UNIVERSITI PUTRA MALAYSIA

MOLECULAR CHARATERISATION OF SALMONELLA SEROV ARS ISOLATED FROM POULTRY

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MOLECULAR CHARATERISATION OF *SALMONELLA* SEROVARS ISOLATED FROM POULTRY

By

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Thesis Submitted in Fulfilment of the Requirement for the Degree of Doctor of Philosophy in the Faculty of Food Science and Biotechnology
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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

MOLECULAR CHARACTERISATION OF *SALMONELLA* SEROVARS ISOLATED FROM POULTRY

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September 2001

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Salmonellosis has received considerable amount of attention because public health concerns in third world and developing countries including Malaysia. In Malaysia, the incidence of non-typhoidal salmonellosis is relatively unknown and has not been given much attention. Data collected shows that the incidence of *Salmonella* species isolated from animals and food sources have increased in recent years especially from poultry sources. One hundred and fourty seven *Salmonella* isolates belonging to 14 serovars were examined in the present study.

Molecular techniques namely random amplified polymorphic DNA-PCR (RAPD-PCR), enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR), polymerase chain reaction-ribotyping and pulsed field gel electrophoresis (PFGE) were used to subtype all the 147 *Salmonella* isolates at strains level. Among these techniques, RAPD-PCR was the most superior in distinguishing all *Salmonella* serovars, followed by PFGE, ERIC-PCR and PCR-ribotyping. The PCR-based methods offered several advantages over PFGE due to its speed, economics, ease to perform, results can be generated within 24 hours and large of samples can be

analysed simultaneously. In contrast, PFGE method was technically tedious, demanding and time consuming.

Extensive genetic polymorphism or diversity was observed among strains of *Salmonella* serovars. In molecular typing for epidemiological studies, the level of the percentage of similarity in cluster analysis is arbitrarily adopted based on the clustering of the strains analysed. In this study, 70% of similarity level was chosen to analyse results obtained in PCR-based and PFGE analysis. Cluster obtained by the PCR-based and PFGE typing techniques showed some disagreements in discriminating strains. Several strains that have different RAPD patterns did not necessarily had similar PFGE, ERIC or PCR-ribotyping patterns.

From the present study, it can be concluded that, it is impossible to rely on a single method to determine genetic relatedness among *Salmonella* serovars for effective epidemiological studies. This study clearly suggests that only a combination of different techniques is able to provide a comprehensive epidemiology of *Salmonella* serovars, and thus the need for a careful selection of the typing techniques. It has been identify the DNA-based typing methods is most effective for epidemiological studies.

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

PENCIRIAN MOLEKULAR KE ATAS *SALMONELLA* SEROVAR YANG DIASINGKAN DARI AYAM

Oleh

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Salmonellosis perlu mendapat perhatian kerana kepentingannya terhadap kesihatan umum kepada dunia ketiga dan negara-negara yang sedang membangun termasuk Malaysia. Di Malaysia, insiden terhadap salmonellosis bukan-tifoid kurang diketahui dan tidak mendapat perhatian umum. Bagaimanapun dari data terkumpul menunjukkan, *Salmonella* banyak diasingkan dari binatang dan makan-makanan yang berasaskan kepada sumber-sumber ayam. Seratus empat puluh tujuh isolat yang mengandungi 14 serotaip telah diselidik di dalam pengkajian ini.

Teknik-teknik molekular seprti amplifikasi rawak polymorpik DNA (RAPD-PCR), enterobakterial repetitif intergenik konsensus-tindakbalas rantaian polimerase (ERIC-PCR), tindakbalas rantaian polimerase (PCR)-ribotiping dan gel elektroforesis medan denyut (PFGE) telah digunakan untuk menentukan kesemua 147 *Salmonella* serotaip sehingga ke tahap sub-strain. Dari kesemua teknik yang telah digunakan, analisis RAPD-PCR menunjukkan kaedah yang terbaik untuk mendiskriminasikan individu-individu *Salmonella*, jika dibandingkan dengan PFGE,

ERIC-PCR dan PCR-ribotiping. Kaedah yang berasaskan tindakbalas rantaian polimerase (PCR) ini, memberikan beberapa kelebihan berbanding dengan kaedah PEGE,kerana kaedah PCR memberikan keputusan yang lebih cepat (dalam tempoh 24 jam), ekonomi, mudah dan jumlah bilangan individu *Salmonella* yang dapat dianalisa secara serentak adalah besar. Kaedah PFGE pula memerlukan, keperluan teknikal yang agak cerewet, mengambil masa yang agak panjang untuk mendapatkan keputusan ujikaji dan jumlah bilangan individu *Salmonella* yang dapat dianalisa adalah terhad..

Kesemua *Salmonella* serovar yang dikaji menunjukkan polimorfisma genetik yang tinggi. Pengkajian tiping molekular untuk kajian epidemiologi di dalam kajian ini, menggunakan peratus keserupaan yang berupa kebangkalian yang berdasarkan kluster di dalam analisis. Dalam pengkajian ini, 70% tingkat keserupaan telah dipilih dan digunakan untuk menganalisa kesemua keputusan yang berasaskan kepada PCR dan analisis PFGE. Kluster yang didapati, dari teknik yang berasaskan PCR dan gel elektroforesis medan denyut (PFGE) menunjukkan keputusan yang berbeza di dalam mendiskriminasikan strain. Seperti, beberapa strain yang menunjukkan corak amplifikasi rawak polymorpik DNA (RAPD) yang sama tidak semestinya menunjukkan corak yang sama didalam gel elektroforesis medan denyut, enterobakterial repetitif intergenik consensus-PCR (ERIC) dan PCR-ribotaiping.

Dari pengkajian ini, keesimpulannya, adalah mustahil bergantung kepada satu metod sahaja untuk menentukan pertalian genetik secara berkesan kepada kesemua serovar *Salmonella*. Dalam kajian ini jelas memmjukkan bahawa, kombinasi dari

pelbagai teknik taiping yang berbeza dapat memberikan gambaran yang lebih tepat terhadap epidemiologi *Salmonella* serovar yang dikaji. Pemilihan teknik taiping perlu dilakukan secara berhati-hati untuk memberikan gambaran epidemilogi yang lebih tepat, serta berkesan. Teknik yang berkesan bagi memberikan gambaran epidemeologi yang lebih tepat di dalam kajian ini adalah dengan menggunakan kaedah taiping DNA.

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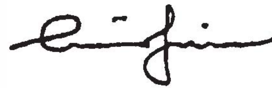

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DECLARATION

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.

SAHILAH ABD. MUTALIB

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LIST OF ABBREVIATIONS

DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminatetra-acetic acid
ERIC	Enterobacterial repetitive intergenic consensus
IMR	Institute for Medical Research
IS	Insertion sequences
MARDI	Malaysian Agricultural Research and Development Institute
MLEE	Multilocus enzyme electrophoresis
%	Percentage
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphisms
SDS	Sodium dodecyl sulphate
TBE	Tris-Boric-EDTA
UV	Ultra violet
v/v	Volume per volume
w/v	Weight per volume

CHAPTER 1

INTRODUCTION

1.1 Introduction

The genus *Salmonella* is a public health concern since 1885 and they are also known as etiological agents of different diseases collectively referred to as salmonellosis. In food industry, processed foods are not permitted to contain any *Salmonella* cells. The reason is that, salmonellae are responsible for severe, acute gastroenteritis (food poisoining) and enteric fever (typhoid fever).

Non-thyphoidal salmonellosis and typhoid fever continue to plague human populations in both developed and third world countries. According The World Health Organisation (W. H. O.) (Calva *et al.,* 1997) reports, acute gastroentiritis and diarrhoea due to non-typhoidal Salmonellae are increasing with a rate of 1.3 billion cases and 3 million deaths. Whereas, 16.6 millions cases of typhoid fever with 600,000 deaths (Calva *et al.,* 1997).

Salmonellae are ubiquitous among domestic and warm-blooded animals and almost all serovars cause illness in man (Kwang *et al.*, 1996). More than 2200 serovars have been identified and they were grouped into six subspecies; 1. subsp. *cholerae*-suis; 2. subsp. *salamae*; 3a. subsp. *arizonae*; 3b. subsp. *diarizonae*; 4. subsp. *houtenae*; 5. subsp. *bongori*; 6. subsp. *indica*. (John, 1998).

Poultry (chicken, ducks, turkeys and geese) are the most important reservoir of Salmonellae in the human food chain (D'Aoust. 1989; John, 1998). The consumption of poultry meat in Malaysia was 570,000 tonnes, representing 69% of total meat consumption in 1995 (Van Der Suis, 1995). It is postulated that the demand of consumption of poultry meat will increase to 907,000 tonnes in year 2000 or 74% of total meat consumption. Poultry can acquire *Salmonella* in a number of ways: contaminated breeder flocks, contaminated hatchery environment, litter, feed and contaminated water. When poultry arrives at the abattoir, both intestinal tract and the outside of the bird are contaminated with *Salmonella*. Scalding, particularly when soft scalds (52-54°C) are used, does not eliminate *Salmonella* and the organism can be found in scald water and on the carcasses leaving the scald. Defeathering spreads *Salmonella* between carcasses and further contamination can occur at evisceration. Though immersion chilling can bring about significant reduction in the number of *Enterobacteriaceae* on carcasses, the incidence of *Salmonella* is often increasing (D'Aoust. 1989). Eggs are contaminated with salmonellae when laid and there have numerous outbreaks of gastroenteritis credited to *Salmonella*-contaminated eggs such as *Salmonella enteritidis* (Stubbs *et al*., 1994; Thong *et al*, 1995a).

The conventional analysis in an outbreak of microorganism such as *Salmonella*, involves a series of basic operation: (i) selection and collection to analytical laboratory; (ii) preparation of samples by mixing, blending or suspension in an appropriate diluent followed by dilution; (iii) culturing and isolation of the

microorganisms; (iv) enumeration of microbial populations; and (v) detection and identification of isolates to genus, species or strain level (John, 1998). These conventional procedures, generally, are relatively slow to give a result and are demanding of labour and requirements for materials. Increasing consumer interest in food safety combined with modern approaches to food processing and quality assurance have created strong demands for fast, automated, cost-effective and more reliable methods for determining microbiological quality.

An outbreak caused by *Salmonella* requires a rapid isolation and identification in order to identify the reservoir or the vector. Therefore, improved epidemiological surveillance is essential and bacterial typing is of great value in epidemiologic investigations. To date several typing methods base on genotypic approaches have been developed, involving the application of nucleic analysis of chromosomal DNA or extra-choromosomal elements (plasmid) or insertion sequences (IS) in investigating of infectious disease outbreaks. These various methods have been used to improve the identification of *Salmonella* that caused food-borne infection and also to examine sets of bacterial isolates that allow discrimination below the species level.

Phenotypic markers such as bacteriophage typing, antigen (serotyping), bacteriocin and antibiotic susceptibility have been of limited utility for subtyping isolates of *Salmonella* spp. (Lin *et al*., 1996). In more recent years, the use of DNA-related techniques such as plasmid profiling, PCR-ribotyping, ribotyping, IS*200* profiles, pulsed field gel electrophoresis (PEGE), random amplified polymorphic

DNA (RAPD or AP-PCR (Arbitrarily primed-PCR) and repetitive element PCR fingerprinting such as ERIC (Enterobacterial repetitive intergenic consensus) (Rodrigue *et al.*, 1992; Stubbs *et al.*, 1994; Olsen *et al.*, 1994; Lagatolla *et al.*, 1996; Gillings and Holley, 1997), have proved to be useful in discriminating isolates of *Salmonella* spp.. For example, both PFGE and ribotyping have become useful tools for typing and differentiating strains for epidemiological studies of *S. enteritidis*, *Salmonella typhi*, *Salmonella bradenburg*, *Salmonella poona* and *Salmonella branderup* (Baquar *et al.*, 1994; Thong *et al.*, 1995a and 1995b; Liebisch and Schwarz, 1996; Thong and Pang, 1996). Whereas, PFGE, ribotyping (restriction fragment length polymorphisms (RFLP) of 16S rRNA gene), IS*200* typing and plasmid profiling have been used for differentiation of *S. enteritidis* isolated from poultry (Liebisch and Schwarz, 1996).

It has been shown that salmonellosis continue to pose an important health problem in many developing countries. In Malaysia, no reportable data has been obtained for non-typhoidal salmonellosis. However, from the data collected shows that the incidence of *Salmonella* spp. isolated from animals and food sources has increased noticeably in recent years especially from poultry sources (Chye and Mohd, 1994; Arumugaswamy *et al.*, 1994; Jamal, 1997). Contaminated poultry can serves as a vehicle for the transmission of the disease to man.

It is believed that the incidence of salmonellosis continues to increase in Malaysia. The increase may probably cause by interrelated factors. These include the rapid pace of economic development, which increase the movement of large

numbers of migrant workers. The other common factors are inadequate supplies of clean water, consumption of raw or inadequately cooked food, the increasing habit of buying ready-to-eat, which may not be hygienically prepared. Therefore, effective surveillance and the development of rational control strategies are urgently needed to monitor the spread of these important human diseases in Malaysia (Thong *et al.*, 1995b).

The aim of the project was to carry out a study on the genetic diversity of *Salmonella* species isolated from poultry sources using molecular approaches. This would allow discrimination below the species level. Besides that the data obtained will provide some baseline information about the molecular epidemiology of *Salmonella* serovars among the poultry isolates. The genetic diversity among *Salmonella* serovars will also show the clonality and the movement of *Salmonella* strains circulating in different location in Malaysia. These informations are of great value for the purpose of surveillance and control.

1.2 The Objective

One hundred and fourty seven strains of *Salmonella* of 14 serovars were examined as below:

1. To determine genetic relatedness among selected *Salmonella* serovars using molecular techniques such as PCR-based methods: Random amplified polymorphic DNA (RAPD), Enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR), PCR-ribotyping, and Pulsed field gel electrophoresis (PFGE).

CHAPTER 2

LITERATURE REVIEW

2.1 Salmonellosis

The genus *Salmonella* is ubiquitous in nature with human and animals being their primary reservoirs. More than 2200 serotypes of *Salmonella* has been identified and the degree of host adaptation are varies. It affects the pathogenicity for human in three ways. There are serovars adapted in human such as *Salmonella typhi* and *Salmonella paratyphi*. They can cause enteric fever and are not usually pathogenic to animals. Some serovars are ubiquitous which affect both human and arrange of animals such as *Salmonella typhimurium* which cause gastrointestinal infections of varying severity. Thirdly, serovars which highly adapted to animal host such as *Salmonella abortovis* (sheep) and *Salmonella gallinarum* (poultry) usually produce no or very mild symptoms in human (Baird-Parker, 1990; John, 1998). Human salmonellosis can be divided into four syndromes: Gastroenteritis or enterocolitis (food poisoning), Enteric fevers (typhoid-like disease) , bacteremia with or without gastroenteritis and asymthomatic carrier states.

2.2. Morphology

Salmonellae are Gram-negative and non- spore forming rods, usually 0.7 - 1.5 X 2-3 μm in dimensions. They are motile by peritrichous flagella, exception only on *S. gallinarum* and *Salmonella pullorum*. Definite capsules can be demonstrated in