products were then separated on a 1.5 % agarose gel. After gel electrophoresis, the gels were stain by ethidium bromide (0.5 µg/ml) and destain three times by H_2O and then visualized under UV (Gel Doc^{TM} XR System, BIO-RAD, CA, USA).

Table 3.8: The volumes of the components used for the monoplex PCR amplifications targeting *inlC* gene

Component	Stock concentration	Reaction concentration	Volume (µl)
DNA template	--	--	5
Buffer	5 X	1X	5
$MgCl_2$	25 mM	2.5 mM	2.5
dNTPs	10 mM	400 µM	1
Primers (*inlC*-F, R)	100 µM each	2.4 µM each	0.6 µl each
Taq DNA polymerase	5 U/µl	1 U	0.2
ddH_2O	--	--	Make to 25
Total volume	--	--	25

3.8 PCR-based Fingerprinting

3.8.1 Repetitive Extragenic Palindromic (REP)-PCR

REP-PCR was done by the use of REP primer as described by Navia *et al.* (1999). PCR was performed in a total volume of 25 µl which contained 50 µM of each dNTPs, 1× PCR buffer, 2.5 mM $MgCl_2$, 0.6 µM of primer and 1.0 U Taq DNA polymerase (Promega). The PCR reaction was carried out using the following cycling conditions: pre-denaturation at 94°C for 4 min, 35 cycles of denaturation at 94°C for 1 min, primer annealing at 42°C for 1 min, primer extension at 68°C for 8 min and a final extension at 72°C for 8 min. The volumes of the components used for REP-PCR have been shown in Table 3.9. Electrophoresis on 1.5% agarose gel was used to separate the PCR products. After gel electrophoresis, the gels were stain by ethidium bromide (0.5 µg/ml) and destain three times by H_2O and then visualized under UV (Gel Doc^{TM} XR System, BIO-RAD, CA, USA). The gel photos of the REP-PCR products were used for data analysis using BioNumerics 6.0 (Applied Maths, Kortrijk, Belgium).

Table 3.9: The volumes of the components used for REP-PCR

Component	Stock concentration	Reaction concentration	Volume (µl)
DNA template	--	--	5
Buffer	5 X	1X	5
$MgCl_2$	25 mM	2.5 mM	2.5
dNTPs	10 mM	50 µM	0.2
REP primer	10 µM	0.6 µM	0.15 µl
Taq DNA polymerase	5 U/µl	1 U	0.2
ddH_2O	--	--	Make to 25
Total volume	--	--	25

3.8.2 BOX-PCR

BOX-PCR was done by the use of BOX A1R primer as described by Versalovic et al. (1994). PCR was performed in a total of volume of 25 µl which contained 400 µM of each dNTPs, 1× PCR buffer, 3 mM $MgCl_2$, 4 µM of primer and 2.5 U Taq DNA polymerase (Promega). The PCR reaction was carried out using the following cycling conditions: pre-denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 1 min, primer annealing at 40°C for 2 min, primer extension at 72°C for 2 min and a final extension at 72°C for 10 min. The volumes of the components used for BOX-PCR have been shown in Table 3.10. Electrophoresis on 1% agarose gel was used to separate the PCR products. After gel electrophoresis, the gels were stain by ethidium bromide (0.5 µg/ml) and destain three times by H_2O and then visualized under UV (Gel Doc™ XR System, BIO-RAD, CA, USA). The gel photos of the BOX-PCR products were used for data analysis using BioNumerics 6.0 (Applied Maths, Kortrijk, Belgium).

Table 3.10: The volumes of the components used for BOX-PCR

Component	Stock concentration	Reaction concentration	Volume (µl)
DNA template	--	--	5
Buffer	5 X	1X	5
$MgCl_2$	25 mM	3 mM	3
dNTPs	10 mM	400 µM	1
BOX A1R primer	100 µM	4 µM	1 µl
Taq DNA polymerase	5 U/µl	2.5 U	0.5
ddH_2O	--	--	Make to 25
Total volume	--	--	25

3.8.3 RANDOM Amplification of Polymorphic DNA (RAPD)

RAPD was done by the use of 15OPA primer as described by Lee *et al.* (2011). PCR was performed in a total of volume of 25 µl which contained 400 µM of each dNTPs, 1× PCR buffer, 3 mM $MgCl_2$, 4 µM of primer and 2.5 U Taq DNA polymerase (Promega). The PCR reaction was carried out using the following cycling conditions: pre-denaturation at 94°C for 4 min, 45 cycles of denaturation at 94°C for 1 min, primer annealing at 36°C for 1 min, primer extension at 72°C for 2 min and a final extension at 72°C for 4 min. The volumes of the components used for RAPD-PCR have been shown in Table 3.11. Electrophoresis on 1% agarose gel was used to separate the PCR products. After gel electrophoresis, the gels were stain by ethidium bromide (0.5 µg/ml) and destain three times by H_2O and then visualized under UV (Gel Doc^{TM} XR System, BIO-RAD, CA, USA). The gel photos of the BOX-PCR products were used for data analysis using BioNumerics 6.0 (Applied Maths, Kortrijk, Belgium).

Table 3.11: The volumes of the components used for RAPD-PCR

Component	Stock concentration	Reaction concentration	Volume (µl)
DNA template	--	--	5
Buffer	5 X	1X	5
$MgCl_2$	25 mM	3 Mm	3
dNTPs	10 mM	400 µM	1
BOX A1R primer	100 µM	4 µM	1 µl
Taq DNA polymerase	5 U/µl	2.5 U	0.5
ddH_2O	--	--	Make to 25
Total volume	--	--	25

3.9 Pulsed Field Gel Electrophoresis (PFGE)

3.9.1 Preparation of DNA plugs

Sterile swabs moistened with sterile TE buffer were used to transfer colonies from the BHI agar plates to sterile Falcon tubes containing 2 ml TE buffer. The optical density (OD) at 610 nm was measured using the spectrophotometer and adjusted until a reading of 0.80 to1.00 was obtained for each sample by adding TE buffer or cells. This was done to ensure approximately equal concentrations of cells in the plugs. A 200 µl volume of the cell suspensions (OD = 0.80-1.00) were transferred to microcentrifuge tubes and 10 µl lysozyme (20 mg mL^{-1}) was added to each tube, the tube was gently mixed and incubated at 55° – 60 °C for 10 – 20 min in a water bath. A 10 µl volume of a 20 mg ml^{-1} Proteinase K solution was added to each tube. A 200 µl volume of melted 1 % SeaKem Gold agarose was added to the 220 µl cell suspension, mixed and immediately dispensed into plug moulds. The plugs were allowed to solidify in the refrigerator temperature (4°C) for 5 min. The plugs were kept out into 5 ml Proteinase K/Cell Lysis Buffer (5 ml Cell Lysis Buffer and 25 µl Proteinase K stock solution (20 mg ml^{-1}) and incubated in a 54°C water bath for an overnight. The Proteinase K/Cell Lysis Buffer was replaced with 15 ml sterile ddH_2O and incubated in a 50°C shaker

water bath for 15 minutes. This washing procedure was repeated one more time with ddH_2O and six times with TE buffer. Plugs were then transferred to 5 ml sterile TE buffer and stored at 4 °C.

3.9.2 Restriction digestion

A sterile scalpel was used to cut a 2.0–2.5 mm wide slice of the *L. monocytogenes* embedded agarose plug. This plug was placed in a microcentrifuge tube containing 200 µl restriction enzyme solution (20 µl 10× Restriction Buffer, 2 µl BSA, 50 U *Apa*I and 173 µl ddH_2O) for digestion with *Apa*I and incubated at 37°C for an overnight. After enzyme digestion, the restriction enzyme solution was removed and the plugs were immediately loaded for gel electrophoresis. *Salmonella* H9812 was used as a marker in PFGE. The 2.0-2.5 mm wide slice of *Salmonella* H9812 was placed under 200 µl restriction enzyme solution (20 µl 10 × Restriction Buffer, 2 µl BSA, 20 U *Xba*I and 176 µl ddH_2O) in a microcentrifuge tube for digestion with *Xba*I and incubated at 37°C for 2-3 hours.

3.9.3 Pulsed-field gel electrophoresis

The restricted plugs were loaded into the wells of a 1 % Sigma agarose gel with 0.5 × TBE Buffer and the wells filled with melted 1% SeaKem Gold agarose and left to set at room temperature for 45 min. A CHEF DRIII system was used for the electrophoretic separation. Three liters of 0.5 X TBE buffer was poured into the chamber and allowed to cool to 14 °C. The current-switching parameters were as follows: 4 s initial switch time, 40 s final switch time at 6 V, buffer temperature at 14 °C and a total run time of 21 hours. After electrophoresis the gel was removed and stained in ethidium bromide for 30 minutes. The separated PFGE fragments were

visualized under an ultraviolet transilluminator (Gel Doc^{TM} XR System, BIO-RAD, CA, USA) and the captured images were used for analysis.

3.10 Antimicrobial susceptibility testing

Susceptibility tests were performed by standard disk diffusion method on Mueller Hinton Agar (MHA), following the procedures recommended by the Clinical and Laboratory Standards Institute (CLSI, 2010). *Listeria* spp. isolates were streaked on TSAYE agar and incubated at 37°C for 24 hours. Then, several single colonies were removed from the plate to Falcon tubes containing of sterile saline using a sterile cotton swab. The turbidity of the colonies with sterile saline was checked for the development of slight turbidity, against 0.5 MacFarland solutions. The cell suspension was then transferred onto the surface of Mueller-Hinton agar and then spread evenly. Antimicrobial discs (Oxoid Ltd, Basingstoke, UK) containing the following antibiotics were spotted with about 3 cm interval rifampicin 5µg, clindamycin 2µg, vancomycin 30µg, streptomycin 30µg, gentamicin 10µg, tetracycline 30µg, erythromycin 30µg, trimethoprim-sulfamethoxazole 25µg, kanamycin 30µg, amoxicillin-clavulanic acid 30µg (20/10), penicillin G 10 units, and chloramphenicol 30µg. After 16 to 18 hours of incubation at 37°C, the zone of inhibition around each disc was measured, and the results were interpreted as resistant, sensitive and intermediate.

3.11 Data Analysis

The banding patterns generated by RAPD, BOX-PCR and PFGE were analyzed by using BioNumerics 6.0 (Applied Maths, Kortrijk, Belgium) based on the weighted pair group method with the arithmetic average (UPGMA) with a position tolerance of

0.1. PCR fingerprints and PFGE profiles were assigned arbitrary designation, and differences were defined by the Dice coefficient of similarity, F.

All statistical and the Spearman's correlation analyses were performed using SPSS 18.0 (SPSS Inc., Chicago, IL, USA).

CHAPTER 4

RESULTS

4.1 Identification of *Listeria* spp. and *L. monocytogenes* isolates

4.1.1 Identification of *Listeria* spp. isolates by conventional methods

Out of 250 RTE food samples, 66 presumptive *Listeria* spp. isolates were identified by the following biochemical tests: Gram positive, catalase positive, oxidase negative, motility test at 20-25°C positive (umbrella motility), produced acid but not H_2S in TSI, indole negative, and MR-VP positive. The biochemical tests are listed in APPENDIX V. The results are described in Table 4.1 and are listed in APPENDIX I.

Table 4.1: Summary of biochemical reactions of *Listeria* spp. isolates

Tests	Observation
Gram determination Using the 2% KOH	No change in viscosity of the cell suspension
Catalase	Bubbling
Oxidase	No color change
Motility test	Umbrella motility
Glucose (TSI)	Yellow slant and butt
H_2S (TSI)	No blackening
Indole test	yellow color at surface
Methyl Red test	Diffuse red color
Voges Proskauer test	Diffuse red color

4.1.2 Confirmation of *Listeria* spp. and *L. monocytogenes* by Polymerase Chain Reaction

A duplex-PCR was used to detect the presence of *Listeria* spp. (U1/LI1) and *L. monocytogenes* (LM1/LM2) simultaneously (Rossmanith *et al.*, 2006). Primers U1/LI1 and LM1/LM2 amplified the 16S rRNA (938 bp) and Listeriolysin O gene (702 bp), respectively (Figure 4.1). Out of 66 presumptive *Listeria* spp. isolates, 32 and 20 isolates were confirmed as *L. monocytogenes* and non-*L. monocytogenes*, respectively.

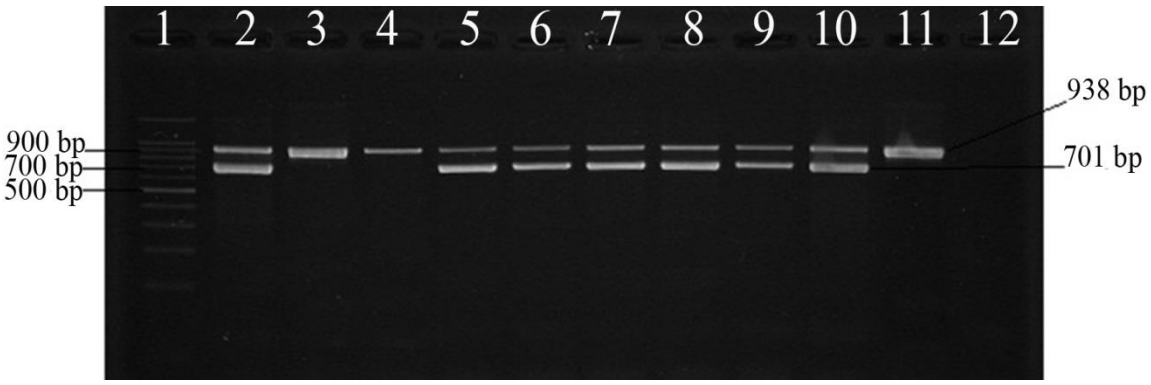


Figure 4.1: The representative agarose gel picture of PCR-amplified products of *Listeria* spp. and *L. monocytogenes. Listeria* spp. is indicated by a single band at 938 bp while *L. monocytogenes* is indicated by two bands, 938 bp and 701 bp. Lane 1, 100 bp molecular size marker; lane 2, positive control (*L. monocytogenes*, ATCC 19117); lanes 3, 4, and 11, Non-*L. monocytogenes*; lanes 5-10, *L. monocytogenes*; lane 12; negative control.

4.1.3 Detection of *Listeria* spp. and *L. monocytogenes* directly from foods homogenates

No positive amplification was observed when the duplex PCR was tested on samples harvested after 4 hours incubation in Half Fraser broth (first enrichment broth culture) and also after 24 in Fraser broth (second enrichment broth culture). *Listeria* spp. and *L. monocytogenes* only were detected after 48 hours incubation in Fraser broth in examined contaminated RTE samples.

Out of 250 foods homogenates, 48 samples showed the 16S rRNA band (938 bp) and considered as positive for *Listeria* spp. However, only 29 food samples were positive for *L. monocytogenes.* The results of detection of *Listeria* spp. and *L. monocytogenes* directly from foods homogenates are listed in APPENDIX I.

4.2 Distribution of *Listeria* species and *L. monocytogenes*

4.2.1 Distribution of *Listeria* spp. and *L. monocytogenes* isolated in RTE foods from street-side hawker stalls

Out of 250 studied RTE food samples, 155 (62%) were purchased from street-side hawker stalls in Selangor state from November 2010 to August 2011. Forty (25.8%) RTE foods were from Chow Kit, 75 (48.4%) from University LRT station, 20 (12.9%) were purchased from cafeterias in University of Malaya and 20 (12.9%) from PJ17 pasar malam (Table 4.2).

Listeria species was isolated from 31 (20%) of the samples (Table 4.2). Among these contaminated samples, 20 (64.5%) harboured *L. monocytogenes*. Ten (50%) of the RTE food samples positive for *L. monocytogenes* were from University LRT station, 4 (20%) were from PJ17 pasar malam (night market), 4 (10%) were from Chow Kit and 2 (10%) were from location University of Malaya.

Among the studied RTE food samples which were purchased from street-side hawker stalls, salad and vegetables showed the maximum present (24.3%) of *Listeria* species. *L. monocytogenes* was isolated from beverages (one out of 5 samples), chicken and chicken products (5 out of 29 samples), salads and vegetables (11 out of 70 samples), eggs and egg products (2 out of 20 samples) and seafood and seafood products (one out of 14 sample) (Table 4.3).

Table 4.2: Distribution of *Listeria* spp. and *L. monocytogenes* isolated in RTE foods from street-side hawker stalls by location

	Total (%)	***Listeria* spp. (%)**	***L. monocytogenes* (%)**
Chow Kit	40 (25.8)	6 (15)	4 (10)
University LRT station	75 (48.4)	16 (21.3)	10 (13.3)
University of Malaya	20 (12.9)	5 (25)	2 (10)
PJ17 Pasar malam	20 (12.9)	4 (20)	4 (20)
Total	155	31 (20)	20 (12.9)

Table 4.3: Distribution of *Listeria* species and *L. monocytogenes* isolated in RTE foods from street-side hawker stalls by food categories

	Total	***Listeria* spp. (%)**	***L. monocytogenes* (%)**
Beverage	5	1(20)	1(20)
Cooked beef and beef products	7	0	0
Cooked chicken and chicken products	29	7(24.1)	5(17.2)
Cooked egg and egg products	20	4(20)	2(10)
Salads and vegetables	70	17(24.3)	11(15.7)
Cooked seafood and seafood products	14	2(14.3)	1(7.1)
Other	10	0	0
Total	155	31(20)	20(12.9)

4.2.2 Distribution of *Listeria* spp. and *L. monocytogenes* isolated in RTE foods from hypermarkets

Out of 250 studied RTE food samples, 95 (38%) were purchased from hypermarkets over the course of six months from August 2011 through January 2012. Fifty six (58.9%) of the RTE food samples were from hypermarket X and 39 (41.1%) were from hypermarket Y. *Listeria* species was isolated from 21 (22.1%) of the 95 studied RTE food samples and 12 (57.1%) of contaminated samples were positive for *L. monocytogenes*. Hypermarkets X and Y had 7 (58.3%) and 5 (41.7%) positive samples for *L. monocytogenes*, respectively (Table 4.4).

Among the examined RTE food samples purchased from hypermarkets, most of food categories were contaminated with *Listeria* spp. However, *L. monocytogenes* was isolated from chicken and chicken products (4 out of 31 samples), eggs and egg products (2 out of 8 samples), packed lunch (one out of 6) salads and vegetables (3 out of 15 samples), and seafood and seafood products (2 out of 26 sample) (Table 4.5).

Table 4.4: Distribution of *Listeria* spp. and *L. monocytogenes* isolated in RTE foods from hypermarkets by location

	Total (%)	***Listeria* spp. (%)**	***L. monocytogenes* (%)**
Hypermarket X	56 (58.9)	10 (17.9)	7 (12.5)
Hypermarket Y	39 (41.1)	11 (28.2)	5 (12.8)
Total	95	21 (22.1)	12 (12.6)

Table 4.5: Distribution of *Listeria* spp. and *L. monocytogenes* isolated in RTE foods from hypermarkets by food categories

	Total	***Listeria* spp. (%)**	***L. monocytogenes* (%)**
Cooked beef and beef products	6	1 (16.7)	0
Cooked chicken and chicken products	31	5 (16.1)	4 (12.9)
Cooked egg and egg products	8	2 (25)	2 (25)
Packed lunch	6	2 (33.3)	1 (16.7)
Salads and vegetable	15	5 (33.3)	3 (20)
Cooked seafood and seafood products	26	6 (23.1)	2 (7.7)
Other	3	0	0
Total	95	21(22.1)	12 (12.6)

4.2.3 Distribution of *Listeria* spp. and *L. monocytogenes* isolated in RTE foods from both Street-side hawker stalls and hypermarkets

Overall, out of 250 samples, *Listeria* spp. was detected in 52 (20.8%) samples. Thirty two (61.5%) out of 52 *Listeria* species were identified to be *L. monocytogenes* (Table 4.6).

Among the 250 studied RTE food samples purchased from street-side hawker stalls and hypermarkets, *Listeria* spp. were isolated from most of food categories and *L. monocytogenes* was detected from salads and vegetables (14 out of 85 samples), chicken and chicken products (9 out of 60 samples), egg and egg products (4 out of 28 samples), seafood and seafood products (3 out of 40 samples) and packed lunch (1 out of 6 samples) (Figure. 4.2).

Table 4.6: Distribution of *Listeria* spp. and *L. monocytogenes* isolated in RTE foods by food categories

	Total	***Listeria* spp. (%)**	***L. monocytogenes* (%)**
Beverages	5	1 (20)	1 (20)
Cooked beef and beef products	13	1 (7.7)	0
Cooked chicken and chicken products	60	12 (20)	9 (15)
Cooked egg and egg products	28	6 (21.4)	4 (14.3)
Packed lunch	6	2 (33.3)	1 (16.7)
Salads and vegetables	85	22 (25.9)	14 (16.5)
Cooked seafood and seafood products	40	8 (20)	3 (7.5)
Other	13	0	0
Total	250	52 (20.8)	32 (12.8)

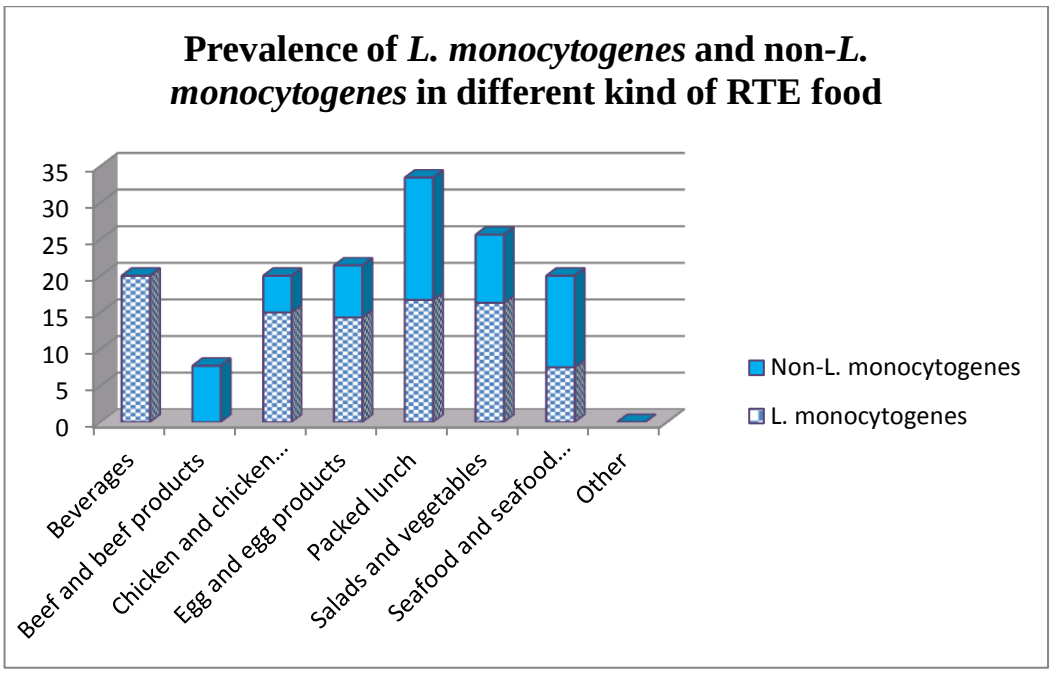


Figure 4.2: Prevalence (number of positive samples/total samples tested) of *L. monocytogenes* and non-*L. monocytogenes* in different kinds of RTE food samples collected from street-side hawker stalls and hypermarkets in different location of Kuala Lumpur and Petaling Jaya. The cultures were confirmed by duplex PCR targeting 16S rRNA and *LLO* gene.

4.3 Distribution of *L. monocytogenes* serogroups

A multiplex PCR was used to serogroup *L. monocytogenes* isolates (Doumith *et al.*, 2004a). Out of 32 *L. monocytogenes* isolates, 28 (87.5%) belonged to lineage I and 4 (12.5%) isolates belonged to lineage II (Table 4.6). The serogrouping results showed that 21 (65.6%) isolates were grouped into serogroup "1/2a, 3a", and 7 (21.9%) and 4 (12.5%) isolates were classified into serogroups "1/2c, 3c" and "4b, 4d, 4e", respectively (Table 4.7) (Figure 4.3).

For the 20 *L. monocytogenes* which were isolated from street-side hawker stalls, the majority serogroup were "1/2a, 3a" (55%), followed by serogroup "1/2c, 3c" (35%) and serogroup "4b, 4d, 4e" (10%). Out of the 12 *L. monocytogenes* isolated from

hypermarkets, 10 (83.3%) isolates belonged to serogroup "1/2a, 3a" and only two (16.7%) isolates were grouped into serogroup "4b, 4d, 4e".

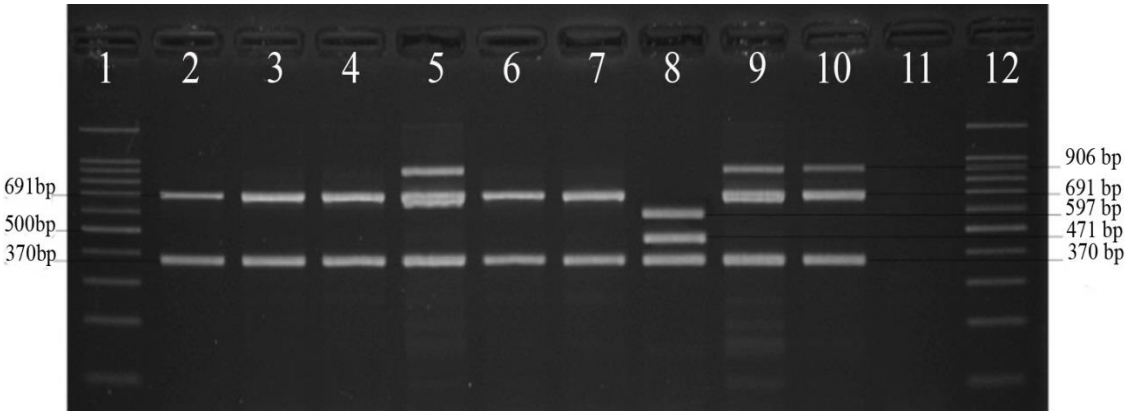


Figure 4.3: A representative gel picture of DNA amplicons generated by multiplex PCR for identification of *L. monocytogenes* serogroups. Serogroup "1/2a, 3a" is indicated by the amplicon of 691 bp, serogroup "1/2c, 3c" by two amplicons 691 bp and 906 bp, serogroup "1/2b, 3b, 7" by amplicon 471 bp and serogroup "4b, 4d, 4e" by two amplicons 471 bp and 597 bp. All members of the *Listeria* genus give an amplicon of 370 bp with prs primers. Lanes 1 and 11, 100 bp molecular size marker; lanes 2 to 4, and 9, serogroup "1/2c, 3c"; lanes 5, 7, and 8, serogroup "4b, 4d, 4e"; lane 6, serogroup "1/2a, 3a"; lane 10, negative control.

Table 4.7: Distribution of *L. monocytogenes* isolates by lineage, serogroup and location

		Total	**Street-side hawker stalls**	**Hypermarket**
Lineage I	Serogroup I.1 (1/2a,3a)	21 (65.6%)	11 (52.4%)	10 (47.6%)
	Serogroup I.2 (1/2c,3c)	7 (21.9%)	7 (100%)	0
Lineage II	Serogroup II.1 (4b,4d,4e)	4 (12.5%)	2 (50%)	2 (50%)

The highest prevalence of serogroups "1/2a, 3a" (42.9%) and "1/2c, 3c" (71.4%) was isolated from salads and vegetables. Serogroup "4b, 4d, 4e" distributed into four food categories of chicken and chicken products, egg and egg products, packed lunch and seafood and seafood products (Table 4.8).

Table 4.8: Distribution of *L. monocytogenes* isolates by serogroup and food categories

Food categories	"1/2a, 3a" (%)			"1/2c, 3c" (%)			"4b, 4d, 4e" (%)		
	HS*	HM**	Total	HS	HM	Total	HS	HM	Total
Beverage	0	0	0	1(14.3)	0	1(14.3)	0	0	0
Chicken and chicken products	4(36.4)	3(30)	7(33.3)	1(14.3)	0	1(14.3)	0	1(50)	1(25)
Egg and egg products	1(9.1)	2(20)	3(14.3)	0	0	0	1(50)	0	1(25)
Packed lunch	0	0	0	0	0	0	0	1(50)	1(25)
Salad and vegetables	6(54.5)	3(30)	9(42.9)	5(71.4)	0	5(71.4)	0	0	0
Seafood and seafood products	0	2(20)	2(9.5)	0	0	0	1(50)	0	1(25)
Total	11(52.4)	10(47.6)	21(100)	7(100)	0	7(100)	2(50)	2(50)	4(100)

*HS: Street-side hawker stalls

**HM: Hypermarket

4.4 Antibiograms

4.4.1 Antibiograms of *L. monocytogenes* isolates

L. monocytogenes isolates showed resistance to 9 (75%) out of the 12 antimicrobial agents tested (Table 4.9). Among 32 *L. monocytogenes* isolates, 18 (56.3%) isolates showed resistance to one or more antimicrobial agents tested, however only one isolate was multi-drug resistant (resistant to more than three classes of antimicrobials). The multi-drug resistant isolate belonged to serogroup "4b, 4d, 4e" and was isolated from fried fish (Table 4.10 and Table 4.11).

The antimicrobial resistance rates for 32 *L. monocytogenes* isolates are as follows: penicillin G, 53.1%; tetracycline, 15.6%; amoxicillin-clavulanic acid, 12.5%; vancomycin, 9.4%; erythromycin, 6.3%; clindamycin, streptomycin, kanamycin and

chloramphenicol, 3.1% each. All the *L. monocytogenes* isolates were susceptible to trimethoprim-sulfamethoxazole, gentamicin and rifampicin.

Out of 17 *L. monocytogenes* isolates which showed resistance to penicillin G, 12 (70.6%) isolates belonged to serogroup "1/2a, 3a", and 2 (11.8%) and 3 (17.6%) isolates belonged to serogroups "1/2c, 3c" and "4b, 4d, 4e", respectively.

The antibiograms of *L. monocytogenes* isolates are listed in APPENDIX VI.

4.4.2 Antibiograms of the non-*L. monocytogenes* isolates

Non-*L. monocytogenes* isolates showed also resistance to nine (75%) out of the twelve antimicrobial agents tested. The most common resistance was to tetracycline / clindamycin (60%), penicillin G (45%), chloramphenicol (30%), streptomycin / kanamycin (15%), amoxicillin-clavulanic acid / rifampicin (10%) and vancomycin (5%) (Table 4.12). All the 20 non-*L. monocytogenes* isolates were susceptible to erythromycin, trimethoprim-sulfamethoxazole and gentamicin. The antibiograms of non-*L. monocytogenes* isolates are listed in APPENDIX VI.

Table 4.9: The resistance patterns of *L. monocytogenes* isolates

Antimicrobial group	Antimicrobial		"1/2a, 3a" (21)	"1/2c, 3c" (7)	"4b, 4d, 4e" (4)	Total (32)
Tetracyclines	Tetracycline	S	12 (57.1%)	7 (100%)	3 (75%)	22 (68.8%)
		I	5 (23.8%)	0	0	5 (15.6%)
		R	4 (19%)	0	1(5%)	5 (15.6%)
Macrolides	Erythromycin	S	18 (85.7%)	7 (100%)	3 (75%)	28 (87.5%)
		I	2 (9.5%)	0	0	2 (6.3%)
		R	1 (4.8%)	0	1 (25%)	2 (6.3%)
Penicillins	Penicillin G	S	9 (42.9%)	5 (71.4%)	1 (25%)	15 (46.9%)
		I	0	0	0	0
		R	12 (57.1%)	2 (28.6%)	3 (75%)	17 (53.1%)
	Amoxicillin-clavulanic acid	S	18 (85.7%)	7 (100%)	3 (75%)	28 (87.5%)
		I	0	0	0	0
		R	3 (14.3%)	0	1 (25%)	4 (12.5%)
Aminoglycosides	Streptomycin	S	20 (95.2%)	7 (100%)	3 (75%)	30 (93.8%)
		I	1(4.8%)	0	0	1 (3.1%)
		R	0	0	1 (25%)	1 (3.1%)
	Kanamycin	S	21 (100%)	6 (85.7%)	3 (75%)	30 (93.8%)
		I	0	1 (14.3%)	0	1 (3.1%)
		R	0	0	1 (25%)	1 (3.1%)
	Gentamicin	S	21 (100%)	7 (100%)	4 (100%)	32 (100%)
		I	0	0	0	0
		R	0	0	0	0
Glycopeptides	Vancomycin	S	17 (81%)	3 (42.9%)	3 (75%)	23 (71.9%)
		I	3 (14.3%)	3 (42.9%)	0	6 (18.8%)
		R	1 (4.8%)	1 (14.3%)	1 (25%)	3 (9.4%)
Lincosamides	Clindamycin	S	17 (81%)	5 (71.4%)	1 (25%)	23 (71.9%)
		I	4 (19%)	2 (28.6%)	2 (50%)	8 (25%)
		R	0	0	1 (25%)	1 (3.1%)
Chloramphenicols	Chloramphenicol	S	21 (100%)	6 (85.7%)	4 (100%)	31 (96.9%)
		I	0	0	0	0
		R	0	1 (14.3%)	0	1 (3.1%)
Ansamycin	Rifampicin	S	21 (100%)	7 (100%)	4 (100%)	32 (100%)
		I	0	0	0	0
		R	0	0	0	0
Potentiated sulfonamide	trimethoprim-sulfamethoxazole	S	21 (100%)	7 (100%)	4 (100%)	32 (100%)
		I	0	0	0	0
		R	0	0	0	0

Table 4.10: Multiple antimicrobial resistances *L. monocytogenes* from RTE foods.

Antimicrobials	Chicken and chicken products		Eggs and egg products		Salad and vegetables		Seafood and seafood products		Total	
	n= 3	%	n= 1	%	n= 4	%	n= 1	%	n= 9	%
AMC + Pen G	1	33.3	-	-	1	25	-	-	2	22.2
Pen G + TE	2	66.7	-	-	2	50	-	-	4	44.4
Pen G + C	-	-	-	-	1	25	-	-	1	11.1
AMC + Pen G + VA	-	-	1	100	-	-	-	-	1	11.1
AMC +DA + E + K + Pen G + S + TE + VA	-	-	-	-	-	-	1	100	1	11.1

AMC: Amoxicillin-clavulanic acid, C: Chloramphenicol, E: Erythromycin, K: Kanamycin, P: Penicillin G, S: Streptomycin, TE: Tetracycline, Va: Vancomycin, DA: Clindamycin

Table 4.11: Multiple antimicrobial resistances *L. monocytogenes* by food categories and serogroups

Antimicrobials	Chicken and chicken products (n)	Egg and egg products (n)	Salad and vegetables (n)	Seafood and Seafood products (n)	Total (n)
AMC + Pen G	"1/2a, 3a" (1)	-	"1/2a, 3a" (1)	-	"1/2a, 3a" (2)
Pen G + TE	"1/2a, 3a" (2)	-	"1/2a, 3a" (2)	-	"1/2a, 3a" (4)
Pen G + C	-	-	"1/2c, 3c" (1)	-	"1/2c, 3c" (1)
AMC + Pen G + VA	-	"1/2a, 3a" (1)	-	-	"1/2a, 3a" (1)
AMC + DA + E + K + Pen G + S + TE + VA	-	-	-	"4b, 4d, 4e" (1)	"4b, 4d, 4e" (1)

AMC: Amoxicillin-clavulanic acid, C: Chloramphenicol, E: Erythromycin, K: Kanamycin, P: Penicillin G, S: Streptomycin, TE: Tetracycline, Va: Vancomycin, DA: Clindamycin

Table 4.12: Antimicrobial susceptibility of non-*L. monocytogenes* isolates

		Non-*L. monocytogenes*
Tetracycline	**S**	8(40%)
	I	0
	R	12(60%)
Erythromycin	**S**	13(65%)
	I	7(35%)
	R	0
Penicillin G	**S**	6(30%)
	I	5(25%)
	R	9(45%)
Clindamycin	**S**	1 (5%)
	I	7(35%)
	R	12(60%)
Streptomycin	**S**	15(75%)
	I	2(10%)
	R	3(15%)
Kanamycin	**S**	17(85%)
	I	0
	R	3(15%)
Chloramphenicol	**S**	14(70%)
	I	0
	R	6(30%)
Vancomycin	**S**	10(50%)
	I	9(45%)
	R	1(5%)
Amoxicillin-clavulanic acid	**S**	18(90%)
	I	0
	R	2(10%)
Gentamicin	**S**	20(100%)
	I	0
	R	0
Rifampicin	**S**	17(85%)
	I	1(5%)
	R	2(10%)
trimethoprim-sulfamethoxazole	**S**	20(100%)
	I	0
	R	0

4.5 Detection of virulence genes in *L. monocytogenes*

All the *L. monocytogenes* isolates were examined for presence/absence of virulence genes *inlA, inlB, inlC,* and *inlJ*. All the *L. monocytogenes* isolates showed the presence of the internalin genes (Figure 4.4).

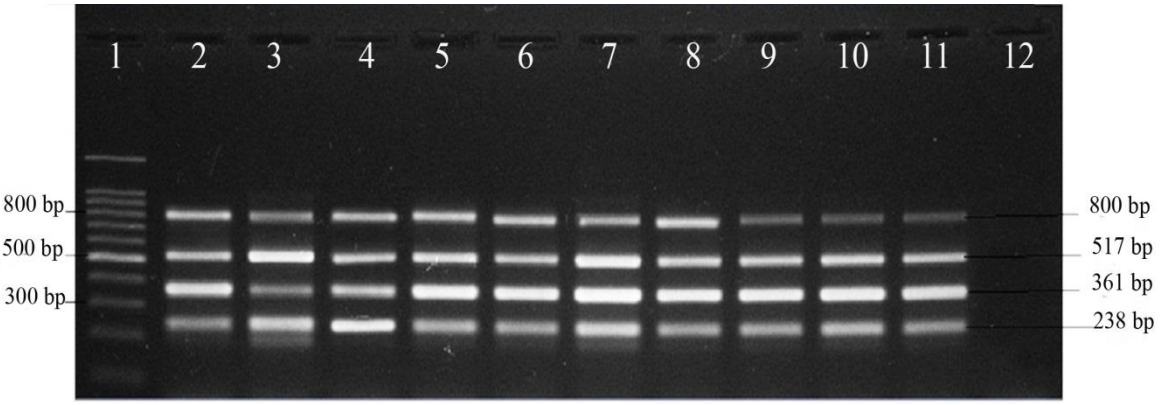


Figure 4.4: The representative agarose gel picture of PCR-amplified products of internalin genes. The 800 bp band shows *inlA*, the 517 bp band displays *inlC*, the 361 bp and 238 bp regions show *inlB* and *inlJ*, respectively. Lane 1, 100bp molecular size markers; lanes 2 to 6 *L. monocytogenes* serogroup "1/2a, 3a"; lanes 6 to 8 *L. monocytogenes* serogroup "1/2c, 3c"; lanes 9 to 11 *L. monocytogenes* serogroup "4b, 4d, 4e"; Lane 12, negative control.

4.6 Validation of the amplicons

Representative amplicons of *inlA, inlB, inlC* and *inlJ* were purified by using MEGA quick-spin PCR and agarose Gel DNA extraction kits (Intron Biotechnology, Korea) and sequenced to validate their identities by comparing against reference strains in NCBI database.

The *inlA, inlB* and *inlC* genes showed 99% similarity with the *inlA, inlB* and *inlC* genes of *L. monocytogenes* strains NRRL_B-57131, A23, and VIMVF110, respectively. The *inlJ* gene displayed 97% similarity with the *inlJ2* gene of *L. monocytogenes* strain L41. The sequencing of the amplicons is listed in APPENDIX VII.

4.7 PCR-Restriction fragment length polymorphism (PCR-RFLP) among selected virulence genes

4.7.1 PCR-RFLP of *inlA*

An 800 bp fragment of *inlA* gene was present in all *L. monocytogenes* isolates. This amplicon was digested with *Xba*I which recognizes the TCTAGA sequence. PCR-RFLP of *Xba*I-digested *inlA* amplicon gave only one profile with two fragments, 144 bp and 656 bp (Figure 4.5).

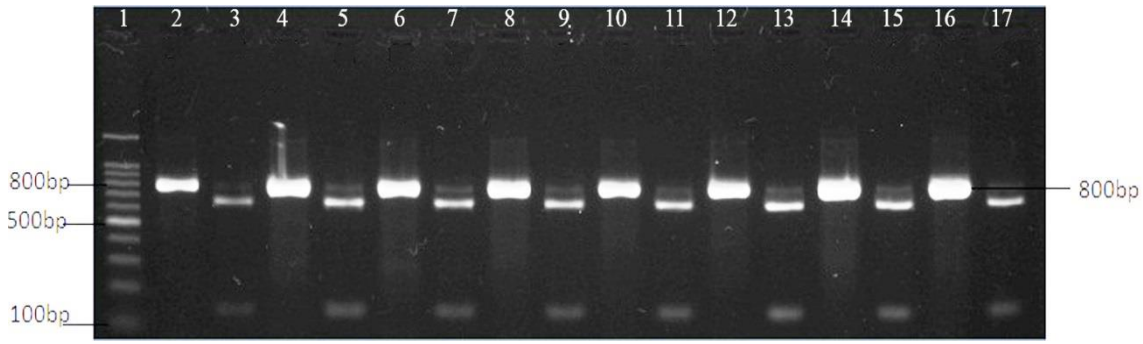


Figure 4.5: The representative agarose gel picture of PCR-amplified products of *inlA* gene and after digestion by *Xba*I. Lane 1, 100 bp molecular size markers; lane 2, LM59 (undigested); lane 3, LM59 (digested by *Xba*I); lane 4, LM61 (undigested); lane 5, LM61 (digested by *Xba*I); lane 6, LM85 (undigested); lane 7, LM85 (digested by *Xba*I) ; lane 8, LM92 (undigested); lane 9, LM92 (digested by *Xba*I); lane 10, LM96 (undigested); lane 11, LM96 (digested by *Xba*I); lane 12, LM107 (undigested); lane 13, LM107 (digested by *Xba*I); lane 14, LM115 (undigested); lane 15, LM115 (digested by *Xba*I); lane 16, LM149 (undigested); lane 17, LM149 (digested by *Xba*I).

4.7.2 PCR-RFLP of *inlC*

A 517 bp fragment of *inlC* gene was present in all *L. monocytogenes* isolates. This amplicon was digested with *Alu*I which recognizes the AGCT sequence. PCR-RFLP of *Alu*I digested *inlC* amplicon gave two profiles containing 2-3 bands; profile I (Al1) showed two fragments, 98 bp and 419 bp while profile II (Al2) showed three fragments, 98 bp, 150 bp and 269 bp (Figure 4.6). Twenty four out of 32 *L. monocytogenes* isolates (75%) belonged to profile Alu1 and 8 (25%) belonged to profile Al2. Eight isolates of profile Al2 were divided into serogroups "1/2a, 3a" (50%) and "4b, 4d, 4e" (50%) (Figure 4.6). DNA sequence analysis of *inlC* showed some mutations in profile II (Alu2).

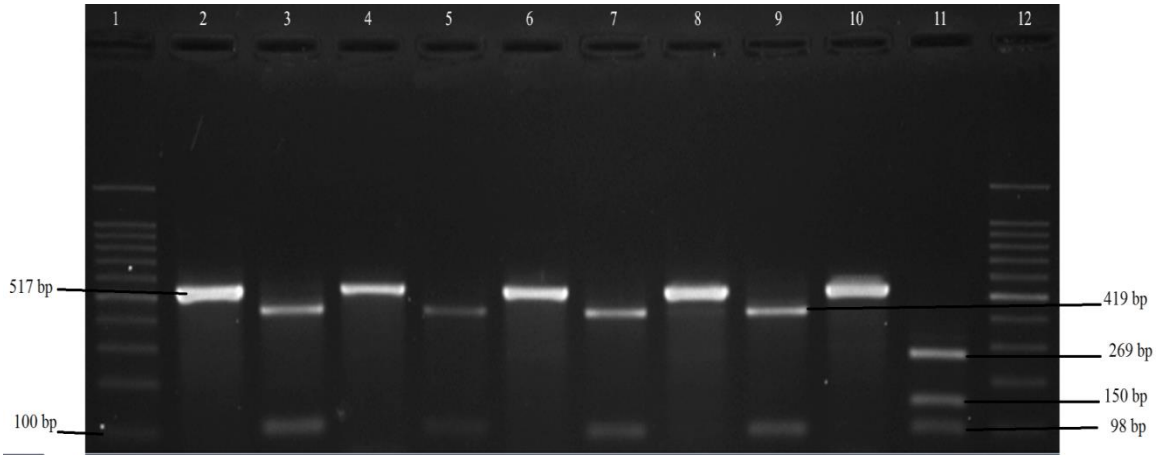


Figure 4.6: The representative agarose gel picture of PCR-amplified products of *inlC* gene and after digestion by *Alu*I. Lanes 1 and 12, 100 bp molecular size markers; lane 2, LM59 (undigested); lane 3, LM59 (Al1; digested by *Alu*I); lane 4, LM96 (undigested); lane 5, LM96 (Al1; digested by *Alu*I); lane 6, LM106 (undigested); lane 7, LM106 (Al1; digested by *Alu*I); lane 8, LM107 (undigested); lane 9, LM107 (Al1; digested by *Alu*I); lane 10, LM115 (undigested); lane 11, L115 (Al2; digested by *Alu*I).

4.8 PCR-based genotyping of *L. monocytogenes*

4.8.1 Repetitive Extragenic Palindromic (REP)-PCR

Molecular typing was done to differentiate the isolates of *L. monocytogenes* isolated from RTE food samples and also to analyze genetic variability among the isolates. Repetitive Extragenic Palindromic (REP)-PCR was performed using REP primer (Navia *et al.*, 1999).

A representative gel photo of REP-PCR is in Figure 4.7. All the 32 *L. monocytogenes* isolates were typeable by REP-PCR into 28 distinct REP profiles (*D*= 0.992). REP profiles consisted of 5 to 18 bands with size ranging from 200 to 2000 bp.

The dendrogram based on the profiles obtained by REP-PCR is shown in Figure 4.8. Thirty two isolates of *L. monocytogenes* were grouped into eight distinctive clusters (based at 80% similarity) by analysis of band-based. Cluster REP-a is represented by nine isolates. The isolates were isolated from different types of foods, at different sampling times and locations. The isolates belonged to serogroup "1/2a, 3a". This cluster shows 81.6% genetic similarity. However, four isolates (LM178 and LM 197; LM192 and LM198) showed 100% genetic similarity. Isolates LM192 and LM198 were from the same location and date sampling times while LM178 and LM197 were from different locations and sampling times.

Cluster REP-b contained three isolates which were isolated from different food categories at different sampling times and locations. The isolates were "4b, 4d, 4e". The genetic similarity was 80% in this cluster. In this cluster, two isolates (LM150 and LM161) displayed 100% genetic similarity. The isolates were detected from different types of foods (chicken and chicken products and packed lunch) at the same sampling date and location (hypermarket X).

Cluster REP-c included two isolates which were grouped into serogroup “1/2c, 3c”. The isolates were isolated from different types of RTE foods and at different sampling times and locations. The isolates showed 88% genetic similarity. Two isolates were represented in cluster REP-d. Both isolates belonged to serogroup “1/2c, 3c”. The isolates were detected from same RTE food (salad) and at the same date of sampling and location (Pasar malam-PJ17). The genetic similarity of this cluster was 88.9%.

Three isolates were grouped into cluster REP-e. Two isolates were “1/2c, 3c” and one isolate was “1/2a, 3a”. The isolates were isolated from different food categories at different sampling times and locations. The cluster showed 81% genetic similarity.

Two isolates of serogroups “1/2a, 3a” and “1/2c, 3c” were grouped into cluster REP-f. The isolates were detected from the same RTE food category at different sampling times and locations. The cluster showed 80% genetic similarity.

Cluster REP-g contained three isolates which were isolated from different food categories and locations and at different dates of sampling and locations. In this cluster all isolates were “1/2a, 3a” and displayed 83.8% genetic similarity. However two isolates (LM44 and LM50) from the same RTE food category and the same location and date of sampling displayed 94.1% genetic similarity in this cluster.

Five isolates of serogroup “1/2a, 3a” were grouped into cluster REP-h. The isolates were detected from different RTE food categories at different sampling times and hypermarkets. Cluster REP-h showed 86.6% genetic similarity. However, two isolates of LM214 and LM231 from different types of foods at the same sampling time and location displayed 100% genetic similarity.

Cluster analysis of the 30 REP profiles clustered the 32 isolates into 8 groups with members of the same serogroup being clustered in the same group and only three exceptions, isolates LM31 (“1/2a, 3a”), LM85 (“1/2c, 3c”) and LM92 (“1/2a, 3a”) were classified into unrelated clusters (Figure 4.9). Among three serogroups, “1/2a, 3a” had

the biggest cluster. Out of 21 *L. monocytogenes* serogroup "1/2a, 3a", 18 REP profiles were observed in different RTE food categories. There was no similarity observed between the REP profiles of 7 *L. monocytogenes* serogroup "1/2c, 3c" recovered from RTE foods. This serogroup was more common in salad and vegetables category (5/7). Among 4 *L. monocytogenes* serogroup "4b, 4d, 4e", 3 REP profiles were detected from different RTE food categories. There was a low genetic diversity among *the L. monocytogenes* serogroup "4b, 4d, 4e" as the 4 isolates were subtyped into 3 REP profiles and differed by < 3 bands. It shows that REP-PCR, as an efficient method of molecular typing for *L. monocytogenes* isolates, was able to distinguish the isolates with different serogroups.

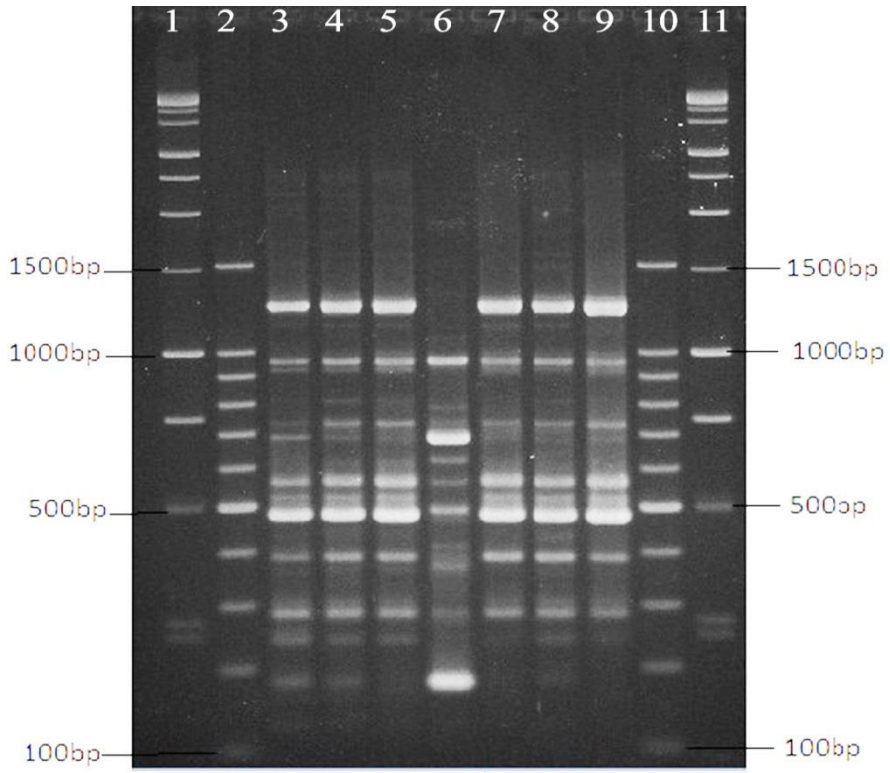
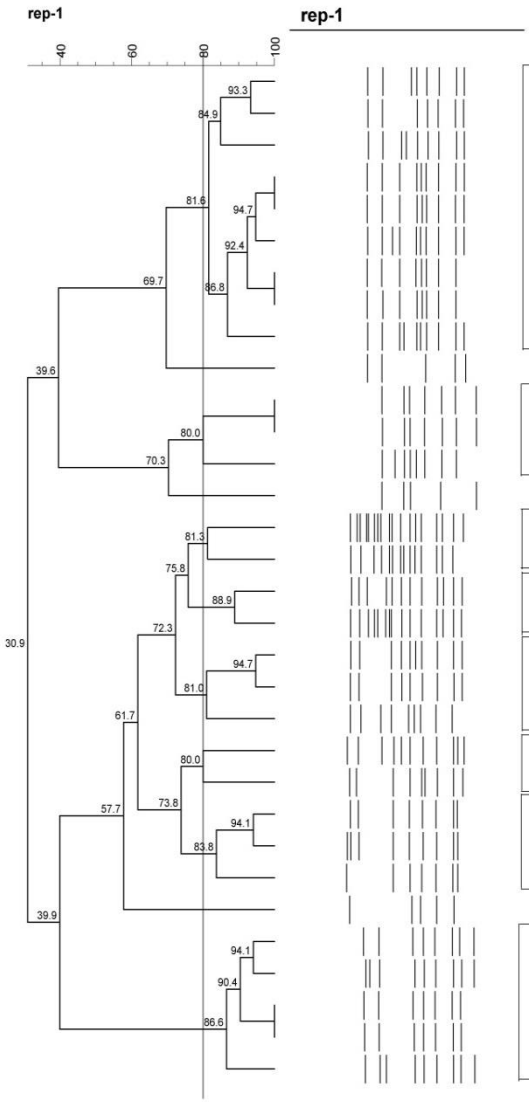


Figure 4.7: The representative gel picture of REP-PCR for *L. monocytogenes* isolated from RTE food samples. The lables and information of the isolates are summarized in the Table 4.13.

Table 4.13: The labels and detailed information of the isolates in Figure 4.7.

Lane	Code	Date of sampling	Source	Serogroup	Rep-profile
1	1kb marker	-	-	-	-
2	100bp marker	-	-	-	-
3	LM163	3/12/2011	Chicken[a]	"1/2a, 3a"	LmREP7
4	LM164	3/12/2011	Salad[b]	"1/2a, 3a"	LmREP5
5	LM178	3/12/2011	Chicken	"1/2a, 3a"	LmREP4
6	LM184	3/12/2011	Egg[c]	"4b, 4d, 4e"	LmREP10
7	LM192	29/12/12	Egg	"1/2a, 3a"	LmREP6
8	LM197	29/12/12	Salad	"1/2a, 3a"	LmREP4
9	LM198	29/12/12	Salad	"1/2a, 3a"	LmREP6
10	100bp marker	-	-	-	-
11	1kb marker	-	-	-	-

[a] Chicken: Cooked chicken and chicken products; [b] Salad: Salad and vegetables;
[c] Egg: Egg and egg products



Key	Source	Serogroup	REP-profile	
LM171	Salad and vegetables	1/2a, 3a	LmREP1	REP-a
LM191	Egg products	1/2a, 3a	LmREP2	
LM177	Chicken products	1/2a, 3a	LmREP3	
LM178	Chicken products	1/2a, 3a	LmREP4	
LM197	Salad and vegetables	1/2a, 3a	LmREP4	
LM164	Salad and vegetables	1/2a, 3a	LmREP5	
LM192	Egg products	1/2a, 3a	LmREP6	
LM198	Salad and vegetables	1/2a, 3a	LmREP6	
LM163	Chicken products	1/2a, 3a	LmREP7	
LM149	Seafood products	1/2a, 3a	LmREP8	
LM150	Chicken products	4b, 4d, 4e	LmREP9	REP-b
LM161	Packed lunch	4b, 4d, 4e	LmREP9	
LM184	Egg products	4b, 4d, 4e	LmREP10	
LM115	Seafood products	4b, 4d, 4e	LmREP11	
LM41	Salad and vegetables	1/2c, 3c	LmREP12	REP-c
LM59	Chicken products	1/2c, 3c	LmREP13	
LM106	Salad and vegetabales	1/2c, 3c	LmREP14	REP-d
LM107	Salad and vegetables	1/2c, 3c	LmREP15	
LM92	Egg products	1/2a, 3a	LmREP16	REP-e
LM96	Beverage	1/2c, 3c	LmREP17	
LM61	Salad and vegetables	1/2c, 3c	LmREP18	
LM31	Salad and vegetables	1/2a, 3a	LmREP19	REP-f
LM85	Salad and vegetables	1/2c, 3c	LmREP20	
LM44	Salad and vegetables	1/2a, 3a	LmREP21	REP-g
LM50	Salad and vegetables	1/2a, 3a	LmREP22	
LM34	Chicken products	1/2a, 3a	LmREP23	
LM15	Salad and vegetables	1/2a, 3a	LmREP24	
LM213	Chicken products	1/2a, 3a	LmREP25	REP-h
LM242	Salad and vegetables	1/2a, 3a	LmREP26	
LM214	Chicken products	1/2a, 3a	LmREP27	
LM231	Seafood products	1/2a, 3a	LmREP27	
LM209	Chicken products	1/2a, 3a	LmREP28	

Figure 4.8: Dendrogram based on the REP-PCR patterns of *L. monocytogenes* isolates. The isolates were typeable by REP-PCR into 28 REP profiles and grouped into 8 distinctive clusters based on 80% similarity. REP-PCR distinguishes each serogroup.

4.8.2 BOX-PCR

BOX-PCR was performed using BOX A1R primer (Versalovic et al., 1994). A representative gel photo of BOX-PCR is in Figure 4.9. All the 32 *L. monocytogenes* isolates were typeable by BOX-PCR into 31 distinct BOX profiles (*D*= 0.998). BOX profiles consisted of 5 to 17 bands with size ranging from 200 to 2000 bp.

The dendrogram based on the profiles obtained by BOX-PCR is shown in Figure 4.10. Thirty two isolates of *L. monocytogenes* were grouped into nine distinctive clusters by analysis of band-based.

Cluster BOX-I was represented by three isolates of serogroup "1/2c, 3c". The isolates were isolated from different food categories at the same sampling time and location. This cluster showed 88.9% genetic similarity. However, two isolates of LM106 and LM107 from the same RET food category displayed 100% genetic similarity. Two isolates of serogroup "1/2a, 3a" from different types of foods were grouped into cluster BOX-II. The isolates were isolated from the same location (hypermarket X) and date of sampling. The cluster showed 85.7% genetic similarity. Cluster BOX-III contained four isolates which were isolated from different RTE food categories (three isolates from salad and vegetables and one isolate from chicken and chicken products) at different sampling times and locations. The genetic similarity was 86.8% in this cluster. Three isolates of LM15, LM171 and LM 164 which isolated from the same RTE food category displayed 87.5% genetic similarity.

Three isolates of serogroup "1/2c, 3c" were represented in cluster BOX-IV. The isolates were detected from different food categories at different sampling times and locations. The genetic similarity of this cluster was 83.3%. However, two isolates (LM61 and LM85) which were detected from the category of salad and vegetable showed 96.6% genetic similarity. Two isolates of serogroup "1/2a, 3a" were grouped

into cluster BOX-V. The cluster showed 81.8% genetic similarity. The isolates were isolated from different food categories at different sampling times and locations.

Four isolates of serogroup "1/2a, 3a" and one isolate of serogroup "1/2c, 3c" were grouped into cluster BOX-VI. The isolates were isolated from different types of foods at different sampling times and locations. The cluster showed 86.7% genetic similarity between these isolates. However, LM31 and LM34 which isolated at the same sampling time and location displayed 97.3% genetic similarity.

Cluster BOX-VII contained two isolates of serogroup "1/2a, 3a". The isolates were detected from different RTE foods at different sampling times and locations. This cluster showed a 81.2% genetic similarity. Two isolates of "1/2a, 3a" from different types of food categories were grouped into cluster BOX-VIII. Although they isolated at different sampling time and locations, they showed 85.7% genetic similarity. Two isolates of serogroup "4b, 4d, 4e" from different types of RTE foods, at different sampling times and locations were grouped into cluster BOX-VIIII. This cluster displayed 90% genetic similarity.

Cluster analysis of the 31 BOX profiles clustered the 32 isolates into 9 groups (based at 80% similarity) with members of the same serogroup being clustered in the same group and only one exception, isolate LM41 (serogroup "1/2c, 3c") was classified into a unrelated cluster (Figure 4.11). Among three serogroups, "1/2a, 3a" had the biggest cluster. Out of 7 *L. monocytogenes* serogroup "1/2c, 3c", 6 BOX profiles were observed in different RTE food categories. There was no similarity observed between the BOX profiles of *L. monocytogenes* serogroups "1/2a, 3a" and "4b, 4d, 4e" isolates recovered from RTE foods. Four isolates with serogroup "4b, 4d, 4e" were clustered together by BOX-PCR (55.9% similarity). There was a low genetic diversity among the *L. monocytogenes* serogroup "4b, 4d, 4e" as the 4 isolates were differed by < 3 bands. It shows that BOX-PCR was able to differentiate the isolates with different serogroups.

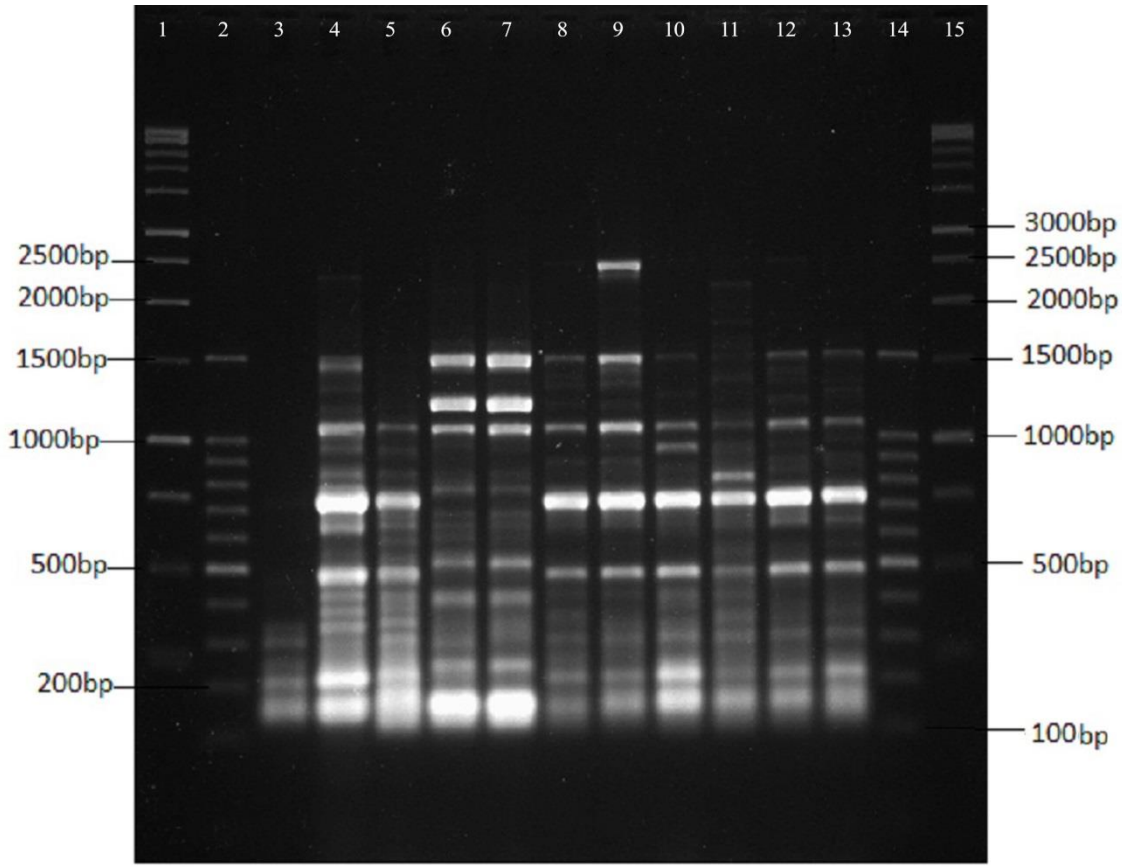


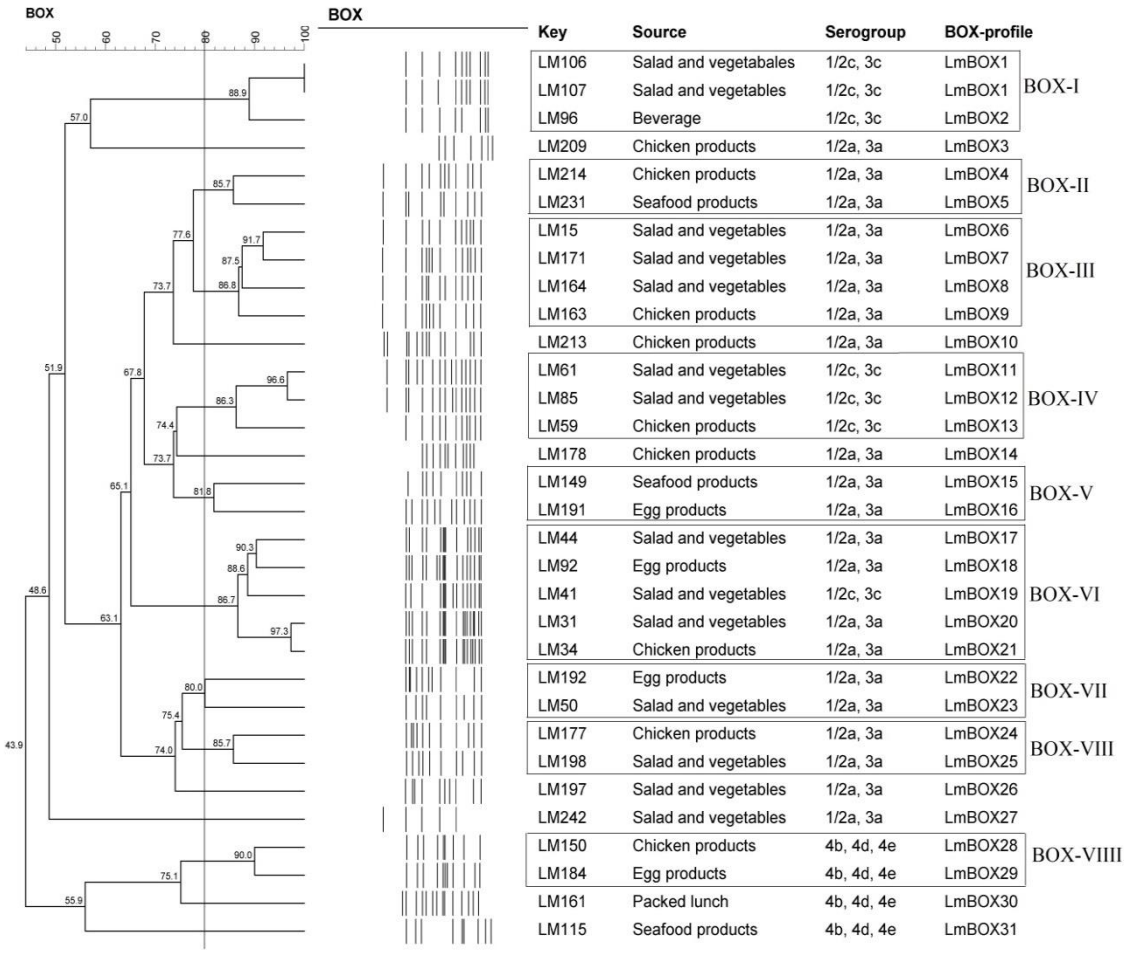
Figure 4.9: The representative gel picture of BOX-PCR for *L. monocytogenes* isolated from RTE food samples. The lables and information of the isolates are summarized in the Table 4.14.

Table 4.14: The labels and detailed information of the isolates in Figure 4.9.

Lane	Code	Date of sampling	Source	Serogroup	Box-profile
1	1kb marker	-	-	-	-
2	100bp marker	-	-	-	-
3	LM242	8/1/2012	Salad[a]	"1/2a, 3a"	LmBOX27
4	LM149	23/8/2011	Seafood[b]	"1/2a, 3a"	LmBOX15
5	LM178	3/12/2011	Chicken[c]	"1/2a, 3a"	LmBOX14
6	LM184	3/12/2012	Egg[d]	"4b, 4d, 4e"	LmBOX29
7	LM150	23/8/2011	Chicken	"4b, 4d, 4e"	LmBOX28
8	LM177	3/12/2012	Chicken	"1/2a, 3a"	LmBOX24
9	LM198	29/12/2011	Salad	"1/2a, 3a"	LmBOX25
10	LM50	10/3/2011	Salad	"1/2a, 3a"	LmBOX23
11	LM191	29/12/2011	Egg	"1/2a, 3a"	LmBOX16
12	LM192	29/12/2011	Egg	"1/2a, 3a"	LmBOX22
13	LM197	29/12/2011	Salad	"1/2a, 3a"	LmBOX26
14	100bp marker	-	-	-	-
15	1kb marker	-	-	-	-

[a] Salad: Salad and vegetables; [b] Seafood: Seafood and seafood products;

[c] Chicken: Cooked chicken and chicken products; [d] Egg: Egg and egg products



Key	Source	Serogroup	BOX-profile	
LM106	Salad and vegetabales	1/2c, 3c	LmBOX1	BOX-I
LM107	Salad and vegetables	1/2c, 3c	LmBOX1	
LM96	Beverage	1/2c, 3c	LmBOX2	
LM209	Chicken products	1/2a, 3a	LmBOX3	
LM214	Chicken products	1/2a, 3a	LmBOX4	BOX-II
LM231	Seafood products	1/2a, 3a	LmBOX5	
LM15	Salad and vegetables	1/2a, 3a	LmBOX6	BOX-III
LM171	Salad and vegetables	1/2a, 3a	LmBOX7	
LM164	Salad and vegetables	1/2a, 3a	LmBOX8	
LM163	Chicken products	1/2a, 3a	LmBOX9	
LM213	Chicken products	1/2a, 3a	LmBOX10	
LM61	Salad and vegetables	1/2c, 3c	LmBOX11	BOX-IV
LM85	Salad and vegetables	1/2c, 3c	LmBOX12	
LM59	Chicken products	1/2c, 3c	LmBOX13	
LM178	Chicken products	1/2a, 3a	LmBOX14	
LM149	Seafood products	1/2a, 3a	LmBOX15	BOX-V
LM191	Egg products	1/2a, 3a	LmBOX16	
LM44	Salad and vegetables	1/2a, 3a	LmBOX17	BOX-VI
LM92	Egg products	1/2a, 3a	LmBOX18	
LM41	Salad and vegetables	1/2c, 3c	LmBOX19	
LM31	Salad and vegetables	1/2a, 3a	LmBOX20	
LM34	Chicken products	1/2a, 3a	LmBOX21	
LM192	Egg products	1/2a, 3a	LmBOX22	BOX-VII
LM50	Salad and vegetables	1/2a, 3a	LmBOX23	
LM177	Chicken products	1/2a, 3a	LmBOX24	BOX-VIII
LM198	Salad and vegetables	1/2a, 3a	LmBOX25	
LM197	Salad and vegetables	1/2a, 3a	LmBOX26	
LM242	Salad and vegetables	1/2a, 3a	LmBOX27	
LM150	Chicken products	4b, 4d, 4e	LmBOX28	BOX-VIIII
LM184	Egg products	4b, 4d, 4e	LmBOX29	
LM161	Packed lunch	4b, 4d, 4e	LmBOX30	
LM115	Seafood products	4b, 4d, 4e	LmBOX31	

Figure 4.10: Dendrogram based on BOX-PCR banding patterns of *L. monocytogenes* isolates. The isolates were typeable by BOX-PCR into 31 BOX profiles and grouped into 9 distinctive clusters based 80% similarity.

4.8.3 Random Amplification of Polymorphic DNA (RAPD)

Random Amplification of Polymorphic DNA (RAPD) was performed using OPA15 primer (Lee *et al.*, 2011). A representative gel photo of RAPD has been presented in Figure 4.11. All the 32 *L. monocytogenes* isolates were typeable by RAPD-PCR into 32 distinct RAPD profiles (*D*= 1). RAPD profiles consisted of 3 to 12 bands with size ranging from 200 to 2000 bp. The dendrogram based on the profiles obtained by RAPD-PCR is shown in Figure 4.12. Thirty two isolates of *L. monocytogenes* were grouped into four distinctive clusters by analysis of band-based.

Cluster RAPD-i was represented by two isolates of serogroup "1/2c, 3c". The isolates were isolated from the same RTE food (salad) at different sampling times and locations. This cluster showed 80% genetic similarity.

Two isolates from the same RTE food categories (salad and vegetables) at different sampling times and locations were grouped into cluster RAPD-ii. The isolates belonged to serogroups "1,2a, 3a" and "1/2c, 3c". The cluster showed 82.4% genetic similarity.

Cluster RAPD-iii contained two isolates which were isolated from different RTE food categories at the same sampling time and location. The isolates were "1/2c, 3c". The genetic similarity was 86.7% in this cluster.

Two isolates of serogroups "4b, 4d, 4e" and "1/2a, 3a" were represented in cluster RAPD-iv. The isolates were detected from the same RTE food category (egg and egg products) at different sampling times and locations. The genetic similarity of this cluster was 83.3%.

Low level of similarity in the RAPD profiles of the isolates indicates a high genetic diversity among the *L. monocytogenes* isolates. The RAPD profiles of LM191 (serogroup "1/2a, 3a") and LM192 (serogroup "1/2a, 3a"), as two cases in point, which have been isolated from a particular type of fried egg and from the same location and at

the same time, shared less than 21.2%. It affirms low level of genetic relatedness among the *L. monocytogenes*. It is also observed in the dendrogram that there is a low level of similarity among the isolates with the same serogroups. Two clusters included two isolates from different serogroups (RAPD-ii and RAPD-iv). It shows that RAPD, as a method of molecular typing for *L. monocytogenes* isolates, was not able to differentiate the isolates with different serogroups.

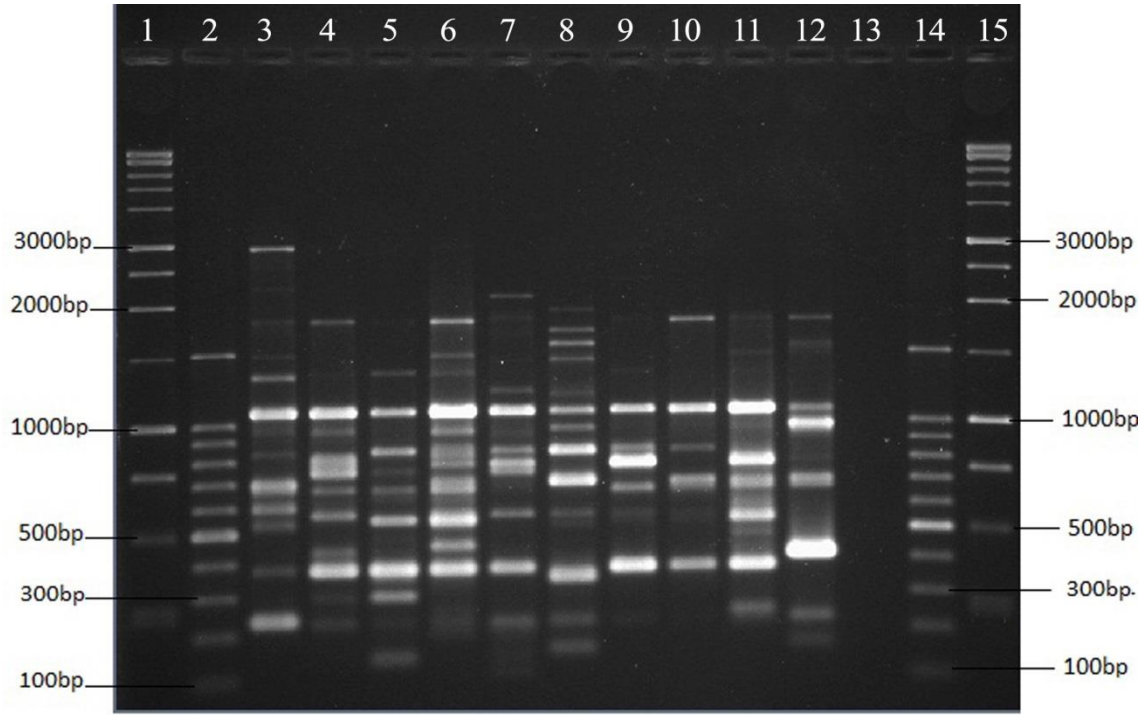


Figure 4.11: The representative gel photo of RAPD for *L. monocytogenes* isolates isolated from RTE food samples. The lables and information of the isolates are summarized in the Table 4.15.

Table 4.15: The labels and detailed information of the isolates in Figure 4.11.

Lane	Code	Date of sampling	Source	Serogroup	RAPD-profile
1	1kb marker	-	-	-	-
2	100bp marker	-	-	-	-
3	L15/12/10	14/12/2010	Salad[a]	“1/2a, 3a”	LmRAPD17
4	L31/02/11	21/2/2011	Salad	“1/2a, 3a”	LmRAPD13
5	L34/02/11	21/2/2011	Chicken[b]	“1/2a, 3a”	LmRAPD9
6	L41/02/11	10/3/2011	Salad	“1/2c, 3c"	LmRAPD10
7	L44/02/11	10/3/2011	Salad	“1/2a, 3a”	LmRAPD16
8	L92/04/11	4/4/2011	Egg[c]	“1/2a, 3a”	LmRAPD24
9	L96/04/11	10/5/2011	Beverage	“1/2c, 3c”	LmRAPD15
10	L106/05/11	10/5/2011	Salad	“1/2c, 3c”	LmRAPD14
11	L107/05/11	10/5/2011	Salad	“1/2c, 3c”	LmRAPD12
12	L115/05/11	10/5/2011	Seafood[d]	“4b, 4d, 4e"	LmRAPD21
13	Negative Control	-	-	-	-
14	100bp marker	-	-	-	-
15	1kb marker	-	-	-	-

[a] Salad: Salad and vegetables; [b] Chicken: Cooked chicken and chicken products;

[c] Egg: Egg and egg products; [d] Seafood: Seafood and seafood products

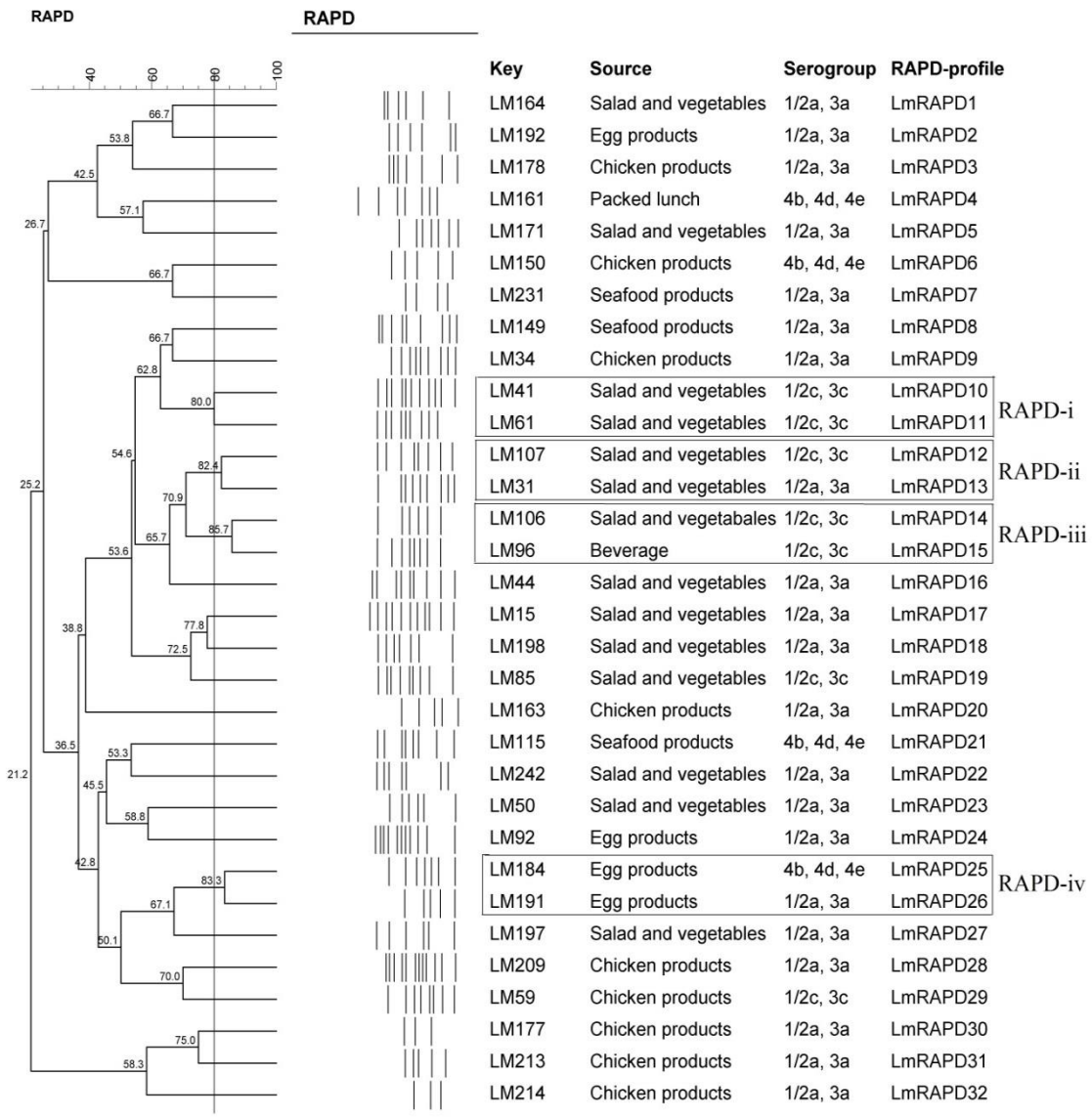


Figure 4.12: Dendrogram based on RAPD banding patterns of *L. monocytogenes*. The isolates were typeable by RAPD-PCR into 32 RAPD profiles and grouped into four distinctive clusters based on 80% similarity.

4.9 Pulsed Field Gel Electrophoresis (PFGE)

PFGE was applied to subtype *L. monocytogenes* isolates. All the 32 *L. monocytogenes* isolates were typable by PFGE into 20 distinct PFGE profiles (*D*= 0.916). PFGE profiles consisted of 12 to 15 bands with size ranging from 28.8 to 668.9 kp (Figures 4.13 to 4.17).

The dendrogram based on the profiles obtained by PFGE is shown in Figure 4.18. Thirty two isolates of *L. monocytogenes* were grouped into seven distinctive clusters by analysis of band-based.

Cluster PFGE-A was represented by four isolates of "1/2c, 3c". All isolates were detected from different types of foods at different sampling times and locations. However, all isolates were isolated from samples which were purchased from hawker street. This cluster showed 80% genetic similarity. Cluster PFGE-B included three isolates which were isolated from different locations. Two isolates and one isolate "1/2c, 3c" and "1/2a, 3a", respectively. The genetic similarity was 90.2% in this cluster. However two isolates of serogroup "1/2c, 3c" which isolated from the same RTE food category (salad and vegetables) displayed 100% genetic similarity.

Two isolates of serogroup "1/2a, 3a" were grouped into cluster PFGE-C. The isolates were detected from different food categories at the same sampling time and location (hypermarket X). The genetic similarity was 92.9% in this cluster.

Two isolates of serogroup "1/2a, 3a" and one isolate of serogroup "1/2c, 3c" were grouped into PFGE-D. The isolates were detected from different types of RTE food categories at different sampling times and locations. The genetic similarity was 91.5% in this cluster.

Cluster PFGE-E contained 12 isolates of serogroup "1/2a, 3a". The isolates were detected from different types of foods at different dates of sampling and locations. The

cluster displayed 92% genetic similarity. In this cluster, 11 isolates (LM209 and LM242; LM164, LM171, LM177, LM178, LM191, LM192, LM197, LM198 and LM213) showed 100% genetic similarity. However these isolates were isolated from different food categories at different sampling times and locations.

Four isolates of "1/2a, 3a" were represented in cluster PFGE-F. The isolates were detected from different RTE food categories at different sampling times and locations. The genetic similarity of this cluster was 80.4%. However the genetic similarity between two isolates (LM31 and LM34) which were isolated from same food categories at the same sampling time and location was 100%.

Four isolates of "4b, 4d, 4e" were represented in cluster pulsotype-G. These isolates were isolated from different types of foods at different sampling times and locations. The genetic similarity was 92.2% in this cluster. Two isolates (LM150 and LM161) which isolated from cooked chicken heart and packed lunch at the same sampling time and location (hypermarket X) showed 100% genetic similarity.

Cluster analysis of the 20 PFGE profiles grouped the 32 isolates into 7 clusters (based at 80% similarity) with members of the same serogroup being cluster in the same group and only two exceptions, isolate LM15 (serogroup "1/2a, 3a") and LM96 (serogroup "1/2c, 3c") were classified into unrelated clusters (Figure 4.14). Among three serogroups, "1/2a, 3a" had the biggest cluster. Out of 21 *L. monocytogenes* serogroup "1/2a, 3a", 11 PFGE profiles were observed in different RTE food categories. Out of 7 *L. monocytogenes* serogroup "1/2c, 3c", 6 PFGE profiles were observed. Four isolates with serogroup "4b, 4d, 4e" showed three different PFGE profiles. Low level of similarity in the pulsotypes of the majority of the isolates indicates a high genetic diversity among the *L. monocytogenes* isolates. The PFGE profiles of LM213 (serogroup "1/2a, 3a") and LM214 (serogroup "1/2a, 3a"), as two cases in point, which

have been isolated from a particular type of chicken, from the same location, and at the same time, shared less than 50.3%. It affirms low level of genetic relatedness among the *L. monocytogenes*. It shows that PFGE, as an efficient method of molecular typing for *L. monocytogenes* isolates, was able to distinguish the isolates with different serogroups.

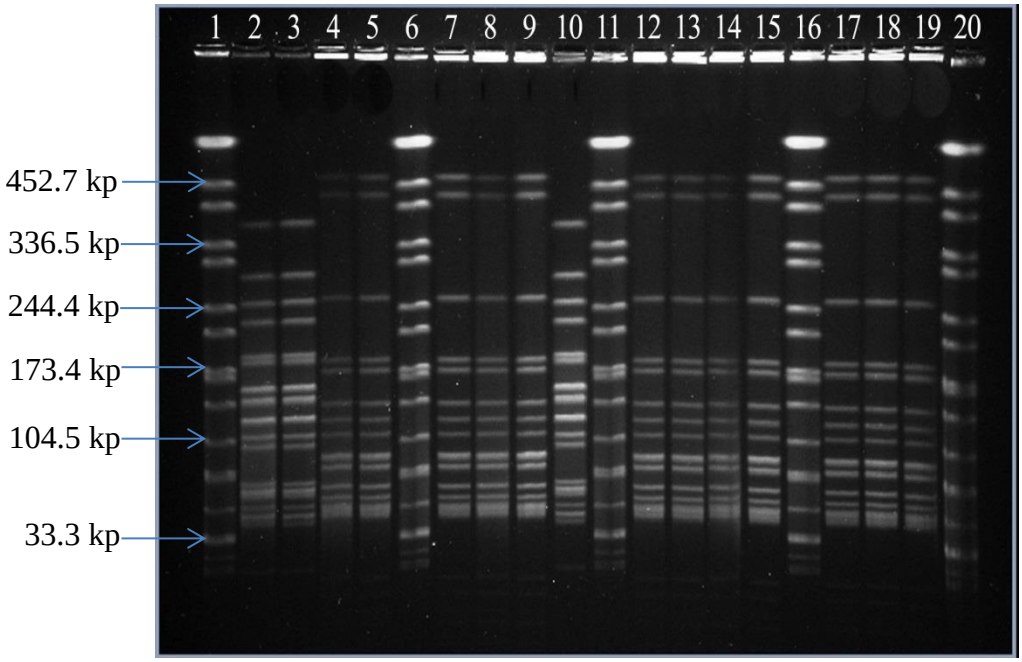


Figure 4.13: The representative gel photo of PFGE for *L. monocytogenes* isolates isolated from RTE food samples. The lables and information of the isolates are summarized in the Table 4.16.

Table 4.16: The labels and detailed information of the isolates in Figure 4.13.

Lane	Code	Date of sampling	Source	Serogroup	Pulsotype
1	H9812-Marker	-	-	-	-
2	LM150	23/8/2011	Chicken[a]	"4b, 4d, 4e"	LmApa18
3	LM161	23/8/2011	Packed lunch	"4b, 4d, 4e"	LmApa18
4	LM163	3/12/2011	Chicken	"1/2a, 3a"	LmApa14
5	LM164	3/12/2011	Salad[b]	"1/2a, 3a"	LmApa13
6	H9812-Marker	-	-	-	-
7	LM171	3/12/2011	Salad	"1/2a, 3a"	LmApa13
8	LM177	3/12/2011	Chicken	"1/2a, 3a"	LmApa13
9	LM178	3/12/2011	Chicken	"1/2a, 3a"	LmApa13
10	LM184	3/12/2011	Egg	"4b, 4d, 4e"	LmApa19
11	H9812-Marker	-	-	-	-
12	LM191	29/12/2011	Egg	"1/2a, 3a"	LmApa13
13	LM192	29/12/2011	Egg	"1/2a, 3a"	LmApa13
14	LM197	29/12/2011	Salad	"1/2a, 3a"	LmApa13
15	LM198	29/12/2011	Salad	"1/2a, 3a"	LmApa13
16	H9812-Marker	-	-	-	-
17	LM209	1/1/2012	Chicken	"1/2a, 3a"	LmApa12
18	LM213	1/1/2012	Chicken	"1/2a, 3a"	LmApa13
19	LM214	1/1/2012	Chicken	"1/2a, 3a"	LmApa7
20	H9812-Marker	-	-	-	-

[a] Salad: Salads and vegetables; [b] Chicken: Chicken and chicken products;
[c] Egg: Egg and egg products

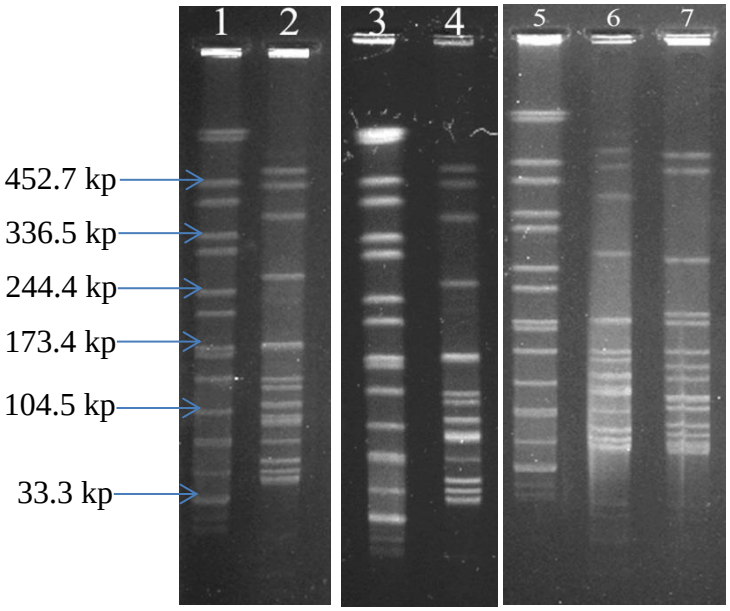


Figure 4.14: The representative gel photo of PFGE for *L. monocytogenes* isolates isolated from RTE food samples. The lables and information of the isolates are summarized in the Table 4.17.

Table 4.17: The labels and detailed information of the isolates in Figure 4.14.

Lane	Code	Date of sampling	Source	Serogroup	Pulsotype
1	H9812-Marker	-	-	-	-
2	LM59	21/03/2011	Chicken[a]	"1/2c, 3c"	LmApa4
3	H9812-Marker	-	-	-	-
4	LM61	21/03/2011	Salad[b]	"1/2c, 3c"	LmApa2
5	H9812-Marker	-	-	-	-
6	LM85	4/4/2011	Salad	"1/2c, 3c"	LmApa5
7	LM92	4/4/2011	Egg[c]	"1/2a, 3a"	LmApa11

[a] Chicken: Chicken and chicken products; [b] Salad: Salads and vegetables; [c] Egg: Egg and egg products

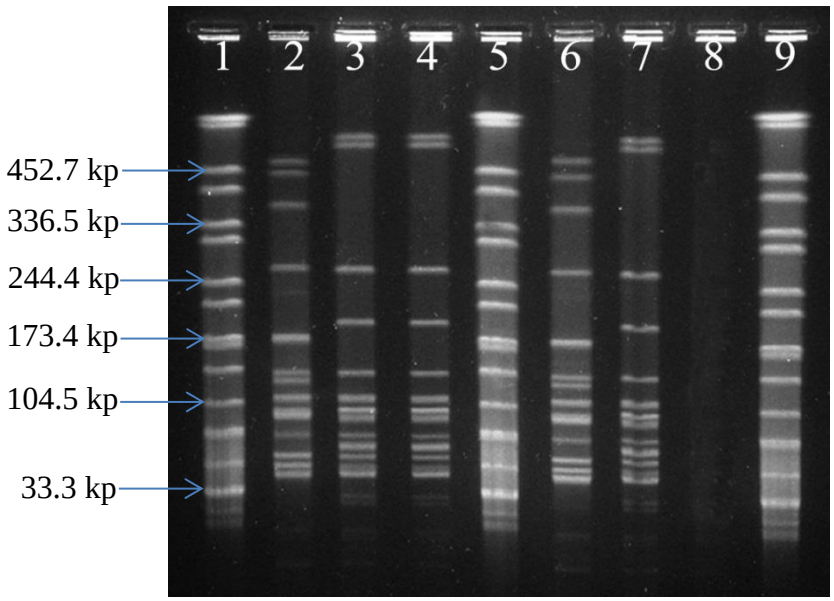


Figure 4.15: The representative gel photo of PFGE for *L. monocytogenes* isolates isolated from RTE food samples. The lables and information of the isolates are summarized in the Table 4.18.

Table 4.18: The labels and detailed information of the isolates in Figure 4.15.

Lane	Code	Date of sampling	Source	Serogroup	Pulsotype
1	H9812-Marker	-	-	-	-
2	LM15	14/12/2010	Salad[a]	"1/2a, 3a"	LmApa6
3	LM31	31/02/2011	Salad	"1/2a, 3a"	LmApa15
4	LM34	31/02/2011	Chicken[b]	"1/2a, 3a"	LmApa15
5	H9812-Marker	-	-	-	-
6	LM41	10/03/2011	Salad	"1/2c, 3c"	LmApa5
7	LM44	10/03/2011	Salad	"1/2a, 3a"	LmApa16
8	-	-	-	-	-
9	H9812-Marker	-	-	-	-

[a] Salad: Salads and vegetables; [b] Chicken: Chicken and chicken products

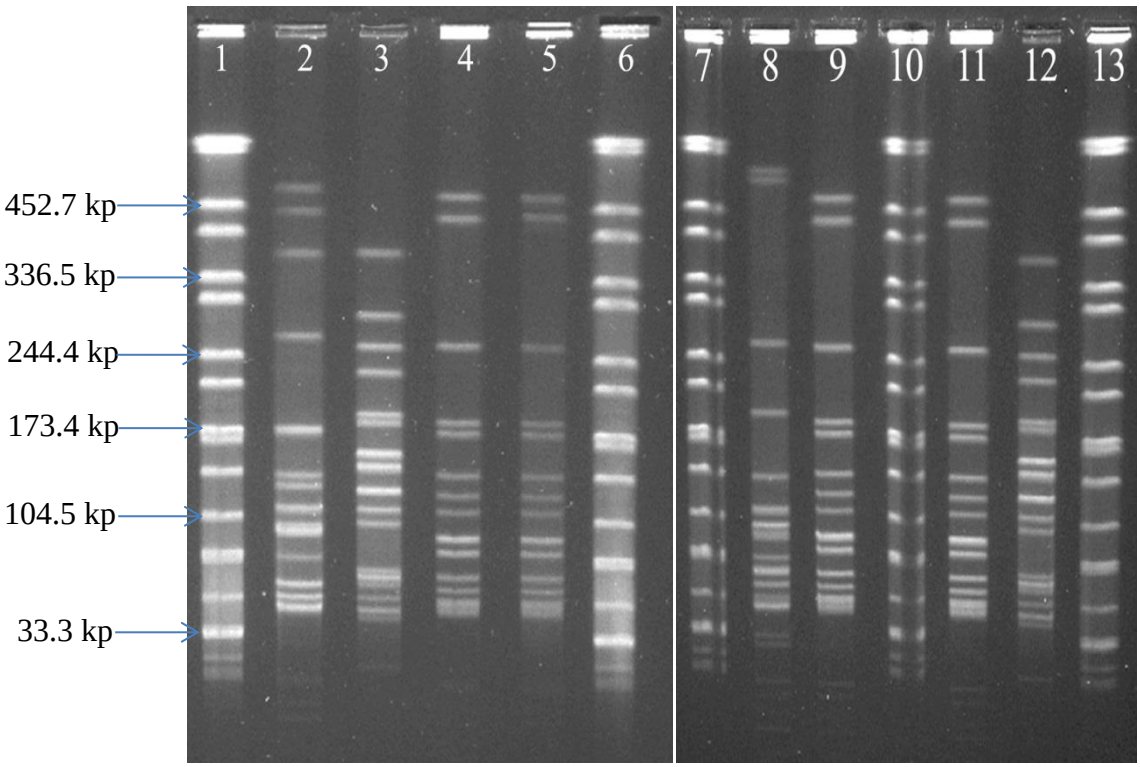


Figure 4.16: The representative gel photo of PFGE for *L. monocytogenes* isolates isolated from RTE food samples. The lables and information of the isolates are summarized in the Table 4.19.

Table 4.19: The labels and detailed information of the isolates in Figure 4.16.

Lane	Code	Date of sampling	Source	Serogroup	Pulsotype
1	H9812-Marker	-	-	-	-
2	LM106	10/05/2011	Salad[a]	"1/2c, 3c"	LmApa1
3	LM161	23/08/11	Packed lunch	"4b, 4d, 4e"	LmApa18
4	LM163	23/08/11	Chicken[b]	"1/2a, 3a"	LmApa14
5	LM149	23/08/11	Seafood[c]	"1/2a, 3a"	LmApa9
6	H9812-Marker	-	-	-	-
7	LM50	10/03/2011	Salad	"1/2a, 3a"	LmApa17
8	LM96	10/05/2011	Beverage	"1/2c, 3c"	LmApa10
9	H9812-Marker	-	-	-	-
10	H9812-Marker	-	-	-	-
11	LM178	03/12/2011	Chicken	"1/2a, 3a"	LmApa13
12	LM184	03/12/2011	Egg[d]	"4b, 4d, 4e"	LmApa19
13	H9812-Marker	-	-	-	-

[a] Salad: Salads and vegetables; [b] Chicken: Chicken and chicken products; [c] Egg: Egg and egg products; [d]Seefood: Seafood and seafood products

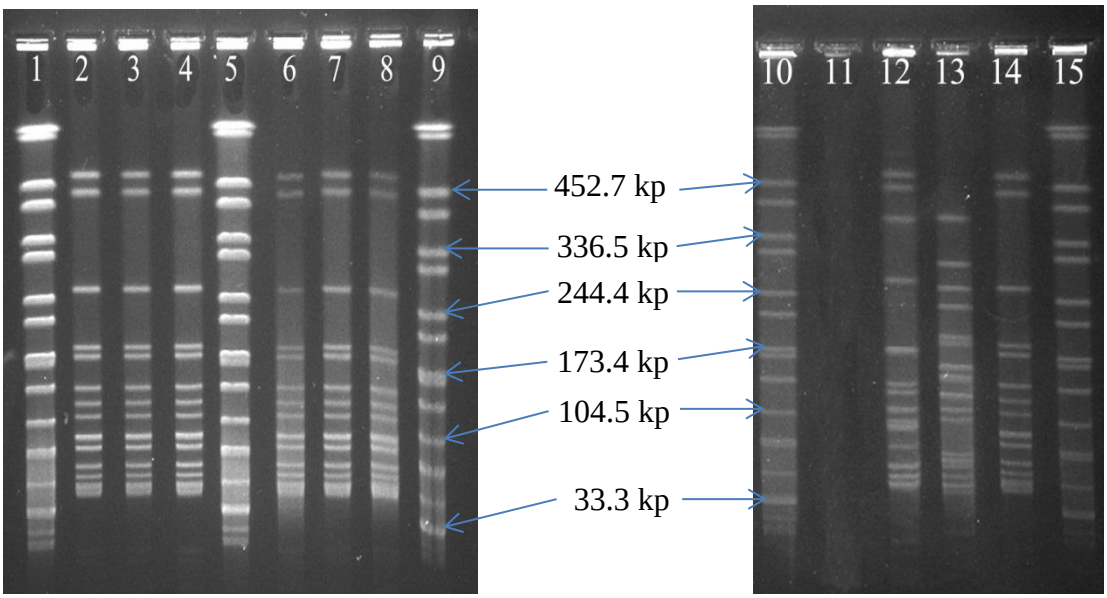
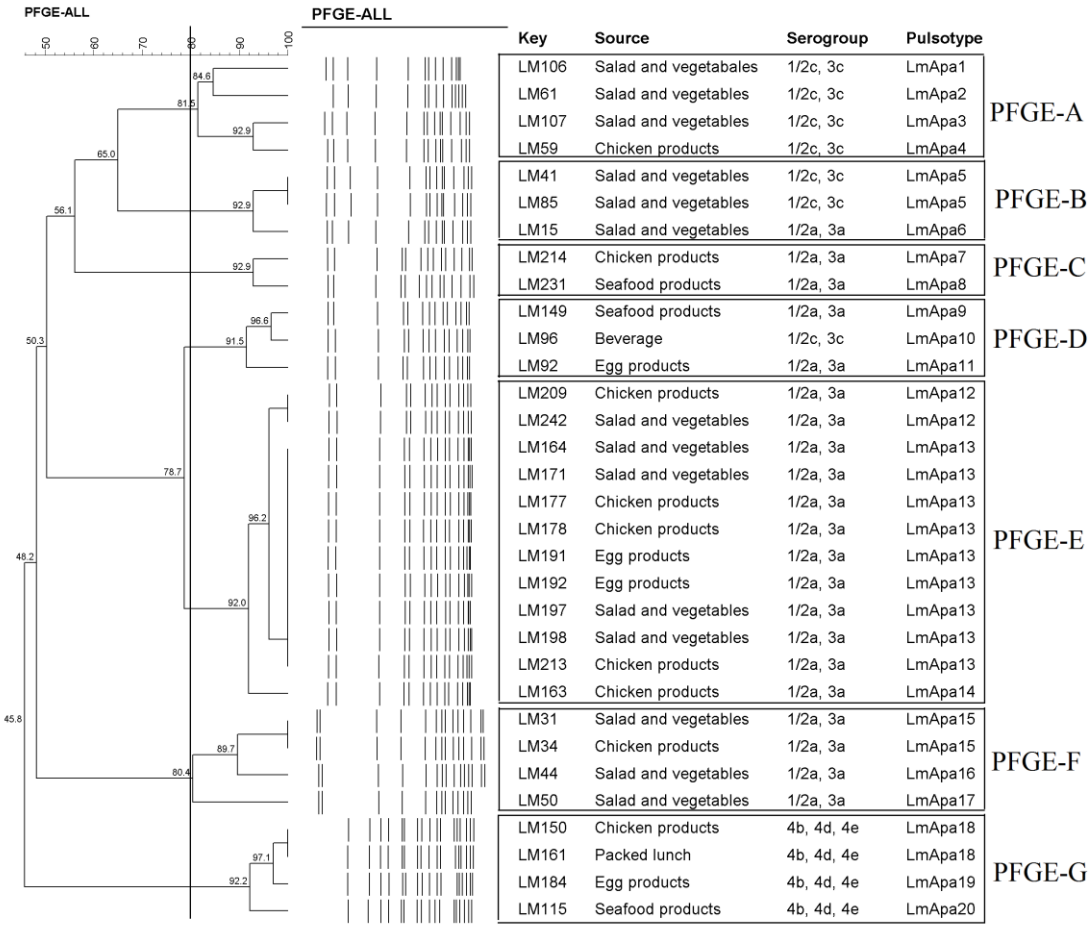


Figure 4.17: The representative gel electrophoresis photo of PFGE for *L. monocytogenes* isolates isolated from RTE food samples. The lables and information of the isolates has been summarized in the Table 4.20.

Table 4.20: The labels and detailed information of the isolates in Figure 4.17.

Lane	Code	Date of sampling	Source	Serogroup	Pulsotype
1	H9812-Marker	-	-	-	-
2	LM198	29/12/2011	Salad[a]	"1/2a, 3a"	LmApa13
3	/LM209	01/01/2012	Chicken[b]	"4b, 4d, 4e"	LmApa12
4	LM242	08/01/2012	Salad	"1/2a, 3a"	LmApa12
5	H9812-Marker	-	-	-	-
6	LM213	01/01/2012	Chicken	"1/2a, 3a"	LmApa13
7	LM214	01/01/2012	Chicken	"1/2a, 3a"	LmApa7
8	LM231	01/01/2012	Seafood[c]	"1/2a, 3a"	LmApa8
9	H9812-Marker	-	-	-	-
10	H9812-Marker	-	-	-	-
11	-	-	-	-	-
12	LM107	10/05/2011	Salad	"1/2c, 3c"	LmApa3
13	LM115	10/05/2011	Seafood	"4b, 4d, 4e"	LmApa20
14	LM149	23/08/11	Seafood	"1/2a, 3a"	LmApa9
15	H9812-Marker	-	-	-	-

[a] Salad: Salads and vegetables; [b] Chicken: Chicken and chicken products; [C] Seefood: Seafood and seafood products



Key	Source	Serogroup	Pulsotype	
LM106	Salad and vegetabales	1/2c, 3c	LmApa1	PFGE-A
LM61	Salad and vegetables	1/2c, 3c	LmApa2	
LM107	Salad and vegetables	1/2c, 3c	LmApa3	
LM59	Chicken products	1/2c, 3c	LmApa4	
LM41	Salad and vegetables	1/2c, 3c	LmApa5	PFGE-B
LM85	Salad and vegetables	1/2c, 3c	LmApa5	
LM15	Salad and vegetables	1/2a, 3a	LmApa6	
LM214	Chicken products	1/2a, 3a	LmApa7	PFGE-C
LM231	Seafood products	1/2a, 3a	LmApa8	
LM149	Seafood products	1/2a, 3a	LmApa9	PFGE-D
LM96	Beverage	1/2c, 3c	LmApa10	
LM92	Egg products	1/2a, 3a	LmApa11	
LM209	Chicken products	1/2a, 3a	LmApa12	PFGE-E
LM242	Salad and vegetables	1/2a, 3a	LmApa12	
LM164	Salad and vegetables	1/2a, 3a	LmApa13	
LM171	Salad and vegetables	1/2a, 3a	LmApa13	
LM177	Chicken products	1/2a, 3a	LmApa13	
LM178	Chicken products	1/2a, 3a	LmApa13	
LM191	Egg products	1/2a, 3a	LmApa13	
LM192	Egg products	1/2a, 3a	LmApa13	
LM197	Salad and vegetables	1/2a, 3a	LmApa13	
LM198	Salad and vegetables	1/2a, 3a	LmApa13	
LM213	Chicken products	1/2a, 3a	LmApa13	
LM163	Chicken products	1/2a, 3a	LmApa14	
LM31	Salad and vegetables	1/2a, 3a	LmApa15	PFGE-F
LM34	Chicken products	1/2a, 3a	LmApa15	
LM44	Salad and vegetables	1/2a, 3a	LmApa16	
LM50	Salad and vegetables	1/2a, 3a	LmApa17	
LM150	Chicken products	4b, 4d, 4e	LmApa18	PFGE-G
LM161	Packed lunch	4b, 4d, 4e	LmApa18	
LM184	Egg products	4b, 4d, 4e	LmApa19	
LM115	Seafood products	4b, 4d, 4e	LmApa20	

Figure 4.18: Dendrogram based on the REP-PCR patterns of *L. monocytogenes* isolates. The isolates were typeable by REP-PCR into 20 pulsotypes and grouped into 7 distinctive clusters based on 80% similarity. PFGE distinguishes each serogroup.

CHAPTER 5

DISCUSSION

Listeria monocytogenes is an important intracellular foodborne pathogen and causes human listeriosis worldwide. Since *L. monocytogenes* is transmitted through contaminated food, the presence of the pathogen in RTE foods which are consumed without heat treatment has public health implications.

The examination of RTE food to detect of *L. monocytogenes* in developed countries is routinely carried out for surveillance of the foodborne pathogen. Colony appearance on selective media was followed by conventional biochemical tests, including Gram determination, catalase, oxidase, TSI, indole, SIM, and MR-VP for detection of *Listeria* species. The conventional biochemical tests which used were not able to distinguish the *L. monocytogenes* from non-*L. monocytogenes* species. Thus, for detection of the species of the isolates, the conventional biochemical tests showed low efficiency. However, the overall findings of these tests indicated that they can be used for detection of *Listeria* at the genus level. Although the majority of the results obtained from the biochemical tests were in agreement with the results of the duplex PCR, the limitation of conventional methods in the detection and differentiation of *Listeria* at species level, confirms that it is required to use these methods together with a molecular method for confirmation.

Conventional methods have limitations in terms of overgrowth of other natural flora present in RTE foods. Molecular methods as rapid detection methods have been shown to be more sensitive and less time-consuming for *L. monocytogenes* detection in foods (Ponniah *et al.*, 2010).

The findings of this study, based on the results obtained from the culturing method and confirmed by the duplex PCR, indicate the prevalence of *L. monocytogenes* and non-*L. monocytogenes* species in 12.8% and 8% of RTE food samples, respectively (Figure 4.1). Other studies in Malaysia also reported high prevalence of *L.*

monocytogenes in different kind of RTE foods. 8.6% of the raw and RTE food samples tested by Marian *et al.* (2012) were found to harbour *L. monocytogenes*. A higher percentage was also reported by Wong *et al.* (2012), in which 22.3% of frozen burger patties collected from Selangor, Malaysia were found to harbour *L. monocytogenes*.

However, the high prevalence of *Listeria* spp. and *L. monocytogenes* in RTE foods was mainly distributed in RTE food type of salad and vegetables. Both salad and vegetables did not go through heat process. This explained why salads and vegetables were highly contaminated with *Listeria* spp. and *L. monocytogenes*. Furthermore, salad dressings are high in nutrients and this could encourage the growth of *Listeria* spp. that was present in the salads. Ponniah *et al.* (2010) showed that 22.5% of the salad vegetables were positive for *L. monocytogenes*. The results obtained from this study and other relevant investigation affirms the potential of *L. monocytogenes* as a major foodborne pathogen. The high prevalence of *L. monocytogenes* in RTE food is considered as potentially hazardous, regarding the probability of pathogenicity among the contaminant strains.

Three serovars 1/2a, 1/2b, and 4b are associated with most of the human listeriosis. Serovar 4b is not the most common subtype isolated from food although it is commonly associated with human listeriosis (50 to 70%) (Martins & Leal Germano, 2011). Thus *L. monocytogenes* with serovar 4b could be more virulent.

As four serovars 3a, 3b, 4d and 4e are rarely isolated from food, serogroups "1/2a, 3a"; "1/2b, 3b"; "1/2c, 3c"; and "4b, 4d, 4e" could be recognized as 1/2a, 1/2b, 1/2c and 4b, respectively as proposed by Zhang *et al.* (2007). Thus, the serovars 1/2a, 1/2c and 4b are most likely associated with our local *L. monocytogenes* isolated from RTE. However, the actual serovar distribution needs to be confirmed by conventional serotyping on a larger sample size.

This is the first study where we could identify the different serogroups (or serovars) of *L. monocytogenes* in Malaysia. The high percentage rate of serogroup "1/2a, 3a" in this study is in agreement with other studies (Nucera *et al.*, 2010; Yan *et al.*, 2010; Wang *et al.*, 2013) which reported a high prevalence of serovar 1/2a in food samples. The presence of epidemiological important serovars 1/2a (or 3a) and 4b (or 4d, 4e) among almost all kinds of food categories indicates possible diverse sources of listeriosis in Selangor, Malaysia.

There have been some reports of multidrug resistant *L. monocytogenes* isolated from listeriosis cases, and foods (Morvan et al., 2010; Adetunji and Isola, 2011; Bhat et al., 2012). Generally, penicillin or ampicillin often in combination with an aminoglycoside is chosen for medical treatment of listeriosis cases (Swaminathan & Gerner-Smidt, 2007). *L. monocytogenes* isolated from food samples were sensitive to penicillin in the most of studies. In a previous study in China, one out of 90 (1%) *L. monocytogenes* isolates showed resistance to penicillin G (Yan *et al.*, 2010). On the other hand, Marian *et al.* (2012) had reported high resistance against penicillin G (100%) from raw and ready-to-eat foods in Malaysia and also Morobe *et al.* (2009) have been found high prevalence percentage of resistance to penicillin G (42%) from various foods in Botswana, which were similar to our finding (53%). This could be attributed to the use of this antibiotic as the first choice drug in the treatment of listeriosis (Hof, 2003; Conter *et al.*, 2009) as well as its use in animals' feed (Rahimi *et al.*, 2010). Mutations in chromosomal genes or gene transfer from transposons and plasmids of other Gram positive bacteria could result in antimicrobial resistance in the pathogen (Harakeh *et al.*, 2009).

Only one isolate from a seafood sample was multidrug resistant (MDR) (resistant to three or more classes of antimicrobial agents). Although *L. monocytogenes* is known to be susceptible to a variety of antimicrobial agents (Altuntas *et al.*, 2012),

the presence of MDR *L. monocytogenes* from food and human cases (Morvan *et al.*, 2010; Bhat *et al.*, 2012) is worrisome.

The results obtained from virulotyping of *L. monocytogenes* isolates confirmed the existence of *inlA, inlB, inlC* and *inlJ* genes among the local isolates. The four genes have indispensable roles in virulence (Liu *et al.*, 2007). The results were similar to previous studies, which *inlA, inlB, inlC* and *inlJ* genes were positive in all or most the examined *L. monocytogenes* isolates from environment (Gelbíčová, & Karpíšková., 2012), food samples (Indrawattana *et al.*, 2011; Lomonaco *et al.*, 2012; Sant'Ana *et al.*, 2012), listeriosis cases in humans (Mammina *et al.*, 2009) and animals (Marien *et al.*, 2007). Hence, the results indicate that pathogenic *L. monocytogenes* isolates are present in RTE foods sold in street-side hawker stalls and hypermarkets in Malaysia and it confirms the high risk of infection for RTE foods consumers.

The PCR-RFLP results showed that the polymorphism of *inlA* and *inlC* genes had limited variation. There was no correlation between the source of *L. monocytogenes* and PCR-RFLP profiles. These results are in agreement with an earlier finding (Strydom *et al.*, 2013). However, in our study, all isolates of serogroup "4b, 4d, 4e" were grouped in one profile (Al2).

The results gained from REP-PCR, BOX-PCR and RAPD indicated genetic diversity among food *L. monocytogenes*. The isolates with in the same serogroup were grouped into the same cluster by REP-PCR and BOX-PCR. Chou and Wang (2006) reported that *L. monocytogenes* isolates were classified by REP-PCR into the clusters based on their serogroups (flagella antigen groups). In contrast, RAPD failed to group the *L. monocytogenes* isolates with in the same serogroup. In earlier study, Aurora *et al.* (2009) reported that different serogroups of *L. monocytogenes* isolates were observed with identical RAPD profiles. This shows the low discriminatory power of RAPD.

Genotyping of *L. monocytogenes* isolates by PFGE indicated that the 32 food *L. monocytogenes* isolates were genetically diverse and heterogeneous and this is in concordance with earlier finding (Yan *et al.,* 2010). No clear genetic relatedness was observed among the isolates isolated from the same type of RTE food, the same location or at the same time. However, the same serogroup isolates were classified into the same cluster. High genetic diversity of *L. monocytogenes* strains had been previously reported in other studies (Zhang *et al.*, 2007; Nucera *et al.*, 2010). Nucera *et al.* (2010) reported 164 different PFGE profiles obtained from 300 *L. monocytogenes* isolates. In another study, Zhang *et al.* (2007) reported 120 different PFGE profiles were observed among 167 *L. monocytogenes* isolates.

More than half of the isolates (53.1%) with the same serogroup were grouped into the same distinct PFGE cluster. Seventeen *L. monocytogenes* isolates that had identical REP-, BOX- and RAPD- profiles were also indistinguishable by their pulsotypes. The majority of *L. monocytogenes* isolates which showed same pulsotypes were isolated from different food categories at different sampling times and location. This could indicate persistence of this organism in this region. On the other hand, eight *L. monocytogenes* isolates (LM150 and LM161; LM197 and LM178; LM192 and LM198; LM214 and LM231) which had same REP profile were distinguishable by their pulsotypes. These isolates were detected from different food categories but were sampled at the same sampling times and location. This could indicate cross-contamination of *L. monocytogenes* isolates between food handlers and RTE foods. Food handlers are considered a significant route in the spread of *L. monocytogenes* in foods through cross-contamination (Cocolin *et al.,* 2009).

There was no correlation between the antibiograms with pulsotypes, PCR-typing, PCR-RFLP profiles, and sources. As in the current study, associations between antimicrobial susceptibility profiles, pulsotypes, PCR typing profiles, PCR-RFLP and

specific food types were not found for isolates of *L. monocytogenes* from different kind of foods (Yan et al, 2010; Strydom et al., 2013; Wang et al., 2013). The apparent lack of association could be due to the small sample size. Hence, for better understanding and control of source of the pathogen contamination, a bigger sample size from wide geographical areas and different kinds of sources be investigated in future study.

In conclusion, the genotyping data showed that the food *L. monocytogenes* isolates were genetically diverse and heterogeneous. Different methods have different levels of discriminatory powers, therefore the application of more than one subtyping approach would provide a more accurate picture of the clonality of *L. monocytogenes*. Furthermore, the presence of the epidemiologically important serovars of *L. monocytogenes* 1/2a (or 3a) and 4b (or 4d, 4e) which are associated with human listeriosisis of public health concern. Hence, surveillance programs are needed to monitor epidemiological information on the pathogen dispersion in different sources in Malaysia.

CHAPTER 6

CONCLUSIONS

1. The prevalence of *Listeria* spp. and *L. monocytogenes* in different kinds of RTE foods sold in street-side hawker stalls and supermarkets in Malaysia and the presence of serogroup 4b (or 4d, 4e) among the isolates pose a high risk of infection for the consumers if such contaminated foods were consumed.
2. All the 32 *L. monocytogenes* isolates were positive for *inlA, inlB, inlC* and *inlJ* genes. The presence of internalin genes (*inlA, inlB, inlC* and *inlJ*) in *L. monocytogenes* isolates from the RTE food samples indicate the virulence potential of the food *L. monocytogenes* isolates.
3. The PCR-RFLP results showed that the polymorphism of *inlA* and *inlC* genes had limited variation. It seems, therefore, that the PCR-RFLP with these primers and restriction endonucleases may not be sufficiently discriminative for polymorphisms and subtyping studies.
4. The presence of multi-drug resistance and the high level of penicillin G resistance (53.1%) could be a public health concern, because it is used in combination with gentamicin for treatment of human listeriosis. However, all the *L. monocytogenes* isolates were susceptible to gentamicin and trimethoprim-sulfamethoxazole.
5. The results obtained from REP-PCR, BOX-PCR, RAPD and PFGE indicated high genetic diversity among the *L. monocytogenes* isolates. REP-PCR, BOX-PCR and PFGE were able to distinguish isolates with different serogroups.

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APPENDIX I

Background data for *Listeria* spp. isolates

Isolates code, date of sampling, type of foods and location of sampling

No	Code	Date of sampling	Source of sample	Food category	Location	Biochemical Tests[a]	PCR confirmation[b]	Direct PCR[c]
1	L09/11/10 (L09)	30/11/2010	Malaysian Salad with vinegar	Salad and vegetables	Chow kit	+	+ (L. sp.)[h]	+ (L. sp.)[h]
2	L10/11/10 (L10)	30/11/2010	Malaysian Salad with vinegar	Salad and vegetables	Chow kit	+	+ (L. sp.)[h]	+ (L. sp.)[h]
3	L15/12/10 (LM15)	14/12/2010	Cabbage	Salad and vegetables	Chow kit	+	+ (L.M)[i]	+ (L.M)[i]
4	L31/02/2011 (LM31)	21/02/2011	Cabbage	Salad and vegetables	University LRT station	+	+ (L.M)[i]	+ (L.M)[i]
5	L34/02/2011 (LM34)	21/02/2011	Satay	Cooked chicken[d]	University LRT station	+	+ (L.M)[i]	+ (L.M)[i]
6	L41/03/11 (LM41)	10/3/2011	Malaysian Salad with vinegar	Salad and vegetables	Chow kit	+	+ (L.M)[i]	-

No	Code	Date of sampling	Source of sample	Food category	Location	Biochemical Tests[a]	PCR confirmation[b]	Direct PCR[c]
7	L44/03/11 (LM44)	10/3/2011	Malaysian Salad with vinegar	Salad and vegetables	Chow kit	+	+ (L.M)[i]	-
8	L50/03/11 (LM50)	10/3/2011	Cucumber	Salad and vegetables	Chow kit	+	+ (L.M)[i]	+ (L.M)[i]
9	L56/03/11 (L56)	21/03/211	Boiled egg	Egg[e]	University LRT station	+	+ (L. sp.)[h]	+ (L. sp.)[h]
10	L57/03/11 (L57)	21/03/211	Boiled egg	Egg[e]	University LRT station	+	+ (L. sp.)[h]	+ (L. sp.)[h]
11	L59/03/11 (LM59)	21/03/211	Chicken	Cooked chicken[d]	University LRT station	+	+ (L.M)[i]	+ (L.M)[i]
12	L61/03/11 (LM61)	21/03/211	Malaysian Salad with vinegar	Salad and vegetables	University LRT station	+	+ (L.M)[i]	+ (L.M)[i]
13	L62/03/11 (L62)	21/03/211	Malaysian Salad with vinegar	Salad and vegetables	University LRT station	+	+ (L. sp.)[h]	+ (L. sp.)[h]
14	L71/03/11 (L71)	21/03/211	Bean sprout	Salad and vegetables	University LRT station	+	+ (L. sp.)[h]	+ (L. sp.)[h]
15	L81/04/11 (L81)	4/4/2011	Chicken	Cooked chicken[d]	University of Malaya	+	+ (L. sp.)[h]	+ (L. sp.)[h]

No	Code	Date of sampling	Source of sample	Food category	Location	Biochemical Tests[a]	PCR confirmation[b]	Direct PCR[c]
16	L83/04/11 (L83)	4/4/2011	Chicken	Cooked chicken[d]	University of Malaya	+	+ (L. sp.)[h]	+ (L. sp.)[h]
17	L85/04/11 (LM85)	4/4/2011	Bean sprout	Salad and vegetables	University of Malaya	+	+ (L.M)[i]	+ (L.M)[i]
18	L88/04/11 (L88)	4/4/2011	Lettuce	Salad and vegetables	University of Malaya	+	+ (L. sp.)[h]	+ (L. sp.)[h]
19	L92/04/11 (LM92)	4/4/2011	Pastry	Egg[e]	University of Malaya	+	+ (L.M)[i]	+ (L.M)[i]
20	L96/05/11 (LM96)	10/5/2011	Orange flavoured drink	Beverage	Pasar malam - PJ 17	+	+ (L.M)[i]	+ (L.M)[i]
21	L106/05/11 (LM106)	10/5/2011	Malaysian Salad with vinegar	Salad and vegetables	Pasar malam - PJ 17	+	+ (L.M)[i]	+ (L.M)[i]
22	L107/05/11 (LM107)	10/5/2011	Malaysian Salad with vinegar	Salad and vegetables	Pasar malam - PJ 17	+	+ (L.M)[i]	-
23	L115/05/11 (LM115)	10/5/2011	Fried fish	Seafood[f]	Pasar malam - PJ 17	+	+ (L.M)[i]	+ (L.M)[i]
24	L139/08/11 (L139)	16/08/2011	Fried fish	Seafood[f]	University LRT station	+	+ (L. sp.)[h]	+ (L. sp.)[h]
25	L149/08/11 (LM149)	23/08/2011	Cuttlefish	Seafood[f]	Jusco - Midvalley	+	+ (L.M)[i]	+ (L.M)[i]

No	Code	Date of sampling	Source of sample	Food category	Location	Biochemical Tests[a]	PCR confirmation[b]	Direct PCR[c]
26	L150/08/11 (LM150)	23/08/2011	Chicken heart	Cooked chicken[d]	Jusco - Midvalley	+	+ (L.M)[i]	+ (L.M)[i]
27	L155/08/11 (L155)	23/08/2011	Potato salad with mayonnaise	Salad and vegetables	Jusco - Midvalley	+	+ (L. sp.)[h]	+ (L. sp.)[h]
28	L161/08/11 (LM161)	23/08/2011	Rice with dishes (Fish, rice, bean, sausage, lettuce, carrot)	Packed lunch	Jusco - Midvalley	+	+ (L.M)[i]	+ (L.M)[i]
29	L163/12/11 (LM163)	3/12/2011	Satay	Cooked chicken[d]	University LRT station	+	+ (L.M)[i]	+ (L.M)[i]
30	L164/12/11 (LM164)	3/12/2011	Salad (Jelantah)	Salad and vegetables	University LRT station	+	+ (L.M)[i]	+ (L.M)[i]
31	L168/12/11 (L168)	3/12/2011	Salad (Jelantah)	Salad and vegetables	University LRT station	+	+ (L. sp.)[h]	+ (L. sp.)[h]
32	L171/12/11 (LM171)	3/12/2011	Salad (Jelantah)	Salad and vegetables	University LRT station	+	+ (L.M)[i]	+ (L.M)[i]
33	L177/12/11 (LM177)	3/12/2011	Sausage	Cooked chicken[d]	University LRT station	+	+ (L.M)[i]	+ (L.M)[i]
34	L178/12/11 (LM178)	3/12/2011	Sausage	Cooked chicken[d]	University LRT station	+	+ (L.M)[i]	+ (L.M)[i]

No	Code	Date of sampling	Source of sample	Food category	Location	Biochemical Tests[a]	PCR confirmation[b]	Direct PCR[c]
35	L184/12/11 (LM184)	3/12/2011	Popia goreng	Egg[e]	University LRT station	+	+ (L.M)[i]	+ (L.M)[i]
36	L189/12/11 (L189)	29/12/2011	Chicken rice	Packed lunch	Carrefour-Midvalley	+	+ (L. sp.)[h]	+ (L. sp.)[h]
37	L191/12/11 (LM191)	29/12/2011	Fried egg	Egg[e]	Carrefour-Midvalley	+	+ (L.M)[i]	+ (L.M)[i]
38	L192/12/11 (LM192)	29/12/2011	Fried egg	Egg[e]	Carrefour-Midvalley	+	+ (L.M)[i]	+ (L.M)[i]
39	L194/12/11 (L194)	29/12/2011	Fish	Seafood[f]	Carrefour-Midvalley	+	+ (L. sp.)[h]	+ (L. sp.)[h]
40	L195/12/11 (L195)	29/12/2011	Fish	Seafood[f]	Carrefour-Midvalley	+	+ (L. sp.)[h]	+ (L. sp.)[h]
41	L197/12/11 (LM197)	29/12/2011	Fruit salad	Salad and vegetables	Carrefour-Midvalley	+	+ (L.M)[i]	+ (L.M)[i]
42	L198/12/11 (LM198)	29/12/2011	Fruit salad	Salad and vegetables	Carrefour-Midvalley	+	+ (L.M)[i]	+ (L.M)[i]
43	L200/12/11 (L200)	29/12/2011	Jumbo sausage	Cooked Beef[g]	Carrefour-Midvalley	+	+ (L. sp.)[h]	+ (L. sp.)[h]
44	L209/01/12 (LM209)	1/1/2012	Chicken liver	Cooked chicken[d]	Jusco - Midvalley	+	+ (L.M)[i]	+ (L.M)[i]

No	Code	Date of sampling	Source of sample	Food category	Location	Biochemical Tests[a]	PCR confirmation[b]	Direct PCR[c]
45	L211/01/12 (L211)	1/1/2012	Fish roll	Seafood[f]	Jusco - Midvalley	+	+ (L. sp.)[h]	+ (L. sp.)[h]
46	L213/01/12 (LM213)	1/1/2012	Chicken	Cooked chicken[d]	Jusco - Midvalley	+	+ (L.M)[i]	+ (L.M)[i]
47	L214/01/12 (LM214)	1/1/2012	Chicken	Cooked chicken[d]	Jusco - Midvalley	+	+ (L.M)[i]	+ (L.M)[i]
48	L227/01/12 (L227)	1/1/2012	Sushi (Smoked salmon)	Seafood[f]	Jusco - Midvalley	+	+ (L. sp.)[h]	+ (L. sp.)[h]
49	L231/01/12 (LM231)	1/1/2012	Sushi (Ebikko)	Seafood[f]	Jusco - Midvalley	+	+ (L.M)[i]	+ (L.M)[i]
50	L242/01/12 (LM242)	8/1/2012	Fruit salad	Salad and vegetables	Carrefour-Midvalley	+	+ (L.M)[i]	+ (L.M)[i]
51	L243/01/12 (L243)	8/1/2012	Fruit salad	Salad and vegetables	Carrefour-Midvalley	+	+ (L. sp.)[h]	+ (L. sp.)[h]
52	L248/01/12 (L248)	8/1/2012	Fried chicken	Cooked chicken[d]	Carrefour-Midvalley	+	+ (L. sp.)[h]	-

[a] Biochemical Tests: The results and presumptions of conventional biochemical tests; [b] PCR confirmation: The results obtained from confirmation of presumptive isolates; [d] Direct PCR: The results obtained from detection of *Listeria* spp. and *L. monocytogenes* directly from food homogenates; [e] Cooked chicken: Cooked chicken and chicken products; [f] Seafood: Cooked seafood and seafood products; [g] Cooked beef: Cooked beef and beef products.

[h] L. sp.: Non-*Listeria monocytogenes*; [i] L. M: *Listeria monocytogenes.*

APPENDIX II

Chemicals / Reagents	Manufacture Suppliers
Absolute alcohol	BDH Lab Supplier, England
Agarose powder (LE Analytical Graade)	Promega, Madison, Wisconsin, US
Agarose, Type I, for PFGE	Sigma Chemical Company, USA
Antibiotic discs	Oxoid Ltd, England
CHROMagar^{TM} *Listeria*	CHROMagar, Paris, France
dNTPs (2.5mM)	iNtRON Biotechnology, South Korea
Ethidium Bromide (EtBr)	Sigma Chemical Company, USA
Ethylenediamine-tetraacetic acid (EDTA)	Sigma Chemical Company, USA
Glycerol	Invitrogen, USA
Listeria Selective agar	Oxoid Ltd, England
Methyl Red-Voges Proskauer (MRVP)	Oxoid Ltd, England
$MgCl_2$ for PCR (25mM)	iNtRON Biotechnology, South Korea
Mueller Hinton agar	Oxoid Ltd, England
Palcam agar	Oxoid Ltd, England
10X PCR buffer	iNtRON Biotechnology, South Korea
Primers	Operon Biotechnologies GmbH, Germany
SIM medium	Oxoid Ltd, England
Sodium Chloride (NaCl)	BDH Lab Supplier, England

Triple Suger Iron Agar (TSI)	Oxoid Ltd, England
Trypticase Soy Agar	Difco, Becton, Dickinson, USA
Yeast Extract	Oxoid Ltd, England

Enzymes and Proteinase	**Manufacture Supplier**
Proteinase K	Promega, Madison, Wisconsin, USA
Taq DNA polymerase	iNtRON Biotechnology, South Korea
*Alu*I	Promega, Madison, Wisconsin, USA
*Xba*I	Promega, Madison, Wisconsin, USA
*Mbo*I	Promega, Madison, Wisconsin, USA

DNA Ladders and Commercial kit Manufacturer Supplier	
100 bp molecular size marker	Promega, Madison, Wisconsin, USA
1 kb molecular size marker	Fermentas, Vilnius, Lithuania
Gel Extraction Kit	iNtRON Biotechnology, South Korea

1% SeaKem Gold (SKG) Agarose

Seakem Gold (SKG) agarose	0.2 g
TE buffer	20.0 ml

0.2 g of SeaKem Gold Agarose powder suspended with 20 ml of TE buffer. Then, it was boiled to dissolve completely.

Agarose gel (1%) for PCR

Agarose gel (Promega)	0.32 g
0.5X TBE buffer	32 ml

The agarose mixture was heated slowly until the agarose particles completely dissolved. Then it was kept in water bath equilibrated to 55-60˚C before pour for casting the gel.

Agarose gel (1%) for PFGE

Agarose gel (Promega)	1.5 g
0.5X TBE buffer	150 ml

The agarose mixture was heated slowly until the agarose particles completely dissolved. Then it was kept in water bath equilibrated to 55-60˚C before pour for casting the gel.

APPENDIX III

Media for bacteria growth

Enrichment Media

Half Fraser Broth

Fraser broth	12.9 g
Distilled water	225 ml

Half Fraser Broth Supplement

Half Fraser Supplement (SR0166E)	1 vial
Ethanol/Sterile distilled water (1:1)	4 ml

The media was autoclaved at 121˚C for 15min. It was then cooled down to 45˚C. Four ml of sterile distilled water/ ethanol (1:1) were added to one vial of the Half Fraser supplement (SR0166E), then the prepared supplement was mixed with the media. The prepared media were kept in dark at the refrigeration temperature (4˚C).

Fraser Broth

Fraser broth	28.7 g
Distilled water	500 g

Fraser Broth Supplement

Half Fraser Supplement (SR0156)	1 vial
Ethanol/Sterile distilled water (1:1)	5 ml

The media was autoclaved at 121˚C for 15min. It was then cooled down to 45˚C. Five ml of sterile distilled water/ ethanol (1:1) were added to one vial of Fraser selective supplement (SR0156), then the prepared supplement was mixed with the media. The prepared media were kept in dark at the refrigeration temperature (4˚C).

Selective Media

CHROMagar™ *Listeria*

The Agar was suspended in the proportion 51.5 g/L in distilled water. The media was then autoclaved at 121˚C for 15min. It was then cooled down in a water bath to 47±2˚C. Nine g of CHROMagar™ *Listeria* supplement was suspended in 40 ml of sterile water. Finally, the supplement was aseptically added to CHROMagar™ *Listeria* and was mixed gently. The prepared media was dispensed into plates and were kept in dark at the refrigeration temperature (at 4 ˚C).

***Listeria* Selective Agar (LSA)**

Listeria Selective Agar Base	27.75 g
Distilled water	500 ml
<u>*Listeria* Selective Supplement</u>	
Listeria Selective Supplement (SR0140)	1 vial
Ethanol/Sterile distilled water (1:1)	5 ml

The media was autoclaved at 121˚C for 15min. It was then cooled down to 45˚C. Five ml of sterile distilled water/ ethanol (1:1) were added to one vial of *Listeria* selective

supplement (SR0140), and then the prepared supplement was mixed with the media. After that the media was dispensed into plates and the plates were kept in dark at the refrigeration temperature (4˚C).

PALCAM agar

PALCAM agar base	34.5 g
Distilled water	500 ml

PALCAM Selective Supplement

PALCAM Selective Supplement (SR150E)	1 vial
Sterile distilled water	2 ml

The media was autoclaved at 121˚C for 15min. It was then cooled down to 45˚C. Two ml of sterile distilled water were added to one vial of PALCAM selective supplement (SR0150), and then the prepared supplement was mixed with the media. After that the media was dispensed into plates and the plates were kept in dark at the refrigeration temperature (4˚C).

Non-selective Medium

Trypticase Soy Agar with 0.6% yeast extract agar (TSAYE):

Trypticase Soy Agar	40.0 g
Yeast Extract	6 g
Distilled water	1 L

The media was autoclaved at 121˚C for 15min. It was cooled down to 45-50˚C and was dispensed into plates. The plates were kept at the refrigerator temperature (at 4 ˚C).

Brain Heart Infusion Agar

BHI agar	52 g
Distilled water	1 L

The suspension was gently brought to boil to dissolve completely. It was then autoclaved at 121˚C for 15min. It was cooled down to 45-50˚C and was dispensed into plates. The plates were kept at the refrigerator temperature (at 4˚C).

Mueller Hinton Agar

MH agar	21.0 g
Distilled water	1 L

The media was then autoclaved at 121˚C for 15min. It was cooled down to 45-50˚C and was dispensed into plates. The plates were kept at the refrigerator temperature (at 4 ˚C).

APPENDIX IV

50% Glycerol

Ultra pure glycerol	50 ml
ddH_2O	50 ml

The mixture was sterilized by autoclave at 121˚C for 15 min and stored at room temperature.

0.85% Saline

NaCl	0.85 g
ddH_2O	100 ml

NaCl was weighted and mixed with 100 ml of ddH_2O. Next, it was sterilized by autoclace and stored at room temperature.

Ethidium Bromide

Ethidium Bromide	30 µl
dH_2O	300 ml

This solution was stored in a dark bottle at room temperature, and diluted to 0.5 µg/ml with distilled water before used.

10X Tris-borated EDTA (TBE), pH 8.3

Trizma base	121.2 g
Orthoboric/Boric acid	61.8 g
EDTA	0.745 g
dH_2O	1000 ml

The above ingredients were dissolved in 500 ml of deionised water by stirring on the heated stirrer plate. pH was adjusted to 8.3 and top up to 1000 ml and autoclaved at 121˚C; 15 psi for 15 min.

0.5X Tris-borate EDTA (TBE)

10X TBE	50 ml
dH_2O	950 ml

100 ml of 10X TBE was aliquoted into clean sterile glassware and top up to 1000 ml by using the deionised water.

1M Tris, pH 8.0 (Molecular weight = 121.14 g)

Tris	36.342 g
dH_2O	250 ml

36.342 g of Tris powder were dissolved in 250 ml of deionised water by stirring on the heated stirrer plate. pH was adjusted to 8.0 and top up to 300 ml and autoclaved at 121˚C; 15 psi for 15 min.

0.5 M EDTA, pH 8.0 (Molecular weight = 372.24 g)

EDTA	55.83 g
dH_2O	250 ml

55.83 g of EDTA powder were dissolved in 250 ml of deionised water by stirring on the heated stirrer plate. pH was adjusted to 8.0 and top up to 300 ml and autoclaved at 121˚C; 15 psi for 15 min.

Tris-EDTA (TE) buffer (10 mM Tris; 1 mM EDTA; pH 8.0)

1M Tris, pH 8.0	10 ml
0.5M EDTA, pH 8.0	2 ml

Top up with deionised water to 1000 ml, and autoclaved 121˚C; 15 psi for 15 min.

Cell Suspension Buffer (100 mM Tris; 100 mM EDTA; pH 8.0

1M Tris, pH 8.0	10 ml
0.5M EDTA, pH 8.0	20 ml
Top up with deionised water to	100 ml

Autoclaved 121˚C; 15 psi for 15 min.

10% Sarcosyl (N-Lauryl-Sarcosine, Sodium Salt) (Molecular weight = 293.39 g)

Sodium N-Lauryl-Sarcosinate solution	10 ml
Top up with deionised water to	100 ml

Autoclaved 121˚C; 15 psi for 15 min.

Cell Lysis Buffer (50mM Tris; 50mM EDTA; pH 8.0; 1% Sarcosine)

1M Tris, pH 8.0	25 ml
0.5M EDTA, pH 8.0	50 ml
10% Sarcosyl (N-Lauryl-Sarcosine, Sodium Salt)	50 ml
Top up with deionised water to	500 ml

Autoclaved 121˚C; 15 psi for 15 min.

Proteinase K 20 mg/ml

Proteinase K powder	200 mg
Deionised water	10 ml

APPENDIX V

Biochemical Tests

Gram determination

The reaction between 3% KOH and a single colony of the bacteria is evaluated. After 60 seconds, when the loop is lifted, Gram positive bacteria (negative KOH reaction) do not show change in viscosity of the cell suspension.

Catalase Test

A single colony is mixed with a drop of 3% hydrogen peroxide on a slide. *Listeria* spp. is catalase positive so will produce immediate bubbling.

Oxidase Test

N, N, N', N'-tetra-methyl-p-phenylenediamine dihydrochloride (≥95%) powder.

Triple Sugar Iron (TxSI)

TSI Agar	5.5 g
ddH_2O	100 ml

Ten ml of the media was dispensed into each test tube. The tubes were autoclaved at 121˚C for 15 min and they were then put slant to harden.

Sulfur reduction – Indol – Motility (SIM) Test

SIM powder was added to distilled water as recommended by the manufacturer and was boiled to dissolve. It was dispensed into sterile universal bottles and the bottles were then autoclaved for 15 min at 121˚C. They were left on the bench at room temperature to harden.

The Methyl Red (MR) and Voges-Proskauer (VP) Tests

MR-VP powder was added into distilled water following the guidelines and was boiled to dissolve, then was dispensed into test tubes (5ml / test tube) and the tubes were autoclaved for 15 min at 121˚C.

APPENDIX VI

Phenotypic and genotypic characteristics of *L. monocytogenes*.

Isolates No.	Date of sampling	Source	Serogroup	REP-type	BOX-type	RAPD-type	Pulsotype	PCR-RFLP *inlC* profile	Antibiograms								
									DA	VA	S	TE	E	K	AMC	P	C
LM15	14/12/2010	Salad[a]	1/2a, 3a	LmREP24	LmBOX6	LmRAPD17	LmApa6	Al1	I	I	S	S	S	S	S	S	S
LM31	21/02/2011	Salad[a]	1/2a, 3a	LmREP19	LmBOX20	LmRAPD13	LmApa15	Al2	S	I	I	S	S	S	R	R	S
LM34	21/02/2011	Chicken[b]	1/2a, 3a	LmREP23	LmBOX21	LmRAPD9	LmApa15	Al2	I	I	S	S	S	S	R	R	S
LM41	10/3/2011	Salad[a]	1/2c, 3c	LmREP12	LmBOX19	LmRAPD10	LmApa5	Al1	I	R	S	S	S	I	S	R	R
LM44	10/3/2011	Salad[a]	1/2a, 3a	LmREP21	LmBOX17	LmRAPD16	LmApa16	Al2	S	S	S	S	S	S	S	S	S
LM50	10/3/2011	Salad[a]	1/2a, 3a	LmREP22	LmBOX23	LmRAPD23	LmApa17	Al2	I	S	S	S	S	S	S	R	S
LM59	21/03/2011	Chicken[b]	1/2c, 3c	LmREP13	LmBOX13	LmRAPD29	LmApa4	Al1	I	S	S	S	S	S	S	S	S
LM61	21/03/2011	Salad[a]	1/2c, 3c	LmREP18	LmBOX11	LmRAPD11	LmApa2	Al1	S	I	S	S	S	S	S	R	S
LM85	4/4/2011	Salad[a]	1/2c, 3c	LmREP20	LmBOX12	LmRAPD19	LmApa5	Al1	S	I	S	S	S	S	S	S	S
LM92	4/4/2011	Egg[c]	1/2a, 3a	LmREP16	LmBOX18	LmRAPD24	LmApa11	Al1	I	R	S	S	S	S	R	R	S
LM96	10/5/2011	Beverage	1/2c, 3c	LmREP17	LmBOX2	LmRAPD15	LmApa10	Al1	S	S	S	S	S	S	S	S	S
LM106	10/5/2011	Salad[a]	1/2c, 3c	LmREP14	LmBOX1	LmRAPD14	LmApa1	Al1	S	S	S	S	S	S	S	S	S
LM107	10/5/2011	Salad[a]	1/2c, 3c	LmREP15	LmBOX1	LmRAPD12	LmApa3	Al1	S	I	S	S	S	S	S	S	S
LM115	10/5/2011	Seafood[d]	4b, 4d, 4e	LmREP11	LmBOX31	LmRAPD21	LmApa20	Al2	R	R	R	R	R	R	R	R	S
LM149	23/08/2011	Seafood[d]	1/2a, 3a	LmREP8	LmBOX15	LmRAPD8	LmApa9	Al1	S	S	S	I	I	S	S	S	S
LM150	23/08/2011	Chicken[b]	4b, 4d, 4e	LmREP9	LmBOX28	LmRAPD6	LmApa18	Al2	I	S	S	S	S	S	S	R	S
LM161	23/08/2011	Packed lunch	4b, 4d, 4e	LmREP9	LmBOX30	LmRAPD4	LmApa18	Al2	I	S	S	S	S	S	S	R	S
LM163	3/12/2011	Chicken[b]	1/2a, 3a	LmREP7	LmBOX9	LmRAPD20	LmApa14	Al1	S	S	S	I	S	S	S	R	S
LM164	3/12/2011	Salad[a]	1/2a, 3a	LmREP5	LmBOX8	LmRAPD1	LmApa13	Al1	S	S	S	R	S	S	S	S	S
LM171	3/12/2011	Salad[a]	1/2a, 3a	LmREP1	LmBOX7	LmRAPD5	LmApa13	Al1	S	S	S	R	S	S	S	R	S

LM177	3/12/2011	Chicken[b]	1/2a, 3a	LmREP3	LmBOX24	LmRAPD30	LmApa13	Al1	S	S	S	I	S	S	S	S	S
LM178	3/12/2011	Chicken[b]	1/2a, 3a	LmREP4	LmBOX14	LmRAPD3	LmApa13	Al1	S	S	S	R	S	S	S	R	S
LM184	3/12/2011	Egg[c]	4b, 4d, 4e	LmREP10	LmBOX29	LmRAPD25	LmApa19	Al2	S	S	S	S	S	S	S	S	S
LM191	29/12/2011	Egg[c]	1/2a, 3a	LmREP2	LmBOX16	LmRAPD26	LmApa13	Al1	S	S	S	I	S	S	S	S	S
LM192	29/12/2011	Egg[c]	1/2a, 3a	LmREP6	LmBOX22	LmRAPD27	LmApa13	Al1	S	S	S	S	S	S	S	S	S
LM197	29/12/2011	Salad[a]	1/2a, 3a	LmREP19	LmBOX26	LmRAPD2	LmApa13	Al1	S	S	S	S	S	S	S	S	S
LM198	29/12/2011	Salad[a]	1/2a, 3a	RmREP6	LmBOX25	LmRAPD18	LmApa13	Al1	S	S	S	S	S	S	S	S	S
LM209	1/1/2012	Chicken[b]	1/2a, 3a	LmREP28	LmBOX3	LmRAPD28	LmApa12	Al1	S	S	S	S	S	S	S	R	S
LM213	1/1/2012	Chicken[b]	1/2a, 3a	LmREP25	LmBOX10	LmRAPD31	LmApa13	Al1	S	S	S	S	R	S	S	R	S
LM214	1/1/2012	Chicken[b]	1/2a, 3a	LmREP27	LmBOX4	LmRAPD32	LmApa7	Al1	S	S	S	S	S	S	S	R	S
LM231	1/1/2012	Seafood[d]	1/2a, 3a	LmREP27	LmBOX5	LmRAPD7	LmApa8	Al1	S	S	S	I	I	S	S	R	S
LM242	8/1/2012	Salad[a]	1/2a, 3a	LmREP26	LmBOX27	LmRAPD22	LmApa12	Al1	S	S	S	R	S	S	S	R	S

DA: Clindamycin, Va: Vancomycin, S: Streptomycin, TE: Tetracycline, E: Erythromycin, K: Kanamycin, AMC: Amoxicillin-clavulanic acid, P: Penicillin G, C: Chloramphenicol.

All isolates were sensitive to rifampicin, gentamicin and trimethoprim-sulfamethoxazole.

S: sensitive, I: intermediate, R: resistant.

[a] Salad: Salads and vegetables, [b] Chicken: Chicken and chicken products, [c] Egg: Egg and egg products, [d] Seafood: Seafood and seafood products.

Antibiograms of Non-*Listeria monocytogenes* isolates

Isolates No.	RD 5	DA 2	VA 30	S 10	CN 10	TE 30	E 15	SXT 25	K 30	AMC 30(20/10)	P 10 units	C 30
L9	S	R	S	I	S	S	I	S	S	S	R	I
L10	S	R	S	S	S	S	I	S	S	S	R	I
L56	S	I	S	S	S	S	I	S	S	S	I	I
L57	S	I	S	S	S	S	S	S	S	S	S	S
L62	S	R	S	S	S	R	S	S	S	S	R	I
L71	S	R	I	S	S	R	S	S	S	S	S	S
L81	S	I	S	S	S	S	S	S	S	S	S	S
L83	S	R	I	S	S	R	S	S	S	S	I	S
L88	S	I	S	S	S	S	S	S	S	S	S	S
L139	S	R	S	R	S	R	I	S	S	S	S	S
L155	S	S	S	S	S	S	S	S	S	S	S	S
L168	S	R	I	S	S	R	S	S	S	S	I	S
L189	S	I	I	S	S	S	S	S	S	S	R	I
L194	S	R	I	S	S	R	S	S	S	S	I	S
L195	S	R	I	S	S	R	S	S	S	S	R	S
L200	R	I	R	S	S	R	I	S	R	R	R	S
L211	I	R	I	R	S	R	I	S	R	R	R	I
L227	R	R	I	R	S	R	I	S	R	S	R	S
L243	S	I	S	S	S	R	S	S	S	S	I	S
L248	S	R	I	I	S	R	S	S	S	S	R	S

RD: Rifampicin; DA: Clindamycin; VA: Vancomycin; S: Streptomyci; CN: Gentamicin; TE: Tetracyclin; E: Erythromycin; SXT:Trimethoprim-sulfamethoxazole; K: Kanamycin; AMC: Amoxicillin-clavulanic acid; P: Pencillin G; C: Chloramphenicol

APPENDIX VII

DNA Sequencing Results

DNA Sequencing Results of *inlA*

gb|GQ414504.1| *Listeria monocytogenes* strain NRRL_B-57131 truncated internalin
A (*inlA*) gene, complete cds
Length=2403

Score = 1402 bits (759), Expect = 0.0
Identities = 766/769 (99%), Gaps = 2/769 (0%)
Strand=Plus/Plus

```
Query  4    AATTACCA-AAGAAACTCCTATTAATCAGATTTTTACAGATACAGCTCTAGCGGAAAAAA  62
            ||||| || |||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  111  AATTA-CACAAGATACTCCTATTAATCAGATTTTTACAGATACAGCTCTAGCGGAAAAAA  169

Query  63   TGAAGACGGTCTTAGGAAAAACGAATGTAACAGACACGGTCTCACAAACAGATCTAGACC  122
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  170  TGAAGACGGTCTTAGGAAAAACGAATGTAACAGACACGGTCTCACAAACAGATCTAGACC  229

Query  123  AAGTTACAACGCTTCAGGCGGATAGGTTAGGGATAAAATCTATCGATGGAGTGGAATACT  182
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  230  AAGTTACAACGCTTCAGGCGGATAGGTTAGGGATAAAATCTATCGATGGAGTGGAATACT  289

Query  183  TGAACAATTTAACACAAATAAATTTCAGCAATAATCAACTTACGGACATAACGCCACTTA  242
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  290  TGAACAATTTAACACAAATAAATTTCAGCAATAATCAACTTACGGACATAACGCCACTTA  349

Query  243  AAAATTTAACTAAGTTAGTTGATATTTTGATGAATAATAATCAAATAGCAGATATAACTC  302
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  350  AAAATTTAACTAAGTTAGTTGATATTTTGATGAATAATAATCAAATAGCAGATATAACTC  409

Query  303  CGCTAGCTAATTTGACGAATCTAACTGGTTTGACTTTGTTCAACAATCAGATAACGGATA  362
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  410  CGCTAGCTAATTTGACGAATCTAACTGGTTTGACTTTGTTCAACAATCAGATAACGGATA  469

Query  363  TAGACCCGCTTAAAAATCTAACAAATTTAAATCGGCTAGAACTATCCAGTAACACGATTA  422
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  470  TAGACCCGCTTAAAAATCTAACAAATTTAAATCGGCTAGAACTATCCAGTAACACGATTA  529

Query  423  GTGATATTAGTGCGCTTTCAGGTTTAACTAGTCTACAGCAATTATCTTTTGGTAATCAAG  482
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  530  GTGATATTAGTGCGCTTTCAGGTTTAACTAGTCTACAGCAATTATCTTTTGGTAATCAAG  589

Query  483  TGACAGATTTAAAACCATTAGCTAATTTAACAACACTAGAACGACTAGATATTTCAAGTA  542
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  590  TGACAGATTTAAAACCATTAGCTAATTTAACAACACTAGAACGACTAGATATTTCAAGTA  649

Query  543  ATAAGGTGTCGGATATTAGTGTTCTGGCTAAATTAACCAATTTAGAAAGTCTTATCGCTA  602
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  650  ATAAGGTGTCGGATATTAGTGTTCTGGCTAAATTAACCAATTTAGAAAGTCTTATCGCTA  709

Query  603  CTAACAACCAAATAAGTGATATAACTCCACTTGGGATTTTAACAAATTTGGACGAATTAT  662
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  710  CTAACAACCAAATAAGTGATATAACTCCACTTGGGATTTTAACAAATTTGGACGAATTAT  769

Query  663  CCTTAAATGGTAACCAGTTAAAAGATATAGGCACATTGGCGAGTTTAACAAACCTTACAG  722
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  770  CCTTAAATGGTAACCAGTTAAAAGATATAGGCACATTGGCGAGTTTAACAAACCTTACAG  829

Query  723  ATTTAGATTTAGCAAATAACCAAATTAGTAATCTAGCACCACTGTCGGG  771
            |||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  830  ATTTAGATTTAGCAAATAACCAAATTAGTAATCTAGCACCACTGTCGGG  878
```

DNA Sequencing Results of *inlB*

gb|FJ932481.1| *Listeria monocytogenes* strain A23 internalin B gene, complete cds

Length=1893

Score = 828 bits (448), Expect = 0.0
Identities = 452/454 (99%), Gaps = 1/454 (0%)
Strand=Plus/Plus

```
Query  13    ATGANGTTGATGG-ACGGTAATAAAAACAAAAGTAGAAGCAGGGACGCGGATAACTGCAC  71
             |||| |||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  980   ATGATGTTGATGGAACGGTAATAAAAACAAAAGTAGAAGCAGGGACGCGGATAACTGCAC  1039

Query  72    CTAAACCTCCGACCAAACAAGGCTATGTTTTTAAAGGATGGTATACTGaaaaaaaTGGTG  131
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1040  CTAAACCTCCGACCAAACAAGGCTATGTTTTTAAAGGATGGTATACTGAAAAAAATGGTG  1099

Query  132   GGCATGAGTGGAATTTTAATACGGATTATATGTCCGGAAATGATTTTACTTTGTACGCAG  191
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1100  GGCATGAGTGGAATTTTAATACGGATTATATGTCCGGAAATGATTTTACTTTGTACGCAG  1159

Query  192   TATTTAAAGCGGAAACGACCGAAAAAGCAGTCAACTTAACCCGCTATGTCAAATATATTC  251
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1160  TATTTAAAGCGGAAACGACCGAAAAAGCAGTCAACTTAACCCGCTATGTCAAATATATTC  1219

Query  252   GCGGGAATGCAGGCATCTACAAACTTCCACGAGAAGATAACTCGCTTAAACAAGGAACTC  311
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1220  GCGGGAATGCAGGCATCTACAAACTTCCACGAGAAGATAACTCGCTTAAACAAGGAACTC  1279

Query  312   TAGCCTCGCACCGCTGTAAAGCTCTAACTGTTGATAGAGAAGCCCGAAATGGCGGAAAAT  371
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1280  TAGCCTCGCACCGCTGTAAAGCTCTAACTGTTGATAGAGAAGCCCGAAATGGCGGAAAAT  1339

Query  372   TATGGTACAGGTTAAAAAATATTGGCTGGACTAAAGCGGAAAACCTTTCCTTAGACCGAT  431
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1340  TATGGTACAGGTTAAAAAATATTGGCTGGACTAAAGCGGAAAACCTTTCCTTAGACCGAT  1399

Query  432   ACGATAAAATGGAATATGACAAAGGGGTTACCGC  465
             |||||||||||||||||||||||||||||||||
Sbjct  1400  ACGATAAAATGGAATATGACAAAGGGGTTACCGC  1433
```

DNA Sequencing Results of *inlC*

gb|EU408950.1| *Listeria monocytogenes* isolate VIMVF110 *InlC* (*inlC*) gene, partial
cds
Length=586

Score = 872 bits (472), Expect = 0.0
Identities = 475/478 (99%), Gaps = 0/478 (0%)
Strand=Plus/Minus

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Query  10   TTACTGNNGTAATATGGAGANATCCATCTTCCATCTGGGTCTTTGACAGTATTTGTTATA  69
            ||||||  |||||||||||| ||||||||||||||||||||||||||||||||||||||
Sbjct  542  TTACTGATGTAATATGGAGATATCCATCTTCCATCTGGGTCTTTGACAGTATTTGTTATA  483

Query  70   TACAATTCTGGTTGGTATTTTACTGGTTCATTCACACATTTCTGACCAGTTAAATCTATC  129
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  482  TACAATTCTGGTTGGTATTTTACTGGTTCATTCACACATTTCTGACCAGTTAAATCTATC  423

Query  130  CAGTTAACTTTCTTCAATCTAGTTAGTCCACCTGTATTTGTTATTTCATTACCATGCAAA  189
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  422  CAGTTAACTTTCTTCAATCTAGTTAGTCCACCTGTATTTGTTATTTCATTACCATGCAAA  363

Query  190  TCTAATACCTCTAGTTTTGATAAAAAACCAAGCATCACAATACTTTTTAGCTTATTATTA  249
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  362  TCTAATACCTCTAGTTTTGATAAAAAACCAAGCATCACAATACTTTTTAGCTTATTATTA  303

Query  250  CGAATAGATAAGATTTCTAGATTTTTCAAATGAATAAGCGAGTCAGTATCTCTGAGTTCG  309
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Sbjct  302  CGAATAGATAAGATTTCTAGATTTTTCAAATGAATAAGCGAGTCAGTATCTCTGAGTTCG  243

Query  310  TTGTTATCTAAAAACAAGCGAGATAAACAAGCACTTGGAATTCCGTTTAAATTTTTCAGT  369
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  242  TTGTTATCTAAAAACAAGCGAGATAAACAAGCACTTGGAATTCCGTTTAAATTTTTCAGT  183

Query  370  CTGTTTCTATTCACAGATAGCTCTTCTAACTTAGTTAGATCCTTTAAAGGACTAAGGTCA  429
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  182  CTGTTTCTATTCACAGATAGCTCTTCTAACTTAGTTAGATCCTTTAAAGGACTAAGGTCA  123

Query  430  CTTATTTGATTATGGGATAGATGAAGTTCTTTTAAATTAGTGAAAAATTGCATTCCCG  487
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  122  CTTATTTGATTATGGGATAGATGAAGTTCTTTTAAATTAGTGAAAAATTGCATTCCCG  65
```

DNA Sequencing Results of *inlJ*

emb|FR686503.1| *Listeria monocytogenes inlJ2* gene for internalin J2, strain L41
Length=2723

Score = 337 bits (182), Expect = 2e-89
Identities = 198/205 (97%), Gaps = 4/205 (2%)
Strand=Plus/Plus

```
Query  9     TTGGAGCGT-AATCCTTT-ACTG-ATTAG-TGTATCTACGCTTTCAAAATTAACTACACT  64
             ||| ||||| |||||||| |||| ||||| ||| ||||||||||||||||||||||||||
Sbjct  977   TTGTAGCGTAAATCCTTTAACTGAATTAGATGTGTCTACGCTTTCAAAATTAACTACACT  1036

Query  65    ACATTGTATACAAACAGATTTATTAGAAATAGACCTAACACACAACACACAATTAATATA  124
             ||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1037  ACACTGTATACAAACAGATTTATTAGAAATAGACCTAACACACAACACACAATTAATATA  1096

Query  125   TTTTCAAGCTGAAGGATGTAGAAAAATAAAAGAGCTTGATGTCACGCATAATACACAATT  184
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1097  TTTTCAAGCTGAAGGATGTAGAAAAATAAAAGAGCTTGATGTCACGCATAATACACAATT  1156

Query  185   ATATTTATTAGACTGCCAAGCCGCT  209
             |||||||||||||||||||||||||
Sbjct  1157  ATATTTATTAGACTGCCAAGCCGCT  1181
```

APPENDIX VIII

PUBLICATIONS

Jamali H., Chai L.C. & Thong K.L. (2013). "Detection and isolation of *Listeria* spp. and *Listeria monocytogenes* in ready-to-eat foods with various selective culture media". Food Control, 32: 19-24.

Manuscript in submission:

Jamali, H., Lim, K.T. & Kwai Lin Thong. Genotypic Characterization and Antimicrobial Resistance of *Listeria monocytogenes* from Ready- to- Eat Foods. Under review.

PRESENTATIONS

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