UNIVERSITI PUTRA MALAYSIA

PREVALENCE AND CHARACTERISATION OF LISTERIA MONOCYTOGENES ISOLATED FROM CHICKEN AND BEEF

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PREVALENCE AND CHARACTERISATION OF *LISTERIA MONOCYTOGENES* ISOLATED FROM CHICKEN AND BEEF

By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

May 2007

DEDICATED TO MY:

MOTHER
LATE FATHER
BROTHERS
LATE BROTHER
SISTERS
WIFE
SON

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

PREVALENCE AND CHARACTERISATION OF *LISTERIA MONOCYTOGENES* ISOLATED FROM CHICKEN AND BEEF

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Chairman: Professor Son Radu, PhD

Faculty : Science and Food Technology

Listeria monocytogenes is an opportunistic haemolytic pathogen of humans and animals involved in several outbreaks and sporadic cases of listeriosis associated with the consumption of contaminated food. Growing antibiotic resistance demands the constant reassessment of antimicrobial efficacy, particularly in countries with wide antibiotic used in veterinary science, where higher resistance prevalence is often found and it has been reported that antibiotic resistant is associated with plasmid in bacteria. The purpose of this study is to characterize *L. monocytogenes* isolated from chicken and beef meat by antibiotic resistance test, plasmid DNA profile and arbitrarily primed (AP)- and repetitive sequence (RS)-PCR analysis and to determine whether there is any correlation between the antibiotic resistant and the incidence of plasmid. A total of 112 samples of chicken meat and 101 samples of beef were collected from different wet markets and night markets in Malaysia. *Listeria* spp. were detected in 39 (34.8%) of the chicken meat and 21 (20.8%) of the

beef samples, respectively. *L. monocytogenes* were detected in 27 (24.1%) and 17 (16.8%) of the chicken meat and beef, respectively. Out of all the *Listeria* spp., 42 and 20 isolates from the chicken meat and beef, respectively, were confirmed as *L. monocytogenes*. The antibiotic susceptibility test with sixteen different types of antibiotics revealed that the highest prevalence of resistance among the *L. monocytogenes* isolates was observed against nalidixic acid (100%). None (0%) of the isolates were resistant to vancomycin. The *L. monocytogenes* strains showed resistance to at least two or more of the antibiotics tested with their multiple antibiotic resistance index (MARI) ranging between 0.13 to 0.63. Thirty-three different patterns of resistance were observed among all the *L. monocytogenes* isolates. Plasmid analysis of the *L. monocytogenes* strains revealed that 24 (38.7%) of the strains carried plasmid DNA ranging in sizes from 1.75 to 104.0 megadalton (MDa). Spearman's rho correlation analysis was utilised to determine the correlation coefficient between MARI and the incidence of plasmid in the *L. monocytogenes* isolates. The result shows that there were no significant correlation between the MARI and the incidence of the plasmid ($r= 0.143$, $p= 0.269$). The AP- and RS-PCR analyses generated diverse PCR patterns with multiple DNA fragments in sizes ranging between 250 and 3000 bp. Dendrograms generated from the PCR patterns clustered the isolates into several groups and subgroups. PCR analyses with primers GEN1-50-02, GEN1-50-10 and repetitive primer clustered the chicken isolates into 3, 13 and 6 main groups; and the beef isolates into 2, 4 and 5 main groups; respectively. These main groups were further clustered into several subgroups. Isolation of *L. monocytogenes* from the beef and chicken meat suggests that there is a risk of acquiring listeriosis through these popular meat sources in Malaysia. *L. monocytogenes* will be killed by cooking and raw or semi raw meat are not

consumed in Malaysia. However, *L. monocytogenes* in raw beef and chicken meat may pose a health hazard in kitchen if contaminating cooked food or other ready to eat food. Considering outbreaks of listeriosis associated with different foods, avoidance of consumption of insufficiently cooked meat by at-risk populations is recommended. Hygiene quality control of meat and its products must be recommended during the slaughtering, transportation carriage, other used devices and stuff carriers. Diligent enforcement of sanitary conditions of food contact surface and handling areas, and personal hygiene practices should reduce the potential contamination of meat products by *L. monocytogenes* at the retail level. Resistance to two or more antibiotics among these isolates was common. It is suggested that incorrect use of these antimicrobial agents for therapeutical purposes in veterinary science may lead to the development of antibiotic resistance. The high MARI value indicated that the *L. monocytogenes* strains were originated from high risk sources of contamination in the geographical area. *L. monocytogenes* can no longer be thought to be uniformly susceptible to antibiotics active against Gram-positive bacteria. Continued surveillance of emerging antimicrobial resistance among *L. monocytogenes* is important to ensure effective treatment of human listeriosis. The non-significant correlation between the antibiotic resistance and the incidence of plasmid suggest that the plasmids could be cryptic plasmids which have no apparent effect on the phenotype, especially the antibiotic resistance of the host. The presence of hemolysin gene in the beef and chicken meat isolates is of public health concern, as this virulence gene is associated with pathogenicity of the bacteria in human listeriosis. Both AP-PCR and RS-PCR, having high discriminatory power, have revealed the high diversity of food related *L. monocytogenes* isolates and their suitability to track down the contamination sources. The results suggest how

complex the epidemiology of the *L. monocytogenes* in the study area, as a result of several strains as opposed to the widespread transmission of a single type. The results do not support that certain genetically related strains are better adapted to a particular food source. The genomic heterogeneity of the *L. monocytogenes* found in this study confirms the usefulness of the AP- and RS-PCR analyses for the rapid differentiation and grouping of this organism.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

KEKERAPAN DAN PENCIRIAN *LISTERIA MONOCYTOGENES* YANG DIPENCILKAN DARI DAGING AYAM DAN DAGING LEMBU

Oleh

SAMUEL LIHAN

Mei 2007

Pengerusi: Profesor Son Radu, PhD

Fakulti : Sains dan Teknologi Makanan

Listeria monocytogenes adalah sejenis patogen hemolitik opportunistik bagi manusia dan haiwan yang terlibat dalam beberapa kes wabak dan kes terpencil listeriosis yang berkaitan dengan memakan makanan yang tercemar. Kerentanan antibiotik yang semakin meningkat memerlukan penilaian semula yang berterusan terhadap keberkesanan antimikrobial, terutamnya di negara yang menggunakan antiobiotik dengan meluas dalam sains veterinar dimana kekekerapan kerentanan tinggi selalunya ditemui dan terdapat laporan yang mengatakan bahawa kerentanan antibiotik berkaitan dengan plasmid dalam bakteria. Kajian ini adalah bertujuan untuk mencirikan *L. monocytogenes* yang dipencilkan dari daging ayam dan daging lembu dengan menggunakan ujian kerentanan antibiotik, analisis profil DNA plasmid dan analisis primer arbitrari (PA)- dan jujukan berulang (JB)-PCR dan menentukan samada terdapat sebarang korelasi antara kerentanan antibiotik dengan kehadiran plasmid. Sebanyak 112 sampel daging ayam dan 101 sampel daging lembu

telah diperolehi dari pelbagai pasar basah dan pasar malam di Malaysia. *Listeria* spp. telah dikesan dalam 39 (34.8%) sampel daging ayam dan 21 (20.8%) sampel daging lembu tersebut. *L. monocytogenes* telah dikesan dalam 27 (24.1%) and 17 (16.8%) sampel daging ayam dan daging lembu, masing-masing. Dari kesemua pencilan *Listeria* spp. ini, 42 dan 20 pencilan dari daging ayam dan daging lembu, masing-masing, telah dikenalpasti sebagai *L. monocytogenes*. Ujian kerentanan antibiotik terhadap 16 jenis antibiotik yang berbeza menunjukkan bahawa kekerapan kerentanan tertinggi dalam pencilan *L. moncytogenes* tersebut diperhatikan terhadap nalidixic acid (100%). Tiada (0%) pencilan yang rentan terhadap vancomycin. *L. monocytogenes* menunjukkan kerentanan terhadap sekurang-kurangnya 2 atau lebih antibiotik dengan indeks kerentanan pelbagai (IKP) dari 0.13 hingga 0.63. Tiga puluh tiga corak kerentanan pelbagai diperhatikan dalam kesemua pencilan tersebut. Analisis plasmid menunjukkan 24 (38.7%) strain membawa plasmid yang bersaiz 1.75 hingga 104.0 megadalton (MDa). Analisis korelasi Spearman's rho telah digunakan untuk menentukan korelasi koefisien antara indeks kerintangan pelbagai dan insiden plasmid dalam pencilan *L. monocytogenes*. Keputusan menunjukkan bahawa tiada perkaitan antara IKP dengan insiden plasmid ($r=0.143$, $p=0.269$). Analisis PA- dan JB-PCR menghasilkan pelbagai corak PCR dengan penghasilan fragmen-fragmen DNA yang bersaiz dari 250 hingga 3000 bp. Dendrogram yang dihasilkan dari corak PCR mengumpulkan pencilan kepada beberapa kumpulan dan subkumpulan. Analisis PCR dengan menggunakan primer GEN1-50-02, GEN1-50-10 dan primer jujukan berulang mengumpulkan pencilan daging ayam kepada 3, 13 dan 6 kumpulan utama manakala pencilan daging lembu dikumpulkan kepada 2, 4 dan 5 kumpulan utama, masing-masing, dengan menggunakan primer-primer yang sama. Kumpulan-kumpulan utama ini seterusnya dibahagikan kepada subkumpulan.

Pemencilan *L. monocytogenes* dari daging lembu dan daging ayam tersebut mencadangkan bahawa terdapat risiko untuk mendapat penyakit listeriosis melalui kedua-dua sumber daging popular tersebut di Malaysia. *L. monocytogenes* akan dibasmi bila makanan dimasak dan daging mentah atau separa masak tidak dimakan oleh penduduk Malaysia. Walaubagaimanapun, *L. monocytogenes* dalam daging lembu dan daging ayam mungkin mendatangkan bahaya kesihatan di dapur kalau makanan yang sudah masak atau makanan yang sedia untuk dimakan dicemari. Dengan mengambilkira wabak penyakit listeriosis berkaitan dengan pelbagai makanan, mengelak dari memakan daging yang tidak dimasak sepenuhnya oleh kumpulan berisiko adalah dicadangkan. Kawalan mutu daging dan hasilan daging adalah dicadangkan semasa penyembelihan, pengangkutan atau peralatan yang dipakai dan pembawa barangan. Penguatkuasaan secara diligen tentang keadaan kebersihan permukaan yang bersentuhan dengan makanan dan kawasan sekitar kerja dan amalan kebersihan individu sepatutnya mengurangkan kemungkinan pencemaran daging dengan *L. monocytogenes* pada peringkat jualan runcit. Kerentanan terhadap dua atau lebih antibiotik dalam pencilan-pencilan ini adalah kerap. Adalah dicadangkan bahawa penyalahgunaan antibiotik untuk teraputik dalam sains verterinar mungkin menjadi pendorong kepada peningkatan kerentanan antibiotik. Nilai IKP yang tinggi menunjukkan bahawa *L. monocytogenes* tersebut berasal dari sumber kontaminasi yang berisiko tinggi dalam kawasan geografi berkenaan. *L. monocytogenes* tidak lagi difikirkan kesemua “susceptible” terhadap antibiotik yang berkesan terhadap bakteria Gram positif. Pemantauan yang berterusan terhadap kemunculan kerentanan antibiotik dalam *L. monocytogenes* adalah perlu untuk memastikan rawatan yang efektif terhadap listeriosis manusia. Korelasi yang tidak bererti antara kerentanan antibiotik dan kehadiran plasmid mencadangkan plasmid

yang dibawa oleh *L. monocytogenes* adalah plasmid kriptik yang mana ianya tidak memberi kesan terhadap fenotip, terutamanya kerentanan antibiotik perumah. Kehadiran gen hemolisin dalam pencilan *L. monocytogenes* dari daging ayam dan daging lembu memerlukan perhatian darisegi kesihatan awam kerana gen virulen ini terlibat dalam kepatogenikan bakteria ini dalam listeriosis manusia. Kedua-dua PA- dan JB-PCR, mempunyai kuasa pengasingan yang tinggi, telah menunjukkan kepelbagaian *L. monocytogenes* yang tinggi dalam makanan dan kesesuaian kedua-duanya digunakan untuk mengesan sumber pencemaran. Keputusan ini mencadangkan bahawa betapa rumitnya epidemiologi *L. monocytogenes* dalam kawasan berkenaan, hasil dari pelbagai strain bertentangan dengan perebakan satu jenis strain. Keputusan ini tidak menyokong bahawa setengah-setengah strain yang rapat dari segi genetik adalah lebih sesuai mengadaptasi terhadap satu jenis sumber makanan. Kepelbagaian genetik *L. monocytogenes* yang dijumpai dalam kajian ini mengesahkan kepentingan analisis PA- dan JB-PCR untuk pengasingan dan pengumpulan organisma ini dengan cepat.

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I certify that an Examination Committee met on 15 May 2007 to conduct the final examination of Samuel Lihan on his Doctor of Philosophy thesis entitled "Prevalence and Characterisation of *Listeria monocytogenes* Isolated from Chicken and Beef " 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. Members of the Examination Committee were as follows:

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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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.

…….
SAMUEL LIHAN

Date:

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%	Percentage
β	Beta
µl	Microliter
µm	Micrometer
16S rRNA	16 subunit ribosomal ribose nucleic acid
A	Adenine or adenosine
Amp	Ampicillin
AP-PCR	Arbitrarily primed-polymerase chain reaction
ATCC	American type culture collection
ATP	Adenosine triphosphate
B	Bacitracin
bp	Base pair
CAMP Test	Christie-Atkins-Munch-Peterson Test
Car	Carbenicillin
CCC	Covalently close circular
CC	Close circular
C	Chloramphenicol or Cytosine
CDC	Centre for Disease Control
cDNA	Complementary DNA
Cfs	Cefsulodin
cm	Centimeter
Cn	Gentamicin
CO_2	Carbon dioxide
dCTP	Deoxycytosine triphosphate
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
dTTP	Deoxythymidine triphosphate
DW	Distilled water
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetra-acetic acid
E	Erythromycine
g	Gram

G	Guanine
H_2SO_4	Sulfuric acid
HACCP	Hazard analysis critical control point
HCl	Hydrochloric acid
HIV	Human immunodefiency virus
HLA	Haemolytic ceftazidime lithium chloride agar
I. U.	International unit
K	Kanamycin
Kb	Kilobase
KDa	Kilodalton
Kg	Kilogram
KH_2PO_4	Potassium phosphate
L. grayii	*Listeria grayii*
L. innocua	*Listeria innocua*
L. ivanovii	*Listeria ivanovii*
L. monocytogenes	*Listeria monocytogenes*
L. seelegeri	*Listeria seeligeri*
L. welshimeri	*Listeria welshimeri*
LB	Luria-Bertani
LEB	*Listeria* enrichment broth
LMBA	*Listeria monocytogenes* basic agar
LPM	Lithium chloride phenylethanol moxlactam medium
Ltd.	Limited
MARI	Multiple antibiotic resistance index
MDa	Megadalton
$MgCl_2$	Magnesium chloride
mg	Miligram
min	Minute
ml	Mililiter
mM	milimolar
MMWR	Morbidity and Mortality Weekly Report
Mox	Latamoxef
MR	Methyl red
mRNA	Messenger RNA

mr	Relative molecular weight
Na_2HPO_4	Di-sodium hydrogen phosphate
NaCl	Sodium chloride
Na	Nalidixic acid
NaOH	Sodium hydroxide
NCCLS	National committee for clinical laboratory standard
Nm	Night market
nm	Nanometer
nt	Nucleotide
^oC	Degree celcius
OD	Optical density
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
P	Penicillin G
R. equi	*Rhodococcus equi*
r.p.m.	Revolutions per minute
RAPD	Random amplified polymorphic DNA
RNA	Ribonucleic acid
S. agalactiae	*Streptococcus agalactiae*
S. aureus	*Staphylococcus aureus*
SDS	Sodium dodecyl sulphate
spp.	Species
ssDNA	Single stranded DNA
S	Streptomycin
Taq	*Thermus aquaticus*
TBE	Tris-Boric acid-EDTA
Te	Tetracycline
TE	Tris-EDTA
TSA	Trypticase soy agar
TSA-YE	Trypticase soya agar-yeast extract
TSB	Trypticase soy broth
TSI	Triple sugar ion
UK	United Kingdom
UPM	Universiti Putra Malaysia