UNIVERSITI PUTRA MALAYSIA

MOLECULAR PROFILING AND ANTIBIOTIC RESISTANCE OF *SALMONELLA ENTERICA* SUBSP. *ENTERICA* ISOLATED FROM INDIGENOUS ULAM AND POULTRY MEAT

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FPSK(M) 2008 5

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By

LEE LEARN HAN

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

May 2008

Dedicated to my loving parents, Lee Guang and Yoke Lin and lovely brothers, Learn Ping and Learn Yong, and sister, Pey Shen and my beloved partner, Kean Ching

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

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Chairman: Cheah Yoke Kqueen, PhD

Faculty: Medicine and Health Sciences

Salmonella enterica subsp. *enterica* formed the major group that represents nearly 60% of the salmonellae. *Salmonella* organisms emerged as a public health problem in many countries as salmonellosis has become the most prevalent foodborne disease worldwide. It has been estimated that approximately 1.4 million cases were reported annually in the developed nations such as USA. In Malaysia, of 8,640 cases of food poisoning reported by the Ministry of Health for the year 1999, 811 (9.4%) were due to *Salmonella*. The purpose of this study was to characterize and study *Salmonella enterica* subsp. *enterica* (*S. enterica*) using multiple antimicrobial resistance and several molecular typing methods including plasmid profiling, PCR-RFLP, RAPD, ERIC-PCR and Multiplex PCR on antibiotic resistant gene. The isolate were recovered from poultry meat (55), four types of indigenous vegetables namely 'selom' (*Oenanthe stolonifera*) (59), 'pegaga' (*Centella asiatica*) (20), 'kesum' (*Polygonum minus*) (41), 'kangkong' (*Ipomoea aquatica*) (14) and processed food (11).

Genomic DNA of the 200 *S. enterica* isolates belonging to 43 different serovars were recovered from poultry meat, various indigenous vegetables and processed food was confirmed by specific and duplex PCR targeting the *iroB* gene that yielded 443 bp and 606 bp amplicons. The PCR amplification of *iroB* gene is a rapid and reliable method for distinguishing between *S. enterica* and other bacterial species.

Plasmids of *S. enterica* varied in sizes from 2 to more than 200 kb. Despite limited knowledge on their function, their presence is frequently used for strain differentiation in epidemiological studies. Plasmid profiling on the 200 *S. enterica* isolates demonstrated high discriminatory capability for serovars differentiation in this study that was clustered into 70 groups based on the number and pattern of the bands.

One of the amplification based techniques used in this study for molecular characterization was PCR-RFLP that incorporated PCR of *iro*B1, *iro*B2 and restriction digest with *Bgl*II and *Alu*I to determine the relatedness of bacterial strains. Results obtained showed that PCR-RFLP has excellent typeablity but low discriminatory power due to its inability to produce different banding patterns.

ERIC sequences are short, highly conserved 126 bp non-coding regions found in the *Enterobacteriaceae*. Its location in bacterial genomes allows discrimination at the genus, species and serovars levels. RAPD is an amplification-based technique using arbitrary primers to detect changes in the DNA sequence at the sites in the genome and enable the discrimination of samples according to sources and serovars. Dendrogram of RAPD and ERIC-PCR were analyzed and comparisons made using

BioNumerics gel analysis software (Applied Maths, Kortrijk, Belgium). Among the 200 isolates of *S. enterica*, RAPD with arbitrary primers OPAR02, OPAR17 and OPAR19 generated 47 clusters and 13 single isolates whereas ERIC-PCR with primers ERIC-1 and ERIC-2 produced 46 clusters and 12 single isolates at 60% similarity level with discriminatory index (*D*) of 0.9726 and 0.9606 respectively. Composite analysis of RAPD and ERIC-PCR profiling simultaneously produced 50 clusters and 18 single isolates at 60% similarity level with highest discriminatory index of 0.9824. These results demonstrated that composite analysis of RAPD (OPAR02, OPAR17 and OPAR19) together with ERIC-PCR are a better tool for differentiation and characterization of *S. enterica* as compared to a single method approach.

The multiplex PCR targeted three different antibiotic resistance genes that was used to detect TEM, PSE-1 and *cmlA/tetR* genes segment encoding resistance towards ampicillin, chloramphenicol and tetracycline, respectively which could reduce labour and cost in analysis of a large number of isolates.

Subsequently antimicrobial resistance was performed using disc diffusion method with a selection of 13 different antimicrobial agents. Total of 66 profiles were generated and multiple antimicrobial resistance (MAR) analysis indicated poultry meat still remains as the main reservoir for multi drug resistant *Salmonella*. In contrast, six isolates from the indigenous vegetables showed the highest MAR index (0.69). This might be due to animal waste fertilizer, irrigation water, contaminated container and improper handling of food by human that contributed to be the sources of *Salmonella* contamination of vegetables. Further investigations need to be

conducted to determine if *Salmonella* isolates in recovered from indigenous vegetables were gaining more antimicrobial resistance. The characterization of MAR enabled the determination of antimicrobial patterns and trends in *Salmonella* from poultry meat and indigenous vegetables in Malaysia.

As a conclusion, the results from this study could provide valuable information on the epidemiology and drug resistance trends of *S.* enterica, and hence contribute towards better surveillance and infection control measures as well as improved public health policy.

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

PROFIL MOLEKUL DAN RINTANGAN TERHADAP AGEN ANTIMICROB BAGI *Salmonella enterica* subsp. *enterica* YANG DIPENCILKAN DARI AYAM DAN ULAM

Oleh

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Febuari 2008

Pengerusi: Cheah Yoke Kqueen, PhD

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Salmonella enterica subsp. *enterica* membentuk kumpulan terbesar yang mewakili hampir 60% salmonella. Kewujudan organisma *Salmonella* yang memberi masalah kesihatan kepada banyak Negara kerana menyebabkan penyakit salmonellosis yang merupakan panyakit berjangkit melalui makanan yang paling utama secara global. Dianggarkan 1.4 juta kes dilaporkan setiap tahun di negara maju seperti Amerika Syarikat. Di Malaysia, 811 (9.4%) daripada 8,640 kes keracunan makanan yang dilaporkan oleh Kementerian Kesihatan pada tahun 1999, merupakan infeksi *Salmonella*. Tujuan penyelidikan ini adalah untuk mengkaji *Salmonella enterica* subsp. *enterica* (*S. enterica*) dengan mengunakan teknik rintangan terhadap pelbagai agen antimicrob dan beberapa teknik analisis molekul termasuk analisis plasmid, PCR-RFLP, RAPD, ERIC-PCR dan Multiplex PCR. Isolat Salmonella adalah dari sampel daging ayam (55) dan ulam seperti 'selom' (*Oenanthe stolonifera*) (59), 'pegaga' (*Centella asiatica*) (20), 'kesum' (*Polygonum minus*) (41), 'kangkong' (*Ipomoea aquatica*) (14) dan makanan proses (11).

DNA genomik dari 200 pencilan *S. enterica* yang terdiri daripada 43 serovar diperolehi dari ayam, pelbagai ulam dan makanan yang telah diproses dan disahkan dengan 'Specific' dan 'duplex PCR' yang menumpu ke atas gen *iroB* dengan menghasilkan 443 bp dan 606 bp. Amplifikasi PCR terhadap gen *iroB* merupakan teknik yang cepat dan berkesan untuk membezakan *S. enterica* dengan bakteria lain.

Saiz plasmid untuk *S. enterica* berada di antara 2 hingga 200 kb. Meskipun oleh kekurangan ilmu pengetahuan terhadap fungsi plasmid, kewujudan plasmid selalu digunakan untuk membezakan strain bakteria berlainan dalam kajian epidemiologi. Analisis profil plasmid yang dihasilkan oleh 200 pencilan *S. Enterica* menunjukkan keupayaan yang tinggi dalam membezakan serovar antara satu sama lain. Dalam kajian ini 70 kumpulan telah diklusterkan mengikut bilangan dan corak band.

Salah satu daripada pemencilan secara molekul dengan kaedah amplifikasi yang digunakan dalam kajian ini adalah PCR-RFLP yang menggabungkan PCR untuk *iro*B1 dan *iro*B2 serta 'restriction digest' dengan *Bgl*II dan *Alu*I merupakan teknik berdasarkan amplifikasi secara specifik. Ia bergabung dengan analisis 'restriction digest' and amplifikasi PCR untuk menentukan hubungan di antara bakteria dalam kajian ini. Lokus yang khusus diamplifikasi dengan primer khusus dan disambung dengan analisis RFLP. Produk PCR dari amplifikasi primer *iroB*1 and *iroB*2 diteruskan dengan percernaan oleh *Bgl*II and *Alu*I untuk menentukan kesamaan antara bakteria. Keputusan yang diperolehi menunjukkan bahawa PCR-RFLP memiliki keupayaan yang tinggi dalam kebolehan mengtipe tetapi daya diskriminasinya rendah kerana ketidakupayaan untuk menghasilkan corak-corak band yang berbeza dalam kajian ini.

ERIC merupakan jujukan yang singkat dan terpelihara dengan saiz 126 bp dari bahagian bukan kod yang dijumpai dalam *Enterobacteriaceae*. Lokasi ERIC bakteria membenarkan diskriminasi pada peringkat genus, spesis dan serovar. RAPD merupakan teknik yang berdasarkan amplifikasi dengan menggunakan primer rambang untuk menentukan perubahan dalam susunan DNA di mana sampel dapat didiskriminasikan berdasarkan sumber dan serovar. Dendrogram dari RAPD dan ERIC-PCR dianalisis dan dibandingkan dengan menggunakan perisian analisis gel BioNumeric (Applied Maths, Kortrijk, Belgium). Daripada 200 pencilan *S. enterica*, RAPD dengan primer rambang iaitu OPAR02, OPAR17 dan OPAR19 dapat menghasilkan 47 kluster dan 13 pencilan tunggal sementara ERIC-PCR dengan primer ERIC-1 dan ERIC-2 menghasilkan 46 kluster dan 12 pencilan tunggal pada tahap keserupaan 60% dengan indeks diskriminasi (*D*) 0.9726 dan 0.9606 masing-masing. Analisis gabungan RAPD dan ERIC-PCR menghasilkan 50 kluster dan 18 pencilan tunggal pada tahap keserupaan 60% dengan indeks diskriminasi yang tertinggi iaitu 0.9824. Keputusan menunjukkan analisis gabungan RAPD (OPAR02, OPAR17 dan OPAR19) bersama dengan ERIC-PCR merupakan sistem analisis yang lebih baik untuk pembezaan dan klasifikasi *S. enterica* berbanding dengan teknik secara tunggal.

Perkembangan mengenai rintangan agen antimikrob antara pelbagai *Salmonella* telah menjadi masalah kesihatan secara global. Penggunaan agen antimikrob dalam manusia, perubatan veterinar, nutrisi dan pertanian secara meluas selalunya memberi implikasi terhadap kewujudan strain *S. enterica* yang mempunyai rintangan terhadap pelbagai ubat. Maka, 'multiplex PCR' yang fokus kepada tiga rintangan gen antibiotik iaitu TEM, PSE-1 dan *cmlA/tetR* yang bertanggungjawab memberi

keupayaan rintangan terhadap ampicillin, chloramphenicol dan tetracycline masing-masing digunakan dalam kajian berkeupayaan menggurangkan kos buruh dan analisis dalam bilangan sampel yang banyak berbanding dengan analisis PCR tunggal untuk setiap gen berasingan.

Tambahan pula, teknik rintangan antimikrob pelbagai (MAR) yang digunakan terhadap 200 pencilan *S. enterica* dengan 13 jenis agen antimikrob memaparkan ayam masih merupakan waduk utama untuk *Salmonella* yang mempunyai rintangan terhadap pelbagai ubat. Sebaliknya, enam pencilan dari ulam menunjukkan indeks MAR yang tertinggi dalam kajian ini. Penyiasiatan yang lebih mendalam diperlukan untuk menentukan samada pencilan *Salmonella* yang diperolehi daripada ulam sedang mangalami process memperolehi lebih rintangan terhadap ajen antimikrob. Klasifikasi MAR membenarkan kita menentukan corak-corak antimikrob dalam *Salmonella* yang dipencilkan daripada ayam dan ulam di Malaysia.

Kesimpulannya, data yang diperolehi dari kajian ini dapat memberi maklumat yang manfaat dalam kajian epidemiologi *Salmonella*, kawalan infeksi yang lebih berkesan dan menyokong polisi kesihatan awam.

ACKNOWLEDGEMENTS

First and foremost, I would like to convey my greatest gratitude and appreciation to the chairman of my supervisory committee, Dr. Cheah Yoke Kqueen for his patience, invaluable guidance, endless motivation, dedicated efforts and continuous support throughout the study. Also heartfelt appreciation is extended for his tremendous effort in teaching me to write proper scientific papers. I am forever indebted to all his teachings and guidance along the path of research.

My special thanks to my co-supervisors, Associate Professor Dr. Sabrina Sukardi for her endless motivation and guidance that kept me going and make this project and thesis a reality. Sincere thanks are extended to my other co-supervisors, Professor Dr. Son Radu and Dr. Noorzaleha Awang Salleh for their advice, suggestions and supports.

My sincere gratitude to all my friends, Sim Jiun Horng, Suzanne Khoo, Crystale Lim, Chee Hong, Phelim Yong, Lee Yean and Fahartani Mahmud for all the help and support given along the journey of my master's research.

My sincere thanks to all the staff of the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, who has contributed in one way or another throughout the research project been conducted.

Last but not least my deepest thanks to my dearest parents, Lee Guang, Yoke Lin, my handsome brothers, Learn Ping and Learn Yong, my beautiful sister, Pey Shen

and my beloved partner, Kean Ching for all their patience, support and encouragement. Thanks and I love you all!

I certified that an Examination Committee has met on 22th May 2008 to conduct the final examination of Lee Learn Han on his Master of Science thesis entitled "Molecular Profiling and Antimicrobial Resistance of *Salmonella enterica* subsp. *enterica* Isolated From Indigenous Vegetables (Ulam) and Poultry Meat" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1981. The Committee recommends that the student be awarded the Master of Science.

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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 for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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Date: 10 July 2008

DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

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LEE LEARN HAN

Date: 24 Jun 2008

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CDC	Center for Disease Control and Prevention
D	Discriminatory index
DNA	Deoxyribonucleic acid
ERIC-PCR	Enterobacterial repetitive intergenic consensus
MAR	Multiple antimicrobial Resistant
NCCLS	National Committee for Clinical Laboratory Standards
PCR	Polymerase chain reaction
PCR-RFLP	PCR-Restriction fragment length polymorphism
PFGE	Pulse field gel electrophoresis
RAPD	Random amplified polymorphic DNA
RPM	Rate per minute
UPGMA	Unweighted pair-group arithmetic average clustering
XLD	Xylose lysine deoxycholate agar