UNIVERSITI PUTRA MALAYSIA

CHARACTERISATION OF SALMONELLA ENTERITIDIS ISOLATES FROM HUMANS, ANIMALS AND ENVIRONMENTAL SOURCES.

MAIMUNAH BT. MUSTAKIM

CHARACTERISATION OF *SALMONELLA ENTERITIDIS* ISOLATES FROM HUMANS, ANIMALS AND ENVIRONMENTAL SOURCES.

By

MAIMUNAH BT. MUSTAKIM

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

August 1998

With special dedication to my loving children :

Hanis Syafiqah

Arinah

Naim

Nazrin

ACKNOWLEDGEMENTS

The magnitude and scope of work involved in this research required the assistance of numerous people, to whom the author indebted, however great or small their respective contribution.

First, the author expresses her great appreciation to Dr. Son Radu, the Committee Chairman, for his kindness, willingness to help, and encouraging advise throughout the research work.

Sincere appreciation was extended to Professor Dr. Gulam Rusul Rahmat Ali and Dr. Zaiton Hassan, the Committee Members, especially for their suggestions and critical reviews of the thesis.

Very special thanks dedicated to all her friends in Microbiology Laboratory : Sahilah, Kak Endang, Zainuri, Zunita, Dayang Fredalina, Loke Chui Fung, Rozila, Wong Wooi Koon, Yuherman, Samuel, Kamil and Razali.

The family : beloved mother, husband and children, their never ending devotions and moral support throughout her education were gratefully acknowledged.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Master of Science.

CHARACTERISATION OF *SALMONELLA ENTERITIDIS* ISOLATES FROM HUMANS, ANIMALS AND ENVIRONMENTAL SOURCES

By

MAIMUNAH MUSTAKIM

August 1998

Chairman : Dr. Son Radu

Faculty : Food Science and Biotechnology

One hundred and five strains of *Salmonella enteritidis* isolated from five different sources including humans, food animals, avian, wild animals and environments were studied. All isolates were characterised for their biotype, plasmid profiles and resistance to antibiotics. Biotyping of the isolates subdivided them into two biotypes i.e., A and E. Subdivision of the isolates by plasmid profiling identified fifteen plasmid patterns i.e., PP0 to PP14. Plasmid pattern PP14 was the most common pattern encountered in 47 (44%) of the total isolates from various sources. Resistance to antibiotics was not uncommon with 104 (99%) of the isolates were resistant to more than one antibiotics. Subdivision by antibiotic resistance pattern exhibited ten antibiograms designated as R1 to R10. A total of 87 (82%) of

the isolates fell into resistant pattern R1 which showed resistant towards erythromycin, penicillin G and triple sulpha. In conjugations studies, the ability of transferring antibiotic resistance was demonstrated in the transconjugants with the concomitant transfer of the respective plasmid and resistant to tetracycline. Sixty seven representative strains of *S. enteritidis* studied for restriction endonuclease digestion polymorphism by pulsed-field gel electrophoresis (REDP-PFGE) exhibited different PFGE fragment patterns. Digestion with low-frequency-cleavage restriction endonucleases, i.e., *Xba*I (5'- TCTAGA-3')and *Spe*I (5'-ACTAGT-3') produced five and three restriction patterns respectively. Inter-relationships of the PFGE patterns among the isolates could be determined by the calculation of similarity index, F. The F values grouped the strains as closely related, unrelated or indistinguishable. The phage-typed strains of *S. enteritidis* were subdivided into one biotypes, eleven plasmid profiles, six resistance patterns and seven restriction polymorphisms. In general, characterisation by DNA-based method of plasmid profiling gave more discrimination compared to other methods. These methods also provide effective adjunct to antibiotic resistance pattern and phage typing for subdivision of *S. enteritidis*.

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

PENCIRIAN PENCILAN *SALMONELLA ENTERITIDIS* DARI SUMBER-SUMBER MANUSIA, HAIWAN DAN PERSEKITARAN

Oleh

MAIMUNAH MUSTAKIM

Ogos 1998

Pengerusi : Dr. Son Radu

Fakulti : Sains Makanan dan Bioteknologi

Seratus lima pencilan *Salmonella enteritidis* diperolehi dari pelbagai sumber termasuk manusia, haiwan ternakan, beburung, haiwan liar dan persekitaran telah dikaji. Kesemua pencilan itu dicirikan secara biotip, pemprofilan plasmid dan sifat kerintangan kepada antibiotik. Secara biotip, pencilan itu telah dibahagikan kepada dua iaitu biotip A dan E. Pembahagian secara plasmid pemprofilan menunjukkan pencilan itu boleh dibezakan dengan lima belas corak plasmid iaitu PP0 - PP14. Corak PP14 tersebar secara meluas di kalangan sumber-sumber yang dikaji. Kerintangan kepada antibiotik adalah perkara biasa bagi pencilan dengan 104 (99%) pencilan adalah rintang kepada sekurang-kurangnya satu jenis antibiotik. Pembahagian secara corak kerintangan menghasilkan sepuluh corak iaitu R1 hingga R10. Sebanyak 87 (82.9%) pencilan itu termasuk di

dalam corak R1 yang menunjukkan kerintangan kepada eritromicin, penicillin G dan 'triple-sulpha'.Di dalam kajian konjugasi, keboḷehan memindahkan sifat-sifat kerintangan telah ditunjukkan di dalam transkonjugan di mana sifat kerintangan telah dipindahkan bersama-sama dengan plasmid. Kajian ke atas enampuluh tujuh wakil isolat-isolat dari pelbagai sumber secara polimorfisma penghadaman enzim restriksi yang dihasilkan melalui electrophoresis gel "pulse-field" menunjukkan corak-corak PFGE yang berbeza. Penghadaman dengan enzim restriksi pemotongan-frekuensi-rendah iaitu *Xba*I (5'-TCTAGA-3') dan *Spe*I (5'-ACTAGT-3') masing-masing menghasilkan lima dan tiga corak restriksi. Hubung-kait strain-strain di dalam sumber masing-masing boleh dikira dari indeks persamaan, F. Dari nilai F itu, strain-strain dikumpulkan kepada berhubungrapat, tiada perhubungan atau sama di antara satu sama lain. Pembahagian strain-strain fajtip menghasilkan satu corak biotip, sebelas corak plasmid dan tujuh polimorfisma penghadaman enzim restriksi. Pada amnya, pencirian dengan kaedah-kaedah berasaskan DNA menunjukkan lebih banyak perbezaan di kalangan strain-strain. Kaedah tersebut juga sangat berfaedah untuk digunakan bersama-sama kaedah antibiogram dan fajtip di dalam pembahagian strain-strain *S. enteritidis*.

CHAPTER I

GENERAL INTRODUCTION

Typhoid, paratyphoid and salmonellosis are collective terms associated to infection and fevers caused by *Salmonella* species. *Salmonella typhi*, the etiologic agent of typhoid fever has long been threatened and led serious illness to human population in both developed and third-world countries. The World Health Organisation (WHO) has estimated that, annually, there are 16.6 million cases of typhoid fever with nearly 600,000 deaths, and 1.3 billion cases of acute gastroenteritis due to non-typhoidal salmonellosis with 3 million deaths. In developing countries, contaminated water supply due to improper disposal of human and animal wastes is the usual mode for transmission of the disease.

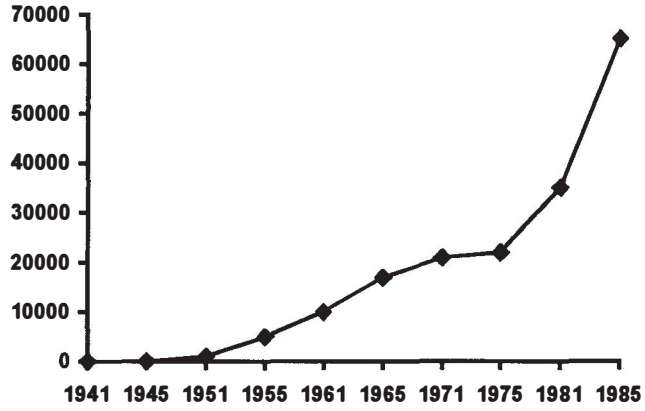
Typhoid fever was recognised by clinical signs and symptoms before the advent of bacteriological era. Beginning with the isolation of *Bacillus cholerae-suis* from pigs with hog cholera in 1885 by Salmon and Smith, followed by isolation of similar bacteria from foodborne intoxication by Gaffky and Paak in 1885 and Gartner in1888 (Le Minor,

1992) or animal disease by Loeffler in 1892, those bacillus were then created a genus and named *Salmonella* by Ligniéres in 1900.

Salmonella infection has been notified as a global problem ranking first or second as a cause of food and waterborne diseases (Gangarosa, 1980). In the United States for example, incidence of salmonellosis increased gradually from below 1000 cases in 1941 to 70,000 cases in 1985. The number has remained between 40,000 to 50,000 cases from 1988 to 1995 [Centre for Disease Control, 1996; **Figure 1 (B)**]. The epidemiological pattern of salmonella infections has changed with time, place and environmental conditions. These changes have resulted due to the changes in food habits in the community as well as changes in food production technology. Also, the emergence of drug resistant salmonella made the situation to be more complicated.

As Salmonella problems have caused unnecessary suffering, death and great economic losses which have led the public health authorities in several countries to institute surveillance programmes in an attempt to control the rapidly increasing incidence of the disease. In the United States, *Salmonella* surveillance was instituted by Centre for Disease Control (CDC) Atlanta, Georgia in 1966 as a co-operative undertaking by 50 states. In England and Wales, a systematic collection of information on *salmonella* infections was started in 1949

(A)



(B)

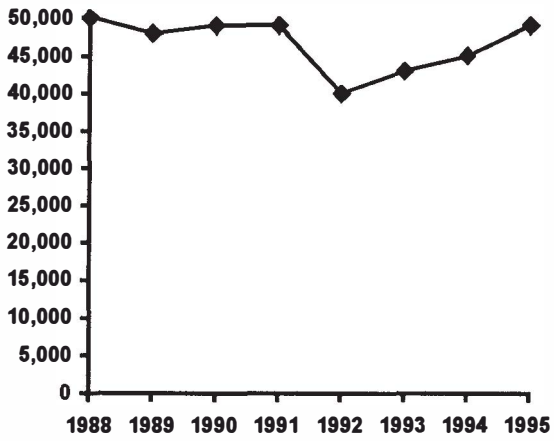


Figure 1 : Comparison of Reported Cases of Salmonellosis excluding Typhoid Fever between the Year 1941 to 1985 (A) and 1988 to 1995 (B).
(Source : A - *Salmonella*, 1992. B - Centre for Disease Control and Prevention, 1996)

by Salmonella Subcommittee of the Public Health Laboratory Services. Surveillance program for typhoid in Malaysia is managed by the Epidemiology Division, Ministry of Health. Generally, salmonella surveillance programme implemented in many countries over the world are important in identifying outbreaks, high risk foods and the mechanism by which transmission occurs.

The genus *Salmonella* belongs to the family Enterobactericeae and comprises of more than 2,200 serotypes. Besides its medical importance, this genus has attracted the interest of a great number of scientists (bacteriologists, immunologists, etc.) for its wide range of variations in the classification systems. These organisms have been subdivided in many ways especially for epidemiological purposes on the basis of biochemical reactions, serological analysis or further amplified by phage typing. Based on biochemical characteristics, Kauffmann subdivided the genera into four major groups: I, II, III and IV (Le Minor, 1992). Then they were further differentiated into seven subspecies i.e., I, II, IIIa, IIIb, IV, V and VI by studies of the thermal stability of hybrids (Le Minor, 1992). Description of serological identification based on the O (somatic), V_i (capsular) and H (flagellar) antigens distinguished the strains into more than 2,200 serotypes (Kauffmann and White scheme). In 1938, Craigie and Yen introduced phage typing for *S.typhi* which proved to be a valuable and sensitive tool in the control of typhoid fever. The ability of phage to distinguished

varieties among identical serotypes led to the development and acceptance of phage typing as a significant epidemiological procedure. With the advent of DNA technology era, several molecular approaches for bacterial identification have been employed in*Salmonella*. The DNA-related techniques such as plasmid analysis, restriction endonuclease digestion polymorphism (REDP), ribotyping and polymerase chain reaction (PCR) were able to identify individual strains with remarkable precision.

Objectives

Nontyphoidal salmonellosis has been recognised as the most frequent foodborne infection world-wide. Incidence of foodborne outbreaks in recent years observed the emergence of *Salmonella enteritidis* as the most commonly isolated serotype in many parts of the world. For instance , the isolation frequency of *S. enteritidis* from humans in Britain, Greece, Spain and the United States showed a significant increase of more than 50% between 1980 and 1994 (Ridley *et al.*, 1996 ; Vatopoulos *et al.*, 1994; Anon, 1991 and Centre for Disease Control, 1993).

Due to the recent prominence of *S. enteritidis*, identification of the strain is essential for an effective investigation of the outbreak cases. Combination of conventional as well as molecular typing methods

have been employed for a precise differentiation of the individual strain. In the present work, S. *enteritidis* strains which are multiply resistant to antimicrobial agents isolated from animals, humans and environmental sources were characterised by using conventional and molecular typing methods.

The specific objectives of the present investigation were as follows:

1. To carry out biochemical tests for biotyping analysis.

2. To characterised the strains by plasmid content, antimicrobial resistant patterns and their correlation.

3. To study the ability to transfer resistance by conjugation studies.

4. To characterise the representative strains of *S. enteritidis* by restriction endonuclease digestion polymorphism generated by pulsed-field gel electrophoresis (REDP-PFGE).

5. To apply the REDP-PFGE technique in determining the genome size.

6. To subdivide the *S. enteritidis* phage typed strains by biotyping, plasmid profiling, antibiotic resistance patterns and REDP-PFGE methods.

CHAPTER II

LITERATURE REVIEW

Introduction

The *Salmonella* was first coined by Lignié̊res in 1900 as an honour to D.E. Salmon, an American bacteriologist who reported on "the hog cholera group of bacteria" in swine. These gram-negative rod-shaped bacteria are confirmed to be the member of the family Enterobacteriaceae and were assigned to genus III in the family (Le Minor, 1984). They were characterised as motile with petrichous flagella, facultatively anaerobic and usually produced gas from glucose fermentation. They were also characterised as positive in hydrogen sulphide production, citrate utilisation and decarboxylation of lysine and ornithine. In contrast, they were negative for indole, urease, phenylalanine and tryptophan.

Although this group of bacteria are ubiquitous in nature, some are strictly host adapted. They have been known to be pathogenic to humans causing enteric fever, gastroenteritis and septicaemia. These pathogens also infect many animal species.

The systematics with respect to classification and nomenclature of *Salmonella* have been in a state of flux for many years. The classification authorities in different countries placed these bacteria in different species or nomenclature that was inconsistent to the international agreement. The existing taxonomic schemes based on the biochemical characteristics and serology (Kaufmann, 1966; Edwards and Ewing 1986; Le Minor, 1992) have been traditionally attributed in the classification of this genus. In general practice, these bacteria were named either for the disease caused or animal involved. For example, the human medical community embraced and used such specific epithets as *S. typhi*, *S. paratyphi* etc. The veterinary medical community were preferable to name the bacteria with the affected animal species such as *Salmonella choleraesuis*, *S. pollorum*, *S. gallinarum*, *S. bovis morbificans* etc. In addition, there were also names associated to geographical areas such as *S. arkansas, S. birmingham, S. jurusalem, S. panama* etc.

The Genus *Salmonella*

The *Salmonella* are natural inhabitants of the intestinal tract of food animals including cattle, swine, sheep, chicken, turkeys and ducks. This bacteria are also found in pet animals such as dogs, cats and turtles. The wild animals like pigeon, reptiles, mice, rats and insects are also natural reservoirs of the *Salmonella*. Usually,

disposals (faeces and urine) of the infected animals have been the main sources of *Salmonella* spread to the environment and then transmit the infection to other susceptible animals or human being.

This genus are classified into three main groups by World Health Organisation (WHO) according to the host and mode of transmission (Guthrie, 1991). The first group consisting of *S. typhi*, *S. paratyphi* A, B and C, only associated to human infections. These serovars are spread primarily either by food or water which have been directly or indirectly contaminated by human waste. The second group is considered to include serovars that are host-adapted in animals. Included in this group are *S. gallinarum* adapted to poultry, *S. dublin* adapted to cattle, *S. abortus equi* adapted to horse, *S. abortus ovis* adapted to sheep, *S. choleraesuis* and *S. typhisuis* adapted to swine. Some of this serovars particularly the dublin and choleraesuis, are pathogenic to human under certain condition. The third group are serovars which show no particular host adaptation and are pathogenic in either humans or animals. Including in this group are *S. enteritidis* and *S. typhimurium* which cause the majority of salmonellosis infection. Salmonellosis in humans caused by organisms from the second and third group would be considered as zoonotic salmonellosis.

Salmonella in Humans

Typhoid fever or enteric fever has plagued the human populations for more than a century. *Salmonella typhi*, the etiologic agent of typhoid fever was one of the earliest pathogenic bacteria to be isolated and characterised (Le Minor, 1992). This fever is an acute illness causing bacteremic or septicemic infections. Paratyphoid fever caused by *S. paratyphi* A, B or C, is pathologically and clinically similar to typhoid, but it forms milder illness. Typhoid infection in humans is initiated by ingestion and survival of the bacteria in the acidic pH of the stomach. With adequate numbers of infectious dose, the bacteria penetrate the mucosa of the small intestine to the midlayer where they are engulfed into the epithelial cells. At this stage, the bacteria will cause inflammatory response in the small bowel and the colon which then produced diarrhoea in the infected persons. A systemic infection characterised by prolonged fever, headache, enlargement of the spleen, rose spots on the abdominal surface, constipation (50% of the cases), diarrhoea (20% of cases) and muscle soreness. The incubation period in such an infection may be as long as 3 weeks following ingestion of the infectious dose of organisms. In the past, the mortality rate in the untreated typhoid fever was as high as 15%, but with antibiotic treatment this has been reduced to less than 1% (Guthrie, 1991).