UNIVERSITI PUTRA MALAYSIA

MOLECULAR CHARACTERIZATION OF *Salmonella enteritidis* ISOLATES BY PULSED FIELD GEL ELECTROPHORESIS AND PLASMID PROFILING

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By

LOKE CHUI FUNG

Thesis Submitted in Fulfilment of the Requirements for the Degree of Master of Science in the Faculty of Food Science and Biotechnology, University Putra Malaysia.

April 1997

Specially dedicated to

my beloved family

and friends……..

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MOLECULAR CHARACTERIZATION OF *SALMONELLA ENTERITIDIS* ISOLATES BY PULSED FIELD GEL ELECTROPHORESIS AND PLASMID PROFILING

By

LOKE CHUI FUNG

APRIL 1997

Chairman : Dr. Raha Abdul Rahim

Faculty : Food Science and Biotechnology

Seventy *Salmonella enteritidis* isolates from poultry and 48 isolates from human sources were analyzed for their restriction polymorphism patterns generated by pulsed field gel electrophoresis (PFGE), plasmid profiles and antimicrobial susceptibility patterns. In the present study, PFGE analysis following digestion with two low-frequency-cleavage restriction endonucleases, *Xba*I (5'-TCTAGA-3') and *Spe*I (5'-ACTAGT-3') generated nine and five distinct fingerprints respectively with *F* values ranging from 0.06 to 0.97. Digestion with high-frequency-cleavage restriction endonucleases, *Mlu*I (5'-ACGCGT-3') and *Pvu*II (5'-CAGCTG-3') revealed less polymorphism in their PFGE patterns with more than 95% of the *S. enteritidis* isolates belonging to a single fingerprint. PFGE restriction analysis with *Sal*I (5'-GTCGAC-3'), *Eco*RI (5'-GAATTC-3') and *Hin*dIII (5'-AAGCTT-3')

revealed identical PFGE pattern (F value = 1). Thus, suggesting that these restriction endonucleases were not suitable in PFGE analysis of *S. enteritidis*. In plasmid profiling, five different plasmid profiles were obtained among the 116 plasmid-containing isolates, of which, plasmid profile of SE38 containing a single serotype-specific plasmid of 60 kb, was the most predominant (83.8%). A significant number of *S. enteritidis* isolates from both sources were resistant to β-lactams antibiotic other than ampicillin, glycopeptides, polypeptide and sulphamethoxazole. The most common pattern encountered was PVaBSu. Resistance to aminoglycosides (gentamicin, kanamycin and streptomycin), quinolones (nalidixic acid), cephalosporins and chloramphenicol was uncommon. Of the 118 isolates, 114 (96.5%) were resistant to at least four antimicrobial agents. Only one isolate from human was susceptible to all 12 antimicrobial agents but none of the isolates expressed resistance to 10 antimicrobial agents. The overall analysis of the present study revealed that PFGE was more suitable and of high discriminatory value in differentiating among *S. enteritidis* isolates than plasmid profiling and antimicrobial susceptibility testing. The similarities among *S. enteritidis* isolates from human and poultry sources, as determined on the basis of restriction polymorphism patterns, plasmid profiles and antimicrobial susceptibility patterns, might suggest a common origin of the *S. enteritidis* isolates and also the possible mode of transmission of *S. enteritidis* isolates from nonhuman sources to human beings.

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PENCIRIAN MOLEKUL BAGI ISOLAT-ISOLAT *SALMONELLA ENTERITIDIS* DENGAN ELEKTROFORESIS GEL 'PULSED FIELD' DAN PEMPROFAILAN PLASMID

Oleh

LOKE CHUI FUNG

APRIL 1997

Pengerusi : Dr. Raha Abdul Rahim

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Tujuh puluh *Salmonella enteritidis* isolat daripada ayam dan 48 daripada sumber manusia telah dianalisis untuk corak polimorfisma penghad yang dihasilkan oleh elektroforesis gel "pulsed field" (PFGE), profail plasmid dan corak kepekaan antimikrob. Dalam kajian ini, analisis PFGE yang diikuti dengan penghadaman dengan dua enzim penghad pemotongan-frekuensi-rendah, *Xba*I (5'-TCTAGA-3') dan *Spe*I (5'-ACTAGT-3') masing-masing menghasilkan sembilan dan lima "fingerprint" dengan nilai *F* dari 0.06 hingga 0.97. Penghadaman dengan enzim penghad pemotongan-frekuensi-tinggi, *Mlu*I (5'-ACGCGT-3') dan *Pvu*II (5'-CAGCTG-3') menunjukkan kurang polimorfisma dalam corak PFGEnya dengan lebih daripada 95% isolat *S.enteritidis* dari satu "fingerprint" tunggal. Analisis penghad PFGE dengan *Sal*I (5'-GTCGAC-3'), *Eco*RI (5'-GAATTC-3') dan *Hind*III (5'-AAGCTT-3')

menunjukkan corak PFGE yang sama (nilai F=1). Maka, dapat dicadangkan bahawa enzim penghad ini tidak sesuai dalam analisis PFGE untuk *S.enteritidis*. Dalam pemprofailan plasmid, lima profail plasmid berlainan telah diperolehi dari 116 isolat yang mengandungi plasmid, yang mana, profail plasmid SE38 yang mengandungi satu plasmid spesifik-serotaip tunggal 60 kb adalah paling pradominan (83.8%). Satu bilangan isolat *S. enteritidis* yang signifikan dari kedua-dua sumber adalah resistan terhadap antibiotik β-laktam melainkan ampisilin, glikopeptida, polipeptida dan sulfametoksazol. Corak yang paling kerap ditemui ialah PVaBSu. Ketahanan terhadap aminoglikosida (gentamisin, kanamisin dan streptomisin), kuinolon (asid nalidisik), sefalosporin dan kloramfenikol adalah jarang. Dari 118 isolat, 114 (96.5%) adalah resistan terhadap sekurang-kurangnya empat agen antimikrob. Hanya satu isolat dari manusia peka terhadap kesemua 12 agen antimikrob tetapi tiada isolat menunjukkan ketahanan terhadap 10 agen antimikrob. Analisis keseluruhan kajian ini menunjukkan bahawa PFGE adalah lebih sesuai dan mempunyai nilai pemisahan yang lebih tinggi dalam membezakan antara isolat *S. enteritidis* jika dibandingkan dengan pemprofailan plasmid dan ujian kepekaan antibiotik, Persamaan antara isolat *S. enteritidis* dari sumber manusia dan ayam, sepertimana yang ditentukan berdasarkan corak polimorfisma penghad, profail plasmid dan corak kepekaan antimikrob, mungkin mencadangkan satu punca yang sama bagi *S. enteritidis* dan juga kemungkinan transmisi isolat *S.enteritidis* dari sumber bukan manusia kepada manusia.

CHAPTER I

GENERAL INTRODUCTION

Salmonellosis is often considered to be the most frequently occurring infectious disease caused by the members of the bacterial genus *Salmonella*, which belong to the family *Enterobacteriaceae*. The emergence of salmonellosis as a world problem has been dealt with elsewhere since 1950's (Oye, 1964). Till now, an estimated annual incidence of 1.3 billion cases and 3 million deaths are reported (Thong *et al.*, 1995). The salmonellae are widely distributed in nature, with man and animals being their primary reservoirs. Because of the ubiquitous distribution of salmonellae in the environment and the broad host adaptability of over 2100 serotypes of this genus (Calnek *et. al.*, 1991), the control of salmonellosis has become an extremely complex problem. The complexity of the problem and the certainty that there is strain diversity within each serotype has necessitated the development of special methods of strain identification for epidemiological purposes.

Salmonella enterica serovar Enteritidis or *S. enteritidis* is one of the subspecies which causes gastroenteritis, a nontyphoidal salmonellosis, with a worldwide distribution in both humans and animals. An increasing number of outbreaks of gastroenteritis has been reported since 1960. It has been suggested that many sporadic cases of salmonellosis may actually be part of the

unrecognized outbreaks which escape from detection because of the lack of efficient epidemiological markers. In both community and nosocomial outbreaks, bacterial epidemic strains have often been defined by using methods such as serotyping, biotyping and antimicrobial resistance patterns. However, these phenotypic determinations have not always been successful in differentiating *S. enteritidis* strains.

Recently, DNA fingerprinting based on the detection by restriction fragment length polymorphism (RFLP) of chromosomal DNA has been used increasingly to improve the identification of foodborne pathogens and also to differentiate strains below the level of serotyping. These molecular techniques include plasmid profiling, ribotyping, PCR-based amplification and the RFLP by using pulsed field gel electrophoresis (PFGE). The recent development of PFGE has provided another approach for obtaining molecular fingerprint which may be useful in epidemiological studies. PFGE has successfully been applied to perform comparative chromosomal DNA analysis of several pathogens for epidemiological investigation and is believed to possess a discriminating capacity greater than those of ribotyping and other probe-based RFLPs methods (Kuhn *et al*., 1995; Liebisch and Schwarz, 1996; Thong *et al*., 1996). The DNA digested patterns produced by restriction endonucleases, such as *Spe*I, *Avr*II and *Xba*I, in PFGE, have revealed clear differences between the bacterial strains (Thong *et al*., 1995). It is suggested that the DNA digested pattern produced by restriction endonucleases may provide a sensitive means of

*Spe*I, *Avr*II and *Xba*I, in PFGE, have revealed clear differences between the bacterial strains (Thong *et al.*, 1995). It is suggested that the DNA digested pattern produced by restriction endonucleases may provide a sensitive means of differentiating individual strains of *Salmonella*. Here, we evaluate three epidemiological methods of subtyping *S. enteritidis* isolates from both poultry and human sources by using plasmid profiles, restriction analysis of chromosomal DNA by PFGE, and antimicrobial susceptibility patterns.

Objectives

The objectives of the present study are to compare the antibiotic susceptibility patterns and the plasmid profiles among *S. enteritidis* isolates, to compare the polymorphisms of chromosomal DNA by PFGE, to distinguish *S.enteritidis* isolates exhibiting the same plasmid profile by PFGE fingerprint, to establish the possible link between the markers used in the study, and to clarify the possible mode of transmission of pathogenic isolates of *S. enteritidis* from nonhuman sources to human beings.

CHAPTER II

LITERATURE REVIEW

Introduction

Salmonellosis is a foodborne disease caused by the members of the genus *Salmonella*. The latter was named after an American bacteriologist and veterinarian, Daniel E. Salmon, in 1900. This genus of organism consists of only one species, *S. enterica* (Ewing, 1986) and is composed of more than 2000 serotypes, which also include the group previously classified as *Arizona hinshawii* (Siebeling *et al*., 1984). All strains of *Salmonella* may be presumed to be pathogenic to human and often animals. However, a few serotypes of *Salmonella* appear to be host-specific, these include *S. typhi,* which causes typhoid fever in human, *S. pullorum* and *S. gallinarum*, which cause pullorum disease and fowl typhoid respectively in poultry. Most serotypes of *Salmonella* can cause gastrointestinal disease when ingested by human. In the past decades, there has been an increased incidence of gastrointestinal infections caused by *S.enteritidis* (Kirby, 1985; Goldberg and Rubun, 1988; Rodrique *et. al*., 1990; Phillips and George, 1994) and now *S. enteritidis* has become the most predominant serotype in many countries including Malaysia.

General Description of *Salmonella*

S. typhi was the first member of the *Salmonella* to be recognized as a pathogen. It was first seen in 1880 by Eberth and isolated by Gaffky in 1884 (Burrows, 1959). Later, other *Salmonella* species associated with the onset of diseases were isolated. Salmon and Smith isolated *S. choleraesuis* in 1885. *S.enteritidis* was isolated by Gaertner in 1888 and in 1892, Loeffler isolated *S.typhimurium* (D'Aoust, 1989).

The genus *Salmonella* is composed of motile bacteria that conform to the definition of the family *Enterobacteriaceae* and the tribe Salmonellae. The family *Enterobacteriaceae*, named by Rahn in 1937, and now are described as intestinal bacteria, possess the following characteristics (Buchanan and Gibbons, 1974).

> "Small Gram-negative rods; motile by peritrichous flagella or non-motile. Capsulated or non-capsulated. Not spore-forming; not acid-fast. Aerobic and facultatively anaerobic. Grow readily on meat extract media but some members have special growth requirements. Chemoorganotropic; metabolism respiratory and fermentative. Acid is produced from the fermentation of glucose, other carbohydrates and alcohol; usually aerogenic but anaerogenic groups and mutants may occur. Catalase positive with the exception of one serotype of *Shigella*; oxidase negative. Nitrates are reduced to nitrites except by some strains of *Erwinia*. G+C content of DNA: 39-59 moles %."

According to the eighth edition of Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1984), the genus *Salmonella* is defined as follows:

> "Rods, usually motile by peritrichous flagella; non-motile mutants may occur and one type (*S. gallinarum* and *S. pullorum*) is always non-motile. Colonies are generally 2-4 mm in diameter but certain types (*S.abortus-equi, S. typhi-suis* and *S. abortus-suis*) produce colonies of about 1 mm. Most strains will grow on defined media without special growth factors and they can use citrate as carbon source. Most strains are aerogenic but *S. typhi*, an important exception, never produces gas."

Salmonella spp. generally grow well at 35-37°C and are susceptible to temperature of lower than 5°C or higher than 49°C (Bryan *et al.*, 1979). However, *Salmonella* can still promote survival even after exposure to a freezing temperature. The growth of *Salmonella* is generally inhibited in the presence of 3-4% sodium chloride. Study by Alford and Palumbo (1969) have revealed that most *Salmonella* were inhibited in the presence of 2-8% sodium chloride where their total cell yield decreased and the lag phase of their growth curve increased. Generally, *Salmonella* can grow well at pH between 6.5 and 7.5, however, growth of *Salmonella* at pH value as low as 4 was reported (Chung and Goepfert, 1970).

Classification of *Salmonella*

The bacterial nomenclature of the genus *Salmonella* has been in the state of change for decades and becomes exceptionally confusing and controversial in many countries because the naming of *Salmonella* has not been co-ordinated to the international agreement. Many serotypes of *Salmonella*

which were found to be somewhat host specific or adapted, were named either for the disease caused or for the animal involved, as if they represented a distinct species within the genus *Salmonella*. Specific epithets such as *S. typhi*, *S. paratyphi*, *S. paratyphi* A, *S. paratyphi* B and *S. paratyphi* C, were named according to a human disease. Whilst, *S. choleraesuis*, *S. pullorum* and *S.abortus ovis* were associated with the affected animal species. In addition, there are some serotypes named after a geographical location, such as *S.newport*, *S. panama*, *S. florida* and *S. indiana*.

According to the Kauffmann-White Scheme (1986), the genus *Salmonella* is subdivided into five serologically defined subgenera I - V, where the identification of *Salmonella* is based on the detection of the specific antigenic components present. It was known that there were two distinct types of antigens present on the *Salmonella* cell surface. The somatic antigen (O = ohne Hauch) is a heat stable, polysaccharide associated with the body of the cell. It is the antigen first determined in *Salmonella* serology using the slide agglutination technique to group the organism. In the Kauffman-White scheme, the *Salmonella* were grouped into different serogroups, namely serogroup A, B, C_1, C_2, C_3, D_1, E_1, E_2, E_3, E_4, F,G_1, G_2, H, I, K, L, N. The assignment of *Salmonella* relied on the antigen specificities and also the occurrence of certain specificities. For example, serogroup A contains O antigen 2, C_1 contains O antigen 7, C_2 contains O antigen 8 (**Table 1**). The somatic antigens of *Salmonella* are comprised of lipid-polysaccharide-polypeptide complexes

Table 1

Antigenic Schema for *Salmonella* (abbreviated)

Organism	Serogroup	Somatic (O) antigen	Flagellar (H) antigen phase 1	phase 2
S. enteritidis				
bioser Paratyphi A	A	1,2,12	a	-
ser Paratyphi B	B	1,4,5,12	b	1,2
variant Odense	B	1,4,12	b	1,2
bioser Java	B	1,4,5,12	b	[1,2]
ser Stanley	B	4,5,12	d	1,2
ser Schwarzengrund	B	1,4,12,27	d	1,7
ser saintpaul	B	1,4,[5],12	e,h	1,2
ser Reading	B	4,5,12	e,h	1,5
ser Chester	B	4,5,12	e,h	e,n,x
ser Sandiego	B	4,5,12	e,h	e,n,z_{15}
ser Derby	B	1,4,5,12	f,g	[1,2]
ser California	B	4,5,12	m,t	-
ser Typhimurium	B	1,4,5,12	i	1,2
variant Copenhagen	B	1,4,12	i	1,2
ser Bredeney	B	1,4,12,27	l,v	1,7
ser Heidelberg	B	[1],4,[5],12	r	1,2
S. chleraesuis	C_1	6,7	c	1,5
bioser Kunzendorf	C_1	6,7	[c]	1,5
S. enteritidis				
ser Braenderup	C_1	6,7	e,h	e,n,z_{15}
ser Montevideo	C_1	6,7	g,m,s	-
ser Oranienburg	C_1	6,7	m,t	-
ser Thompson	C_1	6,7,[14]	k	1,5
ser Infantis	C_1	6,7,[14]	r	1,5
ser Bareilly	C_1	6,7,[14]	y	1,5
ser Tennessee	C_1	6,7	z_{29}	-
ser Muenchen	C_2	6,8	d	1,2
ser Manhattan	C_2	6,8	d	1,5
ser Newport	C_2	6,8	e,h	1,2
ser Blockley	C_2	6,8	k	1,5
ser Litchfield	C_2	6,8	l,v	1,2
ser Tallahassee	C_2	6,8	z_4,z_{32}	-
ser Kentucky	C_3	[8],20	i	z_6
bioser Miami	D_1	1,9,12	a	1,2
S. typhi	D_1	9,12,Vi	d	
S. enteritis				
ser Berta	D_1	9,12	f,g,t	-
ser Enteritidis	D_1	1,9,12	g,m	-

(cont'd)

Table 1 (cont'd)

Organism	Serogroup	Somatic (O) antigen	Flagellar (H) antigen phase 1	phase 2
ser Dublin	D_1	1,9,12	g,p	-
ser Penama	D_1	1,9,12	l,v	1,5
ser Javiana	D_1	1,9,12	l,z_{28}	1,5
bioser Pullorum	D_1	9,12	-	-
ser Anatum	E_1	3,10	e,h	1,6
ser Meleagridis	E_1	3,10	e,h	l,w
ser Give	E_1	3,10	l,v	1,7
ser Newington	E_2	3,15	e,h	1,6
ser Illinois	E_3	[3],[15],34	z_{10}	1,5
ser Senftenberg	E_4	1,3,19	g,s,t	-
ser Simsbury	E_4	1,3,19	z27	-
ser Rubislaw	F	11	[d],r	[d]e,n,x
ser Poona	G_1	[1],13,22,[36]	z	1,6
ser Worthington	G_2	1,13,23	z	l,w
ser Cubana	G2	1,13,23	z_{29}	-
ser Florida	H	1,6,14,25	d	1,7
ser Madelia	H	1,6,14,25	y	1,7
ser Cerro	K	18	z_4,z_{23}	[z_{45}]
ser Siegburg	K	6,14,18	z_4,z_{23}	[1,5]
ser Minnesota	L	21	e,h	e,n,x
ser Urbana	N	30	b	e,n,x

Source: Kauffmann , 1986.

where the specificity is contained in the polysaccharide portion of the molecule and is always termed as endotoxins. As listed in **Table 1**, most strains or serovars of *Salmonella* share the same antigen, nevertheless, they were different in their pathogenic characteristics. For instance, *S. typhi* which is in serogroup D_1, shares the same somatic antigen 12 with serovar from group A, B and D_1, but this serovar is much more pathogenic if compared with others. The flagellar antigen (H = Hauch) is a heat-labile protein, located in the flagella of the organism. Most *Salmonella* have two different "H" antigens. Very few

such as *S. enteritidis bioser Gallinarum* and *Pullorum*, have none as they are non-motile. Others possess three or even four "H" antigens. Occasionally, a third type antigen called the Vi antigen, is present and always associated with the virulent strains. Vi antigen, a capsular antigen, is a heat-labile, envelope antigen, surrounding the cell wall which masks the somatic antigen rendering the organism resistant to the action of O sera. Normally, agglutination of *Salmonella* cells occurs when suspended in sera containing antibodies against either O, H or Vi antigen. Actually, this property was found to be useful in identifying and characterizing *Salmonella* serotypes, and now more than 2000 serologically distinct types of *Salmonella* have been described.

As a matter of expediency in taxonomy, Edwards and Ewing (1972) recommended the use of a three species concept in *Salmonella* nomenclature which recognized the species *S. typhi*, *S. choleraesuis* and *S. enteritidis*, where the latter includes all strains except for the two species mentioned formerly. As an example, *S. typhimurium* as formerly known, was named as *S. enteritidis ser Typhimurium*, while *S. pullorum*, which is an aberrant strain, was named as *S.enteritidis bioser Pullorum*. However, this classification scheme has not been adopted to the Bergey's Manual of Determinative Bacteriology.

According to Le Minor *et al.* (1986), the genus *Salmonella*, based on the DNA relatedness, consists of a single species, *S. choleraesuis*, and possibly as many as seven subspecies, namely *S. choleraesuis* subsp. *Choleraesuis*,