Faculty of Resource Science and Technology

MOLECULAR CHARACTERIZATION OF *Listeria monocytogenes* FROM RAW MILK

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Bachelor of Science with Honours
(Resource Biotechnology)
2013

Molecular Characterization of *Listeria monocytogenes* from Raw Milk

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(28645)

This project is submitted in partial fulfillment of the requirements for the degree of
Bachelor of Science with Honours
(Resource Biotechnology)

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University Malaysia Sarawak

2013

ACKNOWLEDGEMENTS

A thousand thanks to my final year project supervisor, Dr. Samuel Lihan which gave valuable guidance and advices. I am truly appreciating the supports, encouragements and motivation which gave me the strength and enable me to accomplish my final year project in time and in a proper manner.

I would like to thank to my co-supervisor Dr. Lesley Maurice Bilung and all Seniors, Kathleen Michelle Mikal, Felecia anak Collick, Mildred Pemela anak Jalin (Master Students) as well as Leong Sui Sien (PhD student) for the guidance and technical advices while I conducting the experiment for my final year project. Additional thanks also given to Abang Iskandar as our lab assistants for the materials supplied as well as to my course mates (Chai Siong Kiat, Ng Kok Hua, Tang Ping Sia and Foo Tun Xian) who had helped me along the project.

Besides, I also appreciate the support from my parents in term of motivation and positive advices.

DECLARATION

I hereby declare that the thesis submitted for the Degree Program at the University Malaysia Sarawak is my own work except for quotation and citation which have been acknowledged. I also declare that no portion of the work referred in this project has been submitted in support of an application for another degree qualification of this or any other university or institution of higher learning.

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List of Abbreviations

℃	Degree Celcius
µl	Microlitre
actA	Actin-polymerizing factor
ATCC	American Type Culture Collection
bp	base pairs
cm	Centimetre
CTAB	Cetyltrimethylammonium Bromide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide Triphosphate
GC	Content Guanine and Cytosine Content
$(GTG)_5$-PCR	$(GTG)_5$ Polymerase Chain Reaction
hlyA	Hemolysin A
inlA, inlB, inlC	Internalin A, Internalin B, Internalin C
KOH	Potassium Hydroxide
LB	Luria-Bertani
LIPI-1	*Listeria* Pathogenecity Island 1
LLO	Listeriosin O
min	Minutes
mL	Millilitre
mpl	Metallooprotease
NT	Nucleotide
PALCAM	Polymyxin-acriflavine-LiCl-ceftazidime-aesculin-mannitol

PCR	Polymerase Chain Reaction
plcA	Phosphatidylinositol-specific Phospholipase C
plcB	Encodes for lecithinase
prfA	Positive Regulatory Factor A
*p*VGC	*prfA* Virulence Gene Cluster
RAPD	Random Amplified Polymorphic DNA
RNA	Ribonucleic Acid
SDS	Sodium Dodecyl Sulphate
TBE	Tris-boric EDTA
TE Buffer	Tris-EDTA

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ABSTRACT

Listeria monocytogenes has been reported as causative agent of foodborne disease which had gain public health concern as it can cause listeriosis in human especially in pregnant women, immunocompromised adults and infants. Various studies on molecular characterization of *L. monocytogenes* in foods had been done in Malaysia such as chicken, beef and ready-to-eat foods. However, the study of *L. monocytogenes* in raw milk is still not well established. The objectives of this study were to detect, isolate and characterize the presence of *L. monocytogenes* from raw milk by using $(GTG)_5$-PCR. Raw milk was collected from Kota Samarahan and Kuching, Sarawak, and it was transported to laboratory for analysis. Samples were enriched using Tryptone Soy Broth, then plated on PALCAM agar for isolation purposes. Next, the colonies formed on PALCAM agar were confirmed by species-specific PCR using LM1 and LM2 specific primer for detection of *L. monocytogenes*. The 234 bp *hly* gene was successfully amplified by PCR. Confirmed isolates were characterized molecularly using $(GTG)_5$-PCR method with 15-mer primers, GTG. $(GTG)_5$-PCR results were analysed using RAPDistance bioinformatics software. A dendrogram was successfully formed from $(GTG)_5$-PCR binding pattern and genetic distribution of *L. monocytogenes* was obtained. Based on the study, 14 positives *L. monocytogenes* were successfully isolated and characterized using $(GTG)_5$-PCR from the total of 120 isolates. Three clusters A, B and C showing the genetic distribution were formed from RAPDistance based on $(GTG)_5$-PCR binding patterns. In conclusion, 14 *L. monocytogenes* isolates were detected, isolated and characterized into three clusters.

Keywords: *Listeria monocytogenes*, Raw milk, Species-specific PCR, $(GTG)_5$-PCR, Genetic distribution.

ABSTRAK

Listeria monocytogenes telah dilaporkan sebagai agen penyebab penyakit bawaan makanan yang telah mendapat perhatian daripada kesihatan awam kerana boleh menyebabkan listeriosis di kalangan wanita hamil, immunokompromi orang dewasa dan bayi. Pelbagai kajian mengenai pencirian molekul L. monocytogenes dalam makanan telah dijalankan di Malaysia seperti ayam, daging lembu, dan sedia dimakan makanan. Walau bagaimanapun, pengajian L. monocytogenes dalam susu mentah masih tidak mantap. Objektif kajian ini adalah untuk menganalisis, mengesan dan mengasingkan L. monocytogenes daripada susu mentah dengan kaedah melekul. Susu mentah dikumpulkan dari Kota Samarahan dan Kuching, Sarawak, dan telah dihantar ke makmal untuk analisis. Sampel telah diperkayakan dengan "Trytic Soy Broth" dan sebaran atas agar PALCAM untuk pengasingan. Seterusnya, coloni terbentuk atas agar PALCAM telah disahkan oleh PCR dengan menggunakan LM1 dan LM2 sebagai primer untuk mengesan L. monocytogenes. Size 234 bp hly gen telah berjaya diamplifikasikan oleh PCR. $(GTG)_5$-PCR dilakukan untuk mengasingkan cirri-ciri L. monocytogenes yang telah berjaya diamplifikasikan dengan menggunakan 15-mer primer, GTG. Keputusan $(GTG)_5$-PCR telah dianalisi oleh RAPDistance. Dendrogram telah berjaya dibentukan daripada $(GTG)_5$-PCR dan pengedaran genetic L. monocytogenes telah diperolehi. Berdasarkan kajian, 14 positif L.monocytogenes telah berjaya diasingkan dan menghasilkan pengedaran genetik. Tiga kumpulan iaitu A, B dan C telah dibentuk dengan menggunakan RAPDistance berdasarkan data daripada $(GTG)_5$-PCR. Secara kesimpulanya, 14 L. monocytogenes pencilan telah diasingkan dan dibahagikan kepada tiga kumplulan.

Kata Kunci: *Listeria monocytogenes, Susu mentah, Spesies-spesifik PCR, $(GTG)_5$-PCR, Pengedaran genetik.*

1.0 Introduction

Listeria monocytogenes has been reported as causative agent of foodborne disease which had gain public health concern as it can cause listeriosis in human. *L. monocytogenes* was known and described completely when Murray *et al.* in the year of 1926 isolated and reported a Gram-positive, short, non-sporing, rod-shaped bacterium from rabbits and guinea-pigs that caused diseases. Murray *et al*. (1996) claimed that it was given name as *monocytogenes* because it infects white blood cells (monocytes) in the blood.

In late 19th century, Gray and Killinger (1996) explained that *L. monocytogenes* was able to cause infection in humans and animal which include cattle, sheep, birds, rodents and fish. Existence of listeriosis on human was not much appreciated until the huge outbreak in Germany in 1949 and scientists have proven that it was caused by consumption of raw milk (Seeliger, 1961; Gray & Killinger, 1966). In addition, the first to be concerned is the dairy industry as it can easily contaminated by *L. monocytogenes* and most of the outbreak is related to it (Griffiths, 1989; Harvey & Gilmour, 1992; Jacquet *et al*, 1993).

L. monocytogenes is considered as one of the major problem in food safety as it can cause listeriosis to human (Hamdi *et al.*, 2006) and food can be contaminated by *L. monocytogenes* along food chain through farm-to-fork (Farber & Peterkin, 2006). According to Shen *et al*. (2006) fatality rate of *L. monocytogenes* is high and this makes it as an important food-borne pathogen. *L. monocytogenes* produce listeriosin O (LLO) which will cause listeriosis in human mostly in pregnant women, immunocompromised individual and infants (Shen *et al.*, 2006). Besides, Shen *et al.* (2006) has also stated that in non-pregnant, the mortality rate is about 20 to 25%, this range of percentage can be

considered as high and *L. monocytogenes* primarily causes meningoencephalitis and septicemia.

L. monocytogenes has been studied worldwide on various type of food like raw milk in Algiers (Algeria) and Mashhad (Iran) (Hamdi *et al*., 2006; Jami *et al.* 2010), ready-to-eat foods in Florida (Shen *et al*., 2006), fresh and cold-smoked fish in Portugal and England (Vaz-Velho *et al.*, 2001). In Malaysia, *L. monocytogenes* occurred in 75% of the frozen beef, 30.4% of the local meat and 12% from fermented fish (Hassan *et al.*, 2001). Besides, study of *L. monocytogenes* has also been done on various type of food in Malaysia such as chicken and beef (Samuel, 2007), raw salad vegetables (Jeyaletchumi *et al.*, 2010), raw and ready-to-eat foods (Marian *et al*., 2012). However, study and research on *L. monocytogenes* in raw milk in Malaysia is still not well established.

Hence, this study was carried out to determine the occurrence of *L. monocytogenes* in raw milk. The ojectives of this study were to:

i. analyse raw milk samples for the presence of *L. monocytogenes*,
ii. isolate *L. monocytogenes* from raw milk
iii. characterize *L. monocytogenes* in raw milk samples by molecular method.

2.0 Literature Review

2.1 *Listeria* species

Based on Rocourt and Cossart (1997) the genus *Listeria* is part of *Clostridium* sub-branch together with *Staphylacoccus, Streptococcus, Lactobacillus* and *Brochothrix*. The advancement on the basis of DNA-DNA hybridization, 16S rRNA sequencing and multilocus enzyme analysis techniques have classified genus *Listeria* comprises of six species namely *L. monocytogenes, L. innocua, L. ivanovii, L. welshimeri, L. seeligeri* and *L. grayi* (Rocourt & Cossart, 1997). Among *Listeria* species, *L. monocytogenes* and *L. ivanovii* have been identified as pathogens which *L.monocytogenes* is a pathogen for human and *L. ivanovii* is a pathogen for animal (Montville & Matthews, 2008). In addition, Rocourt and Cossart (1997) supported that *L. monocytogenes* and *L. ivanovii* are pathogenic with respect to both the 50% lethal dose in mice. The pathogenicity of *L. monocytogenes* had gain public health concern as it can infect human which lyse monocytes in human blood (Rocourt & Cossart, 1997). Montiville and Matthews (2008) stated that a few biochemical traits can differentiate *Listeria* species. Acid production is the main component to characterize the *Listeria* species for example D-xylose, L-rhamnose, alpha-methyl-D-mannoside and D-mannitol (Montiville & Matthews, 2008). *L. monocytogenes* having the ability to lyse red blood cells and this capability differentiated *L. monocytogenes* from other *Listeria* species (Montiville & Matthews, 2008). Based on the work of researchers which had been done previously, hemolysis was found as a biochemical markers used to differentiate *L. monocytogenes* from other *Listeria* species (Rocourt & Cossart, 1997).

2.2 *L. monocytogenes*

Listeria monocytogenes is one of the sub-species from the genus *Listeria*. *L. monocytogenes* is a global concern, Gram-positive, non-spore forming, facultative anaerobic and rod-shaped intracellular pathogens (Rocourt & Cossart, 1997; Montiville & Matthews, 2008). In the early 1980s, *L. monocytogenes* has been identified as one of the causative microorganism in various outbreak of foodborne disease (Montiville & Matthews, 2008). Montiville and Matthews (2008) stated that *L. monocytogenes* is able to grow from 0 to 45 ℃, pH values on 4.4, water activity (a_w) >0.97 and 6.5% salt concentrations. *L. monocytogenes* genome is approximately 3.0 Mb and the genomics is shown in Table 1.

Table 1: *L. monocytogenes* genomic.

	Listeria monocytogenes
Size of Chromosome (Mb)	≈ 2.94
GC content of protein-coding genes (%)	≈ 38
Total number of protein-coding genes	≈ 2,846

Note. Adapted from National Center for Biotechnology Information, 2009, U.S. National Library of Medicine

Jung *et al.* (2009) claimed that *L. monocytogenes* could contaminate foods which are stored in refrigerator as it has the ability to grow from 0 to 45 ℃. According to Seeliger and Jones (1986), serotype of *Listeria* species is determined by unique combinations of species heat-stable somatic (O) antigen as well as heat-labile flagella (H) antigen. Furthermore, Seeliger and Jones (1986) claimed that there are at least 13 serotypes which have been identified and characterized in *L. monocytogenes* namely 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, and 7. Among these serotypes, only serotypes 1/2a, 1/2b and 4b are accountable for 98% of human listeriosis cases (Wiedmann *et al.*, 1996).

2.3 Pathogenecity of *L. monocytogenes*

Listeria monocytogenes is capable to produce hemolysis (*hlyA*) or listeriolysin O (LLO) that lyse monocytes of animal and human (Montiville & Matthews, 2008) and this capability has classified *L. monocytogenes* as pathogenic microorganism. *L. monocytogenes* has gained global public health concern and it has been associated as a foodborne diseases which cause diseases such as septicemia, meningoencephalitis, miscarriages, meningitis among infants, pregnant women and immunocompromised individuals (Choi & Hong, 2003; Kayser, 2001; Rossmanith *et al.*, 2006). Swaminathan (2007) claimed that mortality rate caused by *L. monocytogenes* is 20 to 25%.

2.4 Virulence genes of *L. monocytogenes*

The main virulence factors responsible for key steps of *L. monocytogenes* is encoded on a 9-kb chromosomal island known as *Listeria* pathogenicity island 1 (LIPI-1) or *prfA* virulence gene cluster (*p*VGC) (Johnson and Ward as cited in Jung *et al*., 2009) as shown in Figure 1.

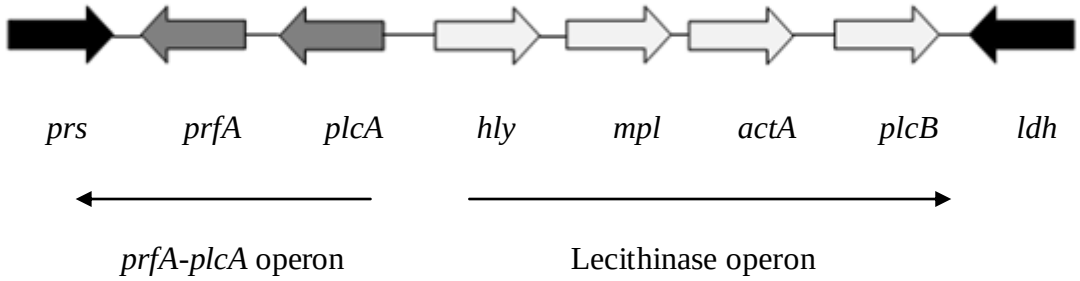

Figure 1: *prfA* Virulence Gene Cluster (*p*VGC) of *L. monocytogenes.* pVGC is in the region of flanked by *prs* and *ldh* loci. *prfA* encodes a positive regulatory factor, *plcA* encodes a phosphatidylinositol-specific phospholipase C, *hly* encodes a hemolysin, *mpl* encodes a metalloprotease, *actA* encodes an actin polymerizing factor and *plcB* encodes a lecithinase.

Note. From "Species-specific detection of *Listeria monocytogenes* using Polymerase Chain Reaction Assays Targeting the *prfA* virulence gene cluster" by H. J. Jung *et al*., 2009, *Bioscience, Biotechnology and Biochemical, 73*(6), p.1414

In the region of *p*VGC, it has six virulence genes which are positive regulatory regulator *prfA*, hemolysin (*hly*), two phospholipases (*plcA* and *plcB*), a metalloprotease (*mpl*) that function as to activates *plcB* and *actA* that works as actin-based motibility (Johnson and Ward, 2009). This *p*VGC cluster is the only one known to date that is involved in the virulence of *Listeria*. Based on Charkraborty *et al.* (2000), *prfA* gene is the key regulator for the virulence of *L. monocytogenes* and it acts as a master regulator in the *p*VGC. Virulence gene of *L. monocytogenes* does not necessary locates in the *p*VGC and there are several other virulence genes that located outside of the cluster (Kuhn & Goebel, 1999). These virulence genes are involved in the production of surface proteins that necessary for internalization of the *L. monocytogenes* to the host cells which include *inlA*, *inlB* and *inlC* genes that codes for internalin A, B and C (Kuhn & Goebel, 1999). In addition, Slutsker and Schuchat (1999) also claimed that *iap* virulence like genes also located outside the cluster and it can be dependent or independent to *prfA* gene. The function of the virulence genes are shown in Table 2.

Table 2: Function of Virulence genes in *prfA* Virulence Gene Cluster (*p*VGC)

Virulence Genes	Name	Functions
prfA	Positive Regulatory Factor A	Transcriptional activator
plcA	Phosphatidylinositol-specific Phospholipase C	Aids in escape from vacuole
plcB	Encodes for lecithinase	Aids in escape from vacuole
hlyA	Hemolysin A or Listeriolysin O (LLO)	Hemolyse monocytes
mpl	Metallooprotease	Maturation of *plcB*
actA	Actin-polymerizing factor	Actin-based motibiliy and cell-to-cell spread
inlA, inlB, inlC	Internalin A, Internalin B, Internalin C respectively	Internalization of *Listeria monocytogenes* to host cells

Note. Adapted from "Pathogenesis of *Listeria monocytogenes*", p. 97-130 by M. Kuhn and W. Goebel, 1999, New York: Marcel Dekker, Inc and "Listeriosis in humans", p. 75-96, by L. Slutsker and A. Schuchat (1999), New York: Marcel Dekker, Inc.

Among these virulence genes, Jung *et al*. (2009) revealed that *hlyA* encodes hemolysin, called as listeriolysin O that is vital for the invasion of the pathogen to the host cells which lyse phagosomes of the host cells and resulting in spreading. Furthermore, Jung *et al*. (2009) also stated that *hlyA*, *plcA* and *plcB* virulence genes and their translational product can be virulence markers to differentiate pathogenic species from non-pathogenic species.

2.5 Outbreak of *L. monocytogenes*

Centers for Disease Control and Prevention (2012) has reported that in September 11, 2012, *L. monocytogenes* outbreak causes a total of 14 persons infected with the listeriosis from 11 states and the District of Columbia. Furthermore, CDC (2012) claimed that the states include California, Colorado, District of Columbia, Maryland, Minnesota, Nebraska, New Jersey, New Mexico, New York, Ohio, Pennsylvania and Virginia. CDC (2012) reported that among the 3 out of 14 person were dead. CDC (2012) explained that the outbreak of listeriosis was linked to imported Frescolina Brand Ricotta Salata Cheese. Besides, Centers for Disease Control and Prevention (2012) also reported that in year 2011, a total of 147 persons infected with any of the 5 outbreak-associated subtypes of *L. monocytogenes* from 28 states which include Alabama, Arkansas, California, Colorado, Idaho, Illinois, Indiana, Iowa, Kansas, Louisiana, Maryland, Missouri, Montana, Nebraska, Nevada, New Mexico, New York, North Dakota, Oklahoma, Oregon, Pennsylvania, South Dakota, Texas, Utah, Virginia, West Virginia, Wisconsin and Wyoming. Tang *et al*., (1994) stated that in Malaysia there has been no report of foodborne listeriosis and Lim (2002) supported that true incidence of foodborne infections in Malaysia is unknown.

2.6 Isolation of *L. monocytogenes*

Listeria enrichment broth is to enrich *L. monocytogenes* present in raw milk samples. After enrichment process, raw milk samples are spreaded on Polymyxin-acriflavine-LiCl-ceftazidime-aesculin-mannitol, PALCAM agar (Oxoid). PALCAM agar is based on the formulation described by Van Netten *et al*. (1989) and is used for isolation of *L. monocytogenes* from foods. Based on Thermo Scientific (2012), PALCAM is highly selective because of lithium chloride, ceftazidime, polymyxin B and acriflavine hydrochloride presents in the agar. *L. monocytogenes* will hydrolyse aesculin which is a chemical composition of PALCAM (Oxoid) and this hydrolysis will result in the formation of a black halo around colonies on PALCAM (Oxoid) (Thermo Scientific, 2012)

2.7 Molecular Confirmation of *L. monocytogenes* using Species-specific Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) is a technique to amplify a small region of DNA to make unlimited amount of copies of the DNA (Russell, 2010).This techniques was discovered by Kary Mullis as a method to amplify strand of DNA using dNTPs, DNA polymerase and primers and this technique involve heating and cooling process which is useful for analysis and manipulation of DNA (McPherson & Muller, 2006). There are 4 components needed in order to perform PCR which is DNA template, DNA polymerase, primers (designated) and dNTPs. The purpose of using Species-specific PCR is to identify *L. monocytogenes* molecularly. For Species-specific PCR, the DNA template used is DNA strain from *L. monocytogenes* and the primers used in this PCR has to be designated based on the molecular maker of *L. monocytogenes* such as listeriolysin O (LLO) virulence gene. Based on Border *et al.* (1990), the primers used for detection of *L. monocytogenes* was

LM1 (5’ CGG AGG TTC CGC AAA AGA TG – 3’) and LM2 (5’ CCT CCA GAG TGA TCG ATG TT -3’) which was specifically design based on the listerioysin O (LLO) virulence gene with expected amplicon of 234 bp. *L. monocytogenes* ATCC (American Type Culture Collection) 15313 as well as 19155 will be the positive control of Species-specific PCR.

2.8 Molecular Typing Methods of *L. monocytogenes*

DNA fingerprinting also can be known as DNA typing or DNA profiling (Russell, 2010). The use of restriction enzyme has enable researches to compare the base sequences of different organism (Tortora *et al.*, 2007). Enzyme is specific as well as restriction enzyme; it will cut at specific and recognize sequence (Tortora *et al.*, 2007). For example like *Eco*RI and *Bam*HI having totally different restriction among each other. As a result, different fragment will be restricted by restriction enzyme and this different fragment will be used as DNA fingerprinting which is benefit to forensic microbiology. Restriction Fragment Length Polymorphic (RFLP) is a type of DNA fingerprinting and Amplified Fragment Length Polymorphic is the PCR-based RFLP. Besides, RAPD is also a type of DNA fingerprinting as it uses 10-mer primer to randomly bind to the DNA of organism and will produce different size of fragment and this fragment will be profiled. Random Amplified Polymorphic DNA (RAPD) markers are short DNA fragments nearly from 8 to 12 base pair and used as primer in PCR (Hodek *et al.,* 2012). Based on Quinn *et al*., (2011), RAPD does not require any specific knowledge on the DNA sequence of the target organism. 10-mer primers (RAPD) will or will not amplify a segment of DNA randomly along the target DNA sequence through PCR. Furthermore, Quinn *et al*., (2011) claimed that RAPD is cost effective and having discriminatory power for many bacterial species. In molecular characterization, Pulsed Field Gel Electrophoresis (PFGE) and RAPD-PCR are

techniques that usually used by the scientist (Cocolin *et al*., 2005). PFGE is not chosen for molecular characterization is due to time consuming and require expensive apparatus although this technique has the most discriminatory power (Franciosa *et al.*, 1998). As for RAPD technique it is widely used and it can monitor bacterial strains on a wide scale and able to determine whole genome diversity (Wagner *et al.*, 1996). This technique will be performed as described by Lee *et al*., (2011). The primers proposed by Lee *et al*., (2011) are 5'-TGT GTG CTG G-3' and 5'-TTC CGA ACC C-3' with the name given as OPA 14 and OPA 15 respectively. OPA 14 and OPA 15 were designed and this primers work best on *L. monocytogenes* ATCC 15313 and 19155 as a result this strain is used as positive control in RAPD-PCR. Molecular typing methods rely solely on PCR include RAPD (Dellaglio *et al*., 2005) and repetitive genomic element PCR (Gevers *et al*., 2001). Repetitive PCR primers amplify repetitive bacterial DNA elements which includes ERIC, BOX or $(GTG)_5$ (Versalovic *et al*., 1991). In many studies, $(GTG)_5$-PCR was proven to be useful for the differentiate of species, subspecies and potentially strain levels (Fernanda *et al.*, 2010).

2.9 Agarose Gel Electrophoresis

Slish (1998) claimed that agarose gel electrophoresis is a technique used to separate DNA fragments by their molecular weight. The technique of electrophoresis is based on the fact that DNA are negatively charged due to its phosphate backbone (Slish, 1998). As a result, a negative pole and positive pole is needed in the technique of electrophoresis. The negative pole will be put near to the sample so that it could push the sample along the agarose gel and for the positive pole is to pull the DNA sample along the agarose gel. Thus, the biggest size of DNA fragment will move slowest and the smallest size fragment will move fastest in the agarose gel. In order to view the banding pattern of the DNA fragments

(sample) it has to be stained with Ethidium Bromide (EtBr) so that the sample will be visible under UV light.

2.10 RAPDistance

RAPDistance is a bioinformatic tool that used to analyse RAPD-PCR or $(GTG)_5$-PCR result. In this study, cluster analysis was done using RAPDistance.

3.0 Materials and Methods

3.1 Raw Milk Samples Collection

Raw milk samples were collected from different areas within Kota Samarahan and Kuching namely Bau, Kampung Haji Baki (Seven miles), Ladang Lapan (Eight miles) and Petra Jaya. The samples were labelled (area and date), stored in the ice box and transported to laboratory in UNIMAS for analysis within 2 to 3 hours. The samples were collected every 2 weeks or 1 month interval. At sampling site, one sample was taken because the raw milk is quite hard to obtain thus the sampling period was prolonged to one month. Five hundred milli-liter of raw milk were collected from each sampling site. Bau and Kampung Haji Baki (Seven miles) raw milk samples were collected on 3rd of November, 2012 whereas Ladang Lapan (Eight miles) and Petra Jaya were collected on 1st of December, 2012. The second batch of sampling was carried out on the 2nd of March 2013 and the third batch sampling was carried out on the 16th of March 2013.

3.2 Enrichment of *Listeria* species from Raw Milk

The samples were enriched using Tryptone Soy Broth (TSB) which was incubated at 30 ℃ for 48 hours with shaking at 120 rpm. Twenty-five mL of the sample were poured into 225 mL of Tryptone Soy Broth.

3.3 Isolation of *L. monocytogenes* among *Listeria* species from Raw Milk

After enrichment using TSB, serial dilution was performed and each dilution was spread on two PALCAM to create replication. The plates were incubated at 37 ℃ for 48 hours under micro-aerophilic conditions. This micro-aerophilic condition can be done by sealing PALCAM plates with Parafilm. The PALCAM plates were examined after 48 hours of