UNIVERSITI PUTRA MALAYSIA

ISOLATION AND MOLECULAR CHARACTERISATION OF LISTERIA MONOCYTOGENES AND LISTERIA INNOCUA FROM POULTRY MEAT

LESLEY MAURICE BILUNG

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By

LESLEY MAURICE BILUNG

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, In Fulfilment of the Requirement for the Degree of Master of Science

April 2002

UPM

DEDICATION

Dedicated to Father of Glory, Lord of Glory and Spirit of Glory

"Blessed are You, Lord God of Israel,
our Father, forever and ever.
Yours, O Lord, is the greatness,
The power and the glory,
The victory and the majesty;
for all that is in heaven and in earth is Yours;
Yours is the kingdom, O Lord,
and You are exalted as head over all.
Both riches and honor come from You,
And You reign over all.
In Your hand is power and might;
In Your hand it is to make great
and to give strength to all.
Now therefore, our God, we thank You
And praise Your glorious name.
But who am I, and who are my people,
That we should be able to offer
so willingly as this?
For all things come from You,
And of Your own we have given You."

1 Chronicles 29:10-14

Abstract of thesis presented to Senate of Universiti Putra Malaysia fulfilment of the requirement for the degree of Master of Science

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April 2002

Chairman: **Associate Professor Dr. Son Radu**

Faculty: **Food Science and Biotechnology**

Thirty isolates of *Listeria monocytogenes* (12) and *Listeria innocua* (18) were isolated from poultry meat. All isolates were confirmed by Microbact (Medvet, Australia) identification kits. All the isolates were subjected to chromosomal and plasmid DNA screening and antibiotic resistance test. Based on the antibiotic resistance profiles, *Listeria monocytogenes* and *Listeria innocua* were differentiated into 10 and 9 profiles respectively. The antibiotyping procedure discriminated the *Listeria monocytogenes* and *Listeria innocua* into 10 and 3 different groups respectively. Most of the isolates were resistant to nalidixic acid (100%), clindamycin (97%), spectinomycin (97%), cefuroxime (93%), cefriaxone (80%), cephalothin (73%), cefotaxime (67%), novobiocin (37%), chloramphenicol (27%), kanamycin (20%), rifampicin (20%), tobramycin (17%), norfloxacin (13%), netilmicin (10%) and

imipenem (3%). The results of the plasmid profiles and antibiotyping show that there is no correlation between them. RAPD-PCR has been used to generate polymorphic genomic fingerprints to discriminate the *Listeria* isolates. Primer GEN15009 was chosen whereby it produced reproducible and typeable results in all isolates examined with the bands ranging from 0.25 to 3.0 kilobase pairs. From the dendrogram generated *L. monocytogenes* were separated from *L. innocua* and the strains in each species were differentiated as well. The data indicate that RAPD-PCR based approaches is a valid means of discriminating strain differences among isolates of *L. monocytogenes* and *L. innocua* and as an adjunct to differentiate among *Listeria* spp.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PEMENCILAN DAN PENCIRIAN MOLEKULAR *LISTERIA MONOCYTOGENES* DAN *LISTERIA INNOCUA* DARI DAGING AYAM

Oleh

LESLEY MAURICE BILUNG

April 2002

Pengerusi: **Profesor Madya Dr. Son Radu**

Fakulti: **Fakulti Sains Makanan dan Bioteknologi**

Tiga puluh pencilan *Listeria monocytogenes* (12 pencilan) dan *Listeria innocua* (18 pencilan) telah dipencilkan dari daging ayam. Semua pencilan telah dianalisis menggunakan Microbact (Medvet, Australia) identification kit. Semua pencilan telah dikaji untuk kerintangan kepada antibiotik dan polimorfik menggunakan analisis random amplification of polymorphic DNA (RAPD). Berdasarkan kepada profil kerintangan antibiotik, *Listeria monocytogenes* dan *Listeria innocua* dibezakan kepada 10 dan 9 profil masing-masing. Kaedah antibiotip mengasingkan *Listeria monocytogenes* dan *Listeria innocua* kepada 10 dan 3 kumpulan-kumpulan yang berbeza-beza. Kebanyakan pencilan rintang terhadap nalidixic acid (100%), clindamycin (97%), spectinomycin (97%), cefuroxime (93%), cefriaxone (80%), cephalothin (73%), cefotaxime (67%), novobiocin (37%), chloramphenicol (27%), kanamycin (20%), rifampicin (20%), tobramycin (17%), norfloxacin (13%), netilmicin (10%) dan impenem (3%).

Keputusan profil plasmid dan antibiotip menunjukkan tidak ada perkaitan di antara mereka. RAPD-PCR telah digunakan untuk menghasilkan corak genomic polimorfik untuk membezakan pencilan - pencilan *Listeria*. Primer GEN15009 dipilih selepas menyaring 10 primer. Primer tersebut menghasilkan keputusan yang pada keseluruhan pencilan yang dikaji dengan saiz jalur di antara 0.25 kb ke 3.0 kb. Daripada dendrogram yang diperolehi *L. monocytogenes* dipisahkan dari *L. innocua* dan pencilan setiap spesis juga dibezakan. Data menunjukkan RAPD-PCR dapat digunakan sebagai satu cara untuk mendiskriminasikan pencilan - pencilan *L. monocytogenes* dan *L. innocua,* dan untuk membezakan spesis *Listeria.*

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Have you not known?
Have you not heard?
The everlasting God, the Lord,
The Creator of the ends of the earth,
Neither faints nor is weary.
His understanding is unsearchable.
He gives power to the weak,
And to those who have no might He increases strength.
He gives the power to the weak,
And to those who have no might He increases strength.
Even the youths shall faints and be weary,
And the young men shall utterly fall,
But those who wait on the Lord
Shall renew their strength;
They shall mount up with wings like eagles,
They shall run and not be weary,
They shall walk and not faint. (Isaiah 40: 28-31)

I've been constantly reminded by God that He is my strength and only in Him I can be fruitful. I do believe that where I am now is because of His grace, love and beautiful plan for me. He's always been my ability, my wisdom, my peace, my joy, my strength, my confidence ..... and He is my everything, He is my all.

My deepest gratitude goes to my supervisor, Associate Professor Dr. Son Radu for trusting me and giving me the opportunity to do my MSc. I treasured and

appreciate his patience, guidance, advices and encouragement through out my study years (since I was doing my BSc). I learn a lot from him.

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Not forgetting my family (dad, mum, Gadreal, Timothy and Rachel) for their unending love, prayers and encouragement. Words could not express my gratitude to all of you.

Special thanks goes to all my cousins (Bhie, Emma, Daniel & team), aunts, uncles, and grandparents (tepu' Liang, the late of tepu' Sheila, tepu' Siren and tepu' Bulan) for their love and prayers.

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I certify that an Examination Committee has met on 17th April 2002 to conduct the final examination of Lesley Maurice Bilung on her Master Science thesis entitled "Isolation and Molecular Characterisation of *Listeria monocytogenes* and *Listeria innocua* from Poultry Meat" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. The Committee Members for the candidate are as follows:

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This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfillment of the requirement for degree of Master of Science.

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Date: 9 JAN 2003

DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations, which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

Lesley Maurice Bilung

Date: 17/6/02

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LIST OF ABBREVIATIONS

AOAC	Association Official Analytical Chemistry
AP-PCR	arbitrary primered- polymerase chain reaction
BLEB	Buffered *Listeria* Enrichment Broth
CAMP	Christie-Atkins-Munch-Peterson
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
EC	European Community
EDTA	Ethylenediamine tetra-acetic acid
ELISAs	enzyme-linked immunosorbent assays
EtBr	Ethidium bromide
FDA	Food and Drug Administration
G	gram
HCl	hydrochloric acid
I	Intermediate
IDF	International Dairy Federation
ISO	International Standard Organisation
KAc	potassium acetate
KCl	potassium chloride
LEB	*Listeria* Enrichment Broth
L-PALCAMY	Polymyxin Acriflavine Lithium Chloride Ceftazidime Aesculin Mannitol Egg Yolk Broth
M	Molar or molarity (moles of solute per liter of solution)
MAR	Multiple Antibiotic Resistance
MBC	Minimal Bactericidal Concentration
MDa	Megadalton
$MgCl_2$	magnesium chloride
mg	milligram
MIC	Minimal Inhibitory Concentration
min	minute(s)
ml	milliliter
mm	millimeter
mM	miliMolar
µg	microgram
µm	micrometer
µl	microliter
mol	mole

N	Normal
NaCl	sodium chloride
NaOH	sodium hydroxide
PCI	Phenol-Chloroform-Isoamyl alcohol
PCR	Polymerase Chain Reaction
PFGE	pulsed-field gel electrophoresis
psi	pound(s) per square inch (Ib/in^2)
R	Resistant
RAPD	Random Amplification of Polymorphic DNA
RNA	ribonucleic acid
rpm	revolution per minute
rRNA	ribosomal ribonucleic acid
S	susceptible
subsp.	subspecies
SDS	Sodium dodecyl sulphate
spp.	species
Taq	Thermus aquaticus DNA (polymerase)
TBE	Tri-Borate EDTA elctrophoresis buffer
Tris	Tris (hydroxymethyl) methylamine
TSA	Tryptone Soy Agar
TSB	Tryptic Soy Broth
U.S	United States
USDA	United States Department of Agriculture
USFDA	United States Food and Drug Administration
UV	ultraviolet
UVM	University of Vermont
V	volts
WHO	World Health Organisation
w/v	weight/volume
v/v	volume/volume
>	more than
°C	degree Celsius

CHAPTER 1

INTRODUCTION

The genus *Listeria* comprises six species: *L. monocytogenes, L. innocua, L. ivanovii, L. welshimeri, L. seeligeri* and *L. grayi.* All species are ubiquitous and potential food contaminants, but only *L. monocytogenes* is a human and animal pathogen, although very rare cases of infection due to *L. ivanovii* and *L. seeligeri* have been described (Boerlin *et al.*, 1992; Rocourt *et al.*, 1992; McLauchlin, 1997). *L. monocytogenes* is gaining recognition as a severe human pathogen which can cause listeriosis, a disease primarily affecting women and their neonates, patients who are immunocompromised and the elderly as well. Listeriosis, mainly caused by the consumption of foodstuffs, can occur as a sporadic disease or as part of an outbreak (Hocking *et al.*, 1997; Giovanacci *et al.*, 1999). The symptoms and clinical manifestation of listeriosis in human pathogens are summarized in Table 1.1 (Marth, 1988). Based on data collected on food consumption and association with listeriosis, the most at risk foods are reported to be ready-to-eat foods such as ready-cooked chicken, sliced ham, pate, processed meat pate, shellfish products, soft and surface ripened cheese and foods held under refrigeration (Sutherland and Poritt, 1997). From the data collected from USDA, 1999 (Table 1.2) reporting the recalls of meat and poultry in the United States from 1994 to 1998, *L. monocytogenes* caused the highest recalls of meat and poultry among the

foodborne pathogens (Kuhn, 1998). Cells of *Listeria* are Gram positive, short, regular rods with rounded ends that can occur singly, in parallel or in short chains arranged to form a V shape. The cells are 0.4 - 0.5 μm in diameter and 0.5 - 2.0 μm in length. The organism is motile by means of few flagella when grown at 20 - 25°C. *Listeria* spp. will grow in most bacterial culture media. Growth is enhanced in the presence of glucose, serum and blood. Table 2.3 shows the biochemical characteristics of the genus *Listeria.* The organisms are widespread throughout nature, being commonly found in the environment and carried by many species of both domestic and wild animals (Sutherland and Poritt, 1997).

The presence of any *Listeria species* in food maybe an indicator of poor hygiene. Moreover the contamination of raw or processed food products by this organism may result in drastic economic losses for the food industry. However, since *L. monocytogenes* is the major human pathogen, it is important to be able to isolate and correctly identify the different *Listeria* species, or at least differentiate *L. monocytogenes* from the other genus (McLauchlin, 1997; Giovanacci *et al.*, 1999). Methodology for the isolation of *Listeria* from food and environmental samples relies on enrichment procedures, followed by selective plating. Traditional isolation methods have partly been replaced by so-called rapid methods, of which latex tests, enzyme-linked immunosorbent assays (ELISAs) and DNA probes are commercially available (Hofstra *et al.*, 1994). For identification on

species level, systems based on biochemical tests are available (Bannerman *et al.*, 1992; Bille *et al.*, 1992).

Listeriosis represents a public health problem since it is fatal in up to 30% of cases (Farber and Peterkin, 1991; Jones and MacGowan, 1995). In general, isolates of *L. monocytogenes*, as well as strains of other *Listeria* spp., are susceptible to a wide range of antibiotics except cephalosporins and fosfomycin (Hof, 1991; Hof, *et al.*, 1997). The treatment of choice for listeriosis remains the administration of ampicillin or penicillin G combined with an aminoglycoside, classically gentamicin (Boisivon *et al.*, 1990; MacGowan *et al.*, 1990; McLauchlin *et al.*, 1991; Franco *et al.*, 1994; Jones and MacGowan, 1995; Lorber, 1997). The association of trimethoprim with a sulfonamide, such as sulfamethoxazole in co-trimoxazole, is considered to be a second-choice therapy. Other strains of *Listeria* spp. isolated from food or the environment or in sporadic cases of human listeriosis resistant to one or several antibiotics have been described (Quentin *et al.*, 1990; MacGowan *et al.*, 1990; Slade and Collins-Thompson, 1990; Poyart - Salmeron *et al.*, 1992; Facinelli *et al.*, 1993; Hadorn *et al.*, 1993; Franco *et al.*, 1994; Charpentier *et al.*, 1995).

The epidemiologic surveillance of diseases caused by several bacteria has been made easier by the study of plasmid profiles (Mayer, 1988). The plasmids may code for antibiotic resistance, toxins, adhesion fimbria, metabolic enzymes and

bacteriocin production (Mayer, 1988). The prevalence of plasmids and their functions in *Listeria* is not well understood (Kolstad *et al.*, 1990). Previous studies had shown that *Listeria* harbour plasmid (Slade and Collins-Thompson, 1990; Kolstad *et al.*, 1992; Peterkin *et al.*, 1991; Lebrun *et al.*, 1992; Isom *et al.*, 1995; Harvey and Gilmour, 2001). The presence of plasmids in poultry bacteria is indicative of a potential human health hazard, since these plasmid-containing bacteria can be transferred to human through the consumption of contaminated poultry meat (Son *et al.*, 1999).

During the last decade, powerful bacterial molecular typing methods such as pulsed-field gel electrophoresis (PFGE), multilocus enzyme electrophoresis (MLEE) and restriction enzyme analysis (REA) have been developed. These methods usually exhibit a higher discriminatory power than phenotyping methods and has proven useful to assess the distribution of *Listeria* strains (Lawrence and Gilmour, 1995; Destro *et al.*, 1996; Ojeniyi *et al.*, 1996; Unnerstad *et al.*, 1996). Genomic fingerprinting via random amplification of polymorphic DNA (RAPD) has been found to be of value with *Listeria* spp. and the usefulness of RAPD analysis for the epidemiological study of *Listeria* isolates has been confirmed by other groups (Mazurier and Wernars, 1992; Lawrence *et al.*, 1993; MacGowan *et al.*, 1993; Czajka and Batt, 1994; Farber and Addison, 1994; Niederhauser *et al.*, 1994; Boerlin *et al.*, 1995).

The ability to identify *Listeria* quickly and reliably can be important in both livestock industry and medicine for establishing the causes of undesirable contaminations and precise determination of pathogenic strains. In Malaysia, there is no report on the outbreak of listeriosis. However, as the isolation rate of *Listeria* in foods is on the increased (Gulam *et al.*, 1991; Saleha *et al.*, 1998; Zaiton *et al.*, 1998) there is an urgent need for precise determination and genetic understanding of the organism. An understanding of genetic variability in *Listeria* is important for the studies of the taxonomy, epidemiology and pathogenicity of this species. The objectives of this study are to:

1. Isolate and identify *Listeria* spp. from poultry meat sample.
2. Determine the antimicrobial resistance patterns and plasmid profiles among the *Listeria* isolates.
3. Detect the DNA sequence diversity of *Listeria* isolates by random amplified polymorphic DNA (RAPD) analysis.

Table 1.1 : Symptoms and clinical manifestation of listeriosis

Symptoms	Clinical manifestations
Fever }	Pyrexia
Convulsions }	Meningitis /meningoencephalitis
Chills } 'Flu'-like symptoms	Septicaemia
Backache }	Spontaneous abortion
Headache }	Granulomatosis infantiseptica
Diarrhoea	(listeriosis of newborn)
Vomiting	Conjunctivitis
Discoloured urine	Oculoglandular listeriosis
	Cutaneous listeriosis
	Pneumonic listeriosis
	Cervicoglandular
	Mild, febrile gastroenteris

Sources: Marth, 1988. Disease characteristics of *Listeria monocytogenes*. *Food Technology* 42: 165-168.

Table 1.2 : US Meat and Poultry Recalls (1994-1998)

Cause	1994	1995	1996	1997	1998
Listeria monocytogenes	16	11	6	3	39
Extraneous material	13	9	2	7	37
E. coli O157:H7	3	5	2	13	29
Processing failures	7	6	6	5	28
Misbranding	1	1	4	6	16
Drug	1	4	2	3	11
Salmonella	0	2	1	3	7
All others	9	4	1	4	20
Total	50	42	42	44	18

Sources: US Department ofAgriculture, *Listeria monocytogenes*, Kuhn, M.L. *Food Processing*, March 1998, p. 16-20.