# PHENOTYPIC AND GENOTYPIC CHARACTERIZATION OF *Salmonella enterica* serovar Typhimurium AND *Salmonella enterica* serovar Enteritidis IN MALAYSIA

**NGOI SOO TEIN**

**FACULTY OF SCIENCE**
**UNIVERSITY OF MALAYA**
**KUALA LUMPUR**

**2018**

# PHENOTYPIC AND GENOTYPIC CHARACTERIZATION OF *Salmonella enterica* serovar Typhimurium AND *Salmonella enterica* serovar Enteritidis IN MALAYSIA

**NGOI SOO TEIN**

**THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY**

**INSTITUTE OF BIOLOGICAL SCIENCES**
**FACULTY OF SCIENCE**
**UNIVERSITY OF MALAYA**
**KUALA LUMPUR**

**2018**

**UNIVERSITY OF MALAYA**

**ORIGINAL LITERARY WORK DECLARATION**

Name of Candidate: NGOI SOO TEIN

Matric No: SHC100014

Name of Degree: DOCTOR OF PHILOSOPHY

Title of Project Paper/Research Report/Dissertation/Thesis ("this Work"):

PHENOTYPIC AND GENOTYPIC CHARACTERIZATION OF *Salmonella enterica* serovar Typhimurium AND *Salmonella enterica* serovar Enteritidis IN MALAYSIA

Field of Study: MOLECULAR MICROBIOLOGY

I do solemnly and sincerely declare that:

(1) I am the sole author/writer of this Work;
(2) This Work is original;
(3) Any use of any work in which copyright exists was done by way of fair dealing and for permitted purposes and any excerpt or extract from, or reference to or reproduction of any copyright work has been disclosed expressly and sufficiently and the title of the Work and its authorship have been acknowledged in this Work;
(4) I do not have any actual knowledge nor do I ought reasonably to know that the making of this work constitutes an infringement of any copyright work;
(5) I hereby assign all and every rights in the copyright to this Work to the University of Malaya ("UM"), who henceforth shall be owner of the copyright in this Work and that any reproduction or use in any form or by any means whatsoever is prohibited without the written consent of UM having been first had and obtained;
(6) I am fully aware that if in the course of making this Work I have infringed any copyright whether intentionally or otherwise, I may be subject to legal action or any other action as may be determined by UM.

Candidate's Signature Date:

Subscribed and solemnly declared before,

Witness's Signature Date:

Name:

Designation:

**PHENOTYPIC AND GENOTYPIC CHARACTERIZATION OF *Salmonella enterica* serovar Typhimurium AND *Salmonella enterica* serovar Enteritidis IN MALAYSIA**


## ABSTRACT

*Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) and *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis) are the two most common non-typhoidal *Salmonella* serovars causing salmonellosis in humans. In Malaysia, these two serovars are often isolated from food and animals but there are limited studies which focused on detailed genotypic and phenotypic characterization of these organisms. The overall goal of this study was to determine the genotypic and phenotypic characteristics of these two major *Salmonella* serovars circulating in Malaysia, in order to understand the epidemiology and pathogenicity of these organisms in this region. A total of 84 *S.* Typhimurium and 111 *S.* Enteritidis strains isolated from food, animals, and humans were examined in this study. Both genotyping methods, namely pulsed-field gel electrophoresis (PFGE) and multiple-locus variable number tandem repeat analysis (MLVA), used in this study showed a limited genetic diversity among the *Salmonella* strains circulating in Malaysia. In fact, the *S.* Enteritidis strains were highly clonal, with most of the strains being single-locus variants, regardless of their sources. Although MLVA was found to be overall less discriminative than PFGE, it was able to complement the latter in further differentiating the strains with identical pulsotypes. Fifty percent of the *S.* Typhimurium strains and 39 % of the *S.* Enteritidis strains were multi-drug resistant. High rates of resistance towards ampicillin, nalidixic acid, streptomycin, sulphonamides, sulfamethoxazole, tetracycline, and trimethoprim were observed among the strains. Upon close examination on the quinolone resistance mechanisms, the major factor that contributed to such resistance was mutations in the *gyrA* gene. Two novel mutations in the *parE* gene, and the plasmid-borne *qnrS1* gene

(found in 30 % of the quinolone-resistant strains) might have contributed to the reduced susceptibility to ciprofloxacin (fluoroquinolone). A high resolution melting curve assay was successfully developed for rapid screening of mutations in the quinolone-resistant determining regions of the gyrase and topoisomerase IV genes, which confer resistance to quinolones and fluoroquinolones. A monophasic variant of *S.* Typhimurium was identified, and the genomic deletion spanning the *fljAB*, *hin*, partial *iroB*, STM2757, and STM2758 genes has resulted in the monophasic phenotype of this strain. The genomes of the endemic *S.* Typhimurium strains were essentially identical except for minor genetic variations in the phage- and plasmid-associated regions. Phylogenetic analyses showed that the *S.* Typhimurium strains in Malaysia were closely related to global *S.* Typhimurium strains with highly virulent phenotypes. Multiple antimicrobial resistance and virulence determinants were identified in the biphasic genomes. In conclusion, *S.* Typhimurium and *S.* Enteritidis strains circulating in Malaysia were genetically homogeneous, with a high rate of multi-drug resistance. The rarely reported monophasic variant of *S.* Typhimurium isolated in Malaysia showed unique *fljAB* operon deletion pattern. Local *S.* Typhimurium strains in Malaysia possess virulence potential to cause severe infections in humans and animals.

**Keywords:** Antimicrobial resistance, comparative genomics, genotyping, mutation screening, resistance mechanisms

**PENCIRIAN FENOTIP DAN GENOTIP *Salmonella enterica* serovar Typhimurium DAN *Salmonella enterica* serovar Enteritidis DI MALAYSIA**

## ABSTRAK

*Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) dan *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis) merupakan dua serovar *Salmonella*-bukan-typhoid yang paling biasa menyebabkan salmonellosis pada manusia. Di Malaysia, kedua-dua serovar ini sering diasingkan daripada makanan dan haiwan, tetapi hanya terdapat kajian yang terhad yang memberikan tumpuan kepada pencirian terperinci genotip dan fenotip organisma-organisma ini. Matlamat keseluruhan kajian ini adalah untuk menentukan ciri-ciri genotip dan fenotip kedua-dua serovar utama *Salmonella* ini di Malaysia, untuk memahami epidemiologi dan patogenik organisma ini di rantau ini. Sebanyak 84 strain *S.* Typhimurium dan 111 strain *S.* Enteritidis yang diasingkan daripada makanan, haiwan, dan manusia telah diperiksa dalam kajian ini. Kedua-dua kaedah pencirian genotip, iaitu pulsed-field gel electrophoresis (PFGE) dan multiple-locus variable number tandem repeat analysis (MLVA), menunjukkan kepelbagaian genetik yang terhad di kalangan strain *Salmonella* di Malaysia. Malah, strain *S.* Enteritidis adalah sangat serupa dari segi genetik, dengan kebanyakan strain merupakan varian tunggal lokus, tidak kira sumber strain tersebut. Walaupun secara keseluruhannya MLVA didapati kurang diskriminasi berbanding dengan PFGE, namun ia dapat melengkapi PFGE dengan membezakan lagi strain dengan pulsotype yang serupa. Lima puluh peratus daripada strain *S.* Typhimurium dan 39 % daripada strain *S.* Enteritidis adalah tahan pelbagai ubat. Kadar rintangan yang tinggi terhadap ampicillin, nalidixic acid, streptomycin, sulphonamides, sulfamethoxazole, tetracycline dan trimethoprim telah diperhatikan di antara strain ini. Pemeriksaan rapi terhadap mekanisme rintangan quinolone menunjukkan faktor utama yang menyumbang kepada rintangan tersebut adalah mutasi dalam gen *gyrA*. Dua mutasi novel dalam gen *parE*, dan gen *qnrS1*

bawaan-plasmid (ditemui dalam 30 % strain yang menunjukkan rintangan terhadap quinolone) mungkin menyumbang kepada pengurangan kecenderungan terhadap ciprofloxacin (fluoroquinolone). High resolution melting curve assay telah berjaya direka untuk mempercepatkan penyaringan mutasi di rantau penentu rintangan quinolone dalam gen gyrase dan topoisomerase IV yang memberi rintangan kepada quinolones dan fluoroquinolones. Satu varian monophasic *S.* Typhimurium telah dikenal pasti, dan penghapusan jujukan genomik yang merangkumi gen-gen *fljAB*, *hin*, separa *iroB*, STM2757, dan STM2758 telah menghasilkan fenotip monophasic bagi varian ini. Jujukan genom bagi kedua-dua *S.* Typhimurium yang endemik adalah sama, kecuali untuk variasi genetik yang kecil dalam kawasan yang berkaitan dengan phage dan plasmid. Analisa filogenetik menunjukkan bahawa strain *S.* Typhimurium di Malaysia berkait rapat dengan strain *S.* Typhimurium yang virulens dari negara-negara lain. Pelbagai penentu-penentu rintangan dan virulens telah dikenal pasti dalam genom biphasic *S.* Typhimurium. Kesimpulannya, strain *S.* Typhimurium dan *S.* Enteritidis di Malaysia menunjukkan ciri homogen dari segi genetik, dengan kadar rintangan pelbagai ubat yang tinggi. Varian monophasic *S.* Typhimurium yang jarang dilaporkan di Malaysia menunjukkan corak penghapusan operon *fljAB* yang unik. *S.* Typhimurium tempatan di Malaysia mempunyai potensi untuk menyebabkan jangkitan yang serius pada manusia dan haiwan.

**Kata kunci:** Mekanisme rintangan, pemeriksaan mutasi, pencirian genotip, perbandingan genomik, rintangan antimikrob

## ACKNOWLEDGEMENTS

First and foremost, I would like to express my deepest gratitude to my supervisor Prof. Dr. Thong Kwai Lin for the continuous support of my PhD study, for her patience, guidance, motivation, and immense knowledge. Her guidance helped me in all the time of research and the writing of this thesis. Besides my supervisor, I would like to thank Dr. Cindy Teh Shuan Ju and Dr. Lim King Ting for being my research mentors. Without their guidance it would be a hard time for me when I first started my research.

My sincere thanks also go to University of Malaya for providing financial support for my study. I would like to thank the University for granting me the UM Fellowship (Skim Biasiswa Universiti Malaya) to support my tuition fees and living expenses. Besides that, this project was also supported by the University of Malaya Postgraduate Research Fund (PS319/2010B), University of Malaya Research Grant (RG017-09BIO), Japan National Institute of Infectious Disease (57-02-03-1015), and University of Malaya High Impact Research Grant (UM.C/625/1/HIR/MOHE/CHAN-02).

I would also like to thank my fellow lab-mates from Lab A407 for the stimulating discussions, for the hard times in research that we went through, and for all the fun we had together. Last but not least, I would like to thank my family for their support and encouragement throughout my study and my life in general.

## TABLE OF CONTENTS

## LIST OF FIGURES

## LIST OF TABLES

## LIST OF SYMBOLS AND ABBREVIATIONS

| | | |
|---|---|---|
| & | : | And |
| ~ | : | Approximately |
| bp | : | Base pair |
| C | : | Categorical coefficient |
| ℃ | : | Degree Celsius |
| F | : | Dice coefficient |
| = | : | Equal to |
| g | : | Gram |
| GC | : | Guanine-plus-cytosine |
| kbp | : | Kilobase pairs |
| < | : | Less than |
| ≤ | : | Less than or equal to |
| Mb | : | Megabases |
| µg | : | Microgram |
| µL | : | Microlitre |
| µmol/L | : | Micromol per litre/Micromolar |
| mL | : | Millilitre |
| mmol/L | : | Millimol per litre/Millimolar |
| min | : | Minute |
| M | : | Molar/Mol per litre |
| MW | : | Molecular weight |
| > | : | More than |
| ≥ | : | More than or equal to |
| ng | : | Nanogram |

| | | |
|---|---|---|
| no. | : | Number |
| n | : | Number of strain |
| % | : | Percent |
| rpm | : | Rotation per minute |
| s | : | Second |
| D | : | Simpson's index of diversity |
| W | : | Wallace coefficient |
| ABC | : | ATP-binding cassette |
| ATP | : | Adenosine triphosphate |
| BLAST | : | Basic Local Alignment Search Tool |
| BRIG | : | BLAST Ring Image Generator |
| CARD | : | Comprehensive Antibiotic Resistance Database |
| CCCP | : | Carbonyl cyanide m-chlorophenylhydrazone |
| CDC | : | Centers for Disease Control and Prevention |
| CDSs | : | Coding sequences |
| CLSI | : | Clinical and Laboratory Standards Institute |
| DNA | : | Deoxyribonucleic acid |
| dNTP | : | Deoxynucleoside triphosphate |
| HIV | : | Human immunodeficiency virus |
| HRM | : | High resolution melting curve |
| MAR | : | Multiple antibiotic resistant index |
| MATE | : | Multidrug and toxic compound extrusion |
| MDR | : | Multi-drug resistant |
| MEGA | : | Molecular Evolutionary Genetic Analysis |
| MFS | : | Major facilitator superfamily |
| $MgCl_2$ | : | Magnesium chloride |

| | | |
|---|---|---|
| MIC | : | Minimum inhibitory concentration |
| ML | : | Maximum likelihood |
| MLST | : | Multilocus sequence typing |
| MLVA | : | Multiple-locus variable number tandem repeat analysis |
| MOH | : | Ministry of Health |
| MST | : | Minimum spanning tree |
| NCBI | : | National Center for Biotechnology Information |
| PCR | : | Polymerase chain reaction |
| PFGE | : | Pulsed-field gel electrophoresis |
| PHASTER | : | Phage Search Tool Enhanced Release |
| QRDR | : | Quinolone-resistant determining region |
| RAST | : | Rapid Annotations using Subsystems Technology |
| RGI | : | Resistance Gene Identifier |
| RND | : | Resistance-nodulation-division |
| SNP | : | Single nucleotide polymorphism |
| SLV | : | Single locus variant |
| ST | : | Sequence type |
| UPGMA | : | Unweighted pair group method with arithmetic mean algorithm |
| US | : | United States of America |
| VNTR | : | Variable number tandem repeat |

## LIST OF APPENDICES

# CHAPTER 1: GENERAL INTRODUCTION

*Salmonella* is a member of the family *Enterobacteriaceae*. The genus *Salmonella* comprises of two species, namely *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* is further divided into six subspecies, namely *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae*, and *indica*. Among these subspecies, *Salmonella enterica* subspecies *enterica* is often the focus of most scientific studies due to their ability to cause infections in humans. To date, over 2600 serovars of *Salmonella enterica* have been described on the basis of their antigenic properties (Issenhuth-Jeanjean *et al.*, 2014). *Salmonella enterica* is an orally-acquired pathogen which often causes enteric fever, gastroenteritis, bacteraemia and chronic asymptomatic carriage in the infected person. *Salmonella* infection causes the majority of foodborne diseases worldwide, both in developed and developing countries. According to laboratory surveillance data, an estimated 93.8 million gastroenteritis cases caused by *Salmonella* occur worldwide on a yearly basis, resulting in 155,000 deaths (Majowicz *et al.*, 2010). Moreover, 86 % of the cases were related to foodborne *Salmonella* infections (Majowicz *et al.*, 2010). *Salmonellosis* is accounted for an estimated 1.2 million illnesses every year in the United States (Adams *et al.*, 2016). In Malaysia, typhoid fever is classified as one of the notifiable diseases by the Ministry of Health (MOH). The majority of foodborne illnesses are generally categorized as food poisoning cases, without stating the etiologic agents in the reports published by the MOH (http://vlib.moh.gov.my/). However, based on past studies carried out by individual research groups in Malaysia, *Salmonella* is frequently isolated from various sources including humans (Modarressi & Thong, 2010; Thong *et al.*, 2011), food (Cheah *et al.*, 2008), and animals (Roseliza *et al.*, 2011). Among all *Salmonella* serovars, Enteritidis and Typhimurium are the two most commonly isolated serovars both worldwide and in Malaysia (Hendriksen *et al.*, 2011; Roseliza *et al.*, 2011). These sources include clinical

specimens such as stools and blood; and food samples including beef, pork, chicken meat, indigenous vegetables, ready-to-eat food, buffalo, poultry and others. However, the majority of local studies were more focused on the isolation and identification of *Salmonella* species rather than detailed characterization of strains using molecular techniques. Hence, focusing on the two major *Salmonella* serovars, namely Enteritidis and Typhimurium, this study seeks to answer the following research questions:

1. *Salmonella* Typhimurium and Enteritidis are the two major non-typhoidal *Salmonella* serovars contributing to most of the salmonellosis cases in human. These two organisms are commonly isolated in Malaysia, often associated with food and animals. What is the genetic relationship of the *Salmonella* Typhimurium and Enteritidis populations that are circulating in Malaysia and the association among strains isolated from different sources?
2. The persistence of antimicrobial resistance, or even multi-drug resistance, and the emergence of quinolone resistance among *Salmonella* have been a major public health concern in most of the developed countries. In Southeast Asian countries, unregulated usage of antibiotics has also raised concern on the emergence of multi-drug resistant *Salmonella* strains. Moreover, *Salmonella* strains that are resistant to quinolones and fluoroquinolones are emerging albeit at low frequencies. What are the antimicrobial resistance patterns among *Salmonella* Typhimurium and Enteritidis populations in Malaysia, and what are the important mechanisms that confer quinolone resistance to these strains?
3. Mutation in the quinolone-resistant determining region (QRDR) of the gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*) genes is the major contributor of quinolone resistance in *Salmonella*. DNA sequencing is able to detect small genetic changes in the QRDRs, but is costly especially when large sample size is screened. The high resolution melting curve (HRM) analysis is

able to detect point mutations in targeted genes, and may be a more affordable alternative for rapid screening of a large strain pool. Could HRM be used for the detection of mutations in the QRDRs of gyrase and topoisomerase IV genes?

4. *Salmonella* Typhimurium is one of the major food pathogens in Malaysia, yet its detailed genomic features and evolution among the strains circulating in this region is still unclear. Moreover, the worldwide increased prevalence of *Salmonella* I 4,[5],12:i:-, a monophasic variant of *Salmonella* Typhimurium was documented since 1990's. This organism is antigenically similar and genetically closely-related to *Salmonella* Typhimurium, thus equally pathogenic as its biphasic counterpart. In Asian countries, the isolation of this organism has been reported in China, Thailand, and Taiwan, but is yet to be documented in Malaysia. What are the genetic features and evolutionary history of this monophasic variant, if present, and the local biphasic *Salmonella* Typhimurium in Malaysia?

Therefore, in order to address these research questions, the following objectives were undertaken in this study:

1. To study the genetic relationship of *Salmonella* Typhimurium and Enteritidis strains isolated from different sources in Malaysia by using molecular approaches (pulsed-field gel electrophoresis and multiple-locus variable number tandem repeat analysis). (Chapter 3)
2. To determine the antimicrobial susceptibility profiles and quinolone resistance mechanisms of *Salmonella* Typhimurium and Enteritidis strains in Malaysia. (Chapter 4)

3. To develop a high resolution melting curve assay for rapid mutation scanning of gyrase and topoisomerase IV genes in *Salmonella*. (Chapter 5)
4. To elucidate the genomic features of the monophasic variant and biphasic *Salmonella* Typhimurium strains isolated in Malaysia via whole genome sequence analysis. (Chapter 6)

In order to provide a clearer picture of the study in a more structured way, this thesis is presented in article style format. This study will be thoroughly discussed in eight chapters. Chapter 1 (General Introduction) begins with the overview of the study, including the research questions imposed and the objectives to be achieved. Chapter 2 (Literature Review) is composed of an extensive background information on *Salmonella* and salmonellosis, the antimicrobial resistance in *Salmonella* population, and a summary of Malaysian *Salmonella* studies. Chapter 3 describes the molecular characterization of *Salmonella* Typhimurium and Enteritidis, and a comparison of the two molecular typing tools used in this study. Chapter 4 focuses on the antimicrobial resistance status among the *Salmonella* strains examined in this study, with special emphasis on quinolone resistance and its mechanisms. Chapter 5 describes the development of a molecular method (HRM assay) which enables rapid detection of point mutations in the genes related to quinolone resistance in *Salmonella*. Chapter 6 discusses the genomic features of a monophasic variant and biphasic *Salmonella* Typhimurium identified in this study. Chapter 7 presents the conclusions from the research findings reported in Chapters 3 to 6, and the direction of future works. Lastly, the reference chapter consists of a list of all references that have been cited in this thesis.

# CHAPTER 2: LITERATURE REVIEW

## 2.1 The *Salmonella* genus

*Salmonella* is a Gram-negative rod-shaped bacterium in the family *Enterobacteriaceae* (Todar, 2008). This organism is a facultative anaerobe that survives in intracellular environment. The genus *Salmonella* comprises of two species, namely *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* is further divided into six subspecies, namely *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae*, and *indica*. To date, approximately 2637 serovars of *Salmonella enterica* have been described on the basis of their antigenic properties defined by the somatic (O) and flagellar (H) antigens displayed on the bacterial cell surface, according to the White-Kauffmann-Le Minor Scheme (Issenhuth-Jeanjean *et al.*, 2014). Besides the somatic and flagellar antigens, some *Salmonella* serovars may contain a surface antigen that masks the O antigens, thus hindering the agglutination process in serological identification. One well-known surface antigen in *Salmonella* is the Vi-antigen, occurring in only Typhi, Paratyphi C, and Dublin. Some *Salmonella* serovars are monophasic, i.e. they produce flagella with the same antigenic specificity at all instances (e.g. Enteritidis and Typhi). However, most of the *Salmonella* serovars are biphasic, i.e. capable of expressing two different H antigens. For example, the serovar Typhimurium is able to alternate its expression of the phase-1 (H:i) or phase-2 (H:1,2) antigens. The phase inversion is often a crucial step in the serological identification process of the biphasic *Salmonella* serovars.

In order to standardize the nomenclature system for the *Salmonella* genus, the World Health Organization (WHO) has suggested that the *Salmonella* serovars are designated by their antigenic formula (Grimont & Weill, 2007). However, for *Salmonella enterica* subspecies *enterica* serovars which account for more than 99.5 % of the isolated *Salmonella* strains, the name of the serovar (often indicates the geographical origin of

the first strain that was identified) is written in non-italicized Roman letters with the first letter capitalized, e.g. *Salmonella enterica* subspecies *enterica* serovar Typhimurium (Grimont & Weill, 2007).

*Salmonella* is mainly found in the intestinal tracts of humans and animals (including both wild animals and domesticated pets). Some serovars are host-specific (e.g. Typhi and Paratyphi A in humans; Gallinarum in avian); while some are ubiquitous, infecting both humans and animals (e.g. Typhimurium, Enteritidis) (Todar, 2008). The dissemination of *Salmonella* in the natural environment, especially in water, is through the excretion of infected humans or animals. Although *Salmonella* may not multiply extensively in the natural environment, they could survive an extended period of time in water and soil if the conditions are favourable.

## 2.2 Salmonellosis and treatment

*Salmonella enterica* is a typical orally-acquired pathogen. Salmonellosis is often resulted from the consumption of food, water, or animal products contaminated with *Salmonella*, or close contact with an infected person or a carrier (Feasey & Gordon, 2014). Salmonellosis in humans includes enteric (typhoid) fever due to invasive bacterial infection; and acute gastroenteritis due to foodborne intoxication. Generally, *Salmonella* serovars can be categorized as typhoidal or non-typhoidal on the basis of their host-specificity and disease manifestations in humans. Typhoidal *Salmonella* serovars are host-specific, causing invasive bacteraemia only in humans. For instance, serovars Typhi and Paratyphi are typhoidal *Salmonella*. Chronic asymptomatic carriage of the pathogen may sometimes occur after a patient recovered from a typhoidal *Salmonella* infection. On the other hand, non-typhoidal *Salmonella* serovars have a wider host range, are ubiquitously found in the environment, and are capable of causing various cross-species infections. On global scale, the most commonly encountered non-

typhoidal *Salmonella* serovars are Enteritidis and Typhimurium (Hendriksen *et al.*, 2011).

The disease manifestation of *Salmonella* depends on host susceptibility and the infectious *Salmonella* serovar (Coburn *et al.,* 2007; Feasey & Gordon, 2014). In healthy individuals, non-typhoidal *Salmonella* infections are usually confined to self-limiting gastroenteritis in patients (Coburn *et al.,* 2007). However, in infants, elderly, and immunocompromised patients, non-typhoidal *Salmonella* infections may cause severe invasive bacteraemia. Acute gastroenteritis in humans often occurs between 6 and 72 hours after the ingestion of greater than 50,000 bacteria in the contaminated food or water. The onset of disease is marked by clinical symptoms such as acute crampy abdominal pain, diarrhoea with or without blood, nausea, and vomiting.

Under normal circumstances, gut-limiting infections by non-typhoidal *Salmonella* often resolve spontaneously in the course of 3-7 days without medical interventions (Coburn *et al.,* 2007). In cases of severe diarrhoea where fluid loss is substantial, patients will be treated with oral or intravenous rehydration. Antimicrobial therapy is only indicated in adults with positive signs of invasive infections, and also in immunocompromised patients, infants, and elderly to prevent invasion (Coburn *et al.*, 2007; Klochko & Wallace, 2014). Evidence has shown that the use of antibiotics in diarrhoeal patients infected by non-typhoidal *Salmonella* faced increased risks of microbiological relapse and prolonged faecal carriage of the pathogen (Onwuezobe *et al.*, 2012). However, the use of antibiotics does not resolve the symptoms or decrease the duration of illness, and sometimes may even prolong the convalescent carriage stage (Coburn *et al.,* 2007; Klochko & Wallace, 2014). Ampicillin, amoxicillin, sulfamethoxazole-trimethoprim, and quinolone are the first-line drugs commonly used in treating invasive salmonellosis (Klochko & Wallace, 2014). In cases of infections

caused by *Salmonella* resistant to the first-line drugs, third-generation cephalosporins and fluoroquinolones will be prescribed instead. When treatment failure occurred due to fluoroquinolone-resistant *Salmonella* infection, the newer generation fluoroquinolone (gatifloxacin), azithromycin, or chloramphenicol (due to a decline of resistant strains in recent years) may be used to treat life-threatening invasive salmonellosis. Nonetheless, problems such as severe adverse side effects and lack of easy access to the drugs may limit the use of these antibiotics in certain countries.

## 2.3 Prevalence and epidemiology

*Salmonella* infection causes the majority of foodborne diseases worldwide, both in developed and developing countries (Hendriksen *et al.,* 2011; Majowicz *et al.,* 2010). According to laboratory surveillance data obtained in 2006, an estimated 93.8 million gastroenteritis cases caused by *Salmonella* occur worldwide on a yearly basis, resulting in 155,000 deaths (Majowicz *et al.,* 2010). Among this figure, 86 % of the cases were related to foodborne *Salmonella* infections. Based on 2010 surveillance data, an estimated 3.4 million cases of invasive non-typhoidal salmonellosis occurred worldwide annually (Ao *et al.,* 2013). In United States, salmonellosis is accounted for 1.2 million illnesses every year (Adams *et al.,* 2016). In developed countries, large-scale salmonellosis outbreaks are often caused by contamination in commercially prepared food or food ingredients (Jackson *et al.,* 2013).

Over the past twelve years, there was an overall increased reporting on the incidence of food poisoning cases in Malaysia (23.89-47.34 cases per 100,000 populations) (Table 2.1). Foodborne diseases in Malaysia have often been associated with outbreaks in schools and institutions, mainly due to unhygienic food handling procedures (Soon *et al.,* 2011). Typhoid fever, which is endemic in this region, is classified as a notifiable disease by the Ministry of Health (MOH). There was an overall decrease in the

reporting of typhoid cases from 2004 to 2014, from 1.93 to 0.70 cases per 100,000 populations, with a relatively low mortality rate (0.00-0.02 cases per 100,000 populations) (Table 2.1). A sudden surge in the incidence rate of typhoid was seen in 2005 and 2015. Other than the four notifiable diseases (cholera, dysentery, hepatitis A, and typhoid) classified as food and waterborne diseases by MOH, the majority (> 90 %) of other cases are generally categorized as food poisoning, without stating the etiologic agents.

**Table 2.1:** Summary of the incidence and mortality rate of food poisoning and typhoid cases in Malaysia in the past 12 years

| Year | Food poisoning (per 100,000 population) | | Typhoid (per 100,000 population) | |
|---|---|---|---|---|
| | **Incidence rate** | **Mortality rate** | **Incidence rate** | **Mortality rate** |
| **2004** | 23.89 | 0.00 | 1.93 | 0.00 |
| **2005** | 17.76 | 0.00 | 4.10 | 0.00 |
| **2006** | 26.04 | 0.01 | 0.77 | 0.02 |
| **2007** | 53.19 | 0.00 | 1.20 | 0.02 |
| **2008** | 62.47 | 0.01 | 0.72 | 0.01 |
| **2009** | 36.17 | 0.01 | 1.07 | 0.00 |
| **2010** | 44.18 | 0.00 | 0.74 | 0.00 |
| **2011** | 56.25 | 0.03 | 0.84 | 0.00 |
| **2012** | 44.93 | 0.00 | 0.75 | 0.00 |
| **2013** | 47.79 | 0.04 | 0.73 | 0.01 |
| **2014** | 58.65 | 0.01 | 0.70 | 0.00 |
| **2015** | 47.34 | 0.01 | 1.42 | 0.03 |

Data retrieved from: http://vlib.moh.gov.my/

Although not specified in MOH reports, *Salmonella enterica* is frequently isolated from various sources in Malaysia, including humans, food, and animals (Cheah *et al.*, 2008; Modarressi & Thong, 2010; Roseliza *et al.*, 2011; Thong *et al.*, 2011), with serovars Enteritidis and Typhimurium being the two most commonly isolated serovars (Hendriksen *et al.*, 2011; Roseliza *et al.*, 2011). The sources of *Salmonella* include clinical specimens such as stool and blood samples; and food samples including beef, pork, chicken meat, indigenous vegetables, ready-to-eat food, buffalo, poultry and

others. The most commonly isolated *Salmonella* serovars in human are Enteritidis, Typhi, and Weltevreden (Hendriksen *et al.*, 2011). In Malaysia, the non-typhoidal *Salmonella* serovar that predominated in human infections had switched from Weltevreden to Enteritidis in the early 1990's (Yasin *et al.*, 1995). In food animals, *Salmonella* serovars Enteritidis and Typhimurium are most commonly encountered, followed by other serovars such as Corvallis, Albany, Agona, Senftenberg, Typhi-suis, and Braenderup (Adzitey *et al.*, 2012; Budiati *et al.*, 2013; Choe *et al.*, 2011; Nor-Faiza *et al.*, 2013; Roseliza *et al.*, 2011). Moreover, serovars Typhimurium, Corvallis, Agona, Biafra, Weltevreden, Senftenberg, and Albany are often isolated from food sources in Malaysia (Cheah *et al.*, 2008; Modarressi & Thong, 2010; Noorzaleha *et al.*, 2003; Tunung *et al.*, 2007). The lack of apparent clinical symptoms in the affected animals may result in the spread of these pathogens among the animal populations, thus entering food production chain and subsequently infect humans. In developing countries such as Malaysia, the dissemination of these pathogens is mainly attributed to inappropriate hygienic practice during food preparation.

## 2.4 Antimicrobial resistance in *Salmonella*

The increasing rate of antimicrobial resistance has been observed among *Salmonella* strains distributed worldwide. In Southeast Asian region, the isolation of multi-drug resistant (MDR) *Salmonella* from farm animals is not uncommon (Van *et al.*, 2012). A high rate of MDR strains was previously documented in *Salmonella* isolated from meats in Malaysia (Khoo *et al.*, 2015). Antimicrobial agents are often used on farm animals for three purposes: therapy, prophylaxis, and performance enhancement (Economou & Gousia, 2015). With animal farming being the major source of meat for human diet, the prevalence of MDR *Salmonella* among the food animals poses threat to human health (Economou & Gousia, 2015; Ricke & Calo, 2015). The emergence and transmission of the antimicrobial resistance mechanisms among bacteria have been extensively studied.

Antimicrobial resistance often arises through mutations or the presence of intrinsic resistance mechanisms in the bacteria, and persists in a population due to selective pressure (Boerlin & Reid-Smith, 2008; Cox & Wright, 2013). Horizontal gene transfer of mobile genetic elements contributes to intra- and inter-species spread of resistance mechanisms (Carattoli, 2013). Moreover, the genes responsible for antimicrobial resistance mostly reside in virulent plasmids (Carattoli, 2013). Hence, the prolonged usage of antibiotics in both clinical and farm settings not only selects for antimicrobial resistance, but also increases the virulence of the bacteria (Preziosi *et al.*, 2012). Earlier studies have proven the co-evolution of virulence and resistance plasmids (Fluit, 2005; Foley & Lynne, 2008). Indeed, a strong correlation was observed between the antimicrobial resistance rate and increased risks of systemic infections caused by *Salmonella* (Ricke & Calo, 2015).

Quinolones, such as nalidixic acid, is one of the first-line drugs prescribed in the treatment of invasive salmonellosis due to its broad spectrum bactericidal activity (Klochko & Wallace, 2014). The mechanism of action of the quinolones is inhibiting the bacterial DNA from unwinding and duplicating in a normal cell division (Hopkins *et al.*, 2005). Fluoroquinolones (e.g. ciprofloxacin) is often prescribed to patients in cases of treatment failure due to bacterial-resistant to first-line drugs (Klochko & Wallace, 2014). As quinolones and fluoroquinolones are fully synthetic drugs, intrinsic resistance towards these antimicrobial agents are not possible (Poirel *et al.*, 2012). However, increasing quinolone resistance and the emergence of resistance to fluoroquinolone among *Salmonella* have been documented worldwide, with the highest disease burden in Southeast Asian region (World Health Organization, 2014). One possible reason for this phenomenon is the extensive use of these antibiotics in animal farming, resulting in a selective pressure on the bacteria to gain resistance towards the antibiotics (Fàbrega *et al.*, 2008; Van *et al.*, 2012). The quinolone-resistant *Salmonella* may enter the food

chain and subsequently infect humans. The spread of quinolone-resistant *Salmonella* results in reduced treatment efficiency in cases of invasive salmonellosis.

To ensure the efficacy of antibiotic treatments, MOH Malaysia has been monitoring the rate of resistance among *Salmonella* strains towards several selected antibiotics (http://www.imr.gov.my). In the past 12 years, an overall increase in resistance to amikacin, chloramphenicol, and ciprofloxacin and a consistently high rate of resistance to tetracycline, ampicillin, and trimethoprim sulfamethoxazole have been observed among clinical *Salmonella* strains in Malaysia (Table 2.2). Nonetheless, resistance to cephalosporins and carbapenems remained low among the *Salmonella* strains. These antimicrobial agents are often used in the treatment of invasive salmonellosis, and therefore the increasing resistance rates could potentially compromise the currently available therapeutic options.

**Table 2.2:** Total number of *Salmonella* strains tested and the percentage of strains resistant to antibiotics from 2003-2014

| Year | | Amikacin | Amoxiclav | Ampicillin | Ampicillin/sulbactam | Cefepime | Cefoperazone | Sulperazone | Cefotaxime | Ceftazidime | Ceftriaxone | Cefuroxime | Cephalexin | Chloramphenicol | Ciprofloxacin | Gentamicin | Imipinem | Meropenem | Netilmicin | Nitrofurantoin | Piperacillin | Piperacillin/Tazobactam | Tetracycline | Trimethoprim/sulfamethoxazole |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **2003** | % | - | - | 16.8 | - | - | - | - | - | - | 0.6 | - | - | 6.9 | 0.7 | - | - | - | - | - | NA | - | 37.3 | 15.8 |
| | No. | - | - | 435 | - | - | - | - | - | - | 360 | - | - | 420 | 409 | - | - | - | - | - | NA | - | 244 | 438 |
| **2004** | % | - | - | 13.7 | - | - | - | - | - | - | 1.9 | - | - | 5 | 0.5 | - | - | - | - | - | NA | - | 41.1 | 15.1 |
| | No. | - | - | 779 | - | - | - | - | - | - | 636 | - | - | 734 | 606 | - | - | - | - | - | NA | - | 333 | 762 |
| **2005** | % | - | - | 26.5 | - | - | - | - | - | - | 3.1 | - | - | 6.3 | 1.1 | - | - | - | - | - | NA | - | 42.9 | 21.6 |
| | No. | - | - | 691 | - | - | - | - | - | - | 511 | - | - | 666 | 658 | - | - | - | - | - | NA | - | 438 | 671 |
| **2007** | % | 0 | 9.1 | 24.8 | 7 | 0 | 1.1 | - | 0.9 | 0.9 | 1.2 | 9.6 | 6.2 | 5.8 | 1.4 | 1.7 | 0 | 0 | 5.3 | 25 | 18.6 | 4.4 | 36.9 | 19.9 |
| | No. | 128 | 110 | 1015 | 57 | 26 | 94 | - | 107 | 112 | 914 | 114 | 16 | 831 | 858 | 119 | 110 | 87 | 19 | 16 | 43 | 68 | 453 | 1011 |
| **2008** | % | 0 | 1.1 | 21.9 | 6.5 | 0 | 2.3 | - | 1.1 | 1.9 | 1.8 | 1.7 | NA | 7 | 0.9 | 5.9 | 0 | 0 | 0 | 0 | 35.3 | 0 | 36.6 | 17.2 |
| | No. | 90 | 94 | 908 | 46 | 36 | 87 | - | 90 | 108 | 821 | 60 | NA | 685 | 867 | 85 | 87 | 90 | 37 | 7 | 17 | 44 | 366 | 888 |
| **2009** | % | 1.8 | 7 | 16.6 | 8.8 | 0 | 4.8 | 1.4 | 5 | 1.8 | 1.9 | 7 | NA | 8.2 | 0.7 | 0 | 0 | 0 | 0 | - | 6.7 | 0 | 42.1 | 13.4 |
| | No. | 57 | 57 | 1170 | 34 | 27 | 83 | 72 | 100 | 111 | 1135 | 43 | NA | 956 | 1122 | 60 | 57 | 55 | 20 | - | 15 | 32 | 482 | 1142 |

**Table 2.2**, continued.

| Year | | Amikacin | Amoxiclav | Ampicillin | Ampicillin/sulbactam | Cefepime | Cefoperazone | Sulperazone | Cefotaxime | Ceftazidime | Ceftriaxone | Cefuroxime | Cephalexin | Chloramphenicol | Ciprofloxacin | Gentamicin | Imipinem | Meropenem | Netilmicin | Nitrofurantoin | Piperacillin | Piperacillin/Tazobactam | Tetracycline | Trimethoprim/sulfamethoxazole |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **2010** | % | 0 | 4.2 | 18.1 | 6.2 | 0 | - | - | - | 2.3 | 2.7 | - | NA | 10.5 | 0.9 | 0 | 0 | 0 | 0 | - | - | 0 | 46.1 | 13 |
| | No. | 71 | 72 | 1361 | 48 | 40 | - | - | - | 131 | 1349 | - | NA | 1171 | 1335 | 87 | 72 | 70 | 31 | - | - | 46 | 555 | 1327 |
| **2011** | % | 3.6 | 5.1 | 22.2 | 8.3 | 1.4 | 3.5 | 0 | 4.5 | 1.6 | 2 | 4.3 | NA | 11.7 | 1.8 | 6.7 | 1.2 | 0 | 1.2 | 0 | 40 | 1.6 | 50.9 | 14 |
| | No. | 165 | 214 | 1841 | 133 | 145 | 142 | 112 | 157 | 182 | 1868 | 93 | NA | 1568 | 1903 | 179 | 162 | 161 | 81 | 6 | 15 | 126 | 697 | 1818 |
| **2012** | % | 1.4 | 3.9 | 20.5 | 4.7 | 0.7 | 2.3 | 0 | 2.1 | 2.4 | 3.1 | 2.2 | NA | 11 | 1.3 | 1.3 | 0 | 0.6 | 1.3 | - | - | 0.9 | 47.3 | 11 |
| | No. | 144 | 388 | 1994 | 127 | 267 | 130 | 112 | 286 | 297 | 1938 | 139 | NA | 1954 | 1787 | 149 | 163 | 158 | 78 | - | - | 117 | 1068 | 1954 |
| **2013** | % | 8.7 | 4.6 | 23.9 | 5 | 0.8 | 0.7 | 0 | 4.9 | 3.6 | 3.3 | 11.1 | NA | 11 | 2 | 9 | 1.2 | 0 | - | - | - | 0.7 | 46 | 12.6 |
| | No. | 161 | 479 | 1954 | 141 | 371 | 152 | 72 | 326 | 329 | 1821 | 162 | NA | 1821 | 1623 | 166 | 161 | 159 | - | - | - | 137 | 1095 | 1914 |
| **2014** | % | 9.8 | 4.9 | 23.8 | 14.9 | 1.3 | - | - | 5.6 | 4.4 | 4.8 | - | NA | 13 | 2.7 | - | - | - | - | - | - | - | 42.6 | 12.3 |
| | No. | 132 | 491 | 2405 | 175 | 386 | - | - | 410 | 408 | 2404 | - | NA | 2182 | 2057 | - | - | - | - | - | - | - | 1180 | 2400 |

- , no data for *Salmonella* spp.; NA, antibiotic is not listed in the MOH report

Data retrieved from: http://www.imr.gov.my/en/component/content/article/75-english-content/national-collabration/1294-nsar.html

## 2.5 Identification and molecular characterization of *Salmonella*

Conventionally, isolation of *Salmonella* is confirmed with a series of biochemical tests, after obtaining presumptive colonies from selective media. The selective media Xylose Lysine Deoxycholate (XLD) agar, Xylose Lysine Tergitol 4 (XLT 4) agar, Bismuth Sulphite agar (BSA), and CHROMagar *Salmonella* are commonly used for selection and differentiation of *Salmonella* isolates. The biochemical tests that are often used in identifying *Salmonella* isolates are catalase, cytochrome oxidase, triple sugar iron, lysine iron, urease, indole, and motility tests (Andrews & Hammack, 2001). Accuracy and reproducibility are of fundamental importance in bacterial identification. Although generally successful, conventional methods sometimes fail to identify *Salmonella* strains due to ambiguous results. Such uncertainty arises because some *Salmonella* strains produce atypical colony morphologies on selective media and chemical reactions in biochemical tests (Andrews & Hammack, 2001). Hence, molecular methods are much relied upon for providing more conclusive results in the identification of *Salmonella* strains.

Many molecular identification techniques have been developed over the years, mostly based on Polymerase Chain Reaction (PCR), due to its advantage in speed, accuracy, and sensitivity. PCR not only identifies *Salmonella* at species level (Alvarez *et al.,* 2004), but is also able to correctly determine strains from different serogroups (Liu *et al.,* 2011), and even identify rough variants of *Salmonella* which could not be serotyped using the conventional slide-agglutination method (Hoorfar *et al.,* 1999). PCR detection of *Salmonella* has often been applied in Malaysian studies (Abatcha *et al.,* 2013; Modarressi & Thong, 2010). Depending on the primer specificity, PCR can further resolve *Salmonella* strains into different serovars (Cardona-Castro *et al.,* 2009). During an outbreak of *Salmonella* infections, rapid identification of the causative agent is essential. Advanced PCR-based technologies such as real-time PCR and loop-

mediated amplification (LAMP) assay have also been developed for identification purpose (Hopkins *et al.*, 2011; Nur-Eliana *et al.*, 2014; Ueda & Kuwabara, 2009).

Various subtyping tools that detect genotypic and phenotypic variations among *Salmonella* strains have been used in outbreak investigations. Phenotyping methods, e.g. serotyping and phage typing, and genotyping methods, e.g. pulsed-field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST), are the commonly used subtyping tools for identifying and characterizing *Salmonella* strains (Wattiau *et al.*, 2011). However, phenotyping methods are often less efficient due suboptimal discriminatory power, low throughput, require technical expertise, and time-consuming.

During recent years, molecular-based technologies have become the primary option for detailed strain subtyping of *Salmonella* strains (Park *et al.*, 2014; Wattiau *et al.*, 2011). In early 2000's, the development and application of various molecular techniques had greatly improved the resolution and speed of strain subtyping in epidemiological studies and outbreak investigations for bacterial pathogens (Liebana, 2002; Lukinmaa *et al.*, 2004; Singh *et al.*, 2006; Van Belkum, 2003). These molecular approaches examine the genetic composition of the organisms instead of their phenotypic characteristics. Hence, molecular techniques could overcome the limitations of phenotyping, and therefore provide the higher resolution essential for epidemiological studies. For several globally applied genotyping methods, such as PFGE, MLST, and multi-locus variable number tandem repeat analysis (MLVA), standardized protocols have been developed to allow reproducible data for inter-centre communication (Hopkins *et al.*, 2011; Maiden *et al.*, 1998; Ribot *et al.*, 2006). These genotyping methods not only could determine the genetic relationship among the *Salmonella* strains, but were also used to pinpoint the possible source of salmonellosis outbreaks. Current gold standard for subtyping *Salmonella* is PFGE. To select for

suitable genotyping tools, multiple factors such as reproducibility, stability, discriminatory power, and typability, of the method need to be considered (Struelens *et al.*, 1996). A summary of the discriminatory power of different genotyping methods used in subtyping Malaysian *Salmonella* strains is shown in Table 2.3.

Current advances in genome technologies, especially the advent of next generation sequencing, have contributed greatly to developments in molecular subtyping of *Salmonella*. Next generation sequencing allows for high throughput genome sequencing, thus drastically reduces the time and cost required (Loman *et al.*, 2012). Moreover, a continually expanding database of genomic sequences of *Salmonella* is available on publicly accessible domains such as NCBI GenBank. Such genomic database enables the sharing of information and subsequently, the improvement of molecular subtyping methods. For instance, comparative genomic analysis was used to identify serogroup-specific genes in *Salmonella* in order to develop a PCR-serogrouping scheme (Liu *et al.*, 2011). Besides that, the whole genome subtyping of *Salmonella enterica* could be achieved by comparing the core genomes in order to investigate molecular evolution, and also to identify potential gene markers for genotyping (Leekitcharoenphon *et al.*, 2012). Whole genome sequencing has been applied to subtype *Salmonella* strains, and is proven superior to PFGE, the classical gold standard for subtyping *Salmonella* (Leekitcharoenphon *et al.*, 2014). However, similar to any other molecular subtyping tool, whole genome sequencing needs to be supported with epidemiological data in order to determine the relatedness of the *Salmonella* strains in a particular outbreak. Due to the relatively high cost of operation, whole genome sequencing has only been used in the phylogenetic study of *Salmonella* in Malaysia, but not for routine subtyping of the organism. The vast amount of data obtained from whole genome sequencing has been used to elucidate the genomic evolution of *Salmonella* Typhi in Malaysia, via a comparison of outbreak, sporadic, and carrier strains (Yap *et*

*al.,* 2014). However, as the cost of new technology tends to decrease gradually over time, the routine application of this high-resolution genotyping tool in Malaysia is possible in the future.

## 2.6 Malaysian *Salmonella* studies

*Salmonella enterica* serovars are commonly isolated from food, humans, and animals in Malaysia. Since early 1990's, several research groups in Malaysia have been actively working on isolation, detection, and characterization of *Salmonella*. In the earlier years, *Salmonella* from clinical sources was the focus of most of the scientific studies in Malaysia. Several molecular tool such as PFGE, plasmid profiling, phage typing, and ribotyping were used in these epidemiological studies of *Salmonella* strains. From year 2000 onwards, reports on the isolation of *Salmonella* from food, animal, and the environment were more frequently published. However, the majority of these studies were more focused on the isolation and identification of *Salmonella* strains rather than on the genetic relationship of strains using molecular techniques. Biochemical tests, conventional serotyping, and the antimicrobial susceptibility test were the main methods used for identification and characterization of the *Salmonella* strains. Nevertheless, several molecular tools, such as PCR-based genotyping and PFGE, have been used to elucidate the genetic relationship among the *Salmonella* strains. Table 2.4 summarizes the published studies in Malaysia on the isolation, identification, and characterization of *Salmonella* from various sources in the past 30 years.

**Table 2.3:** Summary of discriminatory power (Simpson's index of diversity, D) for molecular subtyping methods of *Salmonella* in Malaysia

| Published study | REP-PCR | ERIC-PCR | RAPD | PFGE | MLVA | Combined analysis |
|---|---|---|---|---|---|---|
| Thong *et al.* (1994) | - | - | - | 0.86 | - | - |
| Thong *et al.* (1995) | - | - | - | 0.15 | - | - |
| Thong *et al.* (1995) | - | - | - | 0.96-0.99 | - | - |
| Thong *et al.* (1996) | - | - | - | 0.99 | - | - |
| Thong *et al.* (2002) | - | - | - | 0.93 | - | - |
| Tunung *et al.* (2007) | - | 0.96-0.99 | - | - | - | - |
| Lee *et al.* (2008) | - | 0.78 | 0.92 | - | - | 0.92 |
| Douadi *et al.* (2010) | - | - | - | 0.99 | - | - |
| Tiong *et al.* (2010) | 0.96 | - | - | 0.98 | - | - |
| Modarressi & Thong (2010) | - | - | - | 0.99 | - | - |
| Thong *et al.* (2011) | - | - | - | 0.91 | - | - |
| Thong & Ang (2011) | 0.93 | - | - | 0.99 | - | - |
| Adzitey *et al.* (2013) | - | 0.92-0.98 | - | - | - | - |
| Elemfareji & Thong (2013) | 0.81 (*S.* Enteritidis)<br>0.57 (*S.* Typhi) | - | - | - | - | - |
| Ngoi *et al.* (2013) | - | - | - | 0.99 | 0.76 | - |
| Ngoi & Thong (2013) | - | - | - | 0.96 | 0.82 | - |
| Raja & Gulam (2014) | - | - | 0.99 | - | - | - |

**Table 2.3**, continued.

| Published study | REP-PCR | ERIC-PCR | RAPD | PFGE | MLVA | Combined analysis |
|---|---|---|---|---|---|---|
| Adzitey *et al.*, (2015) | - | - | 0.85 (*S.* Typhimurium)<br>0.64 (*S.* Enteritidis)<br>0.71 (*S.* Albany) | - | - | - |

REP-PCR, Repetitive extragenic palindromic sequences PCR; ERIC-PCR, Enterobacterial repetitive intergenic consensus PCR; RAPD, Random amplified polymorphic DNA fingerprinting; PFGE, Pulsed-field gel electrophoresis; MLVA, Multi-locus variable number tandem repeat analysis

**Table 2.4:** Summary of published studies in Malaysia on the isolation and characterization of *Salmonella* (1983-2015)

| Reference | Year | Source | Predominant serovar | Identification and characterization methods |
|---|---|---|---|---|
| Jegathesan | 1983 | Humans | Typhi | Phage typing |
| Jegathesan | 1984 | Humans | Typhi, Typhimurium, Weltevreden | Conventional serotyping |
| Joseph *et al.*, | 1988 | Animals; food | Blockley, Dublin, Kentucky, Sofia, Typhimurium, Weltevreden | Conventional isolation and biochemical identification; conventional serotyping |
| Thong *et al.* | 1994 | Humans | Typhi | Pulsed-field gel electrophoresis; ribotyping |
| Arumugaswamy *et al.* | 1995 | Food | Agona, Bareilly, Blockley, Chinicol, Enteritidis, Muenchen, Singapore | Conventional isolation and biochemical identification; conventional serotyping |
| Radu *et al.* | 1995 | Animals | *Salmonella* spp. | Antimicrobial susceptibility test; plasmid profiling |
| Thong *et al.* | 1995 | Humans | Enteritidis | Pulsed-field gel electrophoresis; ribotyping |
| Thong *et al.* | 1995 | Humans | Typhi | Pulsed-field gel electrophoresis |
| Yasin *et al.* | 1995 | Humans | Enteritidis, Weltevreden | Conventional serotyping |
| Gulam *et al.* | 1996 | Animals | Blockley, Kentucky, Enteritidis, Muenchen | Conventional isolation and biochemical identification; conventional serotyping |
| Thong *et al.* | 1996 | Environment; humans | Typhi | Pulsed-field gel electrophoresis |
| Yasin *et al.* | 1997 | Humans | Enteritidis, Typhi | Conventional serotyping |
| Lee *et al.*, | 1998 | Humans | Non-typhoidal *Salmonella* | Antimicrobial susceptibility test; conventional isolation and biochemical identification |
| Thong *et al.*, | 1998 | Humans | Enteritidis | Antimicrobial susceptibility test; pulsed-field gel electrophoresis |

**Table 2.4**, continued.

| Reference | Year | Source | Predominant serovar | Identification and characterization methods |
|---|---|---|---|---|
| Thong *et al.* | 2002 | Environment; humans | Weltevreden | Antimicrobial susceptibility test; conventional isolation and biochemical identification; conventional serotyping; pulsed-field gel electrophoresis |
| Noorzaleha *et al.* | 2003 | Food | Agona, Albany, Senftenberg, Weltevreden | Conventional isolation and biochemical identification; conventional serotyping |
| Bakeri *et al.* | 2003 | Humans | Enteritidis | Antimicrobial susceptibility test; plasmid profiling; pulsed-field gel electrophoresis |
| Goh *et al.* | 2003 | Food; humans | Paratyphi B | Antimicrobial susceptibility test; conventional biochemical test (D-tartrate utilization test); pulsed-field gel electrophoresis |
| Lee *et al.* | 2003 | Humans | Non-typhoidal *Salmonella* | Antimicrobial susceptibility test; conventional isolation and biochemical identification; conventional serotyping |
| Thong *et al.*, | 2003 | Laboratory culture collection | *Salmonella* spp. | Pulsed-field gel electrophoresis |
| Puthucheary *et al.* | 2004 | Humans | *Salmonella* spp. | Antimicrobial susceptibility test; conventional isolation and biochemical identification; conventional serotyping |
| Lee *et al.* | 2005 | Humans | Non-typhoidal *Salmonella* | Antimicrobial susceptibility test; conventional isolation and biochemical identification; conventional serotyping |
| Tunung *et al.* | 2007 | Food; humans | *Salmonella* spp. | Antimicrobial susceptibility test; conventional isolation and biochemical identification; conventional serotyping; ERIC-PCR |
| Cheah *et al.* | 2008 | Animals; food | Agona, Weltevreden | Antimicrobial susceptibility test; conventional serotyping |

**Table 2.4**, continued.

| Reference | Year | Source | Predominant serovar | Identification and characterization methods |
|---|---|---|---|---|
| Cheah *et al.* | 2008 | Food | Weltevreden | ERIC-PCR; PCR-restriction fragment length polymorphism; random amplified polymorphic DNA fingerprinting |
| Intan *et al.* | 2008 | Humans | Enteritidis, Paratyphi B | Antimicrobial susceptibility test; conventional isolation and biochemical identification |
| Lee *et al.* | 2008 | Food | Agona, Weltevreden | Antimicrobial susceptibility test; conventional serotyping; ERIC-PCR; random amplified polymorphic DNA fingerprinting |
| Dhanoa & Fatt | 2009 | Humans | Blegdam, Corvallis, Enteritidis, ParatyphiB | Antimicrobial susceptibility test; conventional serotyping |
| Khoo *et al.* | 2009 | Food | *Salmonella enterica* | PCR virulotyping (20 virulence genes) |
| Lim & Thong | 2009 | Laboratory culture collection | *Salmonella enterica* | PCR serotyping |
| Diana *et al.*, | 2010 | Food | Typhi, Typhimurium | Most probable number (MPN) analysis; PCR identification |
| Douadi *et al.* | 2010 | Animals; humans | Typhimurium | Antimicrobial susceptibility test; PCR resistance genes profiling; plasmid profiling; pulsed-field gel electrophoresis |
| Modarressi & Thong | 2010 | Food | Corvallis, Hadar, Enteritidis, Typhimurium | Conventional isolation and biochemical identification; commercial identification kit; PCR identification; pulsed-field gel electrophoresis |
| Nori & Thong | 2010 | Food; humans | Hadar, Enteritidis, Typhi, Typhimurium, Weltevreden | PCR serotyping |
| Tiong *et al.* | 2010 | Humans | Corvallis, Enteritidis, Typhimurium, Paratyphi B, Weltevreden | Antimicrobial susceptibility test; conventional serotyping; pulsed-field gel electrophoresis; REP-PCR |

**Table 2.4**, continued.

| Reference | Year | Source | Predominant serovar | Identification and characterization methods |
|---|---|---|---|---|
| Choe *et al.* | 2011 | Animals | Typhimurium | Antimicrobial susceptibility test; conventional isolation and biochemical identification; conventional serotyping |
| Choo *et al.* | 2011 | Animals | Hadar, Indiana, Muenster, Newington | Conventional isolation and biochemical identification; conventional serotyping |
| Lee & Wee | 2011 | Animals | *Salmonella* spp. | Antimicrobial susceptibility test; conventional isolation and biochemical identification; commercial identification kit; heavy metal tolerance test |
| Pui *et al.* | 2011 | Laboratory culture collection | *Salmonella enterica* | PCR identification |
| Pui *et al.* | 2011 | Food | *Salmonella* spp., Typhi, Typhimurium | Conventional isolation; MPN-multiplex PCR |
| Roseliza *et al.* | 2011 | Animals | Agona, Corvallis, Enteritidis, Indiana, Senftenberg, Typhimurium, Typhi-suis, Weltevreden | Conventional serotyping |
| Thong & Ang | 2011 | Animals; environment; food; humans | Paratyphi B | Conventional biotyping; PCR biotyping; pulsed-field gel electrophoresis; REP-PCR |
| Thong *et al.* | 2011 | Humans | *Salmonella enterica* | Antimicrobial susceptibility test; conventional serotyping; pulsed-field gel electrophoresis |
| Thong & Modarressi | 2011 | Food | *Salmonella enterica* | Antimicrobial susceptibility test; conjugation; PCR resistance genes profiling |

**Table 2.4**, continued.

| Reference | Year | Source | Predominant serovar | Identification and characterization methods |
|---|---|---|---|---|
| Adzitey *et al.*, | 2012 | Animals | Albany, Braenderup, Derby, Enteritidis, Gallinarum, Hadar, London, Newbrunswick, Typhimurium, Weltevreden | Antimicrobial susceptibility test; conventional isolation and biochemical identification; conventional serotyping; plasmid profiling |
| Norazah *et al.* | 2012 | Humans | Paratyphi B | Conventional biotyping; PCR biotyping |
| Yaqub *et al.* | 2012 | Animals | *Salmonella* spp. | Antimicrobial susceptibility test; conventional isolation and biochemical identification |
| Karunakaran *et al.* | 2012 | Humans | Non-typhoidal *Salmonella* | Antimicrobial susceptibility test; minimum inhibitory concentration test (commercial E-test strip); PCR detection of resistance genes |
| Sim *et al.*, | 2012 | Food | *Salmonella* spp. | Plate count |
| Abatcha *et al.* | 2013 | Animals | Corvallis, Mbandaka, Poona, *Salmonella* spp., Typhimurium | Antimicrobial susceptibility test; conventional isolation and biochemical identification; PCR identification |
| Adzitey *et al.* | 2013 | Animals | Albany, Braenderup, Enteritidis, Hadar, Typhimurium | ERIC-PCR |
| Budiati *et al.* | 2013 | Animals; environment | Albany, Agona, Bovis-mobificans, Corvallis, Mikawashima, Stanley, Typhimurium | Antimicrobial susceptibility test; conventional isolation and biochemical identification; conventional serotyping; plasmid extraction |
| Elemfareji & Thong | 2013 | Animals; humans | Enteritidis, Typhi | PCR virulotyping (22 virulence genes), REP-PCR |

**Table 2.4**, continued.

| Reference | Year | Source | Predominant serovar | Identification and characterization methods |
|---|---|---|---|---|
| Nor-Faiza *et al.* | 2013 | Animals | Agona, Braenderup, Corvallis | Conventional isolation and biochemical identification; conventional serotyping |
| Ngoi *et al.* | 2013 | Animals; food; humans | Typhimurium | Antimicrobial susceptibility test; multi-locus variable number tandem repeat analysis; PCR identification; pulsed-field gel electrophoresis |
| Ngoi & Thong | 2013 | Animals; humans | Enteritidis | Antimicrobial susceptibility test; multi-locus variable number tandem repeat analysis; PCR identification; pulsed-field gel electrophoresis |
| Abatcha *et al.* | 2014 | Animals | Agona, Corvallis, Mbandaka, Typhimurium | Antimicrobial susceptibility test; conventional isolation method; PCR identification |
| Cheng *et al.*, | 2014 | Animals | *Salmonella*subspecies *enterica, arizonae, diarizonae, indica* | Antimicrobial susceptibility test; conventional isolation method; commercial identification kit; PCR identification |
| Karunakaran *et al.* | 2014 | Humans | Non-typhoidal *Salmonella* | Minimum inhibitory concentration test (commercial E-test strip); PCR detection of resistance genes |
| Siti *et al.* | 2014 | Animals | *Salmonella* spp. | Conventional isolation and biochemical identification; conventional serotyping |
| Ong *et al.* | 2014 | Animals | Enteritidis, *Salmonella* spp., Typhimurium | Conventional isolation and biochemical identification; conventional serotyping |
| Raja & Gulam | 2014 | Animals; environment | Albany, Braenderup, Corvallis, Enteritidis, Havana, Mbandaka, Typhimurium | Conventional isolation and biochemical identification; random amplified polymorphic DNA fingerprinting |

**Table 2.4**, continued.

| Reference | Year | Source | Predominant serovar | Identification and characterization methods |
|---|---|---|---|---|
| Salwani *et al.* | 2014 | Environment | Typhi | Conventional isolation and biochemical identification; conventional serotyping; pulsed-field gel electrophoresis |
| Yap *et al.* | 2014 | Laboratory culture collection | Typhi | Whole genome sequencing |
| Adzitey *et al.* | 2015 | Animals | Albany, Enteritidis, Typhimurium | Random amplified polymorphic DNA fingerprinting |
| Khoo *et al.* | 2015 | Animals | Typhimurium | Antimicrobial susceptibility test |
| Thong *et al.* | 2016 | Animals; food; humans | Agona, Albany, Corvallis, Emek, Enteritidis, Hadar, Istanbul, Newport, *Salmonella* spp., Typhimurium,Wandsworth, Weltevreden | Conjugation experiment; PCR detection of resistance genes |

# CHAPTER 3: MOLECULAR CHARACTERIZATION OF *Salmonella enterica* serovar Typhimurium AND *Salmonella enterica* serovar Enteritidis IN MALAYSIA (2002-2009)

## 3.1 Introduction

*Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) and *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis) are the two most common non-typhoidal *Salmonella* serovars isolated in Malaysia. Not only are these organisms infect humans, they are often associated with food and animals in this region. Due to their ubiquitous nature, the ability of these two *Salmonella* serovars to cause cross-species infections poses a high risk to public health. However, majority of the *Salmonella* studies previously done in Malaysia had been focusing on source-specific isolation and identification. Hence, there is a lack of comprehensive information on the genetic background of *S.* Typhimurium and *S.* Enteritidis strains originated from different sources.

In order to understand the genetic association among the strains circulating in Malaysia, this study aimed to determine the genetic relatedness of the *S.* Typhimurium and *S.* Enteritidis strains isolated from animals, food, and human sources, by means of molecular approaches. Since multiple-locus variable number tandem repeat analysis (MLVA) has not been previously applied in the subtyping of *Salmonella* strains in Malaysia, the potential of MLVA for detailed strain differentiation would also be evaluated, in comparison with the gold standard, pulsed-field gel electrophoresis (PFGE).

The data generated provide better understanding of the *S.* Typhimurium and *S.* Enteritidis population genetics and transmission dynamics in this region, aiding in stimulating implementation of efficient control measures for disease prevention.

## 3.2 Literature review

In Malaysia, *S.* Typhimurium and *S.* Enteritidis are frequently isolated from infected patients, contaminated food, and animal sources (Modarressi & Thong, 2010; Roseliza *et al.,* 2011; Thong *et al.,* 2011; Tiong *et al.,* 2010). In the early 1990's, *S.* Enteritidis had replaced *S.* Weltevreden as the major non-typhoidal *Salmonella* serovar that caused human infections in Malaysia (Yasin *et al.,* 1995). Based on a study done by the Veterinary Research Institute Malaysia, *S.* Typhimurium is the dominant serovar isolated from animal sources, followed by *S.* Enteritidis (Roseliza *et al.,* 2011). *S.* Typhimurium is predominantly found in bovine and porcine samples, while *S.* Enteritidis is mainly associated with poultry samples (Roseliza *et al.,* 2011). The prevalence of these organisms in the farm animals poses a threat to public health, and in developing countries the dissemination of these pathogenic organisms is mainly attributed to inappropriate hygienic practice during food preparation. Evidence supporting this notion could be found in a study whereby *Salmonella* had been isolated from ready-to-eat food in Malaysia (Modarressi & Thong, 2010).

Detailed strain subtyping is essential for the successful epidemiological investigation of *Salmonella* outbreaks. Pulsed-field gel electrophoresis (PFGE) remains the gold standard for *Salmonella* subtyping. This method provides sufficiently high discriminative power for successful source identification and investigation of outbreaks of *Salmonella* strains from various serovars (Wattiau *et al.,* 2011). The high discriminatory capacity of PFGE in the subtyping of *Salmonella* strains is also observed among different *Salmonella* serovars in Malaysia (Table 2.3). Generally, PFGE is able to discriminate *Salmonella* strains from different sources with a discriminatory index of greater than 0.90 (Simpson's Index of Diversity, D-index). The only exception occurred in an earlier study that investigated the *S.* Enteritidis outbreak strains obtained from a single hospital, wherein the D-index was only 0.15 (Thong *et al.,* 1995). A previous

study performed by Kerouanton *et al.* (2007) showed that PFGE may be less discriminative in subtyping some *Salmonella* serovars, including *S.* Enteritidis. This is probably due to the lack of genetic variations at the endonuclease restriction sites in the genomes of these *Salmonella* serovars, especially when only one restriction enzyme is used; thereby producing limited pulsotypes among the strains. In these studies (Kerouanton *et al.,* 2007; Thong *et al.,* 1995), only one restriction enzyme, i.e. the *Xba*I enzyme, was used as this is the primary restriction enzyme recommended by PulseNet USA (Ribot *et al.,* 2006).

Despite its high discriminatory capacity, PFGE is technically-demanding as various parameters including the amount of bacterial cells, temperature used for lysis and washing, concentration of buffers and enzymes used, pulse time, and run time, can affect the outcome of PFGE analysis (Ribot *et al.,* 2006). Therefore, protocol standardization is essential to obtain reproducible results. Strict adherence to a standardized protocol should be practiced by researchers and technicians to allow reproducible results for inter-laboratory comparison (Ribot *et al.,* 2006; Swaminathan *et al.,* 2001). Nonetheless, other strain subtyping methods, such as multi-locus sequence typing, phage typing, random amplified polymorphic DNA fingerprinting, and plasmid profiling, yielded suboptimal discriminatory power compared to that of PFGE (Fakhr *et al.,* 2005; Foley *et al.,* 2006; Harbottle *et al.,* 2006; Laconcha *et al.,* 1998; Liebisch & Schwarz, 1996). To date, PFGE is still the reference method for the evaluation of new molecular typing methods. In fact, PFGE is the core technology of PulseNet, an international laboratory network that connects worldwide foodborne infection cases to detect outbreaks, via DNA fingerprinting techniques (Swaminathan *et al.,* 2001).

Since 2000's, multiple-locus variable number tandem repeat analysis (MLVA) has been proposed for bacterial subtyping (Van Belkum, 2007). MLVA is a molecular

subtyping technique that utilizes repetitive DNA sequences in the bacterial genome. This method assesses the variability of the genetic entity called variable number tandem repeat (VNTR). VNTRs are present at multiple loci and may vary in terms of nucleotide sequence and unit size. Such variations are often the strain-defining parameters (Van Belkum, 2007). MLVA has gained increasing popularity as an alternative to PFGE for subtyping *Salmonella* strains since 2003 (Bergamini *et al.,* 2011; Boxrud *et al.,* 2007; Lindstedt *et al.,* 2004; Octavia & Lan, 2009; Tien *et al.,* 2011). Most of these studies have documented the high discriminatory power of MLVA in subtyping various *Salmonella* serovars. MLVA subtyping of *Salmonella* was first described for the differentiation of genetically homogeneous *Salmonella* serovars, such as Typhimurium (DT104) and Enteritidis (PT4) (Boxrud *et al.,* 2007; Lindstedt *et al.,* 2003). Furthermore, MLVA has proven to be a rapid, high throughput, and relatively cheap PFGE alternative that could be easily standardized for inter-laboratory comparisons (Hopkins *et al.,* 2011; Kruy *et al.,* 2011; Lindstedt *et al.,* 2007). MLVA is easier to perform than PFGE because the protocol involves only a simple PCR amplification step followed by capillary electrophoresis to accurately determine the amplified fragment lengths, hence providing highly reproducible results (Kruy *et al.,* 2011).

## 3.3 Methodology

### 3.3.1 Bacterial strains

All bacterial strains were obtained from the Laboratory of Biomedical Science and Molecular Microbiology, Institute of Graduate Studies, University of Malaya. From a total of 165 *S.* Typhimurium strains retrieved from the glycerol stock cultures, only 84 viable strains from clinical (n = 48), zoonotic (n = 16), and food (n = 20) sources were recovered. These strains were isolated from 11 States in Malaysia, namely Perlis, Kedah, Penang, Perak, Kelantan, Selangor, Pahang, Melaka, Negeri Sembilan, Johor, Sabah; and the capital city Kuala Lumpur. Majority of the strains (n = 80) were isolated

between 2002 and 2009; while the remaining four strains were isolated in earlier years, in 1970 (n = 3) and 1998 (n = 1). All strains were previously serotyped as *S.* Typhimurium according to the White-Kauffman-Le Minor scheme by the *Salmonella* Reference Laboratory at the Institute for Medical Research, and Veterinary Research Institute in Malaysia. The background information of all *S.* Typhimurium strains used in this study is provided in Appendix A.

A total of 111 clinical (n = 45) and zoonotic (n = 66) *S.* Enteritidis strains isolated between 2003 and 2008 were characterized. The clinical strains were previously isolated from two tertiary hospitals, located in Penang (n = 25) and capital city Kuala Lumpur (n = 20). Four of the clinical strains were isolated from human immunodeficiency virus (HIV)-positive patients. All zoonotic strains were previously obtained from Veterinary Research Institute, Malaysia. All strains were serotyped as *S.* Enteritidis according to the White-Kauffman-Le Minor scheme in the *Salmonella* Reference Laboratory in Institute of Medical Research, and Veterinary Research Institute, Malaysia. Strains used in this study were originally isolated from sporadic cases submitted to the hospitals or from routine screening by the Veterinary Research Institute. The background information of all *S.* Enteritidis strains used in this study is provided in Appendix B.

The identity of all revived *Salmonella* strains was further confirmed with a PCR targeting the DNA sequences specific to the *Salmonella* genus (outer membrane porin protein, OmpC), serovar Typhimurium (DUF2219 domain-containing protein) and Enteritidis (*Salmonella* difference fragment I, SdfI), before being subjected to further analysis (Table 3.1) (Alvarez *et al.*, 2004).

**Table 3.1:** PCR identification of *Salmonella* spp., *S*. Typhimurium, and *S*. Enteritidis

| Target organism | Primer | Primer sequence (5'-3') | Amplicon size (bp) | PCR amplification protocol | Reference |
|---|---|---|---|---|---|
| *S*. Enteritidis | ENT-F | TGTGTTTTATCTGATGCAAGAGG | 304 | PCR amplification was done in a 25 µL multiplex reaction mixture, containing 1× green GoTaq Flexi Buffer, 1.5 mmol/L magnesium chloride (MgCl2), 280 µmol/L deoxynucleoside triphosphate (dNTP) mix, 0.4 µmol/L of each primer pair, 1 U Taq DNA polymerase (Promega, Madison, USA), and approximately 100 ng of bacterial genomic DNA. The PCR reaction mixtures were first incubated at 95 ℃ for 2 min; followed by 35 cycles of 95 ℃ for 1 min, 55 ℃ for 1 min, and 72 ℃ for 2 min; with a final extension step of 72 ℃ for 5 min. | Alvarez *et. al.* (2004) |
| | ENT-R | TGAACTACGTTCGTTCTTCTGG | | | |
| *S*. Typhimurium | TYPH-F | TTGTTCACTTTTTACCCCTGAA | 401 | | |
| | TYPH-R | CCCTGACAGCCGTTAGATATT | | | |
| *Salmonella* spp. | OMPC-F | ATCGCTGACTTATGCAATCG | 204 | | |
| | OMPC-R | CGGGTTGCGTTATAGGTCTG | | | |

### 3.3.2 Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) was performed according to previously described protocol (Thong *et al.,* 2003). The detailed PFGE protocol is appended (Appendix C). *Xba*I enzyme (Promega, Madison, USA) was used for endonuclease restriction of the *Salmonella* genomic DNA. *Xba*I-digested *Salmonella* Braenderup H9812 was used as the DNA size marker. The pulsotypes obtained were subsequently analysed using BioNumerics v6.0 software (Applied Maths, Belgium). A dendrogram was constructed based on the Dice coefficient of similarity (F) and unweighted pair group method with arithmetic mean algorithm (UPGMA) to illustrate the genetic diversity among the tested strains. A band position tolerance of 1.5 % was used for analysis of the PFGE fingerprints. The Dice coefficient of similarity (F) measures the genetic similarity of the strains based on the banding profiles of the individuals in DNA fingerprinting analyses, from a scale of 0 to 1, where F = 0 shows genetic dissimilarity and F = 1 indicates that the strains are genetically clonal (Kosman & Leonard, 2005; Thong *et al.,* 1994). The F-value is calculated using the following formula: $F = 2n_{xy} / (n_x + n_y)$, where $n_x$ is the total number of DNA fragments from strain X; $n_y$ is the total number of DNA fragments from strain Y; and $n_{xy}$ is the total number of DNA fragments that are identical in the two strains (Thong *et al*., 1994).

### 3.3.3 Multiple-locus variable number tandem repeat analysis

Crude DNA for each strain was prepared by suspending a loopful of bacterial colonies in 50 µL of deionized distilled water and boiled at 99 ˚C for 5 min. After a quick centrifugation, 5 µL of the supernatant (~150 ng of DNA template) was used for PCR. The targeted VNTRs for each *Salmonella* serovar, together with the fluorescently-labelled primer sequences and the PCR amplification protocol are shown in Table 3.2.

**Table 3.2:** Details for the PCR amplification step for the MLVA of *S.* Typhimurium and *S.* Enteritidis

| Organism | VNTR | Fluorescently-labeled primer (dye-sequence; 5'-3') | Repeat size (bp) | PCR amplification protocol | Reference |
|---|---|---|---|---|---|
| ***S.* Typhimurium** | STTR3 | Forward: **NED**-CCCCCTAAGCCCGATAATGG<br>Reverse: TGACGCCGTTGCTGAAGGTAATAA | 33 | PCR master mix (25 µL) consisted of 1× colourless GoTaq Flexi Buffer, 2 mmol/L $MgCl_2$, 200 µmol/L dNTP mix, 0.3 µmol/L of each primer pair, 2 U *Taq* DNA polymerase (Promega, Madison, USA), and approximately 100 ng of bacterial genomic DNA. The PCR reaction mixtures were first incubated at 95 ℃ for 15 min; followed by 25 cycles of 94 ℃ for 30s, 63 ℃ for 90 s, and 72 ℃ for 90 s; with a final extension step of 72 ℃ for 10 min. | Lindstedt *et al.* (2004) |
| | STTR6 | Forward: **PET**-TCGGGCATGCGTTGAAA<br>Reverse: CTGGTGGGGAGAATGACTGG | 6 | | |
| | STTR5 | Forward: **FAM**-ATGGCGAGGCGAGCAGCAGT<br>Reverse: GGTCAGGCCGAATAGCAGGAT | 6 | PCR master mix (25 µL) consisted of 1× colourless GoTaq Flexi Buffer, 3 mmol/L $MgCl_2$, 200 µmol/L dNTP mix, 0.3 µmol/L of each primer pair, 1.5 U *Taq* DNA polymerase (Promega, Madison, USA), and approximately 150 ng of bacterial genomic DNA. The thermal cycling condition is identical to that of STTR3 and STTR6. | |
| | STTR9 | Forward: **PET**-AGAGGCGCTGCGATTGACGATA<br>Reverse: CATTTTCCACAGCGGCAGTTTTTC | 9 | | |
| | STTR10pl | Forward: **VIC**-CGGGCGCGGCTGGAGTATTTG<br>Reverse: GAAGGGGCCGGGCAGAGACAGC | 6 | | |

**Table 3.2**, continued.

| Organism | VNTR | Fluorescently-labeled primer (dye-sequence; 5'-3') | Repeat size (bp) | PCR amplification protocol | Reference |
|---|---|---|---|---|---|
| ***S.* Enteritidis** | SENTR4 | Forward: **NED**-GACCAACACTCTATGAACCAATG<br>Reverse: ACCAGGCAACTATTCGCTATC | 7 | PCR master mix (25 µL) consisted of 1× colourless GoTaq Flexi Buffer, 2 mmol/L $MgCl_2$, 200 µmol/L dNTP mix, 0.3 µmol/L of each primer pair, 2 U *Taq* DNA polymerase (Promega, Madison, USA), and approximately 150 ng of bacterial genomic DNA. The PCR reaction mixtures were first incubated at 95 ℃ for 15 min; followed by 28 cycles of 94 ℃ for 30 s, 55 ℃ for 90 s, and 72 ℃ for 90 s; with a final extension step of 72 ℃ for 10 min. | Malorny *et al.* (2008) |
| | SENTR5 | Forward: **FAM**-CACCGCACAATCAGTGGAAC<br>Reverse: GCGTTGAATATCGGCAGCATG | 6 | | |
| | SENTR6 | Forward: **PET**-ATGGACGGAGGCGATAGAC<br>Reverse: AGCTTCACAATTTGCGTATTCG | 7 | | |
| | SENTR7 | Forward: **VIC**-ACGATCACCACGGTCACTTC<br>Reverse: CGGATAACAACAGGACGCTTC | 9 | | |
| | SE7 | Forward: **FAM**-CCGACCCAATAAGGAG<br>Reverse: CTTACCGTTGGTAGTTTGTTA | 60 | PCR master mix (25 µL) was same as SENTR4-7. PCR reaction mixtures were first incubated at 95 ℃ for 15 min; followed by 35 cycles of 94 ℃ for 30 s, 58 ℃ for 90 s, and 72 ℃ for 90 s; with a final extension step of 60 ℃ for 30 min. | Cho *et al.* (2007) |

All *S.* Typhimurium VNTR amplicons were diluted with sterile deionized distilled water in 1:10 ratio; PCR products for SENTR4, SENTR5, SENTR6 and SENTR7 were diluted 1:100; while that of SE7 was diluted 1:10 in sterile deionized distilled water. The diluted sample (1 µL) was mixed with 10 µL Hi-Di™ formamide (Applied Biosystems, USA) and 0.3 µL GeneScan™ 600 LIZ® internal size-standard (Applied Biosystems, USA). The samples were then denatured for 5 min at 95 ℃ and cooled to room temperature before being subjected to capillary electrophoresis using the ABI prism® 3130xl Genetic Analyzer (Applied Biosystems, USA).

An electropherogram was generated for each sample where the presence of a VNTR locus was displayed as a coloured peak. Fragment sizes were analysed using GeneMapper v4.0 software (Applied Biosystems, USA). The peak table generated by this software was subsequently imported to BioNumerics v6.0 software (Applied Maths, Belgium) to assign allele numbers. An allelic profile was constructed for each strain, resembling a string of numbers indicating the number of repeat units in individual locus arranged in the order STTR9-STTR5-STTR6-STTR10pl-STTR3 for *S.* Typhimurium, and SENTR4-SENTR5-SENTR6-SENTR7-SE7 for *S.* Enteritidis. The number 00 indicates the absence of the VNTR locus. The STTR3 locus of *S.* Typhimurium contains two repeat units (27 bp and 33 bp respectively) and therefore the actual amplicon length was used to construct an allele string according to the system adapted by Australian laboratories (Gilbert, 2008).

Cluster analysis of the strains based on categorical coefficient (C) and UPGMA algorithm was performed by using BioNumerics v6.0 software (Applied Maths, Belgium). The categorical coefficient gives an equivalent weight to a multistate character at any locus, regardless of the number of repeats (Ramisse *et al.*, 2004). The minimum spanning tree (MST) was constructed using the highest number of single

locus variants (SLVs) as the priority rule. The genetic relationships of the MLVA types were determined by the allelic diversity of the five VNTR loci examined, and hypothetical type was not created in the MST.

### 3.3.4 Statistical analysis

The subtyping results for *S.* Typhimurium and *S.* Enteritidis strains were analysed and discussed separately, as these two organisms constituted different serovars and combined analyses of the two serovars were not possible for MLVA as different VNTR loci were targeted in each serovar. The discriminatory power of PFGE and MLVA was determined by the Simpson's index of diversity (D) according to Hunter and Gaston (1988). The D-index measures the ability of a typing method to distinguish between unrelated strains, from a scale of 0 to 1, indicating the probability of finding two randomly selected strains from a population falling into different types (Hunter & Gaston, 1988). Allelic diversity of each VNTR locus was calculated as $1-\sum(\text{Allele frequency})^2$ (Nei's diversity index) (Nei, 1987). The Nei's diversity index defines genetic diversity based on allele frequencies in a population. The "null" allele was included in the calculation of allelic diversity. Next, the bidirectional Wallace coefficient (W) was calculated as a quantitative method to determine the correlation between PFGE and MLVA typing of the strains (Wallace, 1983). In cluster analysis of both PFGE and MLVA typing, strains were classified into groups (clusters) based on their genetic similarity to determine the relatedness among strains from different sources. The W-coefficient provides an asymmetric view of concordance by considering clusters from one typing method as the standard for another to compare against. It represents the probability that strains grouped in one PFGE cluster will also share identical MLVA type, and vice versa.

### 3.3.5 DNA hybridization

The absence of a VNTR locus was confirmed by DNA hybridization. Approximately 5 µg genomic DNA was loaded onto a nylon membrane using a PR600 24-slot blot filtration manifold (Hoefer® Inc., Holliston, USA) attached to a vacuum pump R-300 (Boeco, Hamburg, Germany). The digoxigenin (DIG) nonradioactive system (Roche Diagnostics, Indiana, USA) for DNA hybridization was used. Purified PCR products of the VNTR loci were used as DNA templates to synthesize probes by using the PCR DIG Probe Synthesis Kit (Roche Diagnostics, USA). The probes targeted the VNTR loci and their flanking regions. The hybridization temperatures for each locus were optimized (54 ℃ for STTR6 and 40 ℃ for STTR10pl). Subsequently, the probe-target hybrids were visualized by chemiluminescent assay, using a chemiluminescent alkaline phosphatase substrate (CSPD). The nylon membrane was then exposed to X-ray film, which was subsequently developed and fixed. The detailed protocol for the synthesis of probes and the DNA hybridization protocol are shown in Appendix D.

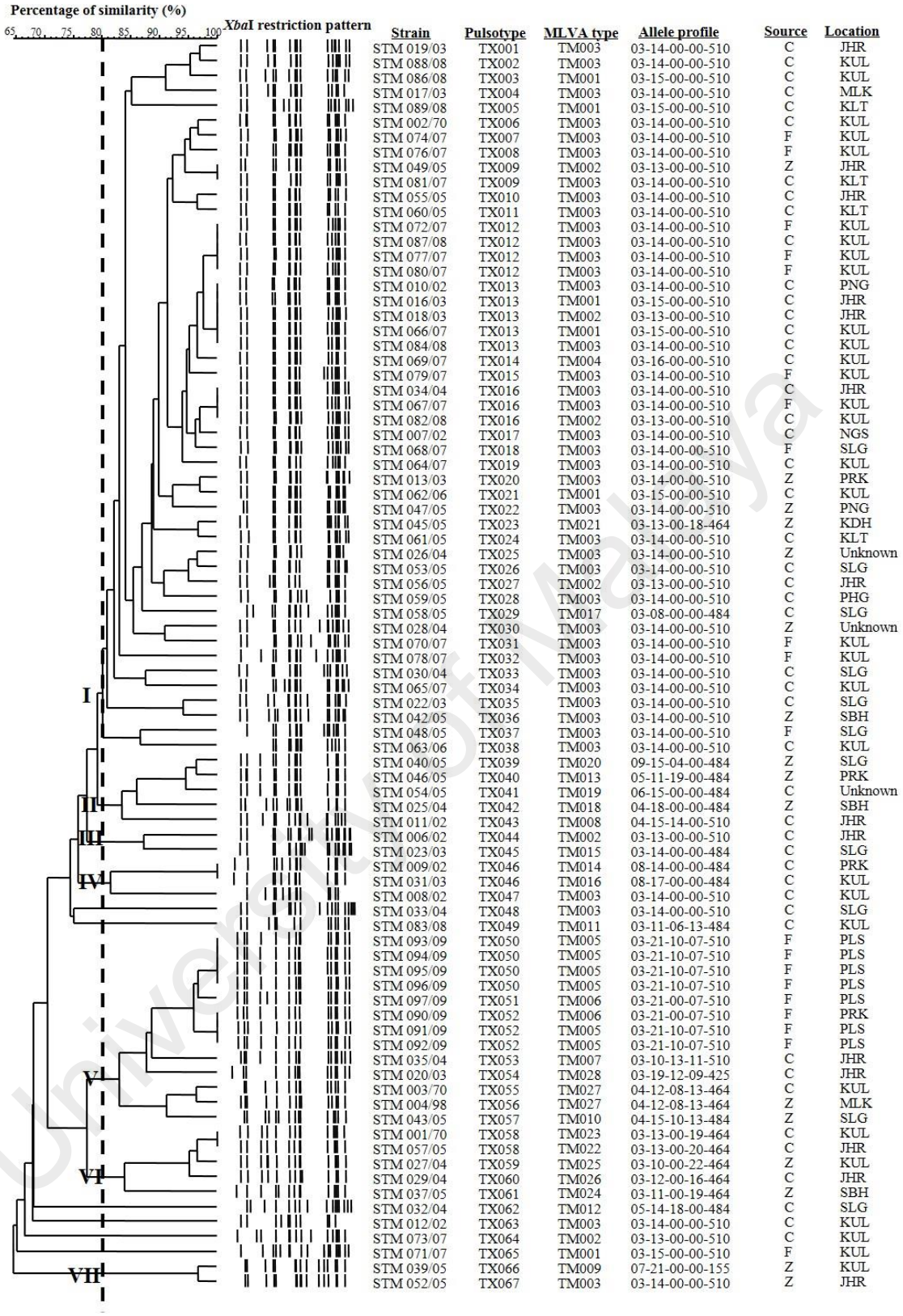
## 3.4 Results

### 3.4.1 PFGE analysis of *S.* Typhimurium and *S.* Enteritidis strains

PFGE analysis of *Xba*I-digested chromosomal DNA from 84 *S.* Typhimurium strains yielded 67 pulsotypes, arbitrarily designated as TX001 to TX067 (Figure 3.1). Each pulsotype contains 12 to 20 DNA fragments and their sizes ranged from 20.5 kbp to 1135 kbp. The genetic diversity (F-value) of the *S.* Typhimurium strains, as determined by the PFGE banding patterns, ranged from 0.60 to 1.00. The TX009 (n = 2), TX012 (n = 4), TX013 (n = 5), TX016 (n = 3), TX046 (n = 2), TX050 (n = 4), TX052 (n = 3), and TX058 (n = 2) were common pulsotypes that appeared in more than one strain. The remaining 60 pulsotypes were unique. The D-index for PFGE typing of *S.* Typhimurium was 0.99. A dendrogram based on 67 pulsotypes revealed seven clusters (clusters I to VII) of closely related *S.* Typhimurium strains, with a genetic similarity of F = 0.80

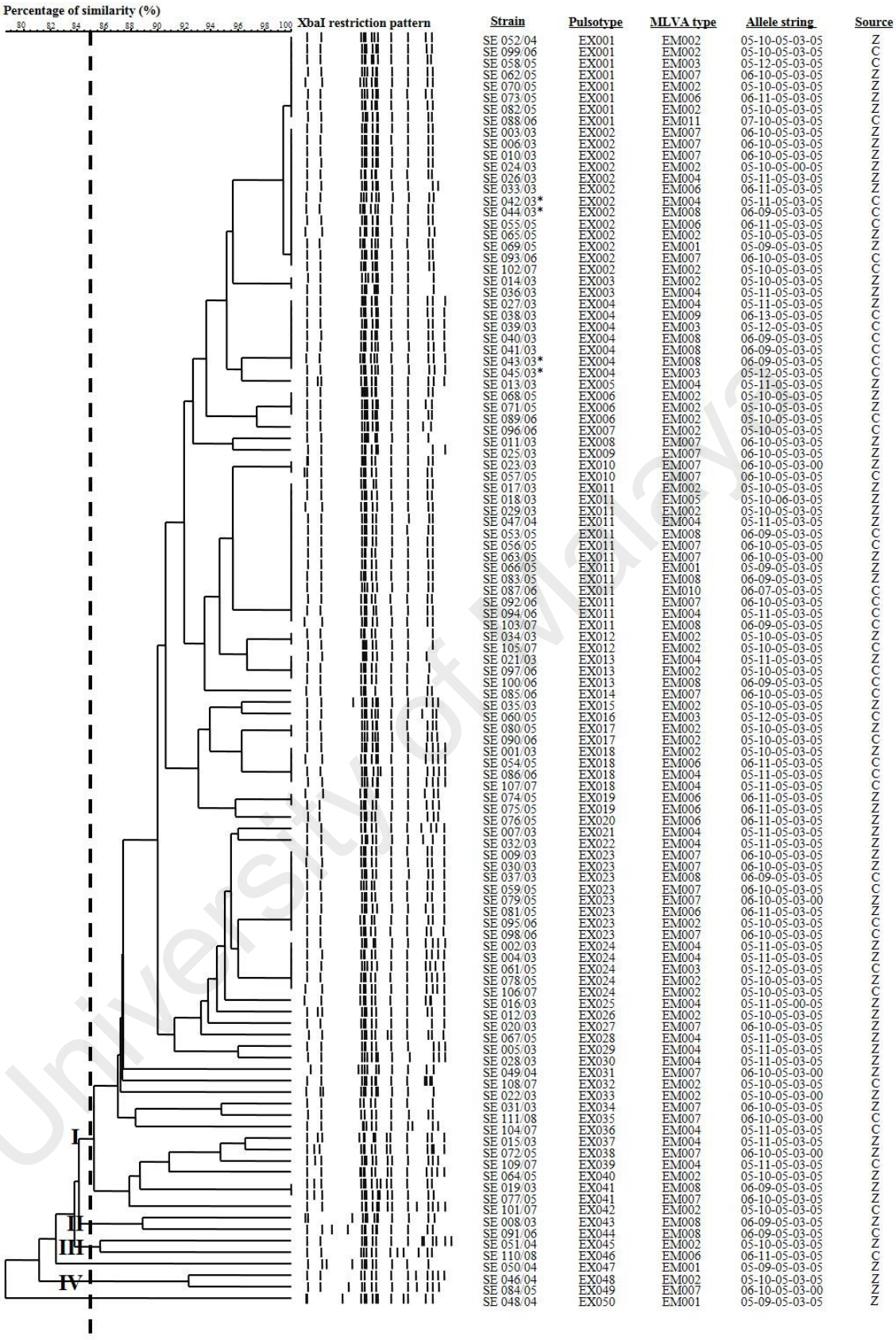
(Figure 3.1). Cluster I (F = 0.80) was a major group consisting 57 % (n = 48) of the *S.* Typhimurium strains. The majority of the strains in this cluster were from clinical (n = 30), followed by food (n = 11) and zoonotic (n = 7) sources between 1970 and 2008. Within this cluster, 44 % of the strains were isolated from the capital city Kuala Lumpur (n = 21). Clusters II (F = 0.83), III (F = 0.87), IV (F = 0.81), VI (F = 0.83), and VII (F = 0.96) were smaller clusters containing two to five strains.

All 111 *S.* Enteritidis strains were typable by PFGE, yielding 50 pulsotypes which were arbitrarily designated as EX001 to EX050 (Figure 3.2). *Xba*I digestion of the bacterial chromosomal DNA produced 10 to 16 fragments with sizes ranging from 20.5 kbp to 1135 kbp. Limited genetic diversity was observed among the *S.* Enteritidis strains ($0.78 \leq F \leq 1.00$). Among the 50 pulsotypes, 15 were represented by two or more strains; while the remaining 35 pulsotypes were non-repeating. Most of the common pulsotypes consisted of both clinical and zoonotic strains (e.g. EX012), indicating the close genetic relationship between *S.* Enteritidis strains from these two sources. The four *S.* Enteritidis strains from HIV-infected patients were genetically similar (F > 0.95), and shared common pulsotype with strains from animals and patients who were not infected by HIV (Figure 3.2). Cluster analysis at 85 % similarity yielded four clusters (I to IV) of closely related *S.* Enteritidis strains. Cluster I is a major group comprised of most of the *S.* Enteritidis strains (n = 103) in this study, including strains from different sources and years (2003-2008). The discriminatory power (D-index) of PFGE was 0.96. Overall, the *S.* Enteritidis strains from different sources in this study showed high genetic similarity ($F \geq 0.80$); with less than three bands difference in their PFGE restriction patterns.




| Strain | Pulsotype | MLVA type | Allele profile | Source | Location |
|---|---|---|---|---|---|
| STM 019/03 | TX001 | TM003 | 03-14-00-00-510 | C | JHR |
| STM 088/08 | TX002 | TM003 | 03-14-00-00-510 | C | KUL |
| STM 086/08 | TX003 | TM001 | 03-15-00-00-510 | C | KUL |
| STM 017/03 | TX004 | TM003 | 03-14-00-00-510 | C | MLK |
| STM 089/08 | TX005 | TM001 | 03-15-00-00-510 | C | KLT |
| STM 002/70 | TX006 | TM003 | 03-14-00-00-510 | C | KUL |
| STM 074/07 | TX007 | TM003 | 03-14-00-00-510 | F | KUL |
| STM 076/07 | TX008 | TM003 | 03-14-00-00-510 | F | KUL |
| STM 049/05 | TX009 | TM002 | 03-13-00-00-510 | Z | JHR |
| STM 081/07 | TX009 | TM003 | 03-14-00-00-510 | C | KLT |
| STM 055/05 | TX010 | TM003 | 03-14-00-00-510 | C | JHR |
| STM 060/05 | TX011 | TM003 | 03-14-00-00-510 | C | KLT |
| STM 072/07 | TX012 | TM003 | 03-14-00-00-510 | F | KUL |
| STM 087/08 | TX012 | TM003 | 03-14-00-00-510 | C | KUL |
| STM 077/07 | TX012 | TM003 | 03-14-00-00-510 | F | KUL |
| STM 080/07 | TX012 | TM003 | 03-14-00-00-510 | F | KUL |
| STM 010/02 | TX013 | TM003 | 03-14-00-00-510 | C | PNG |
| STM 016/03 | TX013 | TM001 | 03-15-00-00-510 | C | JHR |
| STM 018/03 | TX013 | TM002 | 03-13-00-00-510 | C | JHR |
| STM 066/07 | TX013 | TM001 | 03-15-00-00-510 | C | KUL |
| STM 084/08 | TX013 | TM003 | 03-14-00-00-510 | C | KUL |
| STM 069/07 | TX014 | TM004 | 03-16-00-00-510 | C | KUL |
| STM 079/07 | TX015 | TM003 | 03-14-00-00-510 | F | KUL |
| STM 034/04 | TX016 | TM003 | 03-14-00-00-510 | C | JHR |
| STM 067/07 | TX016 | TM003 | 03-14-00-00-510 | F | KUL |
| STM 082/08 | TX016 | TM002 | 03-13-00-00-510 | C | KUL |
| STM 007/02 | TX017 | TM003 | 03-14-00-00-510 | C | NGS |
| STM 068/07 | TX018 | TM003 | 03-14-00-00-510 | F | SLG |
| STM 064/07 | TX019 | TM003 | 03-14-00-00-510 | C | KUL |
| STM 013/03 | TX020 | TM003 | 03-14-00-00-510 | Z | PRK |
| STM 062/06 | TX021 | TM001 | 03-15-00-00-510 | C | KUL |
| STM 047/05 | TX022 | TM003 | 03-14-00-00-510 | Z | PNG |
| STM 045/05 | TX023 | TM021 | 03-13-00-18-464 | Z | KDH |
| STM 061/05 | TX024 | TM003 | 03-14-00-00-510 | C | KLT |
| STM 026/04 | TX025 | TM003 | 03-14-00-00-510 | Z | Unknown |
| STM 053/05 | TX026 | TM003 | 03-14-00-00-510 | C | SLG |
| STM 056/05 | TX027 | TM002 | 03-13-00-00-510 | C | JHR |
| STM 059/05 | TX028 | TM003 | 03-14-00-00-510 | C | PHG |
| STM 058/05 | TX029 | TM017 | 03-08-00-00-484 | C | SLG |
| STM 028/04 | TX030 | TM003 | 03-14-00-00-510 | Z | Unknown |
| STM 070/07 | TX031 | TM003 | 03-14-00-00-510 | F | KUL |
| STM 078/07 | TX032 | TM003 | 03-14-00-00-510 | F | KUL |
| STM 030/04 | TX033 | TM003 | 03-14-00-00-510 | C | SLG |
| STM 065/07 | TX034 | TM003 | 03-14-00-00-510 | C | KUL |
| STM 022/03 | TX035 | TM003 | 03-14-00-00-510 | C | SLG |
| STM 042/05 | TX036 | TM003 | 03-14-00-00-510 | Z | SBH |
| STM 048/05 | TX037 | TM003 | 03-14-00-00-510 | F | SLG |
| STM 063/06 | TX038 | TM003 | 03-14-00-00-510 | C | KUL |
| STM 040/05 | TX039 | TM020 | 09-15-04-00-484 | Z | SLG |
| STM 046/05 | TX040 | TM013 | 05-11-19-00-484 | Z | PRK |
| STM 054/05 | TX041 | TM019 | 06-15-00-00-484 | C | Unknown |
| STM 025/04 | TX042 | TM018 | 04-18-00-00-484 | Z | SBH |
| STM 011/02 | TX043 | TM008 | 04-15-14-00-510 | C | JHR |
| STM 006/02 | TX044 | TM002 | 03-13-00-00-510 | C | JHR |
| STM 023/03 | TX045 | TM015 | 03-14-00-00-484 | C | SLG |
| STM 009/02 | TX046 | TM014 | 08-14-00-00-484 | C | PRK |
| STM 031/03 | TX046 | TM016 | 08-17-00-00-484 | C | KUL |
| STM 008/02 | TX047 | TM003 | 03-14-00-00-510 | C | KUL |
| STM 033/04 | TX048 | TM003 | 03-14-00-00-510 | C | SLG |
| STM 083/08 | TX049 | TM011 | 03-11-06-13-484 | C | KUL |
| STM 093/09 | TX050 | TM005 | 03-21-10-07-510 | F | PLS |
| STM 094/09 | TX050 | TM005 | 03-21-10-07-510 | F | PLS |
| STM 095/09 | TX050 | TM005 | 03-21-10-07-510 | F | PLS |
| STM 096/09 | TX050 | TM005 | 03-21-10-07-510 | F | PLS |
| STM 097/09 | TX051 | TM006 | 03-21-00-07-510 | F | PLS |
| STM 090/09 | TX052 | TM006 | 03-21-00-07-510 | F | PRK |
| STM 091/09 | TX052 | TM005 | 03-21-10-07-510 | F | PLS |
| STM 092/09 | TX052 | TM005 | 03-21-10-07-510 | F | PLS |
| STM 035/04 | TX053 | TM007 | 03-10-13-11-510 | C | JHR |
| STM 020/03 | TX054 | TM028 | 03-19-12-09-425 | C | JHR |
| STM 003/70 | TX055 | TM027 | 04-12-08-13-464 | C | KUL |
| STM 004/98 | TX056 | TM027 | 04-12-08-13-464 | Z | MLK |
| STM 043/05 | TX057 | TM010 | 04-15-10-13-484 | Z | SLG |
| STM 001/70 | TX058 | TM023 | 03-13-00-19-464 | C | KUL |
| STM 057/05 | TX058 | TM022 | 03-13-00-20-464 | C | JHR |
| STM 027/04 | TX059 | TM025 | 03-10-00-22-464 | Z | KUL |
| STM 029/04 | TX060 | TM026 | 03-12-00-16-464 | C | JHR |
| STM 037/05 | TX061 | TM024 | 03-11-00-19-464 | Z | SBH |
| STM 032/04 | TX062 | TM012 | 05-14-18-00-484 | C | SLG |
| STM 012/02 | TX063 | TM003 | 03-14-00-00-510 | C | KUL |
| STM 073/07 | TX064 | TM002 | 03-13-00-00-510 | C | KUL |
| STM 071/07 | TX065 | TM001 | 03-15-00-00-510 | F | KUL |
| STM 039/05 | TX066 | TM009 | 07-21-00-00-155 | Z | KUL |
| STM 052/05 | TX067 | TM003 | 03-14-00-00-510 | Z | JHR |

**Figure 3.1:** Dendrogram showing the cluster analysis of 84 *S.* Typhimurium strains based on PFGE profiles of *Xba*I-digested chromosomal DNA of the bacterial strains. Roman numerals I to VII denote strain clusters with a cut-off value of F = 0.80. The sources and geographical locations of the strains are indicated by letters, whereby 'C' represents clinical strains; 'Z', zoonotic strains; 'F', food strains; JHR, Johor; KDH, Kedah; KLT, Kelantan; KUL, Kuala Lumpur; MLK, Melaka; NGS, Negeri Sembilan; PHG, Pahang; PLS, Perlis; PNG, Penang; PRK, Perak; SBH, Sabah; SLG, Selangor.




| Strain | Pulsotype | MLVA type | Allele string | Source |
|---|---|---|---|---|
| SE 052/04 | EX001 | EM002 | 05-10-05-03-05 | Z |
| SE 099/06 | EX001 | EM002 | 05-10-05-03-05 | C |
| SE 058/05 | EX001 | EM003 | 05-12-05-03-05 | C |
| SE 062/05 | EX001 | EM007 | 06-10-05-03-05 | Z |
| SE 070/05 | EX001 | EM002 | 05-10-05-03-05 | Z |
| SE 073/05 | EX001 | EM006 | 06-11-05-03-05 | Z |
| SE 082/05 | EX001 | EM002 | 05-10-05-03-05 | Z |
| SE 088/06 | EX001 | EM011 | 07-10-05-03-05 | C |
| SE 003/03 | EX002 | EM007 | 06-10-05-03-05 | Z |
| SE 006/03 | EX002 | EM007 | 06-10-05-03-05 | Z |
| SE 010/03 | EX002 | EM007 | 06-10-05-03-05 | Z |
| SE 024/03 | EX002 | EM002 | 05-10-05-00-05 | Z |
| SE 026/03 | EX002 | EM004 | 05-11-05-03-05 | Z |
| SE 033/03 | EX002 | EM006 | 06-11-05-03-05 | Z |
| SE 042/03* | EX002 | EM004 | 05-11-05-03-05 | C |
| SE 044/03* | EX002 | EM008 | 06-09-05-03-05 | C |
| SE 055/05 | EX002 | EM006 | 06-11-05-03-05 | C |
| SE 065/05 | EX002 | EM002 | 05-10-05-03-05 | Z |
| SE 069/05 | EX002 | EM001 | 05-09-05-03-05 | Z |
| SE 093/06 | EX002 | EM007 | 06-10-05-03-05 | C |
| SE 102/07 | EX002 | EM002 | 05-10-05-03-05 | C |
| SE 014/03 | EX003 | EM002 | 05-10-05-03-05 | Z |
| SE 036/03 | EX003 | EM004 | 05-11-05-03-05 | Z |
| SE 027/03 | EX004 | EM004 | 05-11-05-03-05 | Z |
| SE 038/03 | EX004 | EM009 | 06-13-05-03-05 | C |
| SE 039/03 | EX004 | EM003 | 05-12-05-03-05 | C |
| SE 040/03 | EX004 | EM008 | 06-09-05-03-05 | C |
| SE 041/03 | EX004 | EM008 | 06-09-05-03-05 | C |
| SE 043/03* | EX004 | EM008 | 06-09-05-03-05 | C |
| SE 045/03* | EX004 | EM003 | 05-12-05-03-05 | C |
| SE 013/03 | EX005 | EM004 | 05-11-05-03-05 | Z |
| SE 068/05 | EX006 | EM002 | 05-10-05-03-05 | Z |
| SE 071/05 | EX006 | EM002 | 05-10-05-03-05 | Z |
| SE 089/06 | EX006 | EM002 | 05-10-05-03-05 | C |
| SE 096/06 | EX007 | EM002 | 05-10-05-03-05 | C |
| SE 011/03 | EX008 | EM007 | 06-10-05-03-05 | Z |
| SE 025/03 | EX009 | EM007 | 06-10-05-03-05 | Z |
| SE 023/03 | EX010 | EM007 | 06-10-05-03-00 | Z |
| SE 057/05 | EX010 | EM007 | 06-10-05-03-05 | C |
| SE 017/03 | EX011 | EM002 | 05-10-05-03-05 | Z |
| SE 018/03 | EX011 | EM005 | 05-10-06-03-05 | Z |
| SE 029/03 | EX011 | EM002 | 05-10-05-03-05 | Z |
| SE 047/04 | EX011 | EM004 | 05-11-05-03-05 | Z |
| SE 053/05 | EX011 | EM008 | 06-09-05-03-05 | C |
| SE 056/05 | EX011 | EM007 | 06-10-05-03-05 | C |
| SE 063/05 | EX011 | EM007 | 06-10-05-03-00 | Z |
| SE 066/05 | EX011 | EM001 | 05-09-05-03-05 | Z |
| SE 083/05 | EX011 | EM008 | 06-09-05-03-05 | Z |
| SE 087/06 | EX011 | EM010 | 06-07-05-03-05 | C |
| SE 092/06 | EX011 | EM007 | 06-10-05-03-05 | C |
| SE 094/06 | EX011 | EM004 | 05-11-05-03-05 | C |
| SE 103/07 | EX011 | EM008 | 06-09-05-03-05 | C |
| SE 034/03 | EX012 | EM002 | 05-10-05-03-05 | Z |
| SE 105/07 | EX012 | EM002 | 05-10-05-03-05 | C |
| SE 021/03 | EX013 | EM004 | 05-11-05-03-05 | Z |
| SE 097/06 | EX013 | EM002 | 05-10-05-03-05 | C |
| SE 100/06 | EX013 | EM008 | 06-09-05-03-05 | C |
| SE 085/06 | EX014 | EM007 | 06-10-05-03-05 | C |
| SE 035/03 | EX015 | EM002 | 05-10-05-03-05 | Z |
| SE 060/05 | EX016 | EM003 | 05-12-05-03-05 | C |
| SE 080/05 | EX017 | EM002 | 05-10-05-03-05 | Z |
| SE 090/06 | EX017 | EM002 | 05-10-05-03-05 | C |
| SE 001/03 | EX018 | EM002 | 05-10-05-03-05 | Z |
| SE 054/05 | EX018 | EM006 | 06-11-05-03-05 | C |
| SE 086/06 | EX018 | EM004 | 05-11-05-03-05 | C |
| SE 107/07 | EX018 | EM004 | 05-11-05-03-05 | C |
| SE 074/05 | EX019 | EM006 | 06-11-05-03-05 | Z |
| SE 075/05 | EX019 | EM006 | 06-11-05-03-05 | Z |
| SE 076/05 | EX020 | EM006 | 06-11-05-03-05 | Z |
| SE 007/03 | EX021 | EM004 | 05-11-05-03-05 | Z |
| SE 032/03 | EX022 | EM004 | 05-11-05-03-05 | Z |
| SE 009/03 | EX023 | EM007 | 06-10-05-03-05 | Z |
| SE 030/03 | EX023 | EM007 | 06-10-05-03-05 | Z |
| SE 037/03 | EX023 | EM008 | 06-09-05-03-05 | C |
| SE 059/05 | EX023 | EM007 | 06-10-05-03-05 | C |
| SE 079/05 | EX023 | EM007 | 06-10-05-03-00 | Z |
| SE 081/05 | EX023 | EM006 | 06-11-05-03-05 | Z |
| SE 095/06 | EX023 | EM002 | 05-10-05-03-05 | C |
| SE 098/06 | EX023 | EM007 | 06-10-05-03-05 | C |
| SE 002/03 | EX024 | EM004 | 05-11-05-03-05 | Z |
| SE 004/03 | EX024 | EM004 | 05-11-05-03-05 | Z |
| SE 061/05 | EX024 | EM003 | 05-12-05-03-05 | C |
| SE 078/05 | EX024 | EM002 | 05-10-05-03-05 | Z |
| SE 106/07 | EX024 | EM002 | 05-10-05-03-05 | C |
| SE 016/03 | EX025 | EM004 | 05-11-05-00-05 | Z |
| SE 012/03 | EX026 | EM002 | 05-10-05-03-05 | Z |
| SE 020/03 | EX027 | EM007 | 06-10-05-03-05 | Z |
| SE 067/05 | EX028 | EM004 | 05-11-05-03-05 | Z |
| SE 005/03 | EX029 | EM004 | 05-11-05-03-05 | Z |
| SE 028/03 | EX030 | EM004 | 05-11-05-03-05 | Z |
| SE 049/04 | EX031 | EM007 | 06-10-05-03-00 | Z |
| SE 108/07 | EX032 | EM002 | 05-10-05-03-05 | C |
| SE 022/03 | EX033 | EM002 | 05-10-05-03-00 | Z |
| SE 031/03 | EX034 | EM007 | 06-10-05-03-05 | Z |
| SE 111/08 | EX035 | EM007 | 06-10-05-03-00 | C |
| SE 104/07 | EX036 | EM004 | 05-11-05-03-05 | C |
| SE 015/03 | EX037 | EM004 | 05-11-05-03-05 | Z |
| SE 072/05 | EX038 | EM007 | 06-10-05-03-00 | Z |
| SE 109/07 | EX039 | EM004 | 05-11-05-03-05 | C |
| SE 064/05 | EX040 | EM002 | 05-10-05-03-05 | Z |
| SE 019/03 | EX041 | EM008 | 06-09-05-03-05 | Z |
| SE 077/05 | EX041 | EM007 | 06-10-05-03-05 | Z |
| SE 101/07 | EX042 | EM002 | 05-10-05-03-05 | C |
| SE 008/03 | EX043 | EM008 | 06-09-05-03-05 | Z |
| SE 091/06 | EX044 | EM008 | 06-09-05-03-05 | C |
| SE 051/04 | EX045 | EM002 | 05-10-05-03-05 | Z |
| SE 110/08 | EX046 | EM006 | 06-11-05-03-05 | C |
| SE 050/04 | EX047 | EM001 | 05-09-05-03-05 | Z |
| SE 046/04 | EX048 | EM002 | 05-10-05-03-05 | Z |
| SE 084/05 | EX049 | EM007 | 06-10-05-03-00 | Z |
| SE 048/04 | EX050 | EM001 | 05-09-05-03-05 | Z |

**Figure 3.2:** Dendrogram showing cluster analysis of 111 *S.* Enteritidis strains based on PFGE profiles of *Xba*I-digested bacterial chromosomal DNA. Roman numerals I to IV denote strain clusters at F = 0.85; strains isolated from HIV-infected patients were marked with asterisk (*). The sources of the strains are represented in letters, whereby 'C' represents clinical strain, and 'Z' represents zoonotic strains.

### 3.4.2 MLVA typing of *S.* Typhimurium and *S.* Enteritidis strains

The five-loci MLVA assay (STTR9, STTR5, STTR6, STTR10pl, and STTR3) widely applied in European countries were adapted to analyse the 84 *S.* Typhimurium strains. Among the five commonly tested VNTR loci, STTR3, STTR5, and STTR9 were present in all strains. Analysis of loci diversity revealed that the STTR5 locus comprised of highest number of different alleles (n = 12), whereas the loci STTR6 and STTR9 showed lowest genetic diversity (Nei's diversity index of 0.34 and 0.26, respectively). VNTR loci STTR6 and STTR10pl were absent in most of the strains tested, with a typability of only 19.0 % and 23.8 %, respectively (Table 3.3).

**Table 3.3:** The VNTR loci characteristics for S. Typhimurium MLVA assay

| VNTR locus | No. of alleles | Allelic diversity[a] | No. of strains | Typability[b] (%) |
|---|---|---|---|---|
| STTR3 | 5 | 0.41 | 84 | 100.0 |
| STTR5 | 12 | 0.70 | 84 | 100.0 |
| STTR6 | 9 | 0.34 | 16 | 19.0 |
| STTR9 | 7 | 0.26 | 84 | 100.0 |
| STTR10pl | 9 | 0.41 | 20 | 23.8 |

[a] Allelic diversity was calculated as 1-∑(Allele frequency) ²(Nei's diversity index)
[b] Typability of the strains were calculated as the percentage of strains harbouring an allele for the specified VNTR locus in the pool of 84 *S.* Typhimurium strains

Altogether, 28 different allelic profiles (MLVA types) were identified among the *S.* Typhimurium strains, which were arbitrarily designated as TM001 to TM028 (Figure 3.1). The discriminatory power of MLVA subtyping for *S.* Typhimurium strains was 0.76 (D-index). TM003 (allele string 03-14-00-00-510) was the most commonly found MLVA type (48 %), encompassing clinical (n = 23), zoonotic (n = 6), and food (n = 11) isolates (Figure 3.1). MLVA types TM001 (03-15-00-00-510), TM002 (03-13-00-00-510), and TM003 (03-14-00-00-510) are single locus variants (SLVs, varied in STTR5 locus) which predominated (62 %) in *S.* Typhimurium strain collection (Figure 3.1). Limited genetic variations were observed among *S.* Typhimurium strains from different

sources isolated many years apart. For instance, a clinical strain (STM002/70) from 1970 shared identical MLVA type with strains as recent as 2008 (STM084/08, STM087/08, and STM088/08) (Figure 3.1). The genetic relatedness of the *S.* Typhimurium strains inferred by MLVA typing is shown in the MST constructed based on the allelic diversity of all five VNTR loci. The *S.* Typhimurium distributed throughout the tree with no clear branching dominated by strains from a single source, location, or year of isolation (Figure 3.3).

Both STTR6 and STTR10pl loci were largely absent in the *S.* Typhimurium strains in this study. Therefore, DNA hybridization was performed on a selected subset of the strains to confirm the MLVA typing results. A total of 23 *S.* Typhimurium strains, with 20 strains positive for STTR10pl locus were hybridized with DIG-labelled STTR10pl probe. The results of DNA hybridization concurred with the MLVA results, i.e. probe-target hybrids were detected for strains tested positive with STTR10pl locus while no signal was detected for strains without STTR10pl locus (Figure 3.4). Similarly, DNA hybridization using the DIG-labelled STTR6 probe on a selected subset of 23 strains, with 6 strains positive for STTR6 locus, showed that positive signals were observed only in strains with STTR6 amplification in MLVA (Figure 3.5).

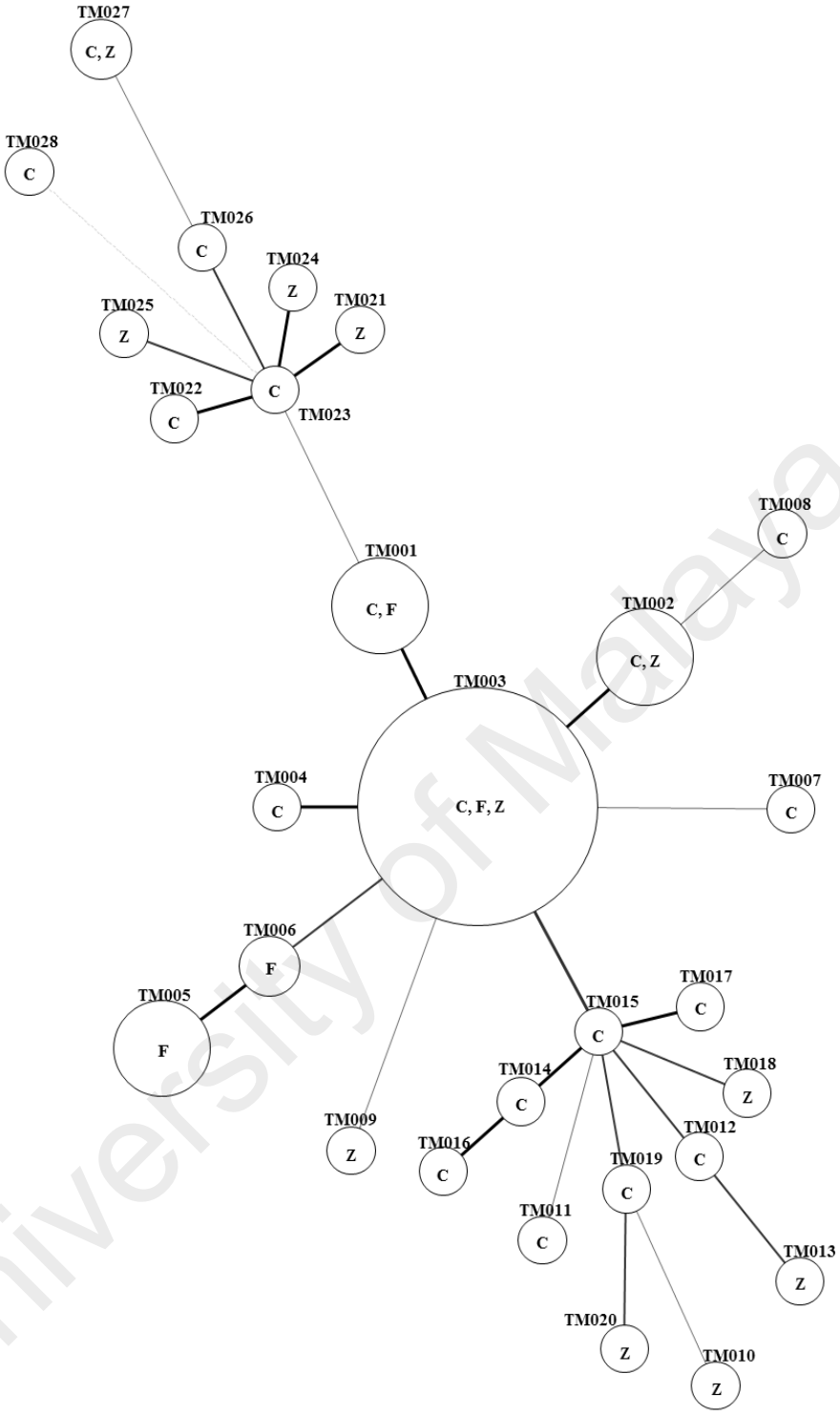





**Figure 3.3:** Minimum spanning tree (MST) for MLVA analysis of the *S.* Typhimurium strains. A circle denotes an MLVA type, and the size of the circle corresponds to the number of strains within the MLVA type. The thickness and dotting of the lines indicated the allelic differences between the MLVA types; thus, a thick line indicates single locus variant, a thin line indicates allelic differences at two different VNTR loci, and a dotted line denotes allelic differences at three or more VNTR loci. The sources of the strains are indicated as letters in the circles, whereby 'C' represents clinical strains; 'Z', zoonotic strains; 'F', food strains; 'C, Z', a mixture of clinical and zoonotic strains; 'C, F', a mixture of clinical and food strains; and 'C, Z, F', a mixture of strains from all three sources.

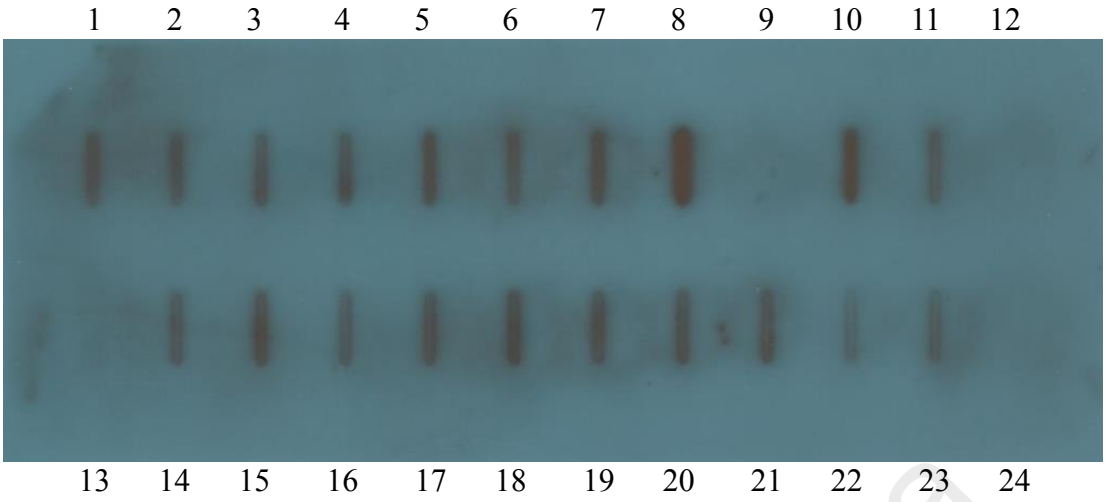



**Figure 3.4:** DNA hybridization film for STTR10pl locus in *S.* Typhimurium. Slot 1 represents STM001/70; slot 2, STM003/70; slot 3, STM004/98; slot 4, STM020/03; slot 5, STM027/04; slot 6, STM029/04, slot 7, STM035/04, slot 8, STM037/05; slot 9, STM042/05; slot 10, STM043/05; slot 11, STM045/05; slot 12, STM047/05; slot 13, STM052/05; slot 14, STM057/05; slot 15, STM083/08; slot 16, STM090/09; slot 17, STM091/09; slot 18, STM092/09; slot 19, STM093/09; slot 20, STM094/09; slot 21, STM095/09; slot 22, STM096/09; slot 23, STM097/09; slot 24, no template control. Slot 9, 12, and 13 show the absence of the STTR10pl locus in the tested *S.* Typhimurium strains.

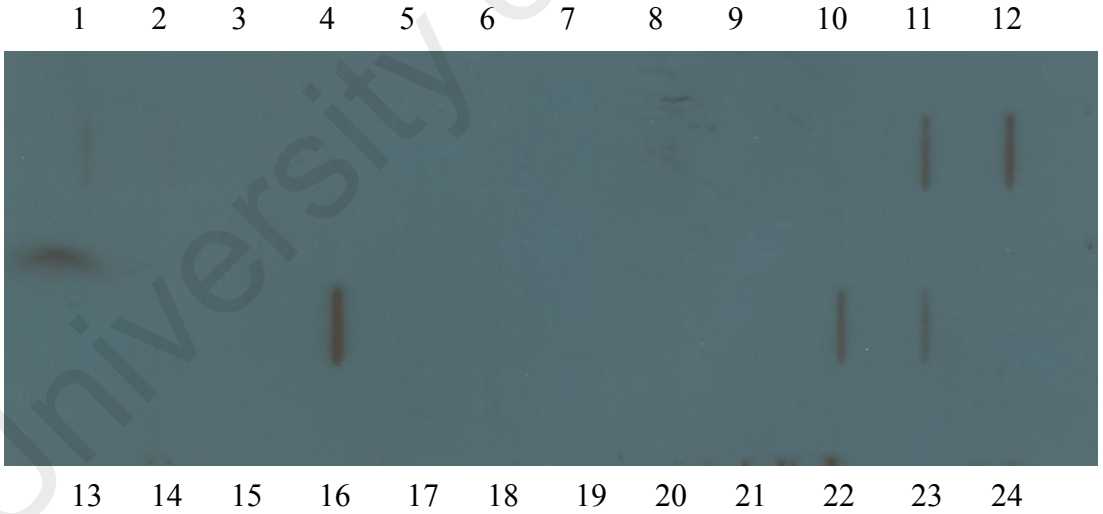



**Figure 3.5:** DNA hybridization film for STTR6 locus in *S.* Typhimurium. Slot 1 represents STM004/98; slot 2, STM006/02; slot 3, STM007/02; slot 4, STM008/02; slot 5, STM013/03; slot 6, STM016/03, slot 7, STM022/03, slot 8, STM028/04; slot 9, STM030/04; slot 10, STM033/04; slot 11, STM032/04; slot 12, STM035/04; slot 13, STM055/05; slot 14, STM068/07; slot 15, STM070/07; slot 16, STM092/09; slot 17, STM091/09; slot 18, STM092/09; slot 19, STM093/09; slot 20, STM094/09; slot 21, STM095/09; slot 22, STM083/08; slot 23, STM091/09; slot 24, no template control. Slot 1, 11, 12, 16, 22, and 23 show the presence of the STTR6 locus in the tested *S.* Typhimurium strains.

All 111 *S.* Enteritidis strains could be typed at the five VNTR loci (SENTR4, SENTR5, SENTR6, SENTR7, and SE7). The allelic diversity of the VNTR loci was very low, with polymorphism indices ranging from zero to 0.63 (Nei's diversity index) (Table 3.4). The loci SENTR7 and SE7 consisted of only a single allele throughout the entire *S.* Enteritidis strains pool. The limited allelic diversity indicated that these loci were conserved among the strains, regardless of their sources and year of isolation.

**Table 3.4:** The VNTR loci characteristics for *S.* Enteritidis MLVA assay

| VNTR locus | No. of alleles | Allelic diversity[a] |
|---|---|---|
| SENTR4 | 3 | 0.50 |
| SENTR5 | 6 | 0.63 |
| SENTR6 | 2 | 0.02 |
| SENTR7 | 1 | 0 |
| SE7 | 1 | 0 |

[a] Allelic diversity was calculated as 1-∑(Allele frequency) ²(Nei's Diversity Index)

Altogether, a total of 11 distinct allelic profiles (MLVA types) were identified (Figure 3.2), and were arbitrarily designated as EM001 to EM011. The discriminatory power of MLVA subtyping for *S.* Enteritidis was 0.82 (D-index). Majority of the *S.* Enteritidis strains (96 %) were closely clustered into seven common MLVA types, five of which were shared by strains isolated from both humans and animals. In the MST constructed (Figure 3.6), no clear branching dominated by strains from a single source, location, or year of isolation was observed. However, the *S.* Enteritidis strains could be separated into two major clusters (Figure 3.6) corresponded to the single allelic change in SENTR4 locus. Within each cluster, the differences between the MLVA types arise from allelic variation in SENTR5 locus. Overall, MLVA subtyping showed that all *S.* Enteritidis strains in this study were genetically closely related, with only single allelic difference between one MLVA types with another. In other word, all members of the *S.* Enteritidis strains pool were SLVs.

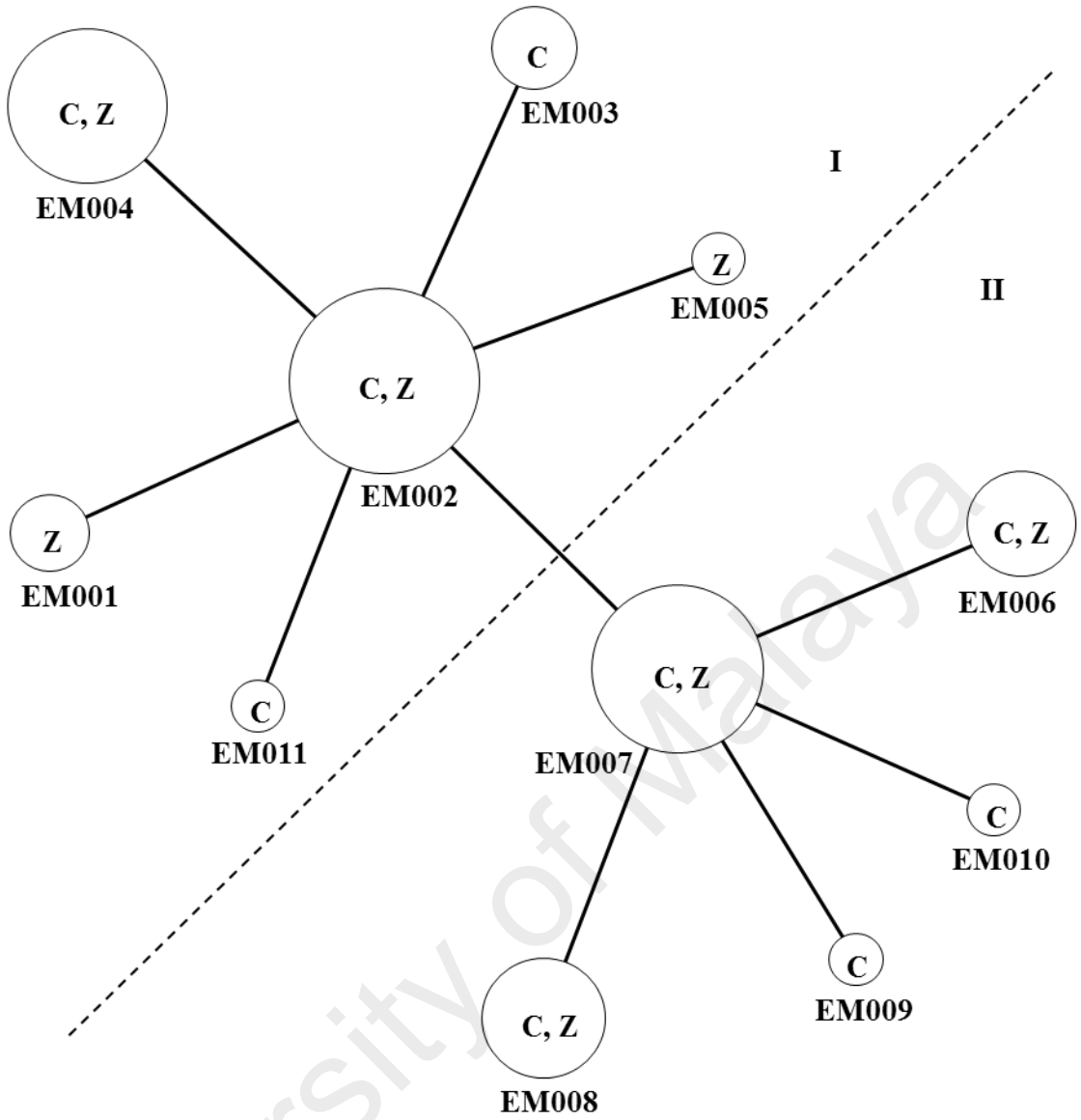



**Figure 3.6:** Minimum spanning tree (MST) for MLVA analysis of the *S.* Enteritidis strains. Each circle denotes an MLVA type. The diameter of the circle corresponds to the number of strains sharing the common MLVA type. The thickness of the lines indicates the allelic differences between the MLVA types. All MLVA types are connected by thick lines, indicating that each MLVA type is an SLV to its immediate neighbour. The sources of the strains are represented in letters, whereby 'Z' represents zoonotic strains, 'C' represents clinical strains, and 'C, Z' represents a mixture of clinical and zoonotic strains. The roman numerals I and II indicate strain clusters.

### 3.4.3 Correlation between PFGE and MLVA cluster analysis

The *S.* Typhimurium and *S.* Enteritidis strains were grouped into clusters based on their genetic similarity as determined by PFGE or MLVA typing. For *S.* Typhimurium, PFGE cluster was defined as a group of strains with pattern similarity of F = 0.80 and above; while in *S.* Enteritidis, strains with more than F = 0.85 pattern similarity were grouped into one PFGE cluster. Meanwhile, the definition of MLVA clusters was restricted to strains with identical MLVA type. The PFGE and MLVA typing of *S.* Typhimurium strains showed a higher concordance compared to that of *S.* Enteritidis. In *S.* Typhimurium, the probability of the strains in any given PFGE cluster having identical MLVA type was relatively higher ($W_{MLVA \rightarrow PFGE}$ = 0.54) compared to *S.* Enteritidis ($W_{MLVA \rightarrow PFGE}$ = 0.19). Meanwhile, for any given MLVA type, the probability of the strains being classified into a particular PFGE cluster was relatively high in both *S.* Typhimurium ($W_{PFGE \rightarrow MLVA}$ = 0.80) and *S.* Enteritidis ($W_{PFGE \rightarrow MLVA}$ = 0.76). The probability whereby the differences in the W-values arise by chance was less than 0.1 %. In other words, *Salmonella* strains of a particular MLVA type were most probably grouped in the same PFGE cluster with a 95 % confidence interval; but strains in a particular PFGE cluster might not necessarily share identical MLVA type. Thus, MLVA complemented PFGE in subtyping *S.* Typhimurium and *S.* Enteritidis by further discriminating highly similar PFGE pulsotypes.

## 3.5 Discussion

Non-typhoidal salmonellosis is one of the common foodborne infections in both developed and developing countries, including Malaysia. Considering the public health importance of *S.* Typhimurium and *S.* Enteritidis in Malaysia, there was a need to investigate the genetic relationship among strains originated from animal, food, and human sources in order to elucidate the possible transmission routes of these pathogens. The data generated showed that a genetically homogeneous *S.* Typhimurium population

has persisted in Malaysia, and a very limited genetic diversity was also observed among the *S.* Enteritidis strains.

Results showed that MLVA was less discriminative (D = 0.76) than PFGE (D = 0.99) in subtyping *S.* Typhimurium strains. Despite that, there was a correlation observed among the strain clusters determined by these two molecular typing methods. This is supported by the observation that the same strains grouped into a single PFGE cluster also exhibit SLVs based on MLVA typing. For example, PFGE cluster I coincides with the SLVs TM001, TM002, TM003, and TM004; while TM005 and TM006 (SLVs) corresponded with the PFGE cluster V. Both typing methods suggested a rather homogeneous *S.* Typhimurium population circulated within Malaysia from 2002 to 2009, showing close genetic relationship with the earlier strains (isolated in 1970 and 1998). The MST constructed (Figure 3.3) did not show clear branching of strains exclusively by a single source, location, or year of isolation in the overall distribution of the MLVA types. Because MLVA is reported to be sensitive to the origins of the strains (Lindstedt *et al.*, 2004), this observation further supports the inference made on the genetic homogeneity of local *S.* Typhimurium strains in Malaysia. This is probably due to the fact that non-typhoidal salmonellosis caused by *S.* Typhimurium is endemic in this region (Douadi *et al.,* 2010; Jegathesan, 1984; Tiong *et al.*, 2010). The close genetic relationship between clinical, food, and zoonotic strains was seen in the sharing of similar allelic profiles and pulsotypes among the strains. This suggested that the *S.* Typhimurium from farm animals may have infected humans, with contaminated food as the vehicle of transmission.

The Nei's diversity index indicated that the STTR5, STTR6, STTR9, and STTR10pl showed lower polymorphism in the *S.* Typhimurium population in Malaysia, compared to the values reported by Lindstedt *et al.* (2004). The absence of STTR6 and STTR10pl

in most strains (81.0 % and 76.2 %, respectively) further contributed to the low diversity. To rule out the possibility of false negative for these two loci, DNA hybridization was carried out and the absence of STTR6 and STTR10pl in the *S.* Typhimurium strains was confirmed. The low prevalence of these loci may be attributed to the association of STTR6 with Gifsy-1 prophage and STTR10pl with the pSLT plasmid (Lindstedt *et al.,* 2004). Therefore, postulation was made such that most Malaysian *S.* Typhimurium strains lacked these two genetic entities and future study is needed to elucidate this. Hence there seems to be a difference between Asian and European strains. Notably, strains from South-east Asia (Thailand, Malaysia and Indonesia) were 30 % negative for STTR6 and 97 % negative for STTR10pl (B. A. Lindstedt, personal communication) whereas among the Norway strains, only 16 % were negative for STTR6 and 48 % were negative for STTR10pl (Lindstedt *et al.,* 2004). On the contrary, STTR3 showed higher diversity in Malaysian *S.* Typhimurium strains. Nevertheless, the overall low diversity indices (0.26 - 0.70) for all five VNTR loci suggested that these loci are less discriminative if used as molecular markers for subtyping *S.* Typhimurium in this geographical region. The inclusion of other VNTR loci that are yet to be examined may prove useful for more precise strain mapping.

The *S.* Enteritidis strains isolated from both humans and animals in Malaysia were genetically highly similar. The analysis of the genetic relationship among the strains isolated from different sources suggested that a *S.* Enteritidis population with limited genetic diversity had circulated within Malaysia between year 2003 and 2008. Obvious temporal clustering of the *S.* Enteritidis strains was not observed. Visual inspection of *Xba*I-digested PFGE patterns showed that *S.* Enteritidis strains in Malaysia were similar to those circulating in other countries, thus supported the observation on the worldwide clonal distribution of this pathogen (De Oliveira *et al.,* 2010; Ling & Wang, 2001; Pang *et al.,* 2007). Similar studies had also reported on the spread of genetically identical

clones of *S.* Enteritidis within this geographical region (Bakeri *et al.*, 2003; Boonmar *et al.*, 1998; Ling & Wang, 2001). Furthermore, the sharing of a particular MLVA type or pulsotype among strains of clinical and zoonotic origins suggested that animals do play an important role in dissemination of *Salmonella* through the food chain (Angulo *et al.*, 2004). In fact, the attempt to minimize *Salmonella* infections in humans requires the elimination of *Salmonella* infections in animals, and the prevention of transmission of environmental origins (Silva *et al.*, 2014).

Based on the five VNTR loci assessed, MLVA (D = 0.82) was less discriminative than PFGE (D = 0.96) in the subtyping of *S.* Enteritidis. The loci SENTR7 and SE7 consisted of only a single allele, despite their reported higher allelic polymorphisms (Cho *et al.*, 2007; Malorny *et al.*, 2008). However, considering the homogeneous nature of the local *S.* Enteritidis strains, such uniformity was to be expected. Furthermore, the presence of some concordance between the two typing methods agreed on the genetic homogeneity of *S.* Enteritidis in Malaysia. Interestingly, the clustering of the SLVs based on MLVA was found related to antimicrobial resistance patterns of the *S.* Enteritidis strains to some extent. This notion is supported by the observation that the majority (72 %) of the strains in cluster I was resistant to nalidixic acid; meanwhile cluster II was dominated (62 %) by strains resistant to tetracycline and sulphonamide. However, similar observation was not made among the *S.* Typhimurium strains.

In this study, four *S.* Enteritidis strains isolated from HIV-infected patients were available for analysis. *S.* Enteritidis is the most common endemic serovar causing non-typhoidal salmonellosis in humans (Thong *et al.*, 2011), and is frequently isolated from immunosuppressed patients in Malaysia (Dhanoa & Fatt, 2009). Both typing methods revealed that these strains were genetically indistinguishable from other clinical and zoonotic *S.* Enteritidis strains. This finding agreed with the suggestion that the bacterial

properties of non-typhoidal *Salmonella* isolated from HIV-patient were not necessarily distinctly different from strains of other origins (Preziosi *et al.*, 2012).

The results of PFGE typing of the *S.* Typhimurium and *S.* Enteritidis strains in this study could be interpreted with confidence ($D \geq 0.90$), according to Hunter & Gaston's criterion (1988). Despite the technical difficulties mentioned in the literature review (section 3.2), PFGE is sufficiently discriminative in subtyping the *Salmonella* strains in Malaysia, more so in the *S.* Typhimurium ($D = 0.99$) compared to the genetically clonal *S.* Enteritidis ($D = 0.96$) population. Nonetheless, *Salmonella* strains of identical pulsotype but distinctive allelic profiles were occasionally noticed. This is not surprising as PFGE and MLVA assess different aspects of the genomes. Based on the W-values calculated, MLVA typing was found to be able to further discriminate highly similar PFGE pulsotypes. This observation is in agreement with Best *et al.* (2007), who suggested that MLVA could enhance the discriminatory ability of PFGE, based on their study on *S.* Typhimurium strains isolated from animals and humans. Thus, joint analyses of MLVA and PFGE may provide further insights into the genetic relationships among the bacterial strains. Most of the time, minor differences in PFGE banding patterns or single allelic variations in MLVA types are crucial for strain differentiation especially in cases of outbreak investigations. Therefore, it is important to use multiple-typing approach for detailed mapping of bacterial strains, with combined analyses involving accurate epidemiological information (Hopkins *et al.*, 2007).

One major disadvantage of MLVA subtyping of *Salmonella* strains is the serovar-specificity of this method. A total of 58 MLVA markers have been developed to subtype *Salmonella* strains, whereby different sets of VNTR loci were used to subtype different serovars (Kruy *et al.*, 2011). Some VNTR loci ($n = 15$), are universal among different *Salmonella* serovars (Kruy *et al.*, 2011). However, other VNTR loci are unique

to a certain serovar. On the other hand, PFGE is universal and requires only two or less restriction enzymes (*Xba*I and *Avr*II) for the analysis of all *Salmonella* serovars. However, efforts have been made to develop an MLVA subtyping scheme that can differentiate multiple serovars of *Salmonella*, with promising results (Van Cuyck *et al.*, 2011). Van Cuyck and colleagues (2011) selected 11 VNTR loci from the *S.* Typhimurium LT2 genome and developed an MLVA scheme to differentiate *Salmonella enterica* strains isolated from humans, animals, and food. The MLVA scheme used has successfully identified 31 serovars, with strains from the same serovar tightly clustered in the phylogeny tree generated, except for serovars Derby, Schwarzengrund, Stanley, and Weltevreden (Van Cuyck *et al.*, 2011). Hence, MLVA can be a promising tool for rapidly identifying and genotyping *Salmonella* strains from a sample pool consisting of multiple serovars.

In recent years, there is a drive to apply whole genome sequencing (WGS) for molecular epidemiology studies (Salipante *et al.,* 2015). WGS typing has become the preference among disease surveillance facilities in the developed countries (Revez *et al.*, 2017). Although WGS is the ultimate bacterial subtyping tool as it can reveal minor genetic variations to the scale of single nucleotide change within the bacterial genome (Salipante *et al.,* 2015), nevertheless, it is still not feasible to be applied fully in low-resourced laboratories in developing countries including Malaysia. WGS is still relatively too expensive to be applied as a routine bacterial subtyping tool, compared to other molecular typing methods. Lack of funding, expertise, and facilities are the main prohibitive factors that lead to the limited application of WGS in bacterial epidemiological studies and outbreak investigations. Therefore, a multiple typing approach encompassing the gold standard PFGE and the high throughput MLVA for detailed strain characterization is suggested for future epidemiological investigations. Moreover, these two subtyping methods are inexpensive and require less sophisticated

equipment compared to the WGS typing, hence are suitable to be used in laboratories with limited funding in the developing countries, including Malaysia.

## 3.6 Conclusion

In conclusion, *S.* Typhimurium and *S.* Enteritidis strains isolated from animals, food, and humans in Malaysia were genetically homogeneous. The close genetic proximity between the clinical, food, and zoonotic *Salmonella* strains suggested that animals might be a possible source of these pathogens causing salmonellosis in humans, with contaminated food as the transmission vector. Although MLVA was less discriminative than PFGE, the existence of some correlation between these two typing methods suggests that MLVA complemented PFGE by providing additional information on the genetic relatedness of the strains. Results of this study may improve understanding of the genetic relationship and the transmission dynamics of *S.* Typhimurium and *S.* Enteritidis in Malaysia, thus aiding in stimulating the implementation of regulations and effective control measures for disease prevention.

# CHAPTER 4: ANTIMICROBIAL RESISTANCE PATTERNS AND QUINOLONE RESISTANCE MECHANISMS

## 4.1 Introduction

Antimicrobial resistance among bacteria has been a major threat to public health worldwide. The ability of bacteria to acquire resistance to multiple antibiotics and the increasing resistance to last-resort drugs have raised concern on the beginning of post-antibiotic era, whereby bacterial infections are no longer treatable with currently-available antibiotics (Falagas & Bliziotis, 2007). In developing countries, including Southeast Asian region, unregulated usage of antibiotics has led to emergence and persistence of antimicrobial resistance among the bacterial populations, including *Salmonella*. The emergence of quinolone and fluoroquinolone resistance among *Salmonella* population is also of importance, as these drugs are primarily used in the treatment of invasive salmonellosis in humans (Klochko & Wallace, 2014). Previous studies carried out in Malaysia were focused on determining the antimicrobial resistance patterns of *Salmonella*, including the resistance towards quinolones and fluoroquinolones, as summarized in Table 2.4. However, none of these studies attempted to elucidate the mechanisms and the long-term development of quinolone resistance among *Salmonella*.

The objective of this study was to characterize the antimicrobial resistance patterns among the two most important non-typhoidal *Salmonella* serovars in Malaysia (*S.* Typhimurium and *S.* Enteritidis), and to investigate the major mechanisms of quinolone resistance among the strains.

## 4.2 Literature review

Antimicrobial resistance among bacteria has become a common phenomenon in most parts of the world. The high background burden of infectious diseases in developing

countries and the easy access to antibiotics in most regions may have attributed to the increasing prevalence of antimicrobial-resistant bacteria (Ao *et al.*, 2015; Gelband *et al.*, 2015). Moreover, the increasing international trade and travel has led to epidemic spread of antimicrobial-resistant pathogens on global scale. Non-typhoidal *Salmonella* is recognized as one of the community-acquired pathogens of global concern, besides *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Shigella* spp., and *Neisseria gonorrhoeae* (Gelband *et al.*, 2015). The high rates of resistance of these organisms to first- and second-line drugs have increased the reliance on last-resort drugs. In 2013, the US CDC had published a report on antimicrobial resistance threat in US, categorizing drug-resistant *Salmonella* (Typhi and non-typhoidal *Salmonella*) as a serious threat that requires prompt and sustained action to curb the problem (CDC, 2013b). Approximately 8 % of the non-typhoidal salmonellosis cases reported each year in US was caused by drug-resistant *Salmonella*. The main concern among the non-typhoidal *Salmonella* serovars is their resistance to ceftriaxone, ciprofloxacin, and multi-drug resistance (MDR) (CDC, 2013b). In Malaysia, the rates of resistance of *Salmonella* to several selected antibiotics are monitored by the Ministry of Health (MOH) Malaysia. Over the past decade, clinical *Salmonella* strains in Malaysia showed a consistently high rate of resistance towards the first-line drugs used in the treatment of invasive salmonellosis, namely ampicillin and sulfamethoxazole-trimethoprim (Table 2.2). Increasing resistance towards amikacin, chloramphenicol, and ciprofloxacin was documented among the *Salmonella* strains too. Nonetheless, cephalosporins and carbapenems remain effective as second-line and last-resort drugs for the treatment of invasive salmonellosis due to low rates of resistance.

Increased prevalence of quinolone-resistant *Salmonella* has been documented worldwide, including the Southeast Asian regions (World Health Organization, 2014). In fact, previous studies in Malaysia have documented an increase in nalidixic acid

resistance over the past decade, with a resistance rate averaged around 30 % among the *Salmonella* strains isolated from various sources (Adzitey *et al.*, 2012; Cheah *et al.*, 2008; Douadi *et al.*, 2010; Thong *et al.*, 2011; Thong & Modarressi, 2011; Tiong *et al.*, 2010; Tunung *et al.*, 2007). The bacterial resistance towards ciprofloxacin, a fluoroquinolone antibiotic used in treatment of invasive salmonellosis, is monitored by MOH Malaysia. Based on the data obtained from MOH reports, *Salmonella* resistance to ciprofloxacin, although low, is slowly on the rise since 2003, with resistance rates ranging from 0.7 % to 2.0 % (Table 2.2). The emergence of the quinolone-resistant *Salmonella* strains could have reduced the efficacy of antibiotic treatment for invasive salmonellosis.

Four types of mechanisms leading to quinolone resistance have been identified so far: target enzyme alteration, bacterial cell membrane alteration, over-expression of efflux pumps, and plasmid-mediated quinolone-resistant determinants (Guan *et al.*, 2013). DNA gyrase and topoisomerase IV are the two important enzymes that play an essential role in bacterial DNA replication, and are the primary targets of quinolone and fluoroquinolone antibiotics in their inhibitory actions. Both gyrase and topoisomerase IV are tetrameric enzymes, each encoded by two pairs of genes (*gyrA* and *gyrB* for DNA gyrase; *parC* and *parE* for topoisomerase IV). Mutations in the quinolone resistance-determining regions (QRDRs) of these genes may alter the conformation of the active sites on the enzymes, thus preventing the binding and subsequent inhibitory actions of quinolones. Target enzyme alteration is the most common resistance mechanism found in *Salmonella* strains isolated worldwide (Guan *et al.*, 2013; Rodr ́guez-Mart ́nez *et al.*, 2011).

Decreased quinolone uptake by the bacteria can be achieved by alteration of the cell membrane and increased expression of efflux pumps. *Salmonella* strains may gain

resistance to quinolones through decreased expression of porins, and/or increased expression of non-specific, energy-dependent efflux systems such as the AcrAB-TolC efflux pumps (Guan *et al.*, 2013; Ruiz, 2003). However, these mechanisms produce only low level of resistance towards quinolones, and are only clinically important when occur concurrently with alterations of target enzymes such as DNA gyrase (Guan *et al.*, 2013; Rodríguez-Martínez *et al.*, 2011; Ruiz *et al.*, 2012).

Decreased susceptibility to fluoroquinolones in non-typhoidal *Salmonella* is often mediated by plasmid-borne resistance genes (*qnr*). Since Martínez-Martínez and colleagues (1998) reported the existence of plasmid-mediated quinolone resistance, many studies have identified *qnr* genes from human isolates of *Salmonella* (Sjölund-Karlsson *et al.*, 2009; Strahilevitz *et al.*, 2009; Taguchi *et al.*, 2009). However, past surveillance showed that the occurrence of plasmid-mediated quinolone resistance in *Salmonella* remains low (Asai *et al.*, 2010; Lunn *et al.*, 2010). Although not commonly found in *Salmonella* and confer only low level of resistance towards quinolones, plasmid-mediated quinolone-resistant determinants poses potential risk to public health. These resistance determinants are found on transferable plasmids, thus may contribute to the rapid spread of quinolone resistance among *Salmonella* strains (Poirel *et al.*, 2012). The Qnr peptides which originated from the environment is often found in animal reservoirs, and may be a potential threat to human should the pathogens harbouring this quinolone-resistant determinant spread to human population (Poirel *et al.*, 2012).

## 4.3 Methodology

### 4.3.1 Bacterial strains

A total of 195 *Salmonella* strains (84 *S.* Typhimurium and 111 *S.* Enteritidis strains), genotyped in previous chapter (Chapter 3), were examined for their antimicrobial

resistance patterns. The background information of the strains are provided in Appendix A (*S.* Typhimurium) and Appendix B (*S.* Enteritidis).

### 4.3.2 Antimicrobial susceptibility test

Antimicrobial susceptibility of the strains was tested by using Kirby-Bauer disk-diffusion method (Cavalieri *et al.,* 2005), and the zones of inhibition obtained were interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines (2015). The strains were screened for resistance towards 16 antimicrobial agents, namely amoxicillin-clavulanic acid (AMC, 30 µg), ampicillin (AMP, 10 µg), cefotaxime (CTX, 30 µg), ceftazidime (CAZ, 30 µg), ceftriaxone (CRO, 30 µg), cephalothin (CEF, 30 µg), chloramphenicol (CHL, 30 µg), ciprofloxacin (CIP, 5 µg), compound sulphonamide (SUL, 300 µg), gentamicin (GEN, 10 µg), kanamycin (KAN, 30 µg), nalidixic acid (NAL, 30 µg), streptomycin (STR, 10 µg), tetracycline (TET, 30 µg), trimethoprim (TMP, 5 µg), and trimethoprim-sulfamethoxazole (SXT, 25 µg) (Oxoid$^{TM}$, United Kingdom). The antimicrobial agents were selected to represent the classes of antibiotics that are often prescribed in the treatment of invasive salmonellosis in humans. The *Escherichia coli* ATCC25922 strain was used as the control strain for the antimicrobial susceptibility test. Next, the multiple antibiotic resistant (MAR) index for each strain was calculated. The MAR index of single isolate is the number of antimicrobial agents to which the isolate was resistant divided by the total number of antimicrobial agents to which the isolate was exposed (Krumperman, 1983).

### 4.3.3 Minimum inhibitory concentration of nalidixic acid and ciprofloxacin

All confirmed quinolone-resistant *Salmonella* strains (resistant to nalidixic acid and/or ciprofloxacin) were further subjected to Epsilometer test (Etest; BioM ́erieux, Marcy l'Etoile, France) to determine the minimum inhibitory concentration (MIC) values of nalidixic acid (NAL) and ciprofloxacin (CIP). The MIC breakpoints for the

resistant strains are $MIC_{NAL} \geq 16$ µg/mL and $MIC_{CIP} \geq 1$ µg/mL according to CLSI guidelines (2015). The MIC breakpoint of an antibiotic is a chosen concentration of the antibiotic, at which a bacterial strain is defined as resistant to the antibiotic if it has an MIC value higher than or equal to the breakpoint value.

### 4.3.4 Detection of point mutations in gyrase and topoisomerase IV genes

All strains which were confirmed as quinolone-resistant were then examined for point mutations in the gyrase and topoisomerase IV genes. The QRDRs of *gyrA*, *gyrB*, *parC* and *parE* were amplified via PCR using previously described primers (Table 4.1). Agarose gel electrophoresis was then carried out to confirm the presence of amplicons prior to DNA sequencing. Next, the PCR products were purified using Wizard® SV Gel and PCR Clean-up System (Promega, Madison, USA) and sequenced (First BASE Laboratories, Selangor, Malaysia). Mutations in the target genes were determined by comparison with the wild-type sequence of the respective genes in *S.* Typhimurium LT2 (GenBank accession no.: NC_003197). Sequence alignment was done using BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/).

### 4.3.5 Detection of plasmid-borne *qnrA*, *qnrB*, and *qnrS* genes

The presence of plasmid-borne *qnrA*, *qnrB* and *qnrS* genes in all of the quinolone-resistant *Salmonella* strains were detected via PCR amplification using previously reported primers (Table 4.1). Agarose gel electrophoresis was performed to confirm the presence of the *qnr* genes. Next, the amplified PCR products were purified using QIAQuick Gel Extraction kit (Qiagen, Hilden, Germany) and sequenced (First BASE Laboratories, Selangor, Malaysia). Sequence alignment was done using BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/) to identify the *qnr* variant. The nomenclature of the *qnr* variants was adapted from an online database (http://www.lahey.org/qnrStudies/) (Jacoby *et al.*, 2008).

**Table 4.1:** PCR primers and protocol for quinolone resistance mechanisms study

| Target gene | Primer | Primer sequence (5'-3') | Amplicon size (bp) | Modified PCR amplification protocol | Reference |
|---|---|---|---|---|---|
| ***gyrA*** | gyrA.Sal1 | AAATCTGCCCGTGTCGTTGGT | 344 | PCR amplification was done in a 25 µL monoplex reaction mixture, containing 1× colourless GoTaq Flexi Buffer, 1.5 mmol/L $MgCl_2$, 200 µmol/L dNTP mix, 0.3 µmol/L of each primer pair, 1 U *Taq* DNA polymerase (Promega, Madison, USA), and approximately 100 ng of bacterial genomic DNA. The PCR reaction mixtures were first incubated at 94 ℃ for 2 min; followed by 25 cycles of 94 ℃ for 30 s, 60-63 ℃ (annealing temperature varies for each gene) for 30 s, and 72 ℃ for 45 s; with a final extension step of 72 ℃ for 5 min. The appropriate annealing temperatures for *gyrA*, *gyrB*, *parC*, and *parE* are 60 ℃, 60 ℃, 63 ℃, and 62 ℃, respectively. | Fàbrega *et al.* (2009) |
| | gyrA.Sal2 | GCCATACCTACTGCGATACC | | | |
| ***gyrB*** | SgyrB.1 | GAATACCTGCTGGAAAACCCAT | 446 | | |
| | SgyrB.2 | CGGATGTGCGAGCCGTCGACGTCCGC | | | |
| ***parC*** | parC.Sal1 | AAGCCGGTACAGCGCCGCATC | 395 | | |
| | parC.Sal2 | GTGGTGCCGTTCAGCAGG | | | |
| ***parE*** | SparE.1 | CCTGCGGCCCGGCGTTGCCGGGG | 465 | | |
| | SparE.2 | CGCCCGCCTTCTCTTCTTCCGTCAGCGCG | | | |

**Table 4.1**, continued.

| Target gene | Primer | Primer sequence (5'-3') | Amplicon size (bp) | Modified PCR amplification protocol | Reference |
|---|---|---|---|---|---|
| ***qnrA*** | QnrAm-F | AGAGGATTTCTCACGCCAGG | 580 | The PCR assay was carried out in a 25 µL monoplex reaction mixture, containing 1× colourless GoTaq Flexi Buffer, 1.40 mmol/L Magnesium chloride, 140 µmol/L deoxynucleoside triphosphate mix, 0.30 µmol/L of each primer pair, 0.5 U *Taq* DNA polymerase (Promega, Madison, USA), and 100 ng of bacterial genomic DNA. The PCR thermal cycling conditions are as follows: pre-denaturation incubation of DNA at 95 ℃ for 10 min, followed by 30 cycles of denaturation at 95 ℃ for 1 min, annealing at 56 ℃ for 45 s, extension at 72 ℃ for 1 min; and a final extension at 72 ℃ for 10 min. | Cattoir *et al.* (2007) |
| | QnrAm-R | TGCCAGGCACAGATCTTGAC | | | |
| ***qnrB*** | QnrBm-F | GGMATHGAAATTCGCCACTG | 264 | | |
| | QnrBm-R | TTTGCYGYYCGCCAGTCGAA (M = A or C; H = A or C or T; Y = C or T) | | | |
| ***qnrS*** | QnrSm-F | GCAAGTTCATTGAACAGGGT | 428 | | |
| | QnrSm-R | TCTAAACCGTCGAGTTCGGCG | | | |

## 4.4 Results

### 4.4.1 Antimicrobial resistance patterns of *S.* Typhimurium and *S.* Enteritidis strains

Twenty-seven out of the 84 *S.* Typhimurium strains screened were susceptible to all 16 antimicrobial agents tested. The resistotypes, MDR status, and MAR index for each *S.* Typhimurium strain are listed in Table 4.2. High rates of resistance to AMP (25 %), CEF (26 %), SUL (49 %), TET (51 %), and STR (52 %) were observed among the strains. Approximately 50 % of the *S.* Typhimurium strains were MDR (defined as strains resistant to three or more different classes of antimicrobial agents). Approximately half of the *S.* Typhimurium strains isolated from each source (clinical, 52 %; food, 45 %; zoonotic, 50 %) exhibited MDR phenotype. The MDR strains originated mostly from urban and densely-populated areas such as Kuala Lumpur and Selangor. The MAR indices of the resistant *S.* Typhimurium strains spanned a wide range (0.06 - 0.69), exhibiting resistance against one to eleven different antimicrobial agents. Majority of the *S.* Typhimurium (73 %) were in the low risk phase (MAR index < 0.200), according to Krumperman's definition of the risk of bacterial contamination that is potentially hazardous to humans (Krumperman, 1983). A total of 33 resistotypes were identified among the *S.* Typhimurium strains, and were arbitrarily designated as TR01 to TR33. The resistotypes and their corresponding antimicrobial resistance profiles are shown in Table 4.3 Resistotype TR26 was most dominant among the *S.* Typhimurium, showing simultaneous resistance to STR, SUL, and TET (R-type SSuT). Overall, the *S.* Typhimurium strains remained highly susceptible towards third-generation cephalosporins. Less than five percent of the strains tested were resistant to CTX, CAZ, and CRO (Table 4.3). Quinolone resistance was observed, specifically, 14 % of the *S.* Typhimurium strains tested were either resistant or exhibited reduced susceptibility to NAL. None of the *S.* Typhimurium strains were resistant to CIP.

**Table 4.2:** The resistotypes, multi-drug resistance (MDR) status and multiple antibiotic resistant (MAR) index for the 84 *S.* Typhimurium strains

| Strain | Resistotype[a] | MDR status[a] | MAR index |
|---|---|---|---|
| STM001/70 | TR23 | MDR | 0.25 |
| STM002/70 | TR08 | MDR | 0.50 |
| STM003/70 | TR14 | MDR | 0.44 |
| STM004/98 | TR25 | MDR | 0.19 |
| STM006/02 | TR12 | MDR | 0.50 |
| STM007/02 | $ | $ | 0.00 |
| STM008/02 | TR26 | MDR | 0.19 |
| STM009/02 | $ | $ | 0.00 |
| STM010/02 | $ | $ | 0.00 |
| STM011/02 | $ | $ | 0.00 |
| STM012/02 | $ | $ | 0.00 |
| STM013/03 | TR26 | MDR | 0.19 |
| STM016/03 | $ | $ | 0.00 |
| STM017/03 | TR32 | non-MDR | 0.06 |
| STM018/03 | TR31 | non-MDR | 0.06 |
| STM019/03 | TR17 | MDR | 0.19 |
| STM020/03 | $ | $ | 0.00 |
| STM022/03 | TR26 | MDR | 0.19 |
| STM023/03 | TR18 | MDR | 0.38 |
| STM025/04 | TR28 | non-MDR | 0.13 |
| STM026/04 | TR21 | MDR | 0.25 |
| STM027/04 | $ | $ | 0.00 |
| STM028/04 | TR27 | non-MDR | 0.13 |
| STM029/04 | $ | $ | 0.00 |
| STM030/04 | TR09 | MDR | 0.50 |
| STM031/03 | $ | $ | 0.00 |
| STM032/04 | TR22 | MDR | 0.25 |
| STM033/04 | TR11 | MDR | 0.50 |
| STM034/04 | $ | $ | 0.00 |
| STM035/04 | $ | $ | 0.00 |
| STM037/05 | TR26 | MDR | 0.19 |
| STM039/05 | TR33 | non-MDR | 0.06 |
| STM040/05 | TR26 | MDR | 0.19 |
| STM042/05 | TR33 | non-MDR | 0.06 |
| STM043/05 | TR16 | MDR | 0.44 |
| STM045/05 | TR06 | MDR | 0.56 |
| STM046/05 | TR15 | MDR | 0.44 |
| STM047/05 | $ | $ | 0.00 |
| STM048/05 | TR02 | MDR | 0.69 |
| STM049/05 | $ | $ | 0.00 |
| STM052/05 | $ | $ | 0.00 |
| STM053/05 | $ | $ | 0.00 |

**Table 4.2**, continued.

| Strain | Resistotype[a] | MDR status[a] | MAR index |
|---|---|---|---|
| STM054/05 | TR26 | MDR | 0.19 |
| STM055/05 | TR31 | non-MDR | 0.06 |
| STM056/05 | $ | $ | 0.00 |
| STM057/05 | TR26 | MDR | 0.19 |
| STM058/05 | TR04 | MDR | 0.56 |
| STM059/05 | TR29 | non-MDR | 0.06 |
| STM060/05 | $ | $ | 0.00 |
| STM061/05 | TR13 | MDR | 0.50 |
| STM062/06 | TR07 | MDR | 0.56 |
| STM063/06 | $ | $ | 0.00 |
| STM064/07 | TR24 | MDR | 0.19 |
| STM065/07 | TR05 | MDR | 0.56 |
| STM066/07 | TR33 | non-MDR | 0.06 |
| STM067/07 | TR26 | MDR | 0.19 |
| STM068/07 | TR26 | MDR | 0.19 |
| STM069/07 | TR19 | MDR | 0.38 |
| STM070/07 | TR10 | MDR | 0.50 |
| STM071/07 | TR03 | MDR | 0.63 |
| STM072/07 | TR26 | MDR | 0.19 |
| STM073/07 | TR30 | non-MDR | 0.06 |
| STM074/07 | TR26 | MDR | 0.19 |
| STM076/07 | TR26 | MDR | 0.19 |
| STM077/07 | TR26 | MDR | 0.19 |
| STM078/07 | TR27 | non-MDR | 0.13 |
| STM079/07 | TR27 | non-MDR | 0.13 |
| STM080/07 | TR27 | non-MDR | 0.13 |
| STM081/07 | TR26 | MDR | 0.19 |
| STM082/08 | TR20 | MDR | 0.31 |
| STM083/08 | TR30 | non-MDR | 0.06 |
| STM084/08 | TR24 | MDR | 0.19 |
| STM086/08 | TR01 | MDR | 0.69 |
| STM087/08 | TR24 | MDR | 0.19 |
| STM088/08 | $ | $ | 0.00 |
| STM089/08 | TR13 | MDR | 0.50 |
| STM090/09 | $ | $ | 0.00 |
| STM091/09 | TR32 | non-MDR | 0.06 |
| STM092/09 | $ | $ | 0.00 |
| STM093/09 | $ | $ | 0.00 |
| STM094/09 | $ | $ | 0.00 |
| STM095/09 | $ | $ | 0.00 |
| STM096/09 | $ | $ | 0.00 |
| STM097/09 | $ | $ | 0.00 |

[a] $, sensitive to all antimicrobial agents tested

**Table 4.3:** Antimicrobial resistance profiles of *S.* Typhimurium strains

| Resistotype | Resistance profile[a] | | | | | | | | | | | | | | | No. of isolates |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **TR01** | AMC | AMP | CEF | - | CAZ | - | CHL | - | GEN | KAN | STR | TET | SXT | SUL | - | 1 |
| **TR02** | AMC | AMP | CEF | - | - | - | CHL | NAL | - | KAN | STR | TET | SXT | SUL | TMP | 1 |
| **TR03** | - | AMP | CEF | - | - | - | CHL | NAL | GEN | KAN | STR | TET | SXT | SUL | - | 1 |
| **TR04** | AMC | AMP | CEF | CTX | CAZ | CRO | - | - | - | - | STR | TET | - | SUL | - | 1 |
| **TR05** | - | AMP | CEF | - | - | - | CHL | - | GEN | KAN | STR | TET | SXT | SUL | - | 1 |
| **TR06** | - | AMP | CEF | - | - | - | CHL | - | - | KAN | STR | TET | SXT | SUL | TMP | 1 |
| **TR07** | - | AMP | - | - | - | - | CHL | - | GEN | KAN | STR | TET | SXT | SUL | TMP | 1 |
| **TR08** | AMC | AMP | CEF | - | - | - | CHL | - | - | KAN | STR | TET | - | SUL | - | 1 |
| **TR09** | AMC | AMP | CEF | CTX | CAZ | CRO | CHL | - | - | - | - | - | - | SUL | - | 1 |
| **TR10** | - | AMP | CEF | - | - | - | CHL | - | - | KAN | STR | TET | SXT | SUL | - | 1 |
| **TR11** | - | AMP | CEF | CTX | CAZ | CRO | - | - | - | - | STR | TET | - | SUL | - | 1 |
| **TR12** | - | AMP | CEF | - | - | - | - | NAL | - | - | STR | TET | SXT | SUL | TMP | 1 |
| **TR13** | - | AMP | - | - | - | - | CHL | - | - | KAN | STR | TET | SXT | SUL | TMP | 2 |
| **TR14** | AMC | AMP | CEF | - | - | - | CHL | - | - | KAN | - | TET | - | SUL | - | 1 |
| **TR15** | AMC | - | CEF | - | - | - | - | - | - | KAN | STR | TET | - | SUL | TMP | 1 |
| **TR16** | - | AMP | - | - | - | - | - | NAL | - | - | STR | TET | SXT | SUL | TMP | 1 |
| **TR17** | - | AMP | - | - | - | - | CHL | - | - | KAN | STR | TET | SXT | - | - | 1 |
| **TR18** | - | AMP | - | - | - | - | CHL | - | - | - | STR | - | SXT | SUL | TMP | 1 |
| **TR19** | - | - | CEF | - | - | - | - | - | - | - | STR | TET | SXT | SUL | TMP | 1 |
| **TR20** | - | - | CEF | - | - | - | - | - | - | - | STR | TET | - | SUL | TMP | 1 |
| **TR21** | AMC | AMP | CEF | - | - | - | - | - | - | - | - | - | - | SUL | - | 1 |
| **TR22** | - | AMP | - | - | - | - | - | NAL | GEN | - | - | TET | - | - | - | 1 |
| **TR23** | - | - | CEF | - | - | - | - | - | - | - | STR | TET | - | SUL | - | 1 |

**Table 4.3**, continued.

| Resistotype | Resistance profile[a] | | | | | | | | | | | | | | | No. of isolates |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **TR24** | - | - | CEF | - | - | - | - | - | - | - | STR | TET | - | - | - | 3 |
| **TR25** | - | - | - | - | - | - | - | - | GEN | - | STR | TET | - | - | - | 1 |
| **TR26** | - | - | - | - | - | - | - | - | - | - | STR | TET | - | SUL | - | 14 |
| **TR27** | - | - | - | - | - | - | - | - | - | - | STR | - | - | SUL | - | 4 |
| **TR28** | - | - | - | - | - | - | - | - | - | - | - | TET | - | SUL | - | 1 |
| **TR29** | - | AMP | - | - | - | - | - | - | - | - | - | - | - | - | - | 1 |
| **TR30** | - | - | CEF | - | - | - | - | - | - | - | - | - | - | - | - | 2 |
| **TR31** | - | - | - | - | - | - | - | NAL | - | - | - | - | - | - | - | 2 |
| **TR32** | - | - | - | - | - | - | - | - | - | - | STR | - | - | - | - | 2 |
| **TR33** | - | - | - | - | - | - | - | - | - | - | - | TET | - | - | - | 3 |
| **$** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 27 |

[a] The resistance profiles excluded antimicrobial agents that exhibited intermediate resistance. AMC, amoxicillin-clavulanic acid; AMP, ampicillin; CEF, cephalothin; CTX, cefotaxime; CIP, ciprofloxacin; CAZ, ceftazidime; CHL, chloramphenicol; CRO, ceftriaxone; GEN, gentamicin; KAN, kanamycin; STR, streptomycin; TET, tetracycline; SXT, trimethoprim-sulfamethoxazole; NAL, nalidixic acid; SUL, compound sulphonamides; TMP, trimethoprim; $, sensitive to all antimicrobial agents tested

Ninety-five (86 %) out of 111 *S.* Enteritidis strains exhibited resistance to at least one antimicrobial agent. Resistance to NAL (45 %), TET (41 %), AMP (33 %), SUL (29 %), SXT (23 %), and TMP (23 %) were observed most frequently. All *S.* Enteritidis strains were susceptible to CIP. Similar to the *S.* Typhimurium, most of the *S.* Enteritidis strains were susceptible to cephalosporins, with a low resistance rate of 11 %, 2 %, 2 %, and 3 % to CEF, CTX, CAZ, and CRO, respectively. Resistance to CHL (1 %), GEN (1 %), KAN (2 %), STR (3 %), and AMC (6 %) was less frequently observed too. Based on their antimicrobial resistance profiles, the 95 antimicrobial-resistant *S.* Enteritidis strains were grouped into 23 resistotypes, arbitrarily designated as ER01 to ER23. The most frequently encountered resistotypes were ER14 (simultaneously resistant to AMP and NAL) and ER08 (simultaneously resistant to SUL, SXT, TET, and TMP); occurring in 23 % of the resistant *S.* Enteritidis strains. Approximately 39 % (n = 43) of the *S.* Enteritidis strains screened for antimicrobial resistance was MDR. The MAR indices for the resistant strains ranged from 0.06 to 0.38, exhibiting resistance against one to six different antimicrobial agents. Most of the *S.* Enteritidis strains (73 %) were in the low risk phase (MAR index < 0.200). The resistotypes, MDR status, and MAR index for each *S.* Enteritidis strain are shown in Table 4.4. The details on resistotypes and their corresponding antimicrobial resistance profiles are shown in Table 4.5.

**Table 4.4:** The resistotypes, multi-drug resistance (MDR) status, and multiple antibiotic resistant (MAR) index for the 111 *S.* Enteritidis strains

| Strain | Resistotype[a] | MDR status[a] | MAR index |
|---|---|---|---|
| SE 001/03 | ER14 | non-MDR | 0.13 |
| SE 002/03 | ER14 | non-MDR | 0.13 |
| SE 003/03 | ER08 | MDR | 0.25 |
| SE 004/03 | ER14 | non-MDR | 0.13 |
| SE 005/03 | ER14 | non-MDR | 0.13 |
| SE 006/03 | ER08 | MDR | 0.25 |
| SE 007/03 | ER23 | non-MDR | 0.06 |
| SE 008/03 | ER22 | non-MDR | 0.06 |
| SE 009/03 | $ | $ | 0.00 |
| SE 010/03 | $ | $ | 0.00 |
| SE 011/03 | ER08 | MDR | 0.25 |
| SE 012/03 | ER20 | non-MDR | 0.06 |
| SE 013/03 | ER20 | non-MDR | 0.06 |
| SE 014/03 | ER22 | non-MDR | 0.06 |
| SE 015/03 | ER20 | non-MDR | 0.06 |
| SE 016/03 | ER22 | non-MDR | 0.06 |
| SE 017/03 | ER22 | non-MDR | 0.06 |
| SE 018/03 | $ | $ | 0.00 |
| SE 019/03 | $ | $ | 0.00 |
| SE 020/03 | $ | $ | 0.00 |
| SE 021/03 | ER22 | non-MDR | 0.06 |
| SE 022/03 | ER14 | non-MDR | 0.13 |
| SE 023/03 | ER08 | MDR | 0.25 |
| SE 024/03 | $ | $ | 0.00 |
| SE 025/03 | ER08 | MDR | 0.25 |
| SE 026/03 | ER14 | non-MDR | 0.13 |
| SE 027/03 | ER14 | non-MDR | 0.13 |
| SE 028/03 | ER14 | non-MDR | 0.13 |
| SE 029/03 | $ | $ | 0.00 |
| SE 030/03 | ER05 | MDR | 0.31 |
| SE 031/03 | $ | $ | 0.00 |
| SE 032/03 | ER14 | non-MDR | 0.13 |
| SE 033/03 | ER14 | non-MDR | 0.13 |
| SE 034/03 | $ | $ | 0.00 |
| SE 035/03 | ER21 | non-MDR | 0.06 |
| SE 036/03 | ER04 | MDR | 0.31 |
| SE 037/03 | ER13 | MDR | 0.19 |
| SE 038/03 | ER08 | MDR | 0.25 |
| SE 039/03 | ER12 | MDR | 0.19 |
| SE 040/03 | $ | $ | 0.00 |
| SE 041/03 | ER08 | MDR | 0.25 |
| SE 042/03 | $ | $ | 0.00 |

**Table 4.4**, continued.

| Strain | Resistotype[a] | MDR status[a] | MAR index |
|---|---|---|---|
| SE 043/03 | ER12 | MDR | 0.19 |
| SE 044/03 | ER08 | MDR | 0.25 |
| SE 045/03 | $ | $ | 0.00 |
| SE 046/04 | ER14 | non-MDR | 0.13 |
| SE 047/04 | ER14 | non-MDR | 0.13 |
| SE 048/04 | ER14 | non-MDR | 0.13 |
| SE 049/04 | ER09 | MDR | 0.19 |
| SE 050/04 | ER20 | non-MDR | 0.06 |
| SE 051/04 | ER18 | non-MDR | 0.13 |
| SE 052/04 | ER18 | non-MDR | 0.13 |
| SE 053/05 | ER08 | MDR | 0.25 |
| SE 054/05 | ER09 | MDR | 0.19 |
| SE 055/05 | ER02 | MDR | 0.38 |
| SE 056/05 | ER03 | MDR | 0.38 |
| SE 057/05 | ER08 | MDR | 0.25 |
| SE 058/05 | ER22 | non-MDR | 0.06 |
| SE 059/05 | $ | $ | 0.00 |
| SE 060/05 | ER22 | non-MDR | 0.06 |
| SE 061/05 | ER22 | non-MDR | 0.06 |
| SE 062/05 | $ | $ | 0.00 |
| SE 063/05 | ER08 | MDR | 0.25 |
| SE 064/05 | ER14 | non-MDR | 0.13 |
| SE 065/05 | ER14 | non-MDR | 0.13 |
| SE 066/05 | ER20 | non-MDR | 0.06 |
| SE 067/05 | ER14 | non-MDR | 0.13 |
| SE 068/05 | ER20 | non-MDR | 0.06 |
| SE 069/05 | ER07 | MDR | 0.25 |
| SE 070/05 | ER20 | non-MDR | 0.06 |
| SE 071/05 | ER22 | non-MDR | 0.06 |
| SE 072/05 | ER08 | MDR | 0.25 |
| SE 073/05 | ER14 | non-MDR | 0.13 |
| SE 074/05 | ER14 | non-MDR | 0.13 |
| SE 075/05 | ER09 | MDR | 0.19 |
| SE 076/05 | ER10 | MDR | 0.19 |
| SE 077/05 | ER08 | MDR | 0.25 |
| SE 078/05 | ER14 | non-MDR | 0.13 |
| SE 079/05 | ER23 | non-MDR | 0.06 |
| SE 080/05 | ER14 | non-MDR | 0.13 |
| SE 081/05 | ER14 | non-MDR | 0.13 |
| SE 082/05 | ER22 | non-MDR | 0.06 |
| SE 083/05 | ER08 | MDR | 0.25 |
| SE 084/05 | ER14 | non-MDR | 0.13 |
| SE 085/06 | ER08 | MDR | 0.25 |

**Table 4.4**, continued.

| Strain | Resistotype[a] | MDR status[a] | MAR index |
|---|---|---|---|
| SE 086/06 | ER06 | MDR | 0.25 |
| SE 087/06 | ER08 | MDR | 0.25 |
| SE 088/06 | ER08 | MDR | 0.25 |
| SE 089/06 | ER17 | non-MDR | 0.13 |
| SE 090/06 | ER06 | MDR | 0.25 |
| SE 091/06 | ER08 | MDR | 0.25 |
| SE 092/06 | ER08 | MDR | 0.25 |
| SE 093/06 | ER08 | MDR | 0.25 |
| SE 094/06 | $ | $ | 0.00 |
| SE 095/06 | ER20 | non-MDR | 0.06 |
| SE 096/06 | ER22 | non-MDR | 0.06 |
| SE 097/06 | ER20 | non-MDR | 0.06 |
| SE 098/06 | ER08 | MDR | 0.25 |
| SE 099/06 | ER11 | MDR | 0.19 |
| SE 100/06 | ER08 | MDR | 0.25 |
| SE 101/07 | ER10 | MDR | 0.19 |
| SE 102/07 | ER15 | non-MDR | 0.13 |
| SE 103/07 | ER19 | non-MDR | 0.13 |
| SE 104/07 | ER01 | MDR | 0.38 |
| SE 105/07 | ER10 | MDR | 0.19 |
| SE 106/07 | ER10 | MDR | 0.19 |
| SE 107/07 | ER10 | MDR | 0.19 |
| SE 108/07 | ER16 | non-MDR | 0.13 |
| SE 109/07 | ER10 | MDR | 0.19 |
| SE 110/08 | ER19 | non-MDR | 0.13 |
| SE 111/08 | $ | $ | 0.00 |

[a] $, sensitive to all antimicrobial agents tested

**Table 4.5:** Antimicrobial resistance profiles of *S.* Enteritidis strains

| Resistotype | Resistance profile[a] | | | | | | | | | | | | | | | No. of isolates |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **ER01** | - | AMP | CEF | CTX | CAZ | CRO | - | - | - | NAL | - | - | - | - | - | 1 |
| **ER02** | AMC | AMP | CEF | - | - | - | GEN | KAN | - | NAL | - | - | - | - | - | 1 |
| **ER03** | - | - | - | CTX | - | CRO | - | - | - | - | - | TET | SUL | SXT | TMP | 1 |
| **ER04** | - | - | - | - | CAZ | CRO | - | - | - | - | CHL | TET | SUL | - | - | 1 |
| **ER05** | - | - | CEF | - | - | - | - | - | - | - | - | TET | SUL | SXT | TMP | 1 |
| **ER06** | AMC | AMP | - | - | - | - | - | - | - | NAL | - | - | SUL | - | - | 2 |
| **ER07** | - | - | - | - | - | - | - | - | STR | NAL | - | TET | SUL | - | - | 1 |
| **ER08** | - | - | - | - | - | - | - | - | - | - | - | TET | SUL | SXT | TMP | 22 |
| **ER09** | AMC | AMP | - | - | - | - | - | - | - | NAL | - | - | - | - | - | 3 |
| **ER10** | - | AMP | CEF | - | - | - | - | - | - | NAL | - | - | - | - | - | 6 |
| **ER11** | - | - | - | - | - | - | - | KAN | - | NAL | - | TET | - | - | - | 1 |
| **ER12** | - | - | - | - | - | - | - | - | STR | - | - | TET | SUL | - | - | 2 |
| **ER13** | - | - | - | - | - | - | - | - | - | - | - | TET | SUL | - | TMP | 1 |
| **ER14** | - | AMP | - | - | - | - | - | - | - | NAL | - | - | - | - | - | 22 |
| **ER15** | - | - | CEF | - | - | - | - | - | - | NAL | - | - | - | - | - | 1 |
| **ER16** | - | - | CEF | - | - | - | - | - | - | - | - | TET | - | - | - | 1 |
| **ER17** | - | - | - | - | - | - | - | - | - | NAL | - | - | SUL | - | - | 1 |
| **ER18** | - | - | - | - | - | - | - | - | - | NAL | - | TET | - | - | - | 2 |
| **ER19** | - | - | - | - | - | - | - | - | - | - | - | TET | - | SXT | - | 2 |
| **ER20** | - | - | - | - | - | - | - | - | - | NAL | - | - | - | - | - | 9 |
| **ER21** | - | - | CEF | - | - | - | - | - | - | - | - | - | - | - | - | 1 |

**Table 4.5**, continued.

| Resistotype | Resistance profile[a] | | | | | | | | | | | | | | | No. of isolates |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **ER22** | - | - | - | - | - | - | - | - | - | - | - | TET | - | - | - | 11 |
| **ER23** | - | AMP | - | - | - | - | - | - | - | - | - | - | - | - | - | 2 |
| **$** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 16 |

[a] The resistance profiles excluded antimicrobial agents that exhibited intermediate resistance. AMC, amoxicillin-clavulanic acid; AMP, ampicillin; CEF, cephalothin; CTX, cefotaxime; CAZ, ceftazidime; CRO, ceftriaxone; GEN, gentamicin; KAN, kanamycin; STR, streptomycin; NAL, nalidixic acid; CHL, chloramphenicol; TET, tetracycline; SUL, compound sulphonamide; SXT, trimethoprim-sulfamethoxazole; TMP, trimethoprim; $, sensitive to all antimicrobial agents tested

### 4.4.2 Nalidixic acid and ciprofloxacin resistance among the *Salmonella* strains

A total of 62 (31.8 %) *Salmonella* strains were categorized as "nalidixic acid-resistant but susceptible to ciprofloxacin" ($NAL^{R}CIP^{S}$) phenotype based on disk diffusion method. The MIC values of NAL ($MIC_{NAL}$) for the $NAL^{R}CIP^{S}$ strains ranged from 6 to > 256 µg/mL (Appendix E). More than half of the *Salmonella* strains were highly resistant to NAL (median $MIC_{NAL}$ value = 256 µg/mL) (Table 4.6). *Salmonella* strains from clinical and zoonotic origins did not vary greatly in their resistance towards NAL (Table 4.6). Overall, the MIC values of CIP ($MIC_{CIP}$) for all $NAL^{R}CIP^{S}$ strains ranged from 0.016 to 0.380 µg/mL, with a median $MIC_{CIP}$ value of 0.064 µg/mL (Table 4.6). Interestingly, 18 (29 %) disk diffusion-confirmed CIP susceptible strains (from clinical and zoonotic sources) showed reduced susceptibility to CIP based on the E-test results ($0.12 \leq MIC_{CIP} \leq 0.5$ µg/mL; as determined by the CLSI MIC breakpoint for clinical resistant *Salmonella* spp.).

**Table 4.6:** Minimum inhibitory concentration (MIC) values for nalidixic acid and ciprofloxacin of the *S.* Typhimurium and *S.* Enteritidis strains isolated from different sources

| Source | No. of strains | $MIC_{NAL}$ (µg/mL) | | | $MIC_{CIP}$ (µg/mL) | | |
|---|---|---|---|---|---|---|---|
| | | Range | Median | Geometrical mean | Range | Median | Geometrical mean |
| Clinical | 24 | 6-256 | 256 | 156.4 | 0.016-0.380 | 0.094 | 0.136 |
| Food[a] | 2 | 8-64 | NA | NA | 0.023 | NA | NA |
| Zoonotic | 36 | 16-256 | 256 | 190.9 | 0.016-0.190 | 0.064 | 0.081 |
| **Total** | **62** | **6-256** | **256** | **172.6** | **0.016-0.380** | **0.064** | **0.100** |

[a] NA, calculation of median and geometric mean is not possible as the sample size is too small

### 4.4.3 Mutations in the quinolone resistance-determining regions of gyrase and topoisomerase genes

DNA gyrase is encoded by two genes, namely *gyrA* and *gyrB*. The quinolone resistance-determining region (QRDR) of *gyrA* is located at Ala67 - Gln106, spanning a region of approximately 120 bp (Hopkins *et al.*, 2005). Among the 62 $NAL^R$ *Salmonella* strains examined, missense mutations were found in 83.9 % (n = 52) of the strains. Codon Asp87 had the highest incidence of missense mutations (conversion of aspartic acid to tyrosine, asparagine, and glycine), followed by codon Ser83 (conversion of serine to phenylalanine, isoleucine, and tyrosine) (Table 4.7). Overall, mutation in *gyrA* QRDR was more frequently found in *Salmonella* strains from clinical and zoonotic origins compared to food-derived strains. More than half (66.7 %) of the clinical and all zoonotic strains contained missense mutations in the *gyrA* QRDR; whereas both of the food strains showed wild type sequence in the same region (Table 4.7).

The QRDR of *gyrB* is located at Asp426 to Lys447 (approximately 66 bp) (Hopkins *et al.*, 2005). All tested strains did not harbour missense mutation in the *gyrB*. However, silent mutations beyond the QRDR of *gyrB* were common among the strains. One *S.* Typhimurium strain was found harbouring a silent mutation at codon Lys447. Majority (96.8 %) of the strains screened contain multiple mutations outside the QRDR of *gyrB* (Table 4.7), in codons Leu398, Asp399, Ala401, Gly402, Cys410, Leu451, Leu462, Ser464, Leu470, Ile471, and Leu488. These silent mutations were found together in blocks of seven or more point mutations.

Topoisomerase IV in the bacterial cells is encoded by *parC* and *parE* genes. The QRDR of *parC* gene spans a region of approximately 120 bp, from Ala64 to Gln103 (Hopkins *et al.*, 2005). All but one *Salmonella* strains were of wild type sequence for *parC* QRDR. One *S.* Typhimurium strain was found to contain multiple silent mutations

within (Val67, His75, His77, Asp101, Gly102) and beyond QRDR (Gly104, Ala117, Ser123, Gly137, and Thr150).

The QRDR of *parE* encompasses the region between Asp420 and Lys441 (approximately 66 bp) (Hopkins *et al.*, 2005). Only one missense mutation (Met438Ile; NCBI GenBank accession no.: KR004177) was found within the QRDR of *parE*. About 97 % of the *S.* Enteritidis were found to have a block of five silent mutations at codons Thr447, Glu449, Glu460, His509, and Leu523 outside the QRDR. A block of six silent mutations (Leu414, Val417, Glu449, Glu460, Ile464, and His509) outside the QRDR region was discovered in a *S.* Typhimurium strain. A missense mutation (Val521Phe; NCBI GenBank accession no.: KR004176) outside the *parE* QRDR was found in one *S.* Enteritidis strain. Similar to *gyrA*, majority of the clinical (66.7 %) and all zoonotic strains contained mutations in *parE* (83.9 %). None of the *Salmonella* strains isolated from food harboured any type of point mutation within and beyond the QRDR of *parE*. The QRDR mutations in each of the $NAL^{R}CIP^{S}$ *Salmonella* strains are listed in Appendix E.

### 4.4.4 Presence of plasmid-borne *qnr* genes

Plasmid-borne *qnrS* was detected in 30.6 % (n = 19; clinical, n = 10; food, n = 1; zoonotic, n = 8) of the tested strains (n = 62) (Table 4.7). Sequence analysis showed that all *qnrS* genes present in the sample pool were the *qnrS1* variant. Representative *qnrS1* and QRDR mutations DNA sequence alignment results are in Appendix F. Meanwhile, *qnrA* and *qnrB* were absent in all quinolone-resistant strains.

**Table 4.7:** Mutations within and beyond QRDR of gyrase and topoisomerase IV genes and the presence of *qnrS1* gene in the 62 nalidixic acid-resistant *Salmonella*

| Gene | Type of mutation[a] | | Total no. of strains | No. of strains | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | Clinical | Food | Zoonotic | S. Enteritidis | S. Typhimurium |
| ***gyrA*** | QRDR | Ser83Ile | 2 | | | 2 | 2 | |
| | | Ser83Phe | 2 | 1 | | 1 | 1 | 1 |
| | | Ser83Tyr | 1 | 1 | | | 1 | |
| | | Asp87Asn | 3 | | | 3 | 3 | |
| | | Asp87Gly | 5 | 3 | | 2 | 4 | 1 |
| | | Asp87Tyr | 39 | 11 | | 28 | 39 | |
| | Wild type | | 10 | 8 | 2 | | | 10 |
| ***gyrB*** | QRDR | Lys447 | 1 | | | 1 | | 1 |
| | Non-QRDR | Leu398, Asp399, Ala401, Gly402, Cys410, Leu451, Leu462, Ser464, Leu470, Ile471, Leu488 | 1 | | | 1 | | 1 |
| | | Leu398, Ala401, Gly402, Cys410, Leu451, Leu462, Ser464 | 59 | 22 | 2 | 35 | 50 | 9 |
| | Wild type | | 2 | 2 | | | | 2 |

**Table 4.7**, continued.

| Gene | Type of mutation[a] | | Total no. of strains | No. of strains | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | Clinical | Food | Zoonotic | S. Enteritidis | S. Typhimurium |
| ***parC*** | QRDR | Val67, His75, His77, Asp101, Gly102 | 1 | | | 1 | | 1 |
| | Non-QRDR | Thr57Ser, Gly104, Ala117, Ser123, Gly137, Thr150 | 1 | | | 1 | | 1 |
| | Wild type | | 61 | 24 | 2 | 35 | 50 | 11 |
| ***parE*** | QRDR | Met438Ile | 1 | 1 | | | | 1 |
| | Non-QRDR | Leu414, Val417, Glu449, Glu460, Ile464, His509 | 1 | | | 1 | | 1 |
| | | Thr447, Glu449, Glu460, His509, Leu523 | 49 | 14 | | 35 | 49 | |
| | | Val521Phe, Thr447, Glu449, Glu460, His509, Leu523 | 1 | 1 | | | 1 | |
| | Wild type | | 10 | 8 | 2 | | | 10 |
| ***qnrS1*** | | | 19 | 10 | 1 | 8 | 10 | 9 |

[a] Wild type, no mutations within and beyond QRDR; Missense mutation, change in amino acid codon (e.g. Ser83Ile, isoleucine replaced serine at codon 83); Silent mutation, no change in amino acid codon (e.g. Lys447, silent mutation occurred at codon 447 which codes for lysine)

## 4.5 Discussion

Generally, the data generated from this study showed that both *S.* Typhimurium and *S.* Enteritidis were highly resistant to ampicillin, tetracycline and sulphonamide. This antimicrobial resistance pattern is common among *Salmonella* populations circulating in Southeast Asian region (Van *et al.,* 2012). Besides that, *S.* Typhimurium were also highly resistant to streptomycin and cephalothin; while *S.* Enteritidis were mostly less susceptible to nalidixic acid, trimethoprim, and sulfamethoxazole-trimethoprim. This observation showed that the two most prevalent non-typhoidal *Salmonella* serovars in Malaysia were generally resistant to the commonly-prescribed first-line drugs used in the treatment of invasive salmonellosis. Moreover, high percentage of MDR was observed among the *Salmonella* strains examined in this study. The prevalence of MDR phenotypes in Malaysia may further compromise the currently available therapeutic options in the treatment of *Salmonella* infections.

Both *S.* Typhimurium and *S.* Enteritidis strains in this study displayed high level of resistance to antimicrobials in the tetracycline and sulphonamide classes. High level of resistance to tetracycline had been frequently observed elsewhere among zoonotic *Salmonella* strains (Fashae *et al.,* 2010; Sanpong *et al.,* 2010), as these antibiotics were often used extensively in agriculture (Aarestrup, 2005; Angulo *et al.,* 2004). Similarly, the high rates of resistance to ampicillin and nalidixic acid, especially among the zoonotic *Salmonella* strains, suggested the common use of these antibiotics in animal farming as the driver for development of resistance, as noted in previous studies (Van *et al.,* 2012). In developing countries including Malaysia, there is a need for the regulation of antimicrobial use in agriculture and the enforcement of control measures, in order to prevent the clonal spread of drug-resistant bacterial strains (Angulo *et al.,* 2004).

The resistance to chloramphenicol remained high in *S.* Typhimurium (17 %) but not in *S.* Enteritidis (1 %). The worldwide decrease in chloramphenicol resistance has made this drug efficient in the treatment of invasive salmonellosis caused by fluoroquinolone-resistant *Salmonella* (Klochko & Wallace, 2014). However, a slow rise in chloramphenicol resistance among *Salmonella* has been observed in Malaysia over the past decade (Table 2.2). High rate of resistance to this antibiotic has persisted among the *Salmonella* populations in several Southeast Asian countries (Van *et al.,* 2012). Hence, chloramphenicol may not be a suitable therapeutic option in the treatment of life-threatening invasive salmonellosis in Malaysia.

In comparison with *S.* Typhimurium, the *S.* Enteritidis strains were generally more susceptible to the antimicrobial agents of the aminoglycoside and cephalosporin families. The *S.* Typhimurium strains were found to be highly resistant to streptomycin, while only 3 % of *S.* Enteritidis were resistant to the same antibiotic. High rates of resistance towards streptomycin have also been documented among *Salmonella* in Southeast Asian countries (Van *et al.,* 2012). Previous study in Malaysia has also reported on the isolation of *S.* Typhimurium that were highly resistant to streptomycin (Khoo *et al.,* 2015). *S.* Typhimurium strains in this study showed a two-fold-higher rate of resistance towards cephalothin (a first generation cephalosporin) than the *S.* Enteritidis. However, both *S.* Typhimurium and *S.* Enteritidis remained highly susceptible to third-generation cephalosporins (CAZ, CRO, and CTX). The overall high susceptibility to aminoglycosides and cephalosporins seems to be the common characteristic of *S.* Enteritidis in this geographical region, where similar findings were reported (Ling & Wang, 2001; Padungtod & Kaneene, 2006). The limited dataset of this study might not represent the true incidence of antimicrobial resistance among *Salmonella* in Malaysia. Nevertheless, the overall low MAR indices and the genetic

stability of the *S.* Typhimurium and *S.* Enteritidis over the years implied that the tested antimicrobial classes may remain effective against these organisms.

Half of the *S.* Typhimurium strains were MDR, and were found in animals, food, and human sources from various locations in Malaysia, including strains isolated from earlier years (1970 and 1998). However, due to the 30-years gap in the years of isolation, conclusion could not be made that whether the MDR phenotypes had persisted since 1970, or there was a relapse between 1970 and 2002. More strains isolated between these two years should be included to provide better understanding on the prevalence of MDR *S.* Typhimurium in Malaysia over the years. Unlike *S.* Typhimurium which showed uniform distribution of MDR strains isolated from different sources, *S.* Enteritidis showed a higher rate of MDR among clinical strains compared to zoonotic strains. This indicated that the clinical strains posed a higher health risk to humans. This was in agreement with previous studies which suggested that a more resistant bacteria is always more virulent, as these two factors often co-evolved (Fluit, 2005; Foley & Lynne, 2008; Preziosi *et al.*, 2012; Ricke & Calo, 2015). Hence, these more virulent strains found their way into human hosts and resulted in severe disease manifestations and hospitalization.

Previous reports on occurrence and characterization of MDR *Salmonella* in Malaysia were mainly source-specific as summarized in Table 2.4. Therefore, the present study provides a more extensive analysis by encompassing strains from clinical, zoonotic, and food sources. The food- and clinically-derived MDR *S.* Typhimurium strains were mostly originated from developed and densely-populated areas such as Kuala Lumpur and Selangor. The close genetic proximity among these strains suggested the probable dissemination of the pathogen from food to humans. This phenomenon is not uncommon in Malaysia since the isolation of pathogenic *Salmonella* serovars from

ready-to-eat food was previously reported (Modarressi & Thong, 2010). Additionally, half of the zoonotic strains screened in this study showed MDR phenotypes suggested that the occurrence of MDR strains in food, especially meat and poultry products might be due to the use of antimicrobial agents in animal feeds at livestock farms. Previous studies showed that food animals indeed serve as a reservoir of MDR *Salmonella* (Economou & Gousia, 2015; Ricke & Calo, 2015; Van *et al.*, 2012).

The most common type of MDR *S.* Typhimurium strains in Malaysia was R-type SSuT (27.4 %), followed by a hexa-resistant pattern R-type ACKSSuT (11.9 %; strains showed simultaneous resistance to AMP, CHL, KAN, STR, SUL, and TET). The resistance to these conventional antimicrobial agents has also been reported elsewhere (Antunes *et al.*, 2006; Ethelberg *et al.*, 2004; Heir *et al.*, 2002). More than half of the MDR *S.* Enteritidis strains showed simultaneous resistance to tetracycline, trimethoprim-sulfamethoxazole, sulphonamide, and trimethoprim. Resistance to these antimicrobial agents are commonly attributed by resistance genes such as *dfr*, *tet*, and *sul* genes. The presence of these resistance genes on the mobile genetic elements allowed dissemination of these factors via horizontal gene transfer among the bacteria. Previous study has reported the presence of these genes in *S.* Typhimurium isolated from humans and animals in Malaysia (Douadi *et al.*, 2010). These resistance genes were found associated with mobile genetic elements such as plasmids and integrons. Hence, the resistance genes might have disseminated among the *Salmonella* strains circulated in Malaysia as these two serovars are the most commonly encountered non-typhoidal *Salmonella* in this country.

An overall high rate of quinolone resistance (32 %) was observed among the *Salmonella* strains. Despite the fact that increasing number of non-classical quinolone-resistant phenotype (resistant to ciprofloxacin but susceptible to nalidixic acid) being

isolated from travellers who had visited Malaysia (Lindgren *et al.,* 2009), all quinolone-resistant *Salmonella* strains in this study showed conventional phenotype, i.e. resistant to nalidixic acid but not to ciprofloxacin. Nonetheless, reduced susceptibility to fluoroquinolone (CIP) was observed among the quinolone-resistant strains, albeit at low frequency. Quinolone resistance was more prevalent among the *S.* Enteritidis (45 %) compared to *S.* Typhimurium (14 %). Notably, nalidixic acid resistance among veterinary *S.* Enteritidis has increased tremendously compared to earlier study in Malaysia (Radu *et al.,* 1995). When compared with our neighbouring countries, high level of resistance to nalidixic acid and ciprofloxacin had been observed among clinical and zoonotic *S.* Enteritidis strains in Thailand, but not in Singapore (Hendriksen *et al.*, 2012; Ling & Wang, 2001; Padungtod & Kaneene, 2006). Studies done in Southeast Asian countries have documented a high rate of resistance towards nalidixic acid among *Salmonella* strains isolated in this region (Van *et al.,* 2012). Prevalence of ciprofloxacin-resistant *Salmonella* remains low in most of the Southeast Asian countries, except Thailand and Vietnam where high rates of resistance were observed among the strains isolated from humans and food animals (Van *et al.,* 2012). The high prevalence of fluoroquinolone-resistant *Salmonella* in these countries poses a public health risk to the neighbouring countries, due to the increased international trade and travel which may possibly cause an epidemic spread of the resistant organisms.

The *Salmonella* strains were retrospectively inspected for specific mechanisms that play a role in the development of quinolone resistance. This study focused on the QRDRs mutation and plasmid-borne *qnr* genes as means to understand the major mechanisms of quinolone resistance among *Salmonella* strains in Malaysia. Other quinolone resistance mechanisms such as alteration of membrane porins and over-expression of efflux pumps were not examined as these mechanisms only result in low level of resistance to quinolones. These two mechanisms are of clinical importance only

when combined with target enzyme alterations. Hence the two major quinolone resistance mechanisms, namely target enzyme alterations and plasmid-mediated quinolone resistance were the focus of this study.

In Gram-negative bacteria, the gyrase genes are the primary target of quinolones for antibacterial activities (Guan *et al.,* 2013). Due to the positioning of gyrase at the replication fork of the bacterial genomic DNA, the inhibitory action of quinolone is more effective on DNA gyrase compared to that of topoisomerase IV (Guan *et al.,* 2013). Consequently, this may have created a selective pressure that causes the gyrase genes to mutate more frequently in order to evade the inhibitory action of quinolones. Most classes of *gyrA* mutations observed in this study, in particular those that were detected at codons Ser83 and Asp87, have been described previously (Hopkins *et al.,* 2005). Indeed, mutations at these two codons are the most commonly encountered in *Salmonella* strains isolated worldwide (Hopkins *et al.,* 2005). The two codons are highly mutable owing to their structural locations near the active site of DNA gyrase (Hopkins *et al.,* 2005). Missense mutations at these codons alter the shape of the active site, thus conferring resistance towards quinolones. The routine use of quinolones in the treatment of typhoid fever and non-typhoidal *Salmonella* infections may have contributed to the prevalence of mutations at these sites in the *gyrA* QRDR, that have persisted for many years. It was reported elsewhere that double point mutations for *gyrA*, especially at codon Ser83 and Asp87 would increase the MIC of fluoroquinolones (Eaves *et al.,* 2004; Song *et al.,* 2010; Turner *et al.,* 2006). In addition, Turner and colleagues (2006) have also reported the increase of MIC based on the combination of Ser83Phe in *gyrA* and Gly84Lys in *parC*. Although the combinations mentioned above were not observed in this study, high MIC values for nalidixic acid was seen for strains with single mutation in *gyrA* at Ser83Phe, Asp87Tyr and Asp87Asn.

In consistence with the literature, the missense mutations in the QRDR of *gyrB* were relatively infrequent among the quinolone-resistant *Salmonella* strains in this study. Most of the reported studies worldwide were unable to identify mutations in *gyrB* QRDR (Hopkins *et al.,* 2005; Kim *et al.,* 2011; Lunn *et al.,* 2010). Similarly, none of the *Salmonella* strains in this study contained missense mutations in the *gyrB* QRDR, but a silent mutation was detected in codon Lys447. In contrast to the scarcity of mutations within the QRDR, majority of the quinolone-resistant strains contain multiple silent mutations outside of the QRDR. Codons Leu398, Ala401, Gly402, Cys410, Leu451, Leu462, and Ser464 were the genetic hot spots for base substitutions in almost every quinolone-resistant *Salmonella* strain tested. Song and colleagues had reported the decrease of ciprofloxacin susceptibility caused by mutations at codon Ser464 and 466 in *gyrB* (Song *et al.,* 2010). However, quinolone-resistant *Salmonella* in this study contained only silent mutation at Ser464. This may explain the generally low level of resistance to ciprofloxacin ($MIC_{CIP} \leq 0.38$ μg/mL) among the studied strains. Nevertheless, high levels of fluoroquinolone resistance are often rarely observed among non-typhoidal *Salmonella* serovars (Guan *et al.,* 2013). Furthermore, mutations in *gyrA* are often more prevalent compared to that of *gyrB*, as *gyrA* alterations produce a higher resistance to quinolones (Hopkins *et al.,* 2005). Therefore, mutation in *gyrA* has a selective advantage during the course of resistance development against quinolones, and this may explain why the *gyrB* remained relatively unaltered over the years.

In Gram-negative bacteria such as *Salmonella*, topoisomerase IV is the secondary target of quinolone antibiotic's inhibitory action. Mutations in *parC* and *parE* are rare in Gram-negative bacteria and usually arise later than *gyrA* mutations (Guan *et al.,* 2013). The results of QRDR mutation screening are consistent with previous notion that mutations are uncommon within the QRDR of *parC.* One missense mutation outside *parC* QRDR (Thr57Ser) was detected in a *S.* Typhimurium strain (STM043/05). The

strain also harboured a missense mutation in *gyrA* (Ser83Phe), and exhibited a high level of resistance towards nalidixic acid ($MIC_{NAL}$ > 256 µg/mL), alongside with a more-than-average $MIC_{CIP}$ value (0.094 µg/mL). Ling and colleagues suggested that Thr57Ser mutation had more pronounced effects than that of Ser80Arg on *parC*, and worked in parallel with the *gyrA* mutation for quinolone resistance (Ling *et al.,* 2003). The presence of Thr57Ser mutation in *parC*, together with *qnrS1* in the *S.* Typhimurium strain might have contributed to the increased MIC towards ciprofloxacin.

Similar to *parC*, mutations within the QRDR of *parE* was infrequent. This is not unexpected, given that all quinolone-resistant *Salmonella* in this study had classical phenotype ($NAL^{R}CIP^{S}$). Indeed, mutations in the topoisomerase IV genes, which are often only required for higher level of resistance (e.g. fluoroquinolone resistance), are rare among *Salmonella* strains with classical phenotype (Hopkins *et al.,* 2005). Most of the previously reported mutations in *parE* were found at locations beyond the QRDR, mainly between Glu453 to Val512 (Hopkins *et al.,* 2005). Although mutation in the QRDR of *parE* has not been reported so far, two novel mutations within (Met438Ile) and beyond (Val521Phe) the QRDR of *parE* were discovered in this study (NCBI GenBank accession no.: KR004176 and KR004177, respectively). The *S.* Typhimurium strain (STM018/03) harbouring the Met438Ile mutation does not contain any missense mutation in the gyrase and the *parC* genes. However, the strain harboured a *qnrS1* gene. Hence, this may explain the relatively lower MIC value for nalidixic acid ($MIC_{NAL}$ = 24 µg/mL) compared to other *Salmonella* strains. Interestingly, the STM018/03 strain showed reduced susceptibility to ciprofloxacin ($MIC_{CIP}$ = 0.38 µg/mL). This finding is in agreement with previous notion that mutations in the topoisomerase IV genes confer resistance to fluoroquinolones (Hopkins *et al.,* 2005). Although less distinctive, the *S.* Enteritidis strain with the novel mutation Val521Phe outside the QRDR of *parE* was

also less susceptible to ciprofloxacin ($MIC_{CIP}$ = 0.094 µg/mL) than half of the *Salmonella* in the strain pool (median $MIC_{CIP}$ = 0.064 µg/mL).

Qnr is a protein with pentapeptide repeats that provides protection to DNA gyrase and topoisomerase IV from the inhibitory action of quinolones. This protein is encoded by *qnr* genes that reside in the naturally transferrable plasmid (pMG252 with the size of 56 kbp). Even though plasmid-mediated quinolone resistance determinants produce low-level of resistance, such resistance determinants are known to facilitate and complement the selection of bacteria with resistance to antibiotics using other mechanisms (Guan *et al.*, 2013; Rodr ́guez-Mart ́nez *et al.*, 2011). The most prevalent *qnr* gene detected worldwide has been the *qnrB* gene (Lunn *et al.*, 2010). In this study however, only *qnrS1* was detected. Indeed, *qnrS1* is frequently detected in *Salmonella* strains (Lunn *et al.*, 2010; Veldman *et al.*, 2011). The *Salmonella* strains that harboured *qnrS1* gene without missense mutation in the gyrase genes had a relatively lower $MIC_{NAL}$ values (6 - 32 µg/mL), concurring to previous notion that *qnr* genes do not confer high levels of quinolone resistance. However, only half (n = 9) of the *Salmonella* strains showing reduced susceptibility to ciprofloxacin contained *qnrS1* gene. Other mechanisms at work might have contributed to the reduced susceptibility to fluoroquinolone among the remaining half, as missense mutation was also absent in the topoisomerase IV genes of the strains. Cesaro and colleagues suggested that bacterial strains with a *qnr* gene are less likely to develop topoisomerase mutations because of the protective nature of the Qnr protein (Cesaro *et al.*, 2008). Nevertheless, *parC* mutation (Thr57Ser) and *parE* mutation (Met438Ile) were found co-existing with *qnrS1* gene in the quinolone-resistant *Salmonella* in this study.

Quinolone-resistant *Salmonella* strains from zoonotic sources had a slightly higher average $MIC_{NAL}$ values compared to strains from humans, demonstrating that

*Salmonella* strains infecting humans were less resistant to quinolone compared to strains isolated from food animals. This observation supports the notion that higher quinolone resistance in *Salmonella* may render the organism less virulent. Previous study had shown that acquisition of quinolone resistance may impair the fitness of *Salmonella* strains by reducing its ability to form biofilm (Fàbrega *et al.,* 2014). Moreover, acquisition of high levels of fluoroquinolone resistance was also shown to reduce host cell invasion due to the down-regulation of invasion genes (Fàbrega *et al.,* 2009). Fàbrega and colleagues (2009) related their findings to the relatively low prevalence of fluoroquinolone-resistant clinical *Salmonella* strains, while the prevalence of strains with quinolone-resistant phenotype was steadily increasing among other sources. However, the quinolone-resistant *Salmonella* strains from clinical sources in this study showed a slightly higher average $MIC_{CIP}$ values compared to zoonotic strains. The high rates of resistance among *Salmonella* strains in Malaysia towards the first-line drugs might have increased the reliance on fluoroquinolone in the treatment of invasive salmonellosis, thus creating a selective pressure on the strains to develop fluoroquinolone resistance. Moreover, the presence of the plasmid-mediated *qnrS1* in the zoonotic strains also raises issues on the risk of horizontal transfer of this resistance determinant, with animals as the reservoir, which will eventually lead to increased fluoroquinolone resistance among *Salmonella* circulating in this region.

*Salmonella* strains isolated in earlier years (1970 and 1998) in Malaysia were susceptible to both quinolones and fluoroquinolones. Similar study in 1995 had also documented the high susceptibility of *Salmonella* to nalidixic acid (Radu *et al.,* 1995). This is not surprising as the worldwide prevalence of quinolone-resistant *Salmonella* was on the rise only in the 1990's (Hopkins *et al.,* 2005). Over the past decade, quinolone resistance among *Salmonella* in Malaysia was steadily growing (Adzitey *et al.,* 2012; Cheah *et al.,* 2008; Douadi *et al.,* 2010; Thong *et al.,* 2011; Thong &

Modarressi, 2011; Tiong *et al.*, 2010; Tunung *et al.*, 2007). Similar trend is also observed among the *Salmonella* strains examined in this study. Between 2002 and 2008, the rates of quinolone-resistant *Salmonella* strains were in the range of 14 % - 60 %. The chromosomal-mediated quinolone resistance mechanism (mutations in gyrase and topoisomerase IV genes) was present in the quinolone-resistant strains from 2003 onwards. The common missense mutations which were identified in these *Salmonella* strains were amongst the ones that are frequently encountered in *Salmonella* isolated worldwide (Hopkins *et al.*, 2005). Plasmid-mediated quinolone-resistant determinant (*qnrS1*) was found in *Salmonella* strains isolated in 2002, and was detected in the quinolone-resistant strains isolated every year since. Plasmid-mediated quinolone resistance mechanism had emerged in the late 1990's (Hopkins *et al.*, 2005; Poirel *et al.*, 2012). The detection of *qnrS1* in Malaysian *Salmonella* strain as early as 2002 showed the possible rapid spread of this transferable resistance determinant, probably across continents.

## 4.6 Conclusion

*S.* Typhimurium and *S.* Enteritidis strains isolated in Malaysia were mostly resistant to majority of the first-line drugs used in the treatment of invasive salmonellosis. Moreover, MDR phenotypes were common among the *Salmonella* strains, although majority of them were in the low-risk phase based on the MAR index. Quinolone resistance was commonly observed among *Salmonella* strains, with an emergence in reduced susceptibility towards fluoroquinolone. Mutations in the QRDR of *gyrA* in majority of the strains have contributed to the high level of quinolone resistance, and two novel mutations were detected in *parE*. Only *qnrS1* gene was detected among the *Salmonella* strains. The presence of antimicrobial-resistant *Salmonella* strains in food, animals, and humans points to a successful farm-to-table dissemination of these drug-resistant organisms. However, as this study is a retrospective investigation of

antimicrobial resistance patterns of local *Salmonella* strains, the clinical implications of the data obtained may not be significant, i.e. may not aid the medical practitioners in the selection of antibiotics for effective treatment of salmonellosis. Nonetheless, by elucidating the antimicrobial resistance patterns and related mechanisms of resistance, the information provided herein may help policy makers to develop up-to-date strategies to curb the spread of drug-resistant *Salmonella*.

# CHAPTER 5: DEVELOPMENT OF HIGH RESOLUTION MELTING CURVE ASSAY FOR RAPID DETECTION OF MUTATIONS

## 5.1 Introduction

Target enzymes alteration is one of the main mechanisms that lead to quinolone and fluoroquinolone resistance among *Salmonella* strains. Mutations in the quinolone-resistant determining regions (QRDRs) of the gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*) genes result in the morphological change in the enzymes, preventing the binding of the quinolone molecules and subsequent inhibitory action. DNA sequencing is able to detect small genetic changes in the QRDRs, but is costly especially when large sample size is screened. The high resolution melting curve (HRM) analysis is able to detect point mutations in targeted genes, and may be a more affordable alternative for rapid screening of a large strains pool.

The objective of this study was to develop a HRM assay for rapid screening of mutations in the QRDRs of gyrase and topoisomerase IV genes in *Salmonella*.

## 5.2 Literature review

*Salmonella* is a human pathogen commonly found in developed and developing countries, causing clinical diseases ranging from mild gastroenteritis to septicaemia (Coburn *et al.*, 2007). Quinolones are broad spectrum antimicrobial agents that inhibit bacterial DNA from unwinding and duplicating during cell division and are commonly used in treating *Salmonella* infections. However, the worldwide emergence of quinolone-resistant bacterial strains raises public health concern. Increased incidence of quinolone-resistant *Salmonella* serovars has been documented in Southeast Asian region (Van *et al.*, 2012).

One of the major mechanisms that contribute to quinolone resistance among *Salmonella* is altered protein targets for quinolones (Guan *et al.*, 2013; Ruiz, 2003).

DNA gyrase and topoisomerase IV are two important enzymes involved in bacterial DNA replication. Quinolones bind to gyrase/topoisomerase IV-DNA complex and inhibit DNA replication. This action is responsible for the bacteriostatic and bactericidal property of quinolones. Mutations in the bacterial genes encoding DNA gyrase and topoisomerase IV may confer resistance to quinolones and it has been shown that altered structures of these enzymes prevent binding of quinolones (Michael *et al.*, 2006; Ruiz, 2003).

Both DNA gyrase and topoisomerase IV are tetrameric enzymes. DNA gyrase is encoded by *gyrA* and *gyrB* genes; while topoisomerase IV is encoded by *parC* and *parE* genes. Mutation in quinolone-resistant determining regions (QRDRs) in these genes is associated with quinolone resistance (Guan *et al.*, 2013; Ruiz, 2003). Many studies have reported the presence of such mutations in quinolone-resistant *Salmonella* (Chen *et al.*, 2007; Eaves *et al.*, 2004; Hopkins *et al.*, 2005; Preisler *et al.*, 2006; Weill *et al.*, 2006). Indeed, lower fluoroquinolone susceptibility and quinolone resistance are linked to point mutations in QRDRs of the gyrase genes (Kehrenberg *et al.*, 2007; Lunn *et al.*, 2010). While most studies revealed that codon 87 of *gyrA* gene represents the most frequently mutated amino acid in *Salmonella* (Chen *et al.*, 2007; Eaves *et al.*, 2004; Lunn *et al.*, 2010), point mutations outside of QRDR may also play a role in quinolone resistance (Ruiz, 2003). Nevertheless, QRDR remains the major focus in quinolone resistance studies since this region exhibits high mutation rate.

Although DNA sequencing is the gold standard for detecting genetic changes (Morozova & Marra, 2008), it is relatively costly to sequence all four genes when examining a large sample pool. Furthermore, mutations are rare in *parC* and *parE* genes (Hopkins *et al.*, 2005), therefore DNA sequencing will not be an economically viable option when dealing with such conserved regions. The real-time Polymerase

Chain Reaction (PCR)-based high-resolution melting curve (HRM) analysis is able to detect small genetic variation in PCR amplicons (Liew *et al.*, 2004; Pietzka *et al.*, 2011; Wittwer *et al.*, 2003). The HRM assay is highly sensitive and specific and has been employed in single nucleotide polymorphism (SNP) scanning (Liew *et al.*, 2004; Reed & Wittwer, 2004). During HRM analysis, a DNA intercalating dye is included in the PCR reaction mixture (White & Potts, 2006). This compound interacts specifically with double-stranded DNA and emits fluorescence signal but loses fluorescence when released from the DNA during denaturation. Upon completion of PCR, the DNA samples are subjected to a temperature gradient, and the loss of fluorescence signal resulting from denaturation gives each DNA sample a unique melting curve that is detected by the real-time PCR system (White & Potts, 2006). Hence, different *Salmonella* strains can be discriminated based on DNA sequence, length, GC content or strand composition.

HRM assay has been used for the identification of quinolone- and fluoroquinolone-resistant bacterial pathogens including *Mycobacterium tuberculosis* (Chen *et al.*, 2011; Lee *et al.*, 2012); *Bacillus anthracis*, *Yersinia pestis*, and *Francisella tularensis* (Loveless *et al.*, 2010); *Salmonella* Typhi and Paratyphi A (Slinger *et al.*, 2007). However, only *gyrA* gene was examined in the reported works. Since mutations in *gyrB*, *parC* and *parE* genes of *Salmonella* and their correlation with lower susceptibility to quinolones and fluoroquinolones had also been documented (Hopkins *et al.*, 2005), it is necessary to perform mutation scanning for these genes.

## 5.3 Methodology

### 5.3.1 Bacterial strains

Sixty-two *Salmonella* strains showing resistance or reduced susceptibility towards nalidixic acid (a quinolone antibiotic) were used to develop the HRM assay in this study

(*S.* Typhimurium, n = 12; *S.* Enteritidis, n = 50). Genomic DNA for each bacterial strain was extracted by using Wizard® genomic DNA purification kit (Promega, Madison, USA). The details of all *Salmonella* strains used in this study are listed in the Appendix E. The types of mutation in the QRDRs of the gyrase and topoisomerase IV genes for the *Salmonella* strains were discussed in Chapter 4.

### 5.3.2 Development of the HRM analysis for detection of mutations in the QRDRs of gyrase and topoisomerase IV genes

A homology search for *gyrA*, *gyrB*, *parC*, and *parE* gene sequences of *Salmonella* serovars from the NCBI GenBank database was performed. Sequence alignment was done using the molecular evolutionary genetics analysis software version 5 (MEGA5) (Tamura *et al.*, 2011). Primers were designed using the online tool Primer3Plus (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi), spanning the QRDR of each gene of interest, and were commercially-synthesized (NHK Bioscience Solutions, Kuala Lumpur, Malaysia). Approximately 20 ng of bacterial genomic DNA was added into 1× MeltDoctor$^{TM}$ HRM master mix (Applied Biosystems, California, USA), which was premixed with 0.3 μM of each primer pair. Sterile deionized distilled water was added to make up a final reaction volume of 20 μL. The reproducibility of the assay was determined by performing all reactions in triplicates. In each assay, the DNA sample from a wild type reference strain was used as positive control. The selection of the wild type reference strains for the different target genes were explained in the result section. Negative control (no-template control) for the experiment was the HRM reaction mix without the addition of bacterial DNA. The HRM assay was performed on the 7500 Fast Real-time PCR System (Applied Biosystems, USA). The real-time PCR amplification included a holding stage at 95 ℃ for 10 min, followed by the cycling stage with 40 cycles of 95 ℃ for 15 sec and subsequently 60 ℃ for 1 min. The subsequent melt curve stage consisted of the following steps: 95 ℃ for 15 sec, a melt from 60 ℃ (1 min) to 95

℃ (30 sec) with a ramp rate of 1 %, and 60 ℃ for 15 sec (according to manufacturer's instructions). The amplification plot for each sample was generated by the 7500 software v2.0 (Applied Biosystems, USA) linked to the instrument. The data generated was subsequently imported to the HRM software v2.0.1 (Applied Biosystems, USA) for melting curve analysis. The fluorescence change of each sample was plotted against temperature, and was normalized to produce the aligned melt curves. In order to better illustrate the minor fluorescence changes between the wild type and mutants, a difference plot was generated using the HRM software. In a difference plot, one *Salmonella* strain with wild type sequence confirmed via sequencing was chosen as the reference. The fluorescence difference between each sample and the reference was plotted against temperature. Mutations in the target region would produce a deviated curve in the difference plot.

## 5.4 Results

HRM primers were designed to span the QRDRs of *gyrA* (Ala67 - Gln106), *gyrB* (Asp426 - Lys447), *parC* (Ala64 - Gln103), and *parE* (Asp420 - Lys441) genes. The primer pairs produced amplicons ranging from 150 to 250 bp (Table 5.1), with a melting temperature of approximately 60 ℃. HRM analysis showed distinct melt curves for wild type versus mutant strains, which were confirmed by sequencing. Based on the DNA sequence analysis, two QRDR mutation-free strains were selected as references to generate difference plots (STM006/02 as reference strain for *gyrA*, *parC*, and *parE* genes mutation detection; and STM032/04 as reference strain for *gyrB* gene mutation detection). The QRDRs of *gyrA*, *parC*, and *parE* genes in the reference strain STM006/02 and the *gyrB* QRDR in the reference strain STM032/04 showed 100 % identity with that of the *S.* Typhimurium reference genome LT2 (NCBI GenBank accession no.: NC_003197).

**Table 5.1:** PCR primers sequences for HRM analysis of gyrase and topoisomerase IV genes

| Target gene | Primer | Sequence (5'-3') | Amplicon size (bp) |
|---|---|---|---|
| ***gyrA*** | gyrA-F | CAATGACTGGAACAAAGCCTA | 164 |
| | gyrA-R | AACCGAAGTTACCCTGACCA | |
| ***gyrB*** | gyrB-F | TGTCCGAACTGTACCTGGTG | 198 |
| | gyrB-R | ACTCGTCGCGACCGATAC | |
| ***parC*** | parC-F | CGTCTATGCGATGTCAGAGC | 219 |
| | parC-R | ATCGCCGCGAATGACTTC | |
| ***parE*** | parE-F | TACCGCGCAGGATCTTAATC | 193 |
| | parE-R | GATCGCCACGGAAATATCAT | |

The melting temperature of wild type *gyrA* allele was in the range 82.5 - 82.8 ℃; while that of the mutants (Asp87Tyr, Asp87Asn, Ser83Phe, Ser83Ile, and Ser83Tyr) were slightly lower at 82.0 - 82.4 ℃. Interestingly, *Salmonella* strains with *gyrA* Asp87Gly mutation produced a distinct melting curve compared to other mutants, at slightly higher melting temperature (82.9 - 83.5 ℃). The differences in the melting curves of the wild type versus mutants were distinguishable in both aligned and difference plots (Figure 5.1). The aligned melting curves were set at 100 % at the beginning, and 0 % at the end of melting process. In the difference plot, the melting curves represent the temperature at which the amplicons were completely denatured. The differences in the melting temperature among the samples are clearly illustrated in the difference plot. Mutants with missense mutations Asp87Tyr, Asp87Asn, Ser83Phe, and Ser83Tyr were denatured at similar melting temperature and therefore formed a tight cluster.

The melting temperature for *gyrB* wild type allele was 86.1 - 86.2 ℃; while that of the mutants were 86.2 - 86.7 ℃. One mutant (STM043/05) with three mutations in *gyrB* has a slightly lower melting temperature at 86.0 ℃. The aligned plot did not show distinctive differences when comparing the melt curves of wild type versus mutant *gyrB* alleles. However, they could be clearly distinguished in the difference plot (Figure 5.2).

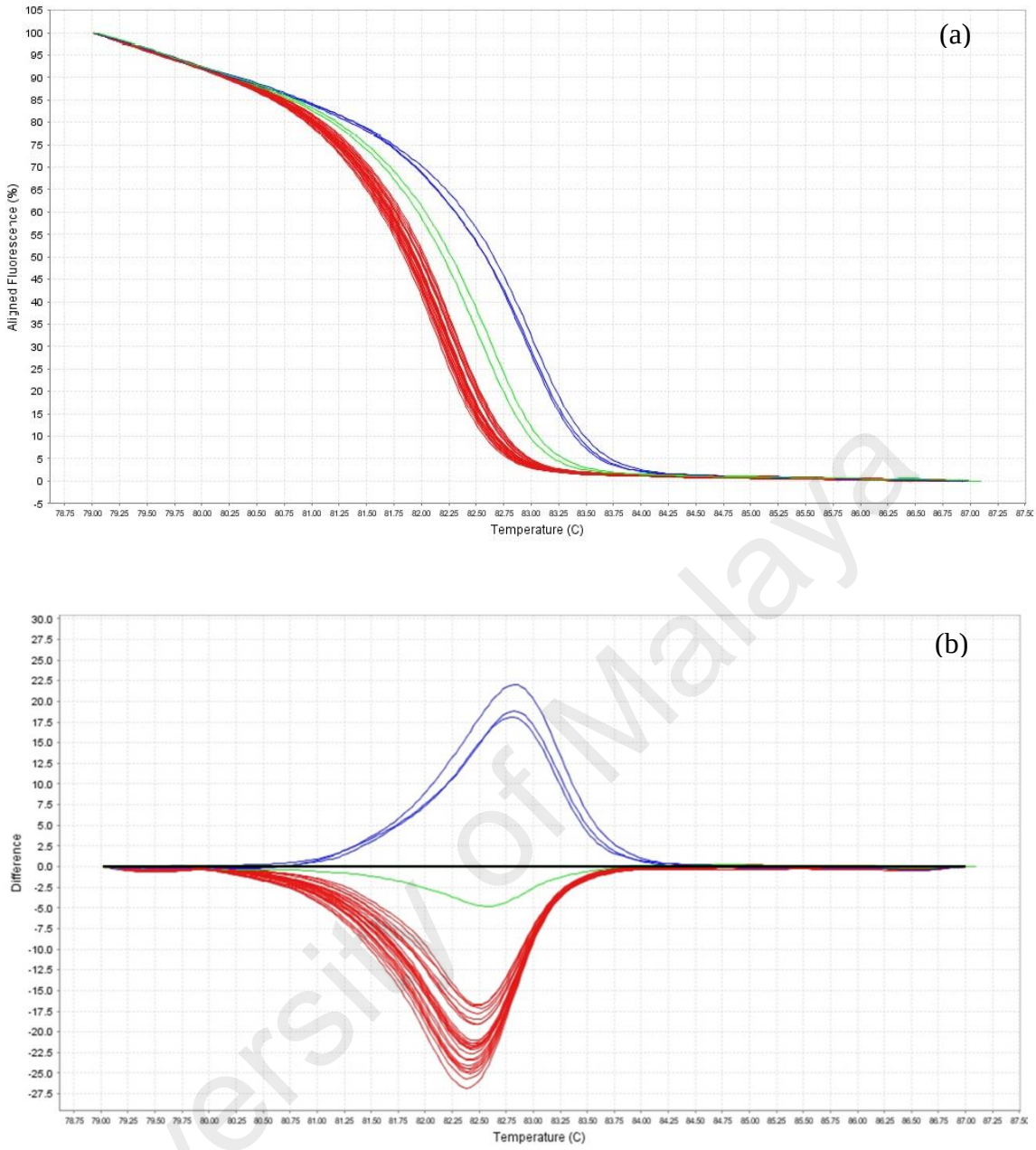



**Figure 5.1:** Representative HRM aligned melting curves (a) and difference plot (b) for mutations in *gyrA* QRDR. The reference strain is indicated by the horizontal black line in the difference plot; and the wild type samples are indicated as green line. The blue curves derived from mutants with the missense mutation Asp87Gly, whereas the red curves belong to other mutants (Asp87Tyr, Asp87Asn, Ser83Phe, and Ser83Tyr).

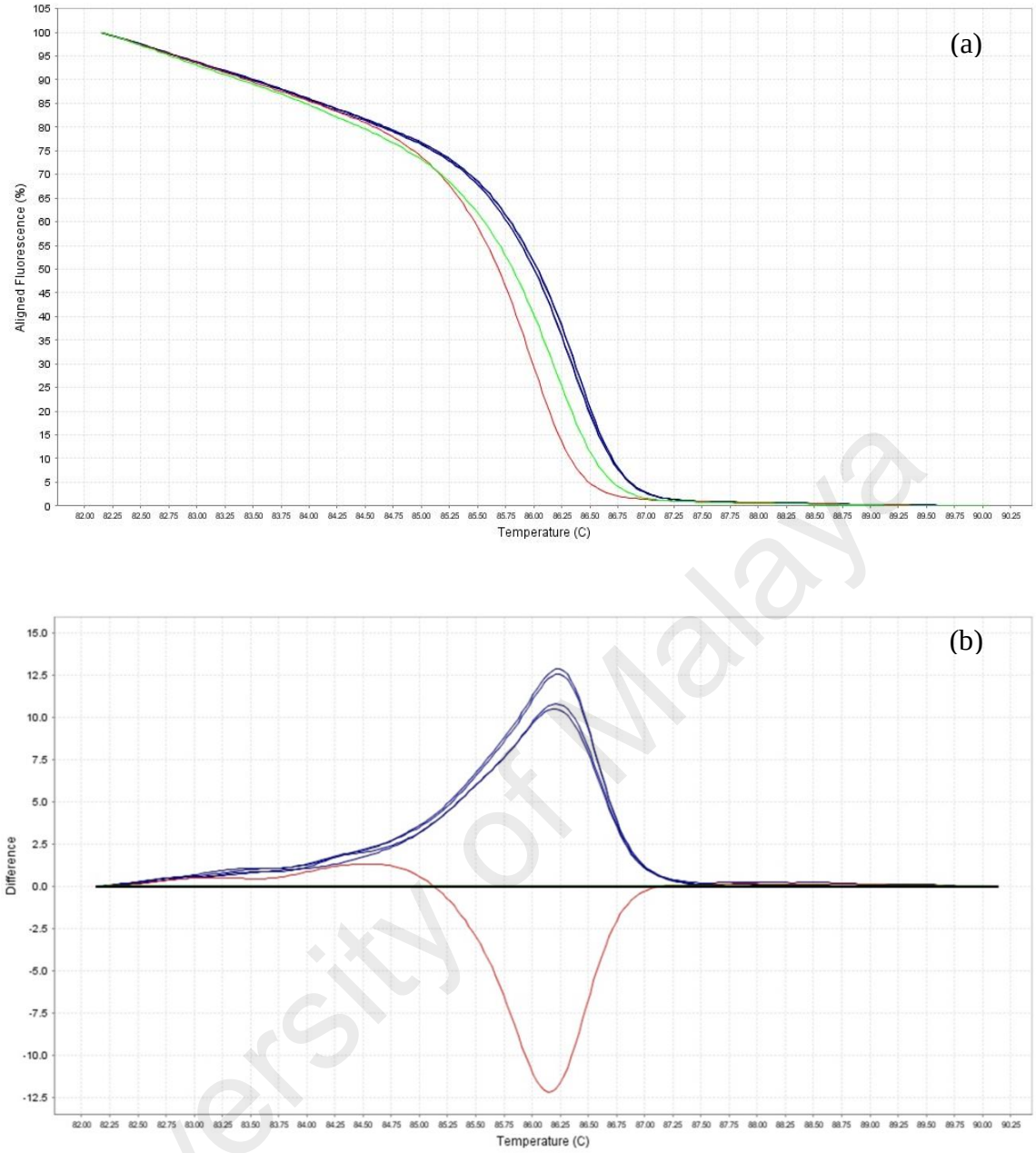



**Figure 5.2:** Representative HRM aligned melting curves (a) and difference plot (b) for mutations in *gyrB* QRDR. The reference strain is indicated by the horizontal black line in the difference plot, while mutants are represented by red (6 mutations within the HRM target region) or blue curves (3 mutations within the HRM target region). All *gyrB* mutants harboured silent mutations.

For *parC* gene, the melting temperature for wild type allele was at 86.1 - 86.5 ℃ (98 % melted below 86.4 ℃); and the mutant at 86.4 ℃. Although the differences in the melting temperature were small, the mutant produced a unique melting curve in the difference plot when compared to the wild type (Figure 5.3).

For *parE* gene, the melting temperature for wild type allele was at 84.6 - 84.9 ℃; whereas that of the mutants containing multiple mutations were at 84.9 - 85.2 ℃. One mutant (STM018/03), which harboured a single mutation in *parE* QRDR (Met438Ile) had a lower melting temperature at 83.9 ℃. The differences in wild type versus mutant melt curves were apparent in both aligned and difference plot (Figure 5.4). All mutant strains contained similar nucleotide changes in the QRDR, resulting in tightly-clustered melting curves. The melting temperatures of the HRM amplicons for the QRDR of each gene in the *Salmonella* strains tested are listed in Table 5.2.

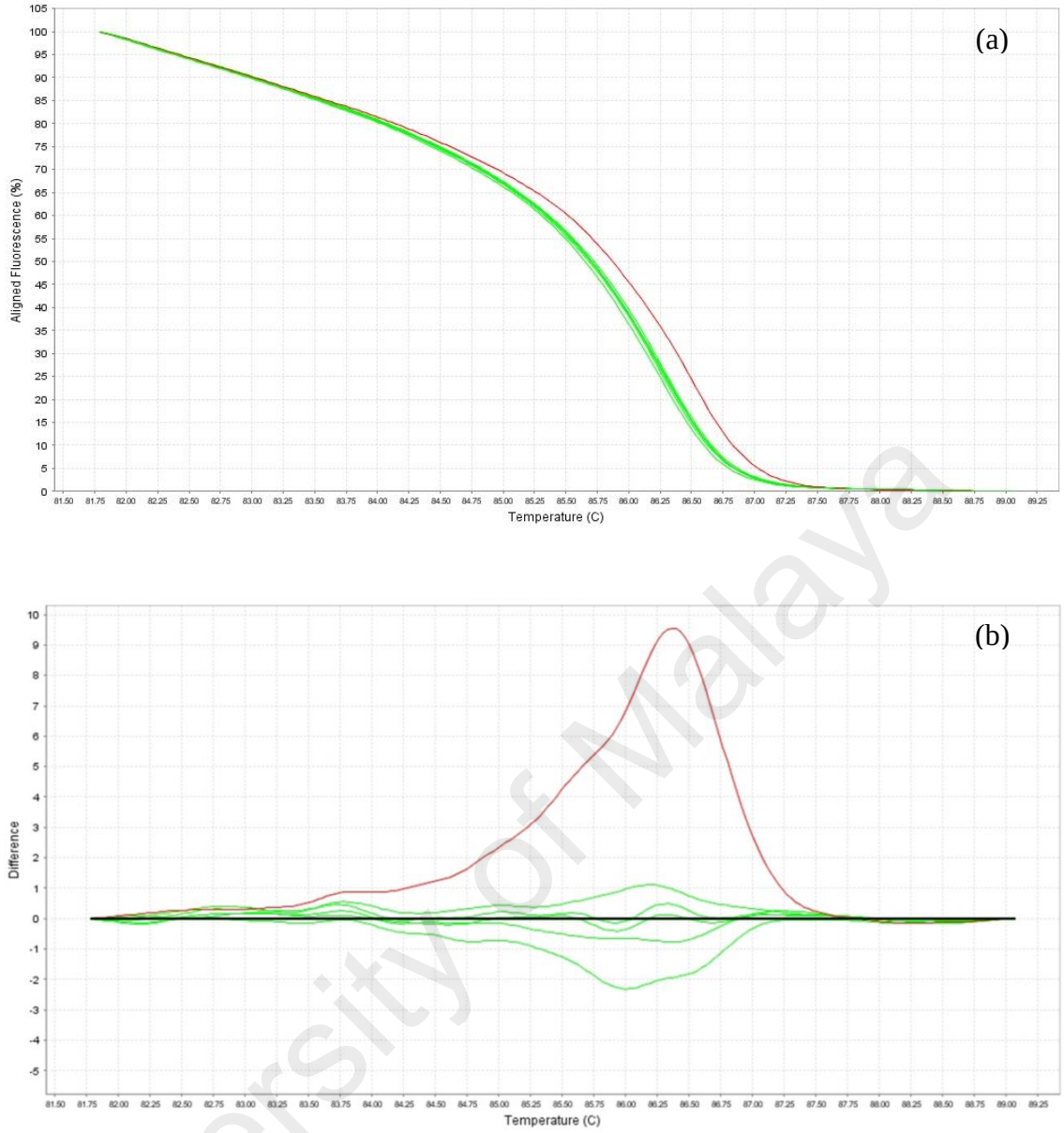



**Figure 5.3:** Representative HRM aligned melting curves (a) and difference plot (b) for mutations in *parC* QRDR. The reference strain is indicated by the horizontal black line in the difference plot. Wild type samples are indicated by green curves and the single mutant is indicated by red curve.

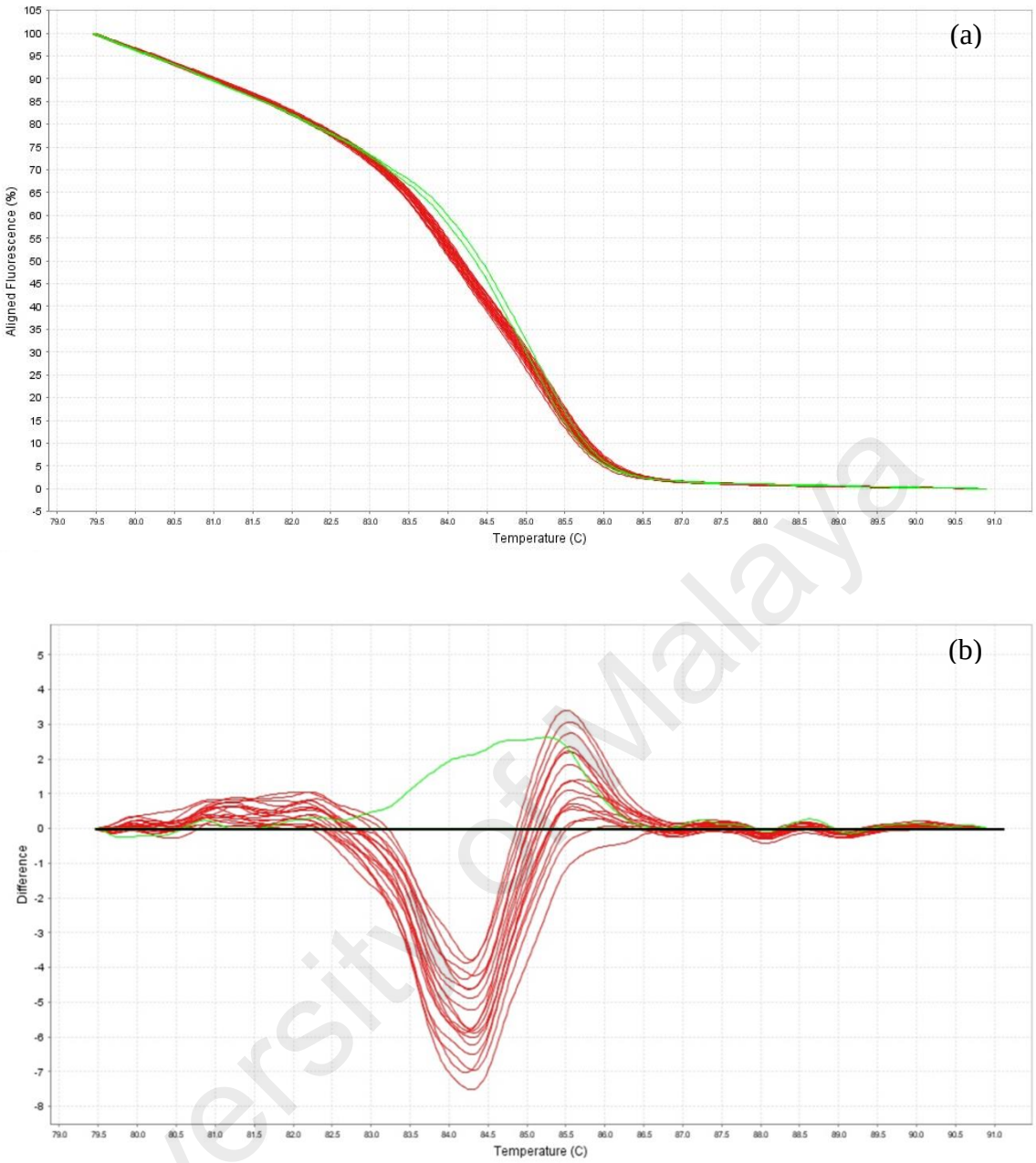



**Figure 5.4:** Representative HRM aligned melting curves (a) and difference plot (b) for mutations in *parE* QRDR. The reference strain is indicated by the horizontal black line in difference plot. Wild type samples are indicated by green curves and mutants are indicated by red curves.

**Table 5.2:** Types of mutation and the corresponding melting temperatures of the HRM amplicons of the QRDRs for gyrase and topoisomerase IV genes

| Strain | *Salmonella* serovar | *gyrA* | | *gyrB* | | *parC* | | *parE* | |
|---|---|---|---|---|---|---|---|---|---|
| | | Mutation[a] | Melting temperature (℃) | Mutation[a] | Melting temperature (℃) | Mutation[a] | Melting temperature (℃) | Mutation[a] | Melting temperature (℃) |
| SE 001/03 | Enteritidis | Asp87Tyr | 82.21 | Silent mutations | 86.41 | None | 86.34 | Silent mutations | 85.02 |
| SE 002/03 | Enteritidis | Asp87Tyr | 82.10 | Silent mutations | 86.42 | None | 86.15 | Silent mutations | 85.22 |
| SE 004/03 | Enteritidis | Asp87Tyr | 82.13 | Silent mutations | 86.51 | None | 86.22 | Silent mutations | 84.98 |
| SE 005/03 | Enteritidis | Asp87Tyr | 82.11 | Silent mutations | 86.53 | None | 86.26 | Silent mutations | 85.22 |
| SE 012/03 | Enteritidis | Asp87Asn | 82.15 | Silent mutations | 86.59 | None | 86.31 | Silent mutations | 84.99 |
| SE 013/03 | Enteritidis | Asp87Asn | 82.27 | Silent mutations | 86.30 | None | 86.37 | Silent mutations | 85.07 |
| SE 015/03 | Enteritidis | Asp87Asn | 82.15 | Silent mutations | 86.55 | None | 86.27 | Silent mutations | 85.06 |
| SE 022/03 | Enteritidis | Asp87Tyr | 82.23 | Silent mutations | 86.57 | None | 86.28 | Silent mutations | 84.91 |
| SE 026/03 | Enteritidis | Asp87Tyr | 82.20 | Silent mutations | 86.32 | None | 86.36 | Silent mutations | 85.10 |
| SE 027/03 | Enteritidis | Asp87Tyr | 82.23 | Silent mutations | 86.22 | None | 86.38 | Silent mutations | 85.18 |
| SE 028/03 | Enteritidis | Asp87Tyr | 82.29 | Silent mutations | 86.29 | None | 86.35 | Silent mutations | 85.14 |

**Table 5.2**, continued.

| Strain | *Salmonella* serovar | *gyrA* | | *gyrB* | | *parC* | | *parE* | |
|---|---|---|---|---|---|---|---|---|---|
| | | **Mutation[a]** | **Melting temperature (℃)** | **Mutation[a]** | **Melting temperature (℃)** | **Mutation[a]** | **Melting temperature (℃)** | **Mutation[a]** | **Melting temperature (℃)** |
| SE 032/03 | Enteritidis | Asp87Tyr | 82.17 | Silent mutations | 86.59 | None | 86.28 | Silent mutations | 84.99 |
| SE 033/03 | Enteritidis | Asp87Tyr | 82.09 | Silent mutations | 86.45 | None | 86.15 | Silent mutations | 84.97 |
| SE 046/04 | Enteritidis | Asp87Tyr | 82.22 | Silent mutations | 86.34 | None | 86.35 | Silent mutations | 84.91 |
| SE 047/04 | Enteritidis | Asp87Tyr | 82.27 | Silent mutations | 86.29 | None | 86.39 | Silent mutations | 85.20 |
| SE 048/04 | Enteritidis | Asp87Tyr | 82.17 | Silent mutations | 86.54 | None | 86.26 | Silent mutations | 85.00 |
| SE 049/04 | Enteritidis | Asp87Tyr | 82.19 | Silent mutations | 86.52 | None | 86.24 | Silent mutations | 84.91 |
| SE 050/04 | Enteritidis | Asp87Gly | 82.96 | Silent mutations | 86.32 | None | 86.28 | Silent mutations | 84.98 |
| SE 051/04 | Enteritidis | Ser83Ile | 82.18 | Silent mutations | 86.34 | None | 86.38 | Silent mutations | 85.22 |
| SE 052/04 | Enteritidis | Ser83Ile | 82.17 | Silent mutations | 86.34 | None | 86.35 | Silent mutations | 84.89 |
| SE 054/05 | Enteritidis | Asp87Tyr | 82.13 | Silent mutations | 86.51 | None | 86.23 | Silent mutations | 84.96 |
| SE 055/05 | Enteritidis | Ser83Phe | 82.31 | Silent mutations | 86.57 | None | 86.29 | Silent mutations | 84.90 |

**Table 5.2**, continued.

| Strain | *Salmonella* serovar | *gyrA* | | *gyrB* | | *parC* | | *parE* | |
|---|---|---|---|---|---|---|---|---|---|
| | | **Mutation[a]** | **Melting temperature (℃)** | **Mutation[a]** | **Melting temperature (℃)** | **Mutation[a]** | **Melting temperature (℃)** | **Mutation[a]** | **Melting temperature (℃)** |
| SE 064/05 | Enteritidis | Asp87Tyr | 82.20 | Silent mutations | 86.57 | None | 86.29 | Silent mutations | 85.00 |
| SE 065/05 | Enteritidis | Asp87Tyr | 82.19 | Silent mutations | 86.32 | None | 86.39 | Silent mutations | 85.02 |
| SE 066/05 | Enteritidis | Asp87Tyr | 82.28 | Silent mutations | 86.34 | None | 86.34 | Silent mutations | 84.98 |
| SE 067/05 | Enteritidis | Asp87Tyr | 82.19 | Silent mutations | 86.28 | None | 86.42 | Silent mutations | 84.96 |
| SE 068/05 | Enteritidis | Asp87Tyr | 82.16 | Silent mutations | 86.51 | None | 86.22 | Silent mutations | 84.86 |
| SE 069/05 | Enteritidis | Asp87Tyr | 82.22 | Silent mutations | 86.37 | None | 86.35 | Silent mutations | 85.00 |
| SE 070/05 | Enteritidis | Asp87Gly | 82.93 | Silent mutations | 86.55 | None | 86.25 | Silent mutations | 85.00 |
| SE 073/05 | Enteritidis | Asp87Tyr | 82.18 | Silent mutations | 86.58 | None | 86.29 | Silent mutations | 84.97 |
| SE 074/05 | Enteritidis | Asp87Tyr | 82.21 | Silent mutations | 86.57 | None | 86.28 | Silent mutations | 85.14 |
| SE 075/05 | Enteritidis | Asp87Tyr | 82.17 | Silent mutations | 86.59 | None | 86.29 | Silent mutations | 85.18 |
| SE 076/05 | Enteritidis | Asp87Tyr | 82.28 | Silent mutations | 86.37 | None | 86.36 | Silent mutations | 85.01 |

**Table 5.2**, continued.

| Strain | *Salmonella* serovar | *gyrA* | | *gyrB* | | *parC* | | *parE* | |
|---|---|---|---|---|---|---|---|---|---|
| | | Mutation[a] | Melting temperature (℃) | Mutation[a] | Melting temperature (℃) | Mutation[a] | Melting temperature (℃) | Mutation[a] | Melting temperature (℃) |
| SE 078/05 | Enteritidis | Asp87Tyr | 82.11 | Silent mutations | 86.53 | None | 86.27 | Silent mutations | 85.05 |
| SE 080/05 | Enteritidis | Asp87Tyr | 82.12 | Silent mutations | 86.57 | None | 86.28 | Silent mutations | 85.03 |
| SE 081/05 | Enteritidis | Asp87Tyr | 82.25 | Silent mutations | 86.31 | None | 86.24 | Silent mutations | 85.22 |
| SE 084/05 | Enteritidis | Asp87Tyr | 82.15 | Silent mutations | 86.53 | None | 86.22 | Silent mutations | 85.19 |
| SE 086/06 | Enteritidis | Asp87Tyr | 82.16 | Silent mutations | 86.32 | None | 86.34 | Silent mutations | 84.96 |
| SE 089/06 | Enteritidis | Asp87Gly | 82.96 | Silent mutations | 86.57 | None | 86.27 | Silent mutations | 84.98 |
| SE 090/06 | Enteritidis | Asp87Tyr | 82.22 | Silent mutations | 86.36 | None | 86.35 | Silent mutations | 85.02 |
| SE 095/06 | Enteritidis | Asp87Gly | 83.02 | Silent mutations | 86.64 | None | 86.36 | Silent mutations | 84.96 |
| SE 097/06 | Enteritidis | Asp87Tyr | 82.19 | Silent mutations | 86.56 | None | 86.29 | Silent mutations | 84.99 |
| SE 099/06 | Enteritidis | Ser83Tyr | 82.26 | Silent mutations | 86.34 | None | 86.34 | Silent mutations | 85.01 |
| SE 101/07 | Enteritidis | Asp87Tyr | 82.11 | Silent mutations | 86.50 | None | 86.27 | Silent mutations | 85.21 |

**Table 5.2**, continued.

| Strain | *Salmonella* serovar | *gyrA* | | *gyrB* | | *parC* | | *parE* | |
|---|---|---|---|---|---|---|---|---|---|
| | | Mutation[a] | Melting temperature (℃) | Mutation[a] | Melting temperature (℃) | Mutation[a] | Melting temperature (℃) | Mutation[a] | Melting temperature (℃) |
| SE 102/07 | Enteritidis | Asp87Tyr | 82.18 | Silent mutations | 86.49 | None | 86.23 | Silent mutations | 85.09 |
| SE 104/07 | Enteritidis | Asp87Tyr | 82.10 | Silent mutations | 86.53 | None | 86.26 | Silent mutations | 85.10 |
| SE 105/07 | Enteritidis | Asp87Tyr | 82.24 | Silent mutations | 86.25 | None | 86.33 | Silent mutations | 84.96 |
| SE 106/07 | Enteritidis | Asp87Tyr | 82.20 | Silent mutations | 86.57 | None | 86.30 | Silent mutations | 85.18 |
| SE 107/07 | Enteritidis | Asp87Tyr | 82.29 | Silent mutations | 86.34 | None | 86.36 | Silent mutations | 85.20 |
| SE 109/07 | Enteritidis | Asp87Tyr | 82.12 | Silent mutations | 86.47 | None | 86.22 | Val521Phe (outside QRDR) | 85.15 |
| STM 006/02 | Typhimurium | None | 82.67 | Silent mutations | 86.31 | None | 86.23 | None | 84.72 |
| STM 018/03 | Typhimurium | None | 82.52 | Silent mutations | 86.26 | None | 86.22 | Met438Ile | 83.88 |
| STM 032/04 | Typhimurium | Asp87Gly | 83.49 | None | 86.19 | None | 86.20 | None | 84.71 |
| STM 033/04 | Typhimurium | None | 83.04 | Silent mutations | 86.30 | None | 86.17 | None | 84.68 |
| STM 034/04 | Typhimurium | None | 83.10 | Silent mutations | 86.30 | None | 86.23 | None | 84.71 |

**Table 5.2**, continued.

| Strain | *Salmonella* serovar | *gyrA* | | *gyrB* | | *parC* | | *parE* | |
|---|---|---|---|---|---|---|---|---|---|
| | | **Mutation[a]** | **Melting temperature (℃)** | **Mutation[a]** | **Melting temperature (℃)** | **Mutation[a]** | **Melting temperature (℃)** | **Mutation[a]** | **Melting temperature (℃)** |
| STM 043/05 | Typhimurium | Ser83Phe | 82.37 | Silent mutations | 85.98 | Silent mutations | 86.37 | Silent mutations | 84.55 |
| STM 048/05 | Typhimurium | None | 83.09 | Silent mutations | 86.30 | None | 86.12 | None | 84.90 |
| STM 053/05 | Typhimurium | None | 82.68 | Silent mutations | 86.31 | None | 86.19 | None | 84.78 |
| STM 055/05 | Typhimurium | None | 82.71 | Silent mutations | 86.25 | None | 86.19 | None | 84.76 |
| STM 056/05 | Typhimurium | None | 82.69 | Silent mutations | 86.26 | None | 86.11 | None | 84.79 |
| STM 057/05 | Typhimurium | None | 82.63 | None | 86.17 | None | 86.14 | None | 84.84 |
| STM 071/07 | Typhimurium | None | 82.56 | Silent mutations | 86.32 | None | 86.33 | None | 84.84 |

[a] Asp, aspartic acid; Phe, phenylalanine; Gly, glycine; Ile, isoleucine; Met, methionine; Asn, asparagine; Ser, serine; Val, valine; Tyr, tyrosine; The first three-letter code indicates the original amino acid, follow by numbers that indicate the position of the amino acid in respective gene, and the last three-letter code indicates the amino acid that substituted the original amino acid in the position mentioned. For example, Asp87Tyr means tyrosine replaced aspartic acid in position 87 in GyrA subunit

## 5.5 Discussion

The HRM primers designed in this study spanned the entire QRDR of all four target genes. The QRDRs are short regions of DNA, making them suitable targets for HRM analysis (optimal amplicon length 150 - 350 bp). The transition of melt curves in HRM analysis of all target regions successfully discriminated the wild types from mutants. Although the melting transitions were not very distinct in aligned melt curves, especially for *gyrB*, the melting transitions of the mutants became apparent when displayed in a difference plot. These transitions are direct consequences of the mutations in the target gene QRDRs, since they are the only genetic variations within the PCR target region.

Point mutations were identified in QRDRs of *gyrA*, *gyrB*, *parC* and *parE*, based on the melting curve analyses of the quinolone-resistant strains. These findings were validated with DNA sequence analysis of the respective amplicons as presented in Appendix E and Appendix F. In consistence with the literature (Hopkins *et al.,* 2005; Lunn *et al.,* 2010), mutations in *gyrA* QRDR of the *Salmonella* strains studied most commonly take place at codon 83 and 87, often resulting in amino acid change and are associated with the nalidixic acid resistance in *Salmonella* strains. Gyrase A-DNA complex is the primary target for quinolones in *Salmonella* (Michael *et al.,* 2006). Hence, prolonged usage of quinolones often selects for development of resistant strains with mutations in *gyrA*, since such mutations confer better resistance to quinolone compared to that of *gyrB* mutants (Hopkins *et al.,* 2005). Therefore, a HRM assay can greatly reduce the time and effort spent in order to screen for this most commonly-encountered mutation in the quinolone-resistant *Salmonella* strains. HRM analysis of the *gyrA* successfully resolved the mutant strains into three distinct melting profiles. The melting profiles of the Asp87Gly mutants were highly divergent from that of the other mutants. The Asp87Gly variants consisted of a single base substitution from

adenosine to guanine. This type of mutation represents class I SNP, which often results in a higher melting temperature shift compared to other classes of SNP.

Mutation was not common in the QRDR of *gyrB* among *Salmonella* strains tested in this study. Indeed, most studies did not identify mutations in *gyrB* of quinolone-resistant *Salmonella* (Hopkins *et al.,* 2005; Kim *et al.,* 2011; Lunn *et al.,* 2010). The HRM primers designed in this study covered a region larger than the QRDR, with an additional 132 bp. Hence, the HRM variants detected in this study consisted of three point mutations outside of QRDRs. However, all nucleotide changes resulted in silent mutations. One strain (STM043/05) consisted of a silent mutation in the QRDR, and an additional three mutations compared to other mutants (Appendix E). This strain consisted of class I SNPs at two sites, thus producing a melting profile different from other variants. The different variants could be resolved by HRM as distinct difference plots.

Topoisomerase is the secondary target of quinolones in Gram-negative bacteria. Mutations within QRDRs of *parC* and *parE* genes are uncommon among *Salmonella* (Hopkins *et al.,* 2005; Kim *et al.,* 2011; Lunn *et al.,* 2010). The strains used in this study showed classical phenotype, i.e. resistant to nalidixic acid but not to ciprofloxacin. These observations are in agreement with previous reports, which suggested that the mutations of these genes are most probably infrequently detected because they are either not important in quinolone resistance, or they are only required for higher level of resistance, e. g. resistant to fluoroquinolones (Hopkins *et al.,* 2005). Therefore, the HRM assay aids in filtering wild type strains for these two genes, thus greatly reducing the cost of operation should a large sample pool is examined. Multiple mutations were detected both within and beyond the QRDRs of *parC* and *parE* genes in the *Salmonella* strains used in this study. However, these were mostly silent mutations. HRM assay

successfully differentiated the mutants from the wild type. The melting profiles of the mutants were highly divergent from that of the wild type, as clearly shown in both aligned melt curves and difference plots (Figure 5.3 and Figure 5.4).

Despite the high efficiency of HRM analysis in mutation screening, this assay could only detect the presence of base substitutions. The precise nucleotide change that occurred and the outcome of the base substitution (missense or silent mutation) could not be confirmed by HRM analysis. Hence, the melting profiles should not be used directly for variants classification without first validating with DNA sequencing. False positive may occur due to synonymous mutations; while false negative occurs due to mutations outside targeted regions, or resistance caused by other mechanisms. To-date, DNA sequencing remains the ultimate proof for mutations that lead to amino acid changes and subsequently an alteration of enzyme structure (Tindall *et al.,* 2009). Nevertheless, melting profiles are useful parameters to distinguish mutants with different characteristics and number of mutations, since individual mutant presents a distinctive melt curve when viewed in the difference plot. Hence, HRM analysis can be considered as an efficient and cost-effective preliminary step in the process of identifying QRDR mutations in *Salmonella*.

## 5.6 Conclusion

In conclusion, HRM analysis allows for detection of mutations in *Salmonella* gyrase and topoisomerase IV genes with sufficient sensitivity. The adoption of this assay for mutation screening prior to DNA sequencing is an attractive option to reduce the cost for mutational studies related to quinolone resistance. Furthermore, HRM analysis is time-saving, allows for high throughput screening, and easy to perform since there is no post-PCR processing or purification steps. Nevertheless, sequencing should be used for final validation and should not be completely replaced by HRM analysis.

# CHAPTER 6: GENOMIC ANALYSES OF ENDEMIC *Salmonella* TYPHIMURIUM AND THE MONOPHASIC VARIANT - *Salmonella* I 4,[5],12:i:-

## 6.1 Introduction

*S.* Typhimurium (antigenic formula 4,[5],12:i:1,2) is one of the most prevalent *Salmonella* serovars causing human infections on global scale (Hendriksen *et al.,* 2011; Majowicz *et al.,* 2010). So far, whole genome sequencing studies of *Salmonella* published in Malaysia have mainly focused on the human-specific serovar *S.* Typhi. Since the mid-90's, worldwide increased prevalence of *Salmonella* I 4,[5],12:i:-, a monophasic variant of *S.* Typhimurium, was documented. This monophasic variant is antigenically similar and genetically closely-related to *S.* Typhimurium. In Asian countries, the isolation of *Salmonella* I 4,[5],12:i:- has been reported in China, Japan, Thailand, and Taiwan, but is yet to be documented in Malaysia.

Hence, the objective of this study was to elucidate the genomic features of the endemic *S.* Typhimurium strains and a monophasic variant which was identified in this study. Whole genome sequence analysis was performed in order to understand the phylogenetic relationship and pathogenicity of the two organisms.

## 6.2 Literature review

### 6.2.1 Whole genome sequencing in epidemiological studies of *Salmonella*

The advent of next generation sequencing has enabled worldwide application of whole genome sequence analysis in bacterial genome studies. The advancement in genomic analysis has greatly improved the epidemiological studies of emerging infectious diseases (Li *et al.,* 2014). Phylogenetic analyses based on whole genome sequences of pathogens provide better insights to the evolution and transmission history of the pathogens (Li *et al.,* 2014). Whole genome sequence analysis showed higher resolution but comparable epidemiological correlation with the current gold standard

pulsed-field gel electrophoresis in subtyping bacterial pathogens (Salipante *et al.,* 2015). The exceptionally high resolution of genomic epidemiology in outbreak investigation has made it possible for targeted interventions to be implemented in outbreak management (Tang & Gardy, 2014).

The high throughput genome sequencing data has allowed rapid and highly accurate serotyping of *Salmonella* species, which is substantial in public health surveillance (Zhang *et al.,* 2015). Indeed, whole genome sequencing has been proven useful in *Salmonella* outbreak investigations and source attribution (Alexander *et al.,* 2016; Ashton *et al.,* 2014; Taylor *et al.,* 2015). Moreover, the high resolution and robust genomic data generated by whole genome sequencing showed strong correlation with epidemiological data, thus making it a gold standard in the evaluation of other molecular typing tools in subtyping *Salmonella* strains (Deng *et al.,* 2015). Besides that, whole genome sequencing has also contributed to the studies of pathogenicity and evolution of *Salmonella* (Cao *et al.,* 2013; Langridge *et al.,* 2015).

Although whole genome sequencing is the recommended molecular tool for epidemiological studies and outbreak investigations in most of the developed countries (Revez *et al.,* 2017; Salipante *et al.,* 2015), it is still not a viable option in majority of the developing countries, including Malaysia. Implementation of whole genome sequencing as the routine bacterial subtyping tool entails a large sum of investments on sequencing facilities, bioinformatics software, and human resources for skilled analysis of the genomic data. Hence, currently in Malaysia, the use of whole genome sequencing is still limited to genomic studies of individual strains, due to the relatively high cost of operation. Most of these genomic studies focused on *Salmonella* Typhi due to the endemic nature of this serovar in Malaysia (Norazah *et al.,* 2017; Baddam *et al.,* 2012; Muhamad Harish *et al.,* 2015; Yap *et al.,* 2012a; Yap *et al.,* 2012b). Whole genome

sequence analysis has shown that the outbreak and carrier strains of *Salmonella* Typhi in Malaysia showed close genetic resemblance with small genetic variations that were most probably related to genes optimisation in the evolution of the organism (Yap *et al.,* 2014). Besides that, the genomic sequence of a multi-drug resistant *Salmonella enterica* serovar Brancaster isolated from poultry in Malaysia was also determined in order to study the antimicrobial resistance genes harboured in the genome this organism (Chin *et al.,* 2017).

### 6.2.2 The prevalence, virulence and resistance patterns of *Salmonella* I 4,[5],12:i:-

*S.* Typhimurium (antigenic profile 4,[5],12:i:1,2) is motile by means of peritrichous flagella. These flagella are made up of either of the two types of flagellar antigens, namely H:i and H:1,2, that are encoded by flagellin genes *fliC* and *fljB*, respectively (Moreno-Switt *et al.,* 2009). The phase transition of biphasic *S.* Typhimurium is achieved by switching between the expressions of the above-mentioned flagellin genes (Aldridge *et al.,* 2006). This phenomenon forms the basis of the identification of *S.* Typhimurium in conventional serotyping via slide-agglutination method.

*Salmonella enterica* serovar I 4,[5],12:i:- is found to be genetically and antigenically similar to the biphasic *S.* Typhimurium, except that it does not express the phase-2 flagellar antigen (H:1,2). This organism is thought to be the monophasic variant of *S.* Typhimurium as the two organisms were often indistinguishable by most of the subtyping methods, including the gold standard pulsed-field gel electrophoresis by producing highly similar and even identical pulsotypes (Amavisit *et al.,* 2005; Zamperini *et al.,* 2007). The *Salmonella* I 4,[5],12:i:- harbours the *mdh* gene which is specific to serovar Typhimurium (Amavisit *et al.,* 2005; Hopkins *et al.,* 2010). Since 2000's, the monophasic variant was more frequently isolated and had become one of the

major *Salmonella* serovars that cause human infections worldwide. The US Centers for Disease Control and Prevention (CDC) has documented an increased reporting of isolation of *Salmonella* I 4,[5],12:i:- since the 1990's to 2011, mostly associated with gastroenteritis, and often found in children from 0 - 4 years old (CDC, 2013a). The emergence of the *Salmonella* I 4,[5],12:i:- in Asian countries has occurred within the same period as in European region. The isolation of this organism has first been reported in Thailand in 1993 (Boonmar *et al.,* 1998). Since then, the occurrence of the *Salmonella* I 4,[5],12:i:- has also been documented in Taiwan, Japan, and China (Chiu *et al.,* 2006; Ido *et al.,* 2014; Moreno-Switt *et al.,* 2009; Yang *et al.,* 2015). The organism has been isolated from various sources other than porcine origins, including poultry and bovine sources.

*Salmonella* I 4,[5],12:i:- strains isolated from the same geographical region often fall into a single genetic lineage, or at least shared a very close common ancestor (Guerra *et al.,* 2000; De la Torre *et al.,* 2003). An earlier study showed that the US and Spanish *Salmonella* I 4,[5],12:i:- strains constituted two distinct clones based on the different genomic deletion patterns surrounding the *fljAB* genes (Soyer *et al.,* 2009). Furthermore, a recent study in China has identified unique genomic deletion patterns in the *Salmonella* I 4,[5],12:i:- isolated from food products in China (Yang *et al.,* 2015). These evidences showed that independent evolution of multiple successful monophasic clones has occurred after the divergence from *S.* Typhimurium ancestors. Besides being genetically clonal, the monophasic variant also showed close genetic relationship with *S.* Typhimurium lineages (Guerra *et al.,* 2000). Majority of the *Salmonella* I 4,[5],12:i:- showed phage pattern similar to *S.* Typhimurium U302 (Amavisit *et al.,* 2005; De la Torre *et al.,* 2003; Echeita *et al.,* 1999; Guerra *et al.,* 2000). However, the *Salmonella* I 4,[5],12:i:- belonging to other phage types, mainly DT193 and DT120, had also been

identified (Barco *et al.,* 2013; Hauser *et al.,* 2010; Hopkins *et al.,,* 2012; Hopkins *et al.,* 2010; Mossong *et al.,* 2007).

Generally, the antimicrobial resistance patterns of *Salmonella* I 4,[5],12:i:- ranged from pan-susceptible to multi-drug resistant (MDR) (Moreno-Switt *et al.,* 2009). The *Salmonella* I 4,[5],12:i:- in Europe are often MDR strains, while strains isolated from US appeared pan-susceptible or only resistant to a few antibiotics (Hopkins *et al.,* 2010; Moreno-Switt *et al.,* 2009). Similar to Europe, majority of the monophasic strains isolated in Asian region were MDR (Amavisit *et al.,* 2005; Huoy *et al.,* 2014; Yang *et al.,* 2015). Interestingly, the resistance gene clusters of the *Salmonella* I 4,[5],12:i:- do not share a common ancestor with MDR *S.* Typhimurium, as seen when a new resistance island is present (Hopkins *et al.,* 2010). The new resistance island is found inserted at the *fljAB* operon region of the European *Salmonella* I 4,[5],12:i:-; while the MDR phenotype of *S.* Typhimurium is often attributed to the *Salmonella* genomic island 1 (SGI-1) (Hermans *et al.,* 2006; Hopkins *et al.,* 2010). On the other hand, the virulence and pathogenicity genes repertoire of *Salmonella* I 4,[5],12:i:- are highly similar to that of *S.* Typhimurium, with minor variations which could be attributed to the virulence plasmids (Hauser *et al.,* 2010; Yang *et al.,* 2015).

## 6.3 Methodology

### 6.3.1 PCR-based identification and characterization of gene deletions in the *fljAB* region of the monophasic variant

The 84 *S.* Typhimurium strains that were previously characterized were scanned for the monophasic variant via PCR serotyping method. Primers used were adapted from a study reported by Cardona-Castro *et al.* (2009) (Table 6.1). The *fliC* primer pairs targeted the phase-1 H:i flagellin gene, while the *fljB* primer pairs targeted phase-2 H:1,2 flagellin gene. PCR was performed according to Table 6.1. The PCR products

were then subjected to gel electrophoresis on a 1.5 % agarose gel (Promega, Madison, USA) and visualized using Gel Doc$^{TM}$ XR imaging system (Bio-Rad, Berkeley, USA) after stained by ethidium bromide solution (Sigma-Aldrich, Missouri, USA).

The confirmed monophasic strain of *S.* Typhimurium was then subjected to PCR identification of *fljA*, *hin*, *iroB*, *iroB*-*iroC* intergenic region, STM2757, and STM2758 genes. The gene-specific primers used in this study were previously described by Soyer *et al.* (2009) and García *et al.* (2013). The primer sequences and optimized PCR conditions were described in Table 6.1. A reference strain of *S.* Typhimurium (ATCC13311) that was available in the laboratory strains collection was used in the PCR detection of *fljAB* operon and the flanking region. The PCR products were then subjected to agarose gel electrophoresis.

**Table 6.1:** PCR primers and thermal-cycling conditions for the identification and characterization of monophasic variant of *S*. Typhimurium

| Target gene | Primer | Primer sequence (5'-3') | Amplicon size (bp) | Modified PCR amplification protocol | Reference |
|---|---|---|---|---|---|
| *fliC*-i | Forward | TACGCCAAAGTTACCGTTACGG | 304 | PCR amplification was done in a 25 µL multiplex reaction mixture, containing 1× colourless GoTaq Flexi Buffer, 1.2 mmol/L $MgCl_2$, 100 µmol/L dNTP mix, 0.2 µmol/L of each primer pair, 1 U *Taq* DNA polymerase (Promega, Madison, USA), and approximately 100 ng of bacterial genomic DNA. The PCR reaction mixtures were first incubated at 94 ℃ for 5 min; followed by 30 cycles of 94 ℃ for 1 min, 58 ℃ for 1 min, and 72 ℃ for 1 min; with a final extension step of 72 ℃ for 10 min. | Cardona-Castro *et al.* (2009) |
| | Reverse | AATCATCGCCTACCTTAACTGCTAA | | | |
| *fljB*-1,2 | Forward | GAATGGTACGGCTTCTGTAACC | 453 | | |
| | Reverse | CCGTCAGCAGCAGTATAACTTG | | | |
| *fljA* | Forward | TTCATTAGGTCCCCTCCGG | 642 | PCR amplification was done in a 25 µL monoplex reaction mixture, containing 1× colourless GoTaq Flexi Buffer, 1.5 mmol/L $MgCl_2$, 200 µmol/L dNTP mix, 0.3 µmol/L of each primer pair (0.45 µmol/L for *fljA* and *iroB*-5' primer pairs), 1 U (1.5 U for *fljA* and *iroB*-5' genes) of *Taq* DNA polymerase (Promega, Madison, USA), and approximately 100 ng of bacterial genomic DNA. The PCR reaction mixtures were first incubated at 95 ℃ for 5 min; followed by 25 cycles of 95 ℃ for 1 min, 55 ℃ for 45 s (62 ℃ for STM2757 gene), and 72 ℃ for 1 min; with a final extension step of 72 ℃ for 10 min. | Soyer *et al.* (2009) |
| | Reverse | ATTCAGCCCCGTGAATTCGGG | | | |
| *hin* | Forward | TGGCTACTATTGGGTATATTCGGG | 570 | | |
| | Reverse | AATTCATTCGTTTTTTTATGCGGC | | | |
| STM2757 | Forward | ATGATGATGGCGTAATGGCGC | 717 | | |
| | Reverse | AAAACGTTCCGGTGCGGCG | | | |
| STM2758 | Forward | GACGGATGGCAAAGGTAAAC | 707 | | García *et al.* (2013) |
| | Reverse | AGCGATTTGTGCCGATAGTG | | | |

**Table 6.1**, continued.

| Target gene | Primer | Primer sequence (5'-3') | Amplicon size (bp) | Modified PCR amplification protocol | Reference |
|---|---|---|---|---|---|
| *iroB* (5' end) | Forward | TGCGTATTCTGTTTGTCGGTCC | 605 | | Garc á *et al.* (2013) |
| | Reverse | TACGTTCCCACCATTCTTCCC | | | |
| *iroB* (3' end) | Forward | ACCGCCCAGCATGAGCATAC | 461 | | |
| | Reverse | CGGGAATAATGCCGCATCCG | | | |
| *iroB-iroC* | Forward | AAGAGCGGGCTTACGAGCAG | 756 | | |
| | Reverse | GCGGCCAGTACGCAAATCAC | | | |

### 6.3.2 Genomic DNA extraction and whole genome sequencing

The monophasic variant and two selected *S.* Typhimurium strains (STM001/70 and STM057/05) isolated from clinical sources were subjected to whole genome sequencing for genomic comparison. The selected *S.* Typhimurium strains were isolated 35 years apart (STM001/70 in 1970; STM057/05 in 2005), but showed identical pulsed-field gel electrophoretic pulsotype and were single locus variants as shown by multiple-locus variable number tandem repeat analysis (Chapter 3: Figure 3.1). The persistence of this genotype over the years and its close genetic proximity with most of the other *S.* Typhimurium strains showed that the two biphasic strains might resemble the endemic *S.* Typhimurium that have circulated in Malaysia over the years. The genomic DNA of the monophasic and biphasic strains was extracted using a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following manufacturer's instructions. The quality of the extracted genomic DNA was then measured using NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, Wilmington, USA) prior to whole genome sequencing via Illumina Miseq platform (GA2x, pipeline version 1.80) by a commercial sequencing vendor (ScienceVision, Selangor, Malaysia).

### 6.3.3 Genomic assembly, annotation, comparative genomics, and phylogenetic analysis

The quality of the sequence reads was assessed and *de novo* assembly was performed by using the CLC Genomics Workbench version 5.1 (CLC Bio, Aarhus, Denmark). The assembled contigs were then ordered according to the *Salmonella* Typhimurium LT2 reference genome (NCBI GenBank accession no.: NC_003197) using Mauve version 2.4.0 (Darling *et al.,* 2010). For each of the assembled genome, the subset of contigs that could not be mapped to the chromosome of *S.* Typhimurium LT2 was identified as "accessory" genome in this study. Genomic annotation was performed using the web server Rapid Annotations using Subsystems Technology (RAST; http://rast.nmpdr.org/)

and the annotated genomes were viewed using SEED viewer (http://pubseed.theseed.org/) (Aziz *et al.,* 2008; Overbeek *et al.,* 2014). The annotated genomes were then mapped against the reference genome *S.* Typhimurium LT2 using RAST server and Mauve software to identify regions with genomic variations. Multiple genome alignment was performed using Mauve software and BLAST Ring Image Generator (BRIG) software (Alikhan *et al.,* 2011). *In-silico* multilocus sequence typing (MLST) analysis for the annotated monophasic and biphasic genomes was performed using the web-based MLST database (http://mlst.warwick.ac.uk/) (Achtman *et al.,* 2012). Phage-associated genes in the assembled genomes were identified by using the web server Phage Search Tool Enhanced Release (PHASTER; http://www.phaster.ca) (Arndt *et al.,* 2016; Zhou *et al.,* 2011). The identified phage-associated regions in the genomes were further verified by examining RAST annotation output and NCBI Basic Local Alignment Search Tool (BLAST; https://blast.ncbi.nlm.nih.gov/Blast.cgi) server was used to manually interrogate individual genes of interest. The web-based Reference sequence Alignment based Phylogeny builder (REALPHY; http://realphy.unibas.ch/fcgi/realphy) was used to perform core genome phylogenetic analysis of the assembled *S.* Typhimurium genomes (Bertels *et al.,* 2014). SNP calling was performed using default input parameters (50 bp read length, 20 bp seed length, 0.95 polymorphism threshold, and zero gap threshold), with *S.* Typhimurium LT2 as reference genome. Next, the generated multiple genome sequence alignments were used to construct an unrooted phylogenetic tree that was inferred via the approximate maximum likelihood (ML) method using FastTreeMP software (Price *et al.,* 2010). The phylogenetic tree was visualized as an unrooted tree using FigTree v1.4.3 software (http://tree.bio.ed.ac.uk/software/figtree/) (Rambaut, 2016). Bootstrap values were indicated at the nodes of the phylogenetic tree generated. Bootstrapping assesses the

accuracy of statistical estimate, useful in providing confidence level in the estimation of phylogenetic trees (Efron *et al.*, 1996).

### 6.3.4 Antimicrobial resistance and virulence determinants identification

The antimicrobial resistant genes present within the assembled genomes were identified using the online tool Resistance Gene Identifier (RGI; https://card.mcmaster.ca/analyze/rgi) (McArthur *et al.*, 2013). The similarity cut-off for the identified genes was selected as perfect and strict matches only, to the curated reference sequences in the Comprehensive Antibiotic Resistance Database (CARD; https://card.mcmaster.ca/home). Essential virulence factors for *S.* Typhimurium pathogenicity were retrieved from the online Virulence Factors for Pathogenic Bacteria database (VFDB; http://www.mgc.ac.cn/VFs/) and aligned to the assembled genomes to identify the virulence determinants present in the organisms (Chen *et al.*, 2005).

### 6.3.5 Nucleotide accession number

The genome sequences of *S.* Typhimurium strains STM001/70 and STM057/05, and *Salmonella* I 4,[5],12:i:- strain STM032/04 reported in this study are deposited in NCBI GenBank under the accession number PQMY00000000, PQMZ00000000, and PQNA00000000, respectively.

## 6.4 Results

### 6.4.1 Identification of monophasic variant strain

Majority of the strains (99 %, n = 83) were confirmed as biphasic *S.* Typhimurium. Only one strain (STM032/04) of clinical origin isolated in 2004 was identified as monophasic variant of *S.* Typhimurium, lacking the *fljB* H:1,2 allele (Figure 6.1).

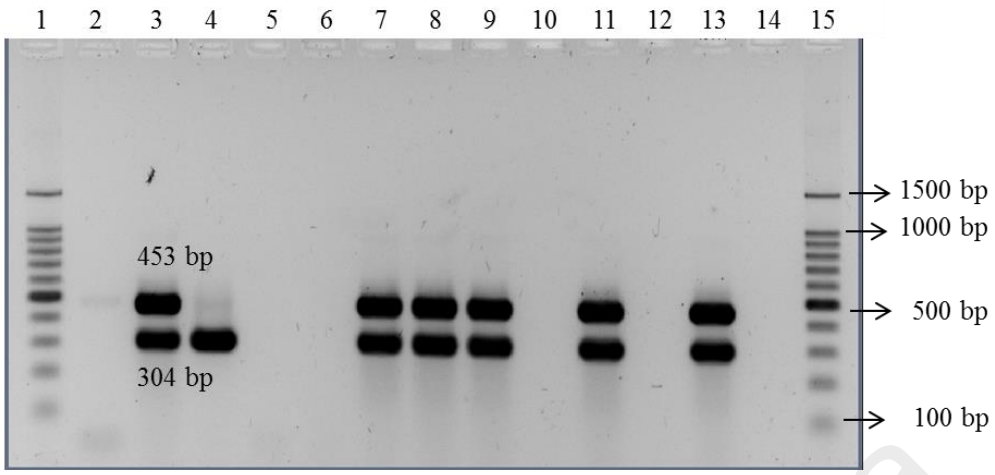



**Figure 6.1:** Representative gel photo for the PCR identification of *fliC* and *fljB* genes in *S.* Typhimurium strains. The amplicon size for *fliC* is 304 bp, and that of *fljB*-1,2 is 453bp. Lanes 1, 15: 100 bp molecular ladders; lane 4: monophasic variant STM032/04; lanes 3, 7, 8, 9, 11: *S.* Typhimurium; lane 13: ATCC13311; lane 2: *Salmonella* subspecies I rough:d:7; lanes 5, 6, 12: *Salmonella* spp. (not Typhimurium); lane 10: *S.* Corvallis.

### 6.4.2 Genomic deletion in the *fljAB* operon and its flanking region in the monophasic STM032/04 strain

PCR analysis showed that the genes related to the expression of phase-2 flagellin were absent in the monophasic strain (Figure 6.2). The *fljAB* operon, which consisted the repressor of phase-1 flagellin gene (*fljA*) and the phase-2 flagellin gene (*fljB*), was absent in the monophasic STM032/04 strain, but present in the biphasic STM057/05 and the control strain *S.* Typhimurium ATCC13311. The DNA invertase gene (*hin*) required for the inversion of the *fljB* controlling region and the putative glycosyl transferase gene *iroB* (STM2773), which flanked the *fljAB* operon, were also absent in the monophasic STM032/04. Interestingly, a truncated region of the *iroB* gene (from nucleotide 655 to 1116) was identified in the genome of the monophasic strain, although the PCR amplification of both 5'- and 3'-end of the *iroB* gene failed to produce amplicons. Genomic sequence analysis of the monophasic strain showed that the *iroC* gene was present, and the presence of amplicon that spanned the *iroB* and *iroC*

intergenic region supported this notion. Two closely-located genes that are also related to the determination of the monophasic variant lineage, namely STM2757 (a cytoplasmic protein that involves in glucose-6-phosphate isomerase activity) and STM2758 (putative phosphotransferase system IIC component that involves in a major carbohydrate active-transport system), were both absent in the monophasic STM032/04 too. All genes were present in the *S.* Typhimurium strains (Figure 6.2). In short, the entire *fljAB* operon was deleted from the monophasic STM032/04 genome, spanning a wide genomic region from gene STM2757, and ended at nucleotide 655 in the *iroB* gene.

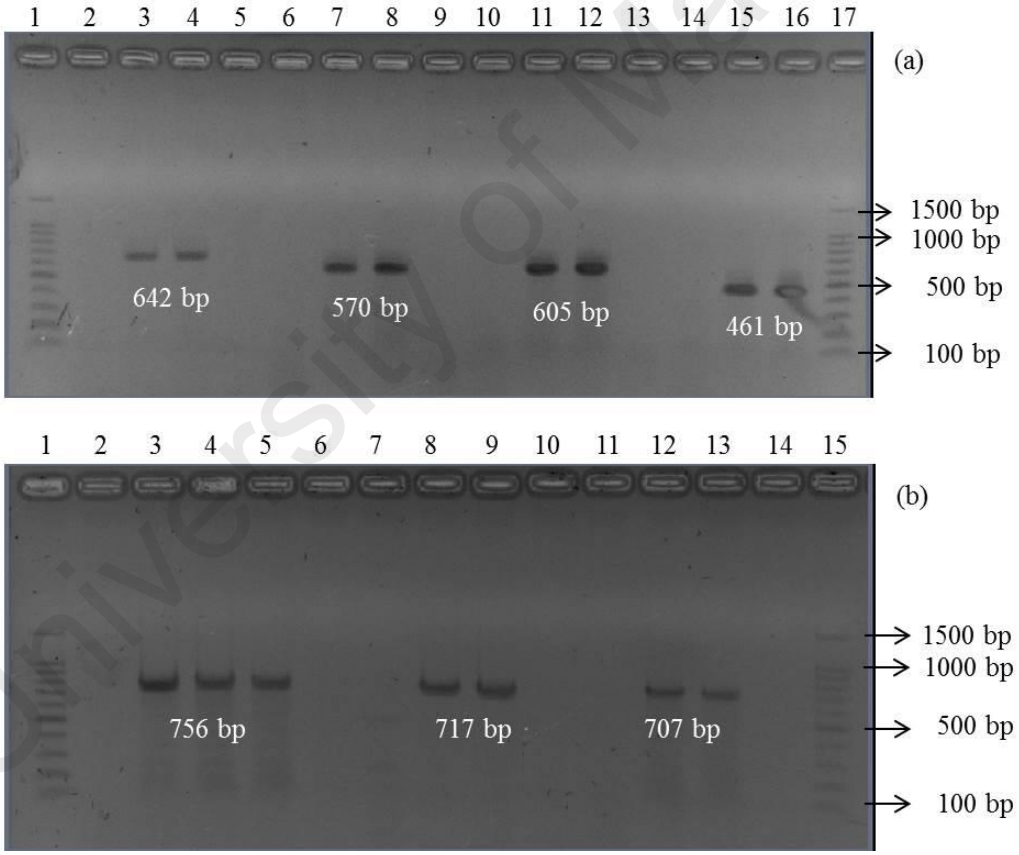



**Figure 6.2:** PCR confirmation of the deletion of *fljAB* operon and its flanking genes. (a) Amplicons of *fljA* (642 bp), *hin* (570 bp), *iroB* (5'-end; 605 bp), and *iroB* (3'-end; 461 bp). Lanes 1, 17: 100 bp molecular ladders; lanes 2, 6, 10, 14: monophasic variant STM032/04; lanes 3, 7, 11, 15: biphasic STM057/05; lanes 4, 8, 12, 16: ATCC13311; lanes 5, 9, 13: no-template-controls for the PCR amplification of respective genes. (b) Amplicons of *iroB*-*iroC* intergenic region (756 bp), STM2757 (717 bp), and STM2758 (707 bp). Lanes 1, 15: 100 bp molecular ladders; lanes 3, 7, 11: monophasic variant STM032/04; lanes 4, 8, 12; biphasic STM057/05; lanes 5, 9, 13: ATCC13311; lanes 2, 6, 10, 14: no-template-controls for the PCR amplification of respective genes.

### 6.4.3 Genome features of the biphasic STM001/70 and STM057/05, and the monophasic variant STM032/04

*De novo* assembly of the biphasic STM001/70 genome produced 278 contigs with total genome coverage of 184-fold, and the N50 value was 60,876 nucleotides. The predicted genome size of STM001/70 was approximately 4.9 Mb (4,890,967 nucleotides), and the guanine-plus-cytosine (GC) content of the strain was 52.2 %. A total of 4855 protein-coding sequences and 93 RNAs were identified in the annotated genome of STM001/70.

*De novo* assembly of the biphasic STM057/05 genome produced 207 contigs with total genome coverage of 138-fold. The N50 value of the assembled genome was 54,347 nucleotides. The predicted genome size of STM057/05 was approximately 4.9 Mb (4,905,734 nucleotides) with a 52.2 % GC-content. A total of 4890 protein-coding sequences and 95 RNAs were identified in the annotated genome of STM057/05.

*De novo* assembly of the monophasic STM032/04 genome generated 114 contigs with total genome coverage of 256-fold, and the N50 value was 104,760 nucleotides. The predicted genome size of the monophasic STM032/04 was approximately 4.8 Mb (4,831,369 nucleotides) with 52.1 % GC-content. The annotated genome of STM032/04 comprised of 4816 protein-coding sequences and 86 RNAs.

*In-silico* MLST results showed that all three strains belong to sequence type ST19, with an allele profile 10-7-12-9-5-9-2 (arranged in the order *aro*C-*dna*N-*hem*D-*his*D-*pur*E-*suc*A-*thr*A). The serovar Typhimurium-specific *mdh* gene was identified in all three assembled genomes.

### 6.4.4 Comparative genomic analysis of the endemic *S.* Typhimurium and the monophasic variant

The assembled genomes were mapped to the *S.* Typhimurium LT2 reference genome and visualized as concentric rings with BRIG software (Figure 6.3). Four complete genomes of historical *S.* Typhimurium strains were retrieved from the NCBI genome database and were included for comparison. These complete genomes include *S.* Typhimurium strain UK-1 (NCBI GenBank accession no.: NC_016863), SL1344 (NC_016810), DT104 (NC_022569), and ATCC13311 (NZ_CP009102). The *S.* Typhimurium STM001/70 and STM057/05 genomes were highly similar to each other, in comparison with the monophasic STM032/04 genome. Genetic variations within the endemic *S.* Typhimurium strains in comparison with the LT2 reference genome were mostly associated with phage regions, as observed in Fels-1, Fels-2, and Gifsy-1 prophages. Similar genetic variations within the Fels-1 and Fels-2 prophage regions were also observed in the other four *S.* Typhimurium reference genomes. Great genetic variation was observed in the genomic island encoding *stb* fimbriae genes in the monophasic STM032/04 genome. Similar to the *S.* Typhimurium, the monophasic STM032/04 genome showed variations in Fels-1 and Fels-2 prophage regions, but not for Gifsy-1. The five *Salmonella* pathogenicity islands (SPIs) commonly found in *S.* Typhimurium were all present in the assembled genomes. In consistence with the PCR results reported in section 6.4.2, a vast genomic deletion spanning the Fels-2 prophage and the *fljAB* operon was observed in the monophasic STM032/04 genome, but not in the *S.* Typhimurium genomes.

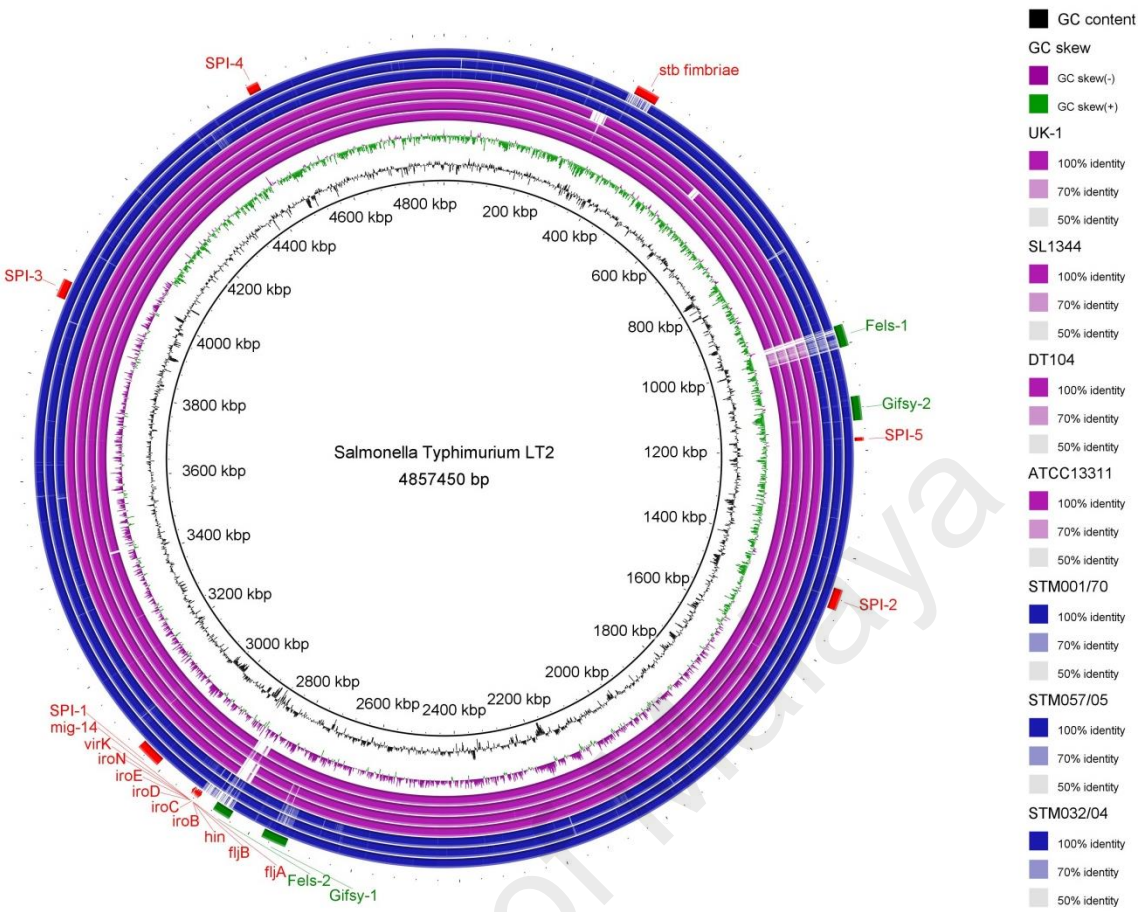



**Figure 6.3:** Genome map of the monophasic variant and *S.* Typhimurium strains. The assembled genomes of the strains examined in this study (blue rings) were mapped to the *S.* Typhimurium LT2 reference genome (not shown in diagram). Four complete genomes that represent the virulent and MDR strains of *S.* Typhimurium were included for genomic comparison (purple rings). From the inner to the outermost ring, the first (innermost) ring shows the genome size in kbp, second and third ring indicate the GC content (black) and GC skew (purple and green) respectively, and the subsequent rings are *S.* Typhimurium strain UK-1, SL1344, DT104, ATCC13311, STM001/70, STM057/05, and the monophasic variant STM032/04. In the outermost ring, the genomic locations of the five major pathogenicity islands (SPI1-5; red), Fels and Gifsy prophages (green), *fljAB* operon, and the flanking genes (red) are shown in arcs.

A total of five phage-associated regions were identified in the core genomes of the *S.* Typhimurium and the monophasic strain respectively (Table 6.2). Among these, three intact prophages were identified in STM001/70, four in STM057/05, and three in the monophasic STM032/04 core genome. Intact region of Gifsy-2 prophage was common among the strains. On the other hand, Gifsy-1 prophage was predicted by PHASTER in the monophasic STM032/04 and *S.* Typhimurium STM001/70 genomes, with the latter

being incomplete prophage. Intact Fels-1 prophage was detected in the monophasic STM032/04 genome, albeit at low phage identity. Multiple alignments of the core genomes showed several common genomic regions shared between the two endemic *S.* Typhimurium strains with high sequence homology, which were not seen in the monophasic genome (Figure 6.4). Most of these common genomic regions were associated with prophages.


**Figure 6.4:** Comparative analyses of the *S.* Typhimurium strains and the monophasic variant STM032/04. Progressive Mauve analysis was performed on the core genome sequences of the strains. The purple regions represent predicted sequence homology among all three genome sequences; while green regions represent sequences that were shared between the *S.* Typhimurium STM001/70 and STM057/05 genomes only. The labelled boxes indicate the relative positions of phage-associated regions on respective genomes, based on PHASTER prediction. The labelling of the phage-associated regions corresponds to the labelling in Table 6.2. The size of the boxes does not indicate the size of the genomic region. Genomic sequences below the line of the STM057/05 genome indicate predicted inversion based on scaffold tiling to the STM001/70 genome.

**Table 6.2:** Phage-associated regions in the core genomes of the *S.* Typhimurium and the monophasic variant

| Strain | Region | Region length (kbp) | Region position[a] (bp) | PHASTER annotation | Phage identity[b] ( %) | PHASTER prediction[c] |
|---|---|---|---|---|---|---|
| **STM001/70** | A1 | 43.8 | 1050341-1094194 | PHAGE_Phage_Gifsy_2_NC_010393 | 88 | Intact |
| | A2 | 64.7 | 2015720-2080432 | PHAGE_Salmon_118970_sal3_NC_031940 | 81 | Intact |
| | A3 | 32.4 | 2792616-2825045 | PHAGE_Phage_Gifsy_1_NC_010392 | 46 | Incomplete |
| | A4 | 35.2 | 2887280-2922483 | PHAGE_Salmon_RE_2010_NC_019488 | 89 | Intact |
| | A5 | 20.3 | 4311612-4331996 | PHAGE_Burkho_BcepMu_NC_005882 | 59 | Incomplete |
| **STM057/05** | B1 | 44.7 | 1025435-1070187 | PHAGE_Phage_Gifsy_2_NC_010393 | 86 | Intact |
| | B2 | 53.7 | 1984923-2038681 | PHAGE_Salmon_118970_sal3_NC_031940 | 78 | Intact |
| | B3 | 32.3 | 2668263-2700638 | PHAGE_Edward_GF_2_NC_026611 | 50 | Intact |
| | B4 | 34.8 | 2763133-2797936 | PHAGE_Salmon_RE_2010_NC_019488 | 91 | Intact |
| | B5 | 21.7 | 4308597-4330329 | PHAGE_Burkho_BcepMu_NC_005882 | 59 | Incomplete |
| **STM032/04** | C1 | 35.6 | 840651-876304 | PHAGE_Salmon_Fels_1_NC_010391 | 37 | Intact |
| | C2 | 32.7 | 967279-1000046 | PHAGE_Phage_Gifsy_2_NC_010393 | 82 | Intact |
| | C3 | 13.3 | 2010490-2023793 | PHAGE_Salmon_SEN34_NC_028699 | 42 | Incomplete |
| | C4 | 38.1 | 2602362-2640484 | PHAGE_Phage_Gifsy_1_NC_010392 | 79 | Intact |
| | C5 | 20.4 | 4166918-4187337 | PHAGE_Burkho_BcepMu_NC_005882 | 57 | Incomplete |

[a] Identified prophages were over multiple contigs that were concatenated for PHASTER analysis;
[b] Phage identity was calculated based on the percentage of phage-associated genes over the total protein-coding sequences identified in the phage region;
[c] A prediction of whether the phage region contains genes necessary for lysogeny

The accessory genome of the *S.* Typhimurium STM001/70 strain consisted of 196 CDS and 14 RNAs; while that of the STM057/05 contained 189 CDS and 13 RNAs. A total of 209 CDS and 7 RNAs were identified in the accessory genome of the monophasic STM032/04. Majority of the CDS identified in the accessory genomes of the strains comprised of plasmid- or phage-related genes, and mobile genetic elements. One DNA segment (contig-66) harbouring tetracycline-resistant determinant (*tetB* gene) was identified in the accessory genome of the monophasic STM032/04 strain. BLAST analysis of the DNA segment showed 99 % query coverage and 100 % identity with part of the *Salmonella* I 4,[5],12:i:- strain 07-2006 resistance region (HG931185). The DNA segment matched to the genomic region that carries Tet-operon and a sodium/glutamate symporter gene (*gltS*), which is flanked by IS1 transposase and IS10 transposase, in the monophasic strain 07-2006 resistance region (Figure 6.5).

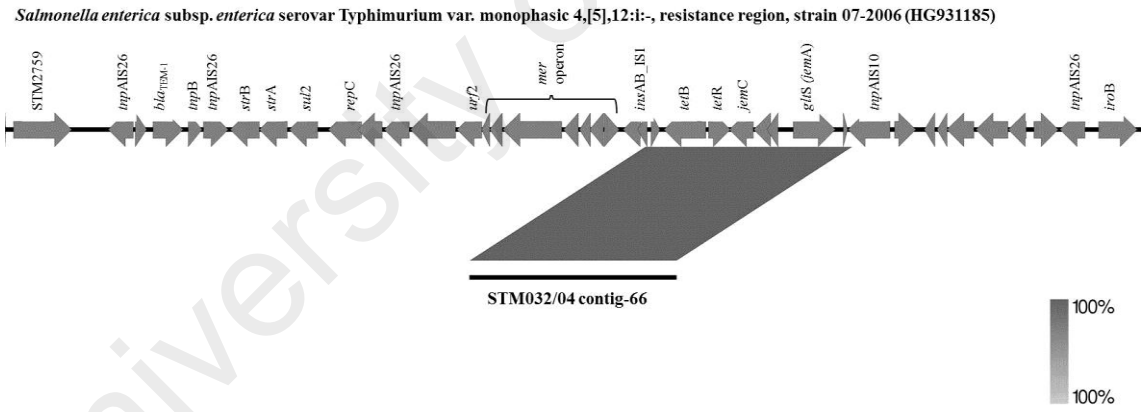



**Figure 6.5:** Genome map of a single DNA segment (contig-66) in the monophasic STM032/04. This monophasic resistance region of *Salmonella* I 4,[5],12:i:- strain 07-2006 (HG931185) is inserted at the *fljAB* operon in the chromosome, between STM2759 and *iroB* genes. The shaded arrows indicate coding sequences in the genomic region (arrows pointing to the left indicate genes on the complement DNA strand). The figure is generated using Easyfig software (Sullivan *et al.,* 2011).

Genomic regions showing great resemblance to the pSLT virulence plasmid (NC_003277) of *S.* Typhimurium LT2 had been identified in the accessory genomes of the examined strains (Figure 6.6). Intact regions of pSLT plasmid were found in the *S.*

Typhimurium STM001/70 and STM057/05 genomes (BLAST analysis showed 100 % query coverage, 99 % nucleotide identity). On the other hand, a short DNA segment resembling an incomplete pSLT plasmid was identified in the monophasic STM032/04 genome. BLAST analysis showed that the plasmid segment spanned the region between *repA2* and *parA* genes in the pSLT plasmid. The plasmid-borne virulence genes *spvRABCD* and the toxin-antitoxin addiction system (*ccdAB*) were identified in this region.

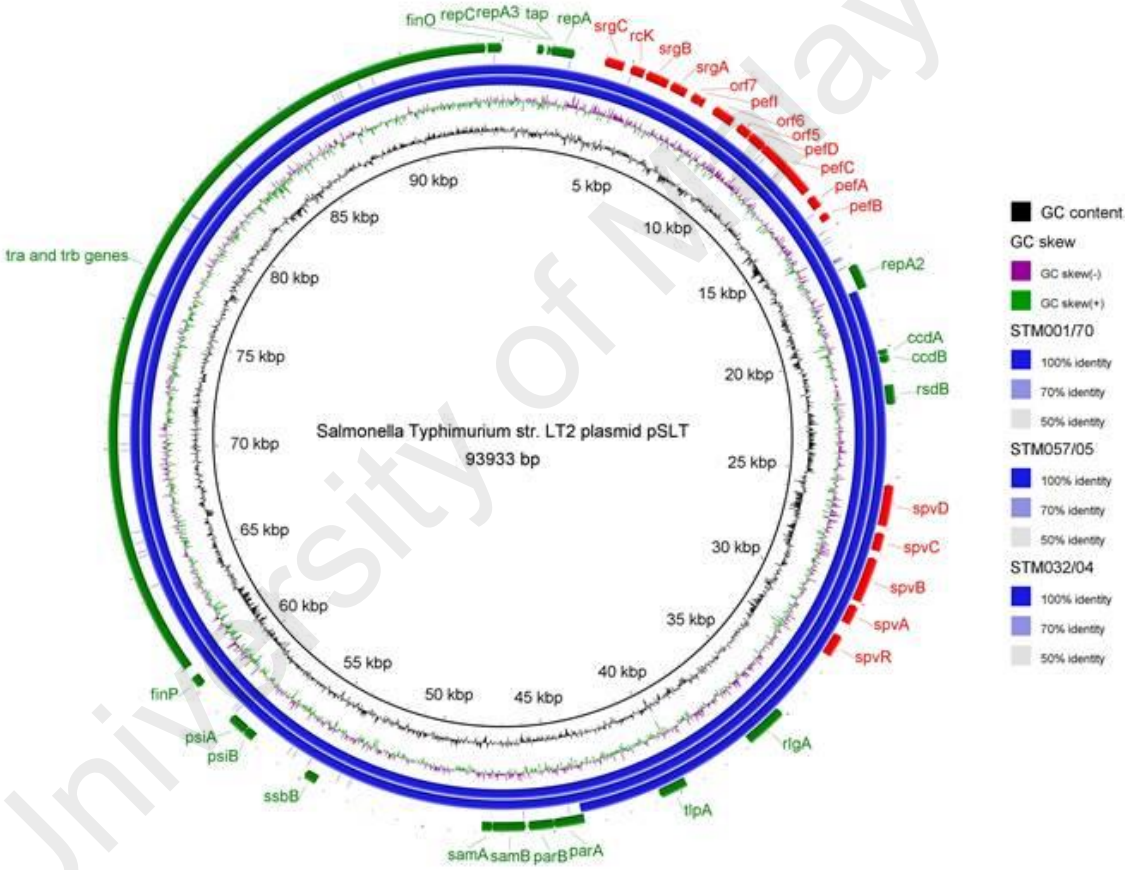



**Figure 6.6:** Genome map of the pSLT plasmid-associated regions. The pSLT plasmid-associated contigs identified in the accessory genomes of the *S.* Typhimurium and monophasic strains were mapped to the pSLT plasmid of the *S.* Typhimurium LT2 (not shown in diagram). From the inner to the outermost ring, the first (innermost) ring shows the genome size in kbp, second and third ring indicate the GC content (black) and GC skew (purple and green) respectively, fourth to sixth ring (blue) represent STM001/70, STM057/05, and STM032/04 respectively. The outermost ring shows the plasmid-borne genes encoding for virulence (red arc), plasmid replication, stability, and conjugative transfer (green arcs).

Besides the pSLT plasmid-associated region, the *S.* Typhimurium STM057/05 also contained a unique DNA segment (contig-65), which harboured several antimicrobial resistance genes (*strAB*, *sul*2, and *tetA*). BLAST analysis of this DNA segment showed 99 % query coverage and 99 % identity with two NCBI GenBank entries (KU852461 and CP004059), both resembling an IncQ plasmid identified in *S.* Typhimurium (pNUC and pSTU288-2). The genetic comparison between contig-65 of STM057/05 and the *S.* Typhimurium pNUC plasmid (KU852461) is visualized as concentric rings in Figure 6.7.

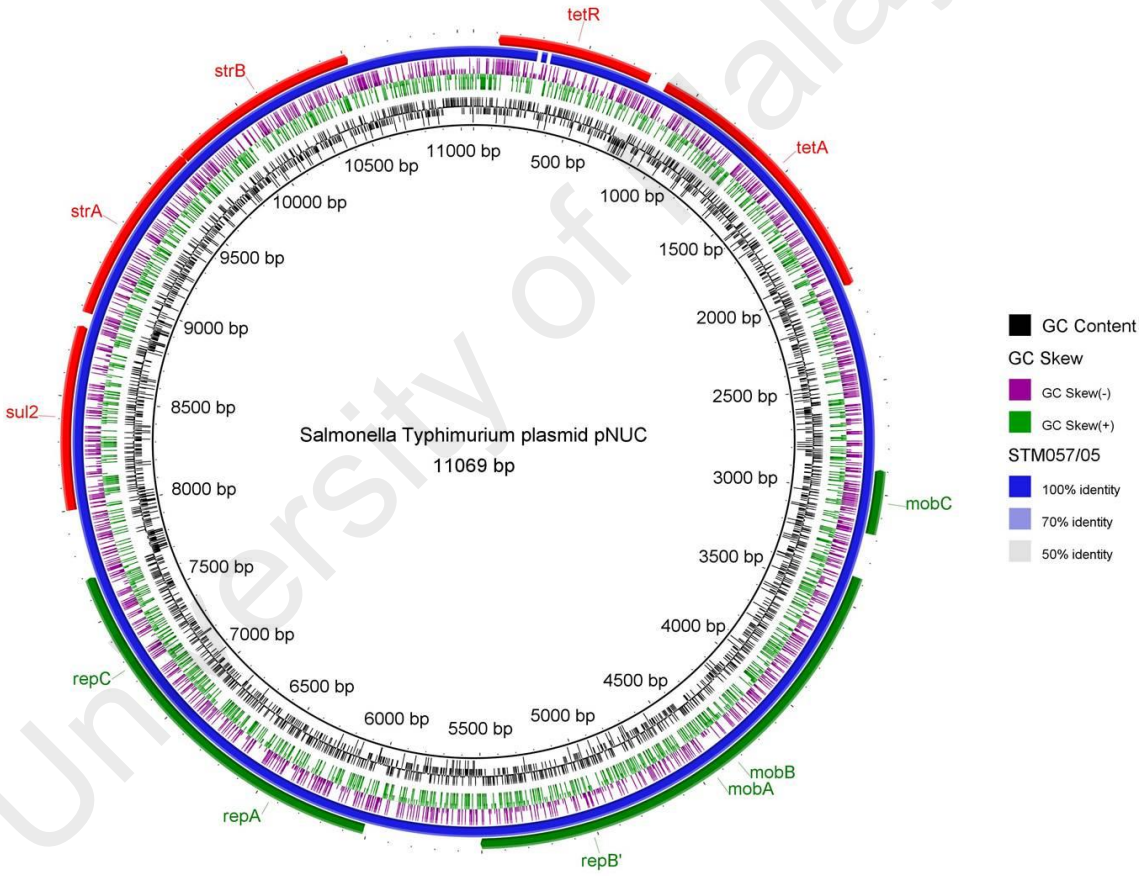



**Figure 6.7:** Genetic features of a DNA segment (contig-65) in theSTM057/05 genome. The antimicrobial resistance genes-encoding DNA segment was mapped to the pNUC plasmid (KU852461) of *S.* Typhimurium (not shown in diagram). From the inner to the outermost ring, the first (innermost) ring shows the genome size in kbp, second and third ring indicate the GC content (black) and GC skew (purple and green) respectively, fourth ring (blue) represents contig-65 in STM057/05, and the outermost ring shows genes related to plasmid replication and mobilization (green arcs), and the antimicrobial resistance genes (red arcs).

In the monophasic STM032/04 accessory genome, DNA fragments that greatly resemble parts of a virulence plasmid pSEEH1578_02 (CP004088) in *Salmonella* Heidelberg strain 41578 (NC_021841) were identified. BLAST analysis of this plasmid-associated genomic region showed 93 % query coverage and 99 % identity with the pSEEH1578_02 virulence plasmid. This plasmid-associated region harboured a type-IV secretion system (T4SS), and plasmid addiction systems (HicAB, RelE/StbE and Relb/StbD). Moreover, a beta-lactamase gene ($bla_{\text{TEM-1}}$) was identified in this genomic region, which was not found in the pSEEH1578_02 plasmid genome. The genetic comparison between the plasmid-associated genomic region of the monophasic STM032/04 and the pSEEH1578_02 plasmid is visualized as concentric rings in Figure 6.8.

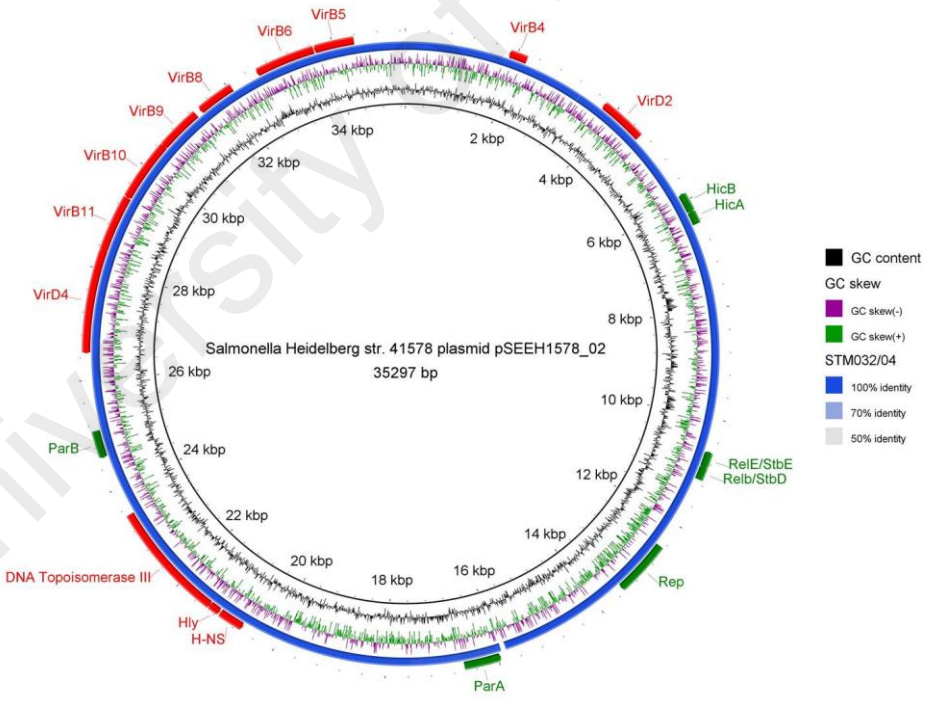



**Figure 6.8:** Genetic features of the plasmid-associated DNA segments identified the accessory genome of the monophasic STM032/04 strain. The DNA segments were mapped to the pSEEH1578_02 plasmid (CP004088) of *S.* Heidelberg strain 41578 (NC_021841) (not shown in diagram). From the inner to the outermost ring, the first (innermost) ring shows the genome size in kbp, second and third ring indicate the GC content (black) and GC skew (purple and green) respectively, fourth ring (blue) represents the concatenated DNA segments of STM032/04, and the outermost ring shows genes related to virulence (red arcs), and plasmid replication and stability (green arcs).

### 6.4.5 Phylogenetic analyses

The monophasic STM032/04 strain was subjected to SNP-based core genome phylogenetic analysis with 16 monophasic *Salmonella* I 4,[5],12:i:- genomes representing clonal strains (CVM23701 for US epidemic clone; STM3910 and SO4698-09 for European clone), outbreak strains (smonpas004260-17 and smonpas003168-1 for atypical nalidixic acid-resistant long-term outbreak strains in Italy; STM87 and STM139 for short-term foodborne outbreak strains in Italy), and strains without any known epidemiological relationship based on available data (Appendix G). The *S.* Typhimurium STM001/70 and STM057/05 were included to investigate the phylogenetic relationship between the monophasic and biphasic strains in Malaysia. The phylogenetic tree generated indicated that the monophasic STM032/04 showed close phylogenetic relationship with the *S.* Typhimurium LT2 reference genome, with 551 SNP sites (Figure 6.9). The European epidemic clones and outbreak strains were closely-clustered, forming distinctive but closely-related clades on the phylogenetic tree. In comparison, the US monophasic strains showed greater genetic diversity than the European strains, forming several closely- or distantly-related clades. The Australian monophasic strain (TW-Stm6) showed close phylogenetic relationship with the European epidemic clones. The *S.* Typhimurium STM001/70 and STM057/05 were essentially identical, forming a distinctive clade separated from the monophasic STM032/04 strain. A total of 1620 and 1633 SNP sites were identified in the core genomes of STM001/70 and STM057/05 respectively, when mapped to *S.* Typhimurium LT2.

When compared with the epidemiologically un-related global strains of *S.* Typhimurium, the endemic strains in Malaysia showed close phylogenetic relationship with the *S.* Typhimurium strains SO2 and D23580, with strong bootstrap support (98 %; i.e. the specific node in the phylogenetic tree showed up in 98 % of the bootstrap

replicates) (Figure 6.10). Specific temporal and geographical distributions among the *S.* Typhimurium strains were not observed (Figure 6.10). Three *Salmonella* I 4,[5],12:i:- strains (STM032/04, CVM23701, and SO4698-09) were included to investigate the phylogenetic relationships among monophasic variants and *S.* Typhimurium. The monophasic strains were found distributed on three branches, showing close phylogenetic relationship with the *S.* Typhimurium strains on the same branch, despite forming a single serovar-specific clade (Figure 6.10).

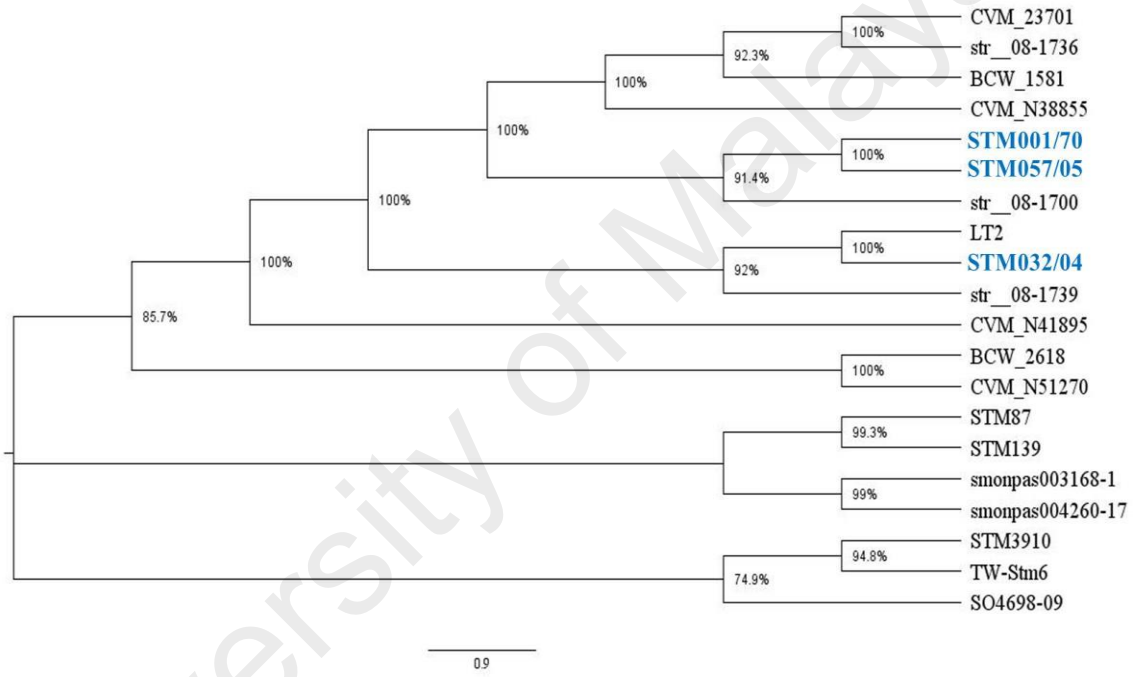



**Figure 6.9:** Phylogenetic tree inferred by approximately-maximum-likelihood method from SNP-based core genomes alignment of the *Salmonella* I 4,[5],12:i:- strains. The multiple genome sequence alignments of 17 global *Salmonella* I 4,[5],12:i:- strains and two endemic *S.* Typhimurium strains (STM001/70 and STM057/05) were generated by the online tool REALPHY using *S.* Typhimurium LT2 as reference. An unrooted phylogenetic tree was inferred by approximately-maximum likelihood method via FastTreeMP software, using Gamma time reversible (GTR) model. Bootstrap support values are indicated at the nodes of the tree. The Malaysian monophasic variant and *S.* Typhimurium strains are labelled in blue letters.

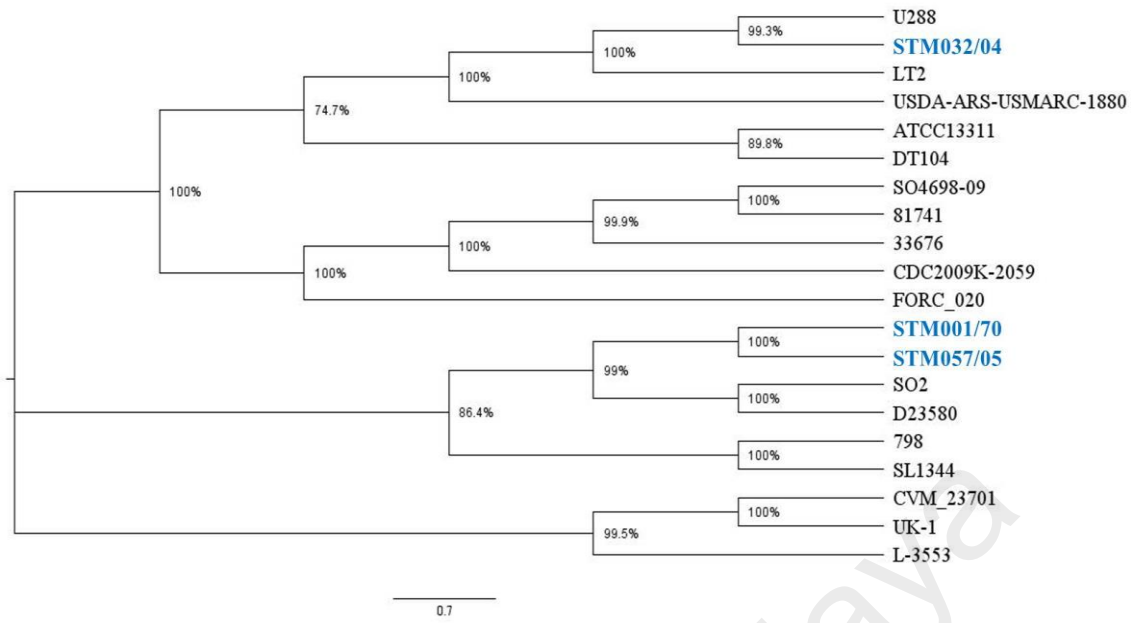



**Figure 6.10:** Phylogenetic tree inferred by approximately-maximum-likelihood method from SNP-based core genomes alignment of the *S.* Typhimurium strains. The multiple genome sequence alignments of 16 global *S.* Typhimurium strains and 3 *Salmonella* I 4,[5],12:i:- strains (STM032/04, CVM23701, and SO4698-09) were generated by the online tool REALPHY using *S.* Typhimurium LT2 as reference. An unrooted phylogenetic tree was inferred by approximately-maximum likelihood method via FastTreeMP software, using GTR model. Bootstrap support values are indicated at the nodes of the tree. The Malaysian monophasic variant and *S.* Typhimurium strains are labelled in blue letters.

### 6.4.6 Antimicrobial resistance and virulence genes identified in the biphasic and monophasic *S.* Typhimurium genomes

The *S.* Typhimurium and monophasic strains examined in this study showed MDR phenotype (Chapter 4). The *S.* Typhimurium STM001/70 exhibited simultaneous resistance to cephalothin, streptomycin, tetracycline, and sulphonamide; STM057/05 was resistant to streptomycin, tetracycline, and sulphonamide; while the monophasic STM032/04 was resistant to ampicillin, gentamicin, tetracycline, and nalidixic acid. Table 6.3 summarizes the antimicrobial resistance genes, efflux pumps contributing to antimicrobial resistance, and regulatory genes identified in the genomes of the *S.* Typhimurium and monophasic strains. Antimicrobial resistance genes that are unique to each individual strain are often identified within the accessory genomes on plasmid-

associated DNA segments. The only exception was the multidrug efflux complex MdsABC, together with its regulatory gene *golS*, which was found in both of the *S.* Typhimurium strains but not the monophasic genome.

Chromosomally-encoded virulence genes essential for fimbrial adherence (*csg*, *bcf*, *fim*, *lpf*, *saf*, *stb*, *stc*, *std*, *stf*, *sth*, *sti*, and *stj* operons), macrophage inducible gene (*mig-14*), magnesium uptake (*mgtBC*), non-fimbrial adherence determinants (*misL*, *ratB*, *shdA*, and *sinH*), SPI-1 and -2 encoded type III secretion system and the translocated effectors, stress protein *sodCI*, and the regulatory system *phoPQ* were all present in the core genomes of the *S.* Typhimurium. Similar observation was made in the monophasic STM032/04 genome, except for the *stb* fimbriae operon, which was not identified in the monophasic genome. All of the pSLT plasmid-encoded virulence factors *pef*, *mig-5*, *rck*, and *spv* operon were identified in both *S.* Typhimurium genomes. On the other hand, only the *spv* operon was detected in the monophasic STM032/04 genome.

**Table 6.3:** Antimicrobial resistance and regulatory genes identified in the monophasic and *S.* Typhimurium genomes

| **Resistance determinants** | **STM001/70[a]** | **STM057/05[a]** | **STM032/04[a]** | **Gene function/Resistance** |
|---|---|---|---|---|
| AAC(3)-IIa | - | - | + | Aminoglycoside |
| AAC(6')-Iaa | + | + | + | Aminoglycoside |
| AcrAB | + | + | + | Efflux pump |
| AcrD | + | + | + | Efflux pump |
| AcrEF | + | + | + | Efflux pump |
| APH(3")-Ib (StrA) | - | + | - | Aminoglycoside |
| APH(6)-Id (StrB) | - | + | - | Aminoglycoside |
| BacA | + | + | + | Peptide antibiotics |
| BaeSR | + | + | + | Regulatory genes |
| CpxRA | + | + | + | Regulatory genes |
| CRP | + | + | + | Regulatory genes |
| EmrD | + | + | + | Efflux pump |
| EmrRAB | + | + | + | Efflux pump |
| GlpT variant | + | + | + | Fosfomycin |
| GolS | + | + | - | Regulatory genes |
| H-NS | + | + | + | Regulatory genes |
| KdpE | + | + | + | Regulatory genes |
| MacAB | + | + | + | Efflux pump |
| MarA | + | + | + | Regulatory genes |
| MdfA | + | + | + | Efflux pump |
| MdsABC | + | + | - | Efflux pump |
| MdtABCD | + | + | + | Efflux pump |
| MdtK | + | + | + | Efflux pump |
| MsbA | + | + | + | Efflux pump |
| MsrB | + | + | + | Efflux pump |
| PatA | + | + | + | Efflux pump |
| Pmr operon | + | + | + | Polymyxin |
| RamA | + | + | + | Regulatory genes |
| RobA | + | + | + | Regulatory genes |
| SdiA | + | + | + | Regulatory genes |
| Sul2 | - | + | - | Sulfonamide |
| $Bla_{TEM-1}$ | - | - | + | Beta-lactam |
| TetA | - | + | - | Tetracycline |
| TetB | - | - | + | Tetracycline |
| TolC | + | + | + | Efflux pump |
| UhpT variant | + | + | + | Fosfomycin |

[a] +, presence of antimicrobial resistance gene;
-, absence of gene

## 6.5 Discussion

A monophasic variant (STM032/04) was identified among the *S.* Typhimurium strains pool in this study. Molecular analysis showed that the genes encoding *fljAB* operon and its flanking regions were absent from the monophasic STM032/04. The genomic features and phylogeny of the monophasic *Salmonella* I 4,[5],12:i:- strain and two endemic *S.* Typhimurium strains isolated in Malaysia are described in this study. All three strains were isolated from human hosts and exhibited MDR phenotype. Comparative whole genome sequence analyses among the strains showed that genetic variations mostly occurred in phage-associated genomic regions. Molecular analysis showed that the genes encoding *fljAB* operon and its flanking regions were absent from the monophasic STM032/04 strain. Phylogenetic analysis of the endemic *S.* Typhimurium (STM001/70 and STM057/05) showed great genomic resemblance between these two strains, despite being isolated 35 years apart. Chromosomal and plasmid-borne virulence and antimicrobial resistance determinants were identified in the monophasic and biphasic genomes.

The monophasic STM032/04 strain was isolated from clinical source in 2004, corresponded with the time of worldwide rapid expansion of monophasic *Salmonella* I 4,[5],12:i:- population (Moreno-Switt *et al.,* 2009). Various *Salmonella* I 4,[5],12:i:- lineages isolated from different geographical regions often showed unique and distinctive deletion patterns in their *fljAB* operon (*fljA*, *fljB*, and *hin*) and the flanking genes (Soyer *et al.,* 2009). The genomic deletion pattern of the *fljAB* operon and its flanking region in the monophasic STM032/04 spanned a wider region compared to all of the known deletion patterns identified so far among the *Salmonella* I 4,[5],12:i:- strains isolated worldwide. Comparable genomic deletion patterns were observed among the Spanish clone (from STM2758 to *iro*B, with conserved STM2757 gene), and the US and China clones (from STM2757 to *flj*B, with conserved *hin* and *iro*B genes)

(Laorden *et al.,* 2010; Soyer *et al.,* 2009; Yang *et al.,* 2015; Zamperini *et al.,* 2007). However, the genetic deletion in the allantoin-glyoxylate operon (STM0517 - STM0529) which is unique to the Spanish clone was not seen in the monophasic STM032/04 genome (Laorden *et al.,* 2010). Genomic deletions among the European monophasic clones often spanned a shorter region (from STM2769 to *hin*, with conserved *iro*B gene) (Lucarelli *et al.,* 2012). The unique genomic deletion pattern seen in the monophasic STM032/04 supported the previous notion that multiple successful monophasic clones have evolved from different *S.* Typhimurium ancestors following different genomic events in various geographical regions (Soyer *et al.,* 2009). However, more monophasic strains should be included for analysis in order to confirm if a single monophasic lineage or multiple clones have arisen in Malaysia.

Previous genotypic characterization of the monophasic STM032/04 based on PFGE and MLVA analyses showed that there was a close genetic relationship between the monophasic variant and other *S.* Typhimurium strains circulating in Malaysia (Chapter 3). This notion was further supported by the sharing of identical MLST type (ST19) between the monophasic variant and the endemic *S.* Typhimurium strains. The sharing of identical STs among *S.* Typhimurium and *Salmonella* I 4,[5],12:i:- is commonly reported among the strains isolated worldwide (Alcaine *et al.,* 2006; Soyer *et al.,* 2009; Yang *et al.,* 2015). Moreover, genomic comparison showed that highly similar genomic regions were shared by the two organisms, except for some genetic variations observed within the prophage regions and the genomic island carrying genes encoding the *stb* fimbriae (Figure 6.3). Such observations concurred with previous premise that monophasic *Salmonella* I 4,[5],12:i:- strains often could not be distinguished from biphasic *S.* Typhimurium using common genotypic or phenotypic typing methods due to the close genetic proximity between the two serotypes (Moreno-Switt *et al.,* 2009). The presence of malate dehydrogenase (*mdh*) gene in the STM032/04 genome confirmed

that this monophasic variant was derived from *S.* Typhimurium via the loss of the phase-2 flagellin (Amavisit *et al.*, 2005; Hopkins *et al.*, 2010).

Comparative analysis showed that the core genomes of the two endemic *S.* Typhimurium strains are essentially identical, and are highly similar to the *S.* Typhimurium LT2 genome, except for minor genetic variations observed in phage-associated regions (Figure 6.3). Moreover, most of the prophages that were identified are unique to the endemic *S.* Typhimurium genomes. As prophages are volatile element of the bacterial genome, the high genetic similarity in the phage regions suggests that STM057/05 falls within the same lineage with the earlier STM001/70 strain, or at least share a recent common ancestor which had acquired the common phage regions prior to an evolutionary divergence. This observation may also indicate that the monophasic STM032/04 strain followed a different evolutionary pathway compared to the endemic *S.* Typhimurium strains that persisted in Malaysia, or might have evolved from a biphasic ancestor that differs from the endemic strains. The acquisition of the prophages might have contributed to the development of virulence and pathogenicity in endemic *S.* Typhimurium strains in Malaysia, as the prophages are known to increase intracellular survival rate (Herrero-Fresno *et al.*, 2014; Klumpp & Fuchs, 2007). The improved competitive fitness of the strains that harboured multiple prophages could warrant the evolutionary success of a phylogenetic lineage (Bossi *et al.*, 2003). This could possibly explain the persistence of the genetically clonal biphasic *S.* Typhimurium strains in Malaysia throughout the 35 years.

Among the four functional prophages unique to serovar Typhimurium (Fels-1 and -2, Gifsy-1 and -2) (McClelland *et al.*, 2001), intact Fels-1, Gifsy-1 and Gifsy-2 prophages were identified in the monophasic STM032/04 genome (Table 6.2). The Gifsy prophages harbour genes that contribute to intracellular replication of *S.* Typhimurium,

which is important in the colonization process of the pathogenic strains (Klumpp & Fuchs, 2007). Intact Gifsy prophages are often reported in the monophasic clones isolated worldwide. The European, US, and China monophasic clones often harbour both Gifsy prophages (García *et al.*, 2013; Soyer *et al.*, 2009; Trüpschuch *et al.*, 2010; Yang *et al.*, 2015). The Spanish clone often has a partially-deleted Gifsy-1 prophage (STM2616 and STM2617 genes deleted), while Japanese monophasic strains lack the entire Gifsy-1 prophage, although both harbour intact Gifsy-2 prophage (Ido *et al.*, 2014). Unlike the ubiquity of the Gifsy prophages, Fels-1 and Fels-2 prophages are often absent in the monophasic strains isolated worldwide (García *et al.*, 2013; Ido *et al.*, 2014; Soyer *et al.*, 2009; Trüpschuch *et al.*, 2010; Yang *et al.*, 2015). In fact, Fels-1 prophage is typically absent in most of the *S.* Typhimurium strains other than LT2 (Hermans *et al.*, 2006). Therefore, the identification of an intact Fels-1 prophage in the monophasic STM032/04 genome, despite at low phage identity, is uncommon even among *S.* Typhimurium. The presence of these Typhimurium-specific prophages in the STM032/04 genome indicated that this monophasic variant is in close genetic proximity with the *S.* Typhimurium LT2, or might have evolved from an LT2-like biphasic ancestor.

The presence of plasmid-borne virulence and antimicrobial resistance factors was identified in the accessory genomes of the *S.* Typhimurium and monophasic strain. DNA segments that corresponded to an intact pSLT plasmid, which harbour multiple plasmid-borne virulence genes (*spv*, *rck*, *pef*, *srgA*, and *mig-5*) and the genes essential for IncF plasmid conjugative transfer (*tra* and *trb*), were found in both of the *S.* Typhimurium strains (Figure 6.6). The presence of the complete set of *tra* and *trb* genes in the plasmid-associated regions of the *S.* Typhimurium genomes indicates the capability of independent conjugative transfer by the plasmid (Rychlik *et al.*, 2006). The monophasic STM032/04 harboured an approximately 7.8 kbp pSLT plasmid-associated

region that contained the *spv* genes required for bacterial survival in macrophage, and the toxin-antitoxin addiction system (*ccdAB* genes) that contributes to plasmid stability (Rychlik et al, 2006). The absence of an intact Typhimurium-specific pSLT plasmid in the monophasic STM032/04 genome explains the absence of MLVA allele STTR10pl that is located on the pSLT plasmid (Chapter 3). Similar observation was made among the Spanish monophasic clone, where none of the strains harboured STTR10pl allele (García *et al.,* 2013). Loss of pSLT plasmid in most of the *Salmonella* I 4,[5],12:i:- strains isolated in United Kingdom and Italy, which represented the European epidemic clone, has also been documented (Petrovska *et al.,* 2016).

Besides the pSLT plasmid-associated region, the *S.* Typhimurium STM057/05 contained a unique DNA segment (contig-65) harbouring multiple antimicrobial resistance genes, that showed great resemblance to a recently characterized IncQ1 plasmid (pNUC) identified in the clinical MDR *S.* Typhimurium strains isolated in southern Italy (Oliva *et al.,* 2017). The Italian MDR *S.* Typhimurium carrying this resistance plasmid was isolated between 2006 and 2009, while the STM057/05 strain that bear similar plasmid region was isolated in 2005. Moreover, the prevalent porcine pathogen *S.* Typhimurium U288 in United Kingdom that carries similar plasmid (pSTU288-2) was also commonly isolated since 2000s (Hooton *et al.,* 2014). The temporal relationship of these IncQ1 plasmid-bearing *S.* Typhimurium strains from multiple geographical regions showed that the spread of this successful epidemic plasmid might have started around 2000s, or even earlier than that. This is not surprising as the presence of multiple antimicrobial resistance genes and an efficient plasmid mobilising system enables the epidemiological spread of the plasmid within bacterial hosts, due to the selective advantage offered to the hosts by the resistance plasmid (Carattoli, 2013).

The accessory genome of the monophasic STM032/04 was found containing several DNA segments that greatly resembled the pSEEH1578_02 virulence plasmid (a VirB/D4 plasmid) that carries a type IV secretion system (T4SS), which is capable of disseminating antimicrobial resistance and virulence determinants in a conjugative way (Hoffmann *et al.,* 2014). Moreover, a beta-lactamase gene ($bla_{\text{TEM-1}}$) that could be responsible for the ampicillin-resistant phenotype of the monophasic STM032/04 was identified in this plasmid-associated genomic region, but not in the pSEEH1578_02 virulence plasmid. The co-existence of virulence and antimicrobial resistance determinants on a mobile genetic element such as a plasmid provides selective advantage to the host bacterium (Carattoli, 2013). Besides that, the monophasic STM032/04 also contained DNA segments that carried an aminoglycoside resistance gene (*aac(3)*-IIa in contig-30; genetically similar to part of the IncI1 resistance plasmid pSEEH1578_01 with NCBI GenBank accession no. CP004087) and a tetracycline resistance gene (*tet*B in contig-66; genetically similar to part of the *Salmonella* I 4,[5],12:i:- strain 07-2006 chromosomal resistance region). The hepta-resistant Spanish monophasic clone often carries an IncA/C plasmid that harboured genes conferring resistant to ampicillin, chloramphenicol, gentamicin, streptomycin/spectinomycin, sulphonamides, trimethoprim, and tetracycline; with a T4SS-like secretion system and pSLT virulence genes (García *et al.,* 2011). On the other hand, the R-type ASSuT European clone (resistant to ampicillin, streptomycin, sulphonamides, and tetracycline) is marked by an insertion of 28 kbp region between STM2759 and *iro*B at the chromosome, carrying multiple antimicrobial resistance genes (García *et al.,* 2016). Besides that, emerging European monophasic clones harbouring multiple MDR plasmids with pSLT virulence genes were also documented in recent years (García *et al.,* 2014). In the monophasic STM032/04 genome, the DNA segments bearing antimicrobial resistance and virulence genes were often flanked by mobile genetic

elements. Therefore, further studies are required to confirm if the DNA segments are found on a single plasmid, or resemble part of a chromosomal insertion at the *fljAB* operon. Nonetheless, this shows that the monophasic *Salmonella* I 4,[5],12:i:- in Malaysia is capable of short-term evolution via horizontal gene transfer, thus contributing to increased pathogenicity and posing a higher risk to public health.

Comparative genomic analyses of the prophage regions suggested that the monophasic STM032/04 strain might have evolved from a *S.* Typhimurium LT2-like ancestor on a phylogenetic lineage different from the two endemic *S.* Typhimurium strains in Malaysia. This inference is further supported by the notion of monophasic STM032/04 and *S.* Typhimurium LT2 forming a single clade with strong bootstrap support on the phylogenetic tree, distinctively separated from the endemic *S.* Typhimurium strains. These endemic *S.* Typhimurium strains showed close phylogenetic relationship with a Mexican *S.* Typhimurium strain SO2 (ST302; asymptomatic clinical strain) and strain D23580 (ST313) which represents an epidemic clone in Africa (Silva *et al.,* 2016; Kingsley *et al.,* 2009). The endemic *S.* Typhimurium strains in Malaysia were seen sharing a more recent common ancestor with the highly invasive and virulent *S.* Typhimurium strains (UK-1, SL1344, D23580, and 798) (Kingsley *et al.,* 2009; Luo *et al.,* 2011; Patterson *et al.,* 2012). In contrast, the monophasic STM032/04 strain was more closely related with the known MDR *S.* Typhimurium strains (DT104, ATCC13311, 33676, 81741, and U288) (Hooton *et al.,* 2013; Mather *et al.,* 2013; Silva *et al.,* 2015; Terabayashi *et al.,* 2014). Indeed, a study in Hong Kong reported that core genome SNPs-based phylogenetic analysis showed a certain degree of correlation with the virulence of *S.* Typhimurium strains (Cheng *et al.,* 2015). Close phylogenetic relationship between the *Salmonella* I 4,[5],12:i:- and *S.* Typhimurium supports the notion that the monophasic variant has evolved from a biphasic ancestor (Hopkins *et al.,* 2010).

Phylogenetic analyses suggested that the European monophasic clone may represent a pandemic clone, given the wide range of geographical origins of the organism. Indeed, the Australian monophasic strain consists of a chromosome that resembles the typical R-type ASSuT European clone, and harbors a large conjugative plasmid that contributes to antimicrobial resistance and virulence of the organism (Dyall-Smith *et al.,* 2017). Moreover, a newly emerged monophasic clone that resembles the typical European clone, which also carries plasmid-mediated resistance genes, has been reported in US (Elnekave *et al.,* 2018). The plasmid-borne antimicrobial resistance and virulence factors might have provided selective advantage for the European clones, contributing to their epidemiological success. Multiple lineages of *Salmonella* I 4,[5],12:i:- observed among the strains isolated in US supports previous notion that the monophasic variants might have emerged through multiple independent evolutionary events (Soyer *et al.,* 2009).

The biphasic and monophasic strains of *S.* Typhimurium examined in this study showed MDR phenotypes. Similar antimicrobial resistance genes repertoire was identified in the *S.* Typhimurium genomes, except for the *strAB*, *sul2*, and *tet* genes which were only found in the STM057/05. These genes were located on a DNA segment that resembled the resistance plasmid pNUC, and contributed to the simultaneous resistance of the STM057/05 strain towards streptomycin, sulphonamide, and tetracycline. The $bla_{\text{TEM-1}}$, *aac*, and *tet* genes identified in the monophasic STM032/04 genome might have been responsible for the ampicillin, gentamicin, and tetracycline-resistant phenotype of the strain. Indeed, the identification of these resistance genes are common among MDR *Salmonella* I 4,[5],12:i:- strains isolated worldwide (Garcia-Russell *et al.,* 2009; Garc á *et al.,* 2011; Guerra *et al.,* 2001; Hopkins *et al.,* 2010; Lucarelli *et al.,* 2012; Moreno-Switt *et al.,* 2009). Point mutation in the *gyrA* gene of the STM032/04 strain was responsible for the nalidixic acid-

resistant phenotype of the organism (Appendix E). Report on missense mutation at codon Asp87 of *gyrA* has also been documented in quinolone-resistant *Salmonella* I 4,[5],12:i:- isolated in Switzerland (Abgottspon *et al.,* 2014). However, nalidixic acid resistance was not commonly observed among the *Salmonella* I 4,[5],12:i:- strains isolated worldwide. Majority of the monophasic clones showed defined MDR patterns, mainly resistant to ampicillin, streptomycin, sulphonamides, and tetracycline; and occasionally resistant to chloramphenicol and gentamicin (Cartwright *et al.,* 2016; Gallati *et al.,* 2013; Hauser *et al.,* 2010; Huoy *et al.,* 2014; Laorden *et al.,* 2010; Mossong *et al.,* 2007; Mulvey *et al.,* 2013; Seixas *et al.,* 2016; Tavechio *et al.,* 2004). However, isolation of quinolone- and fluoroquinolone-resistant *Salmonella* I 4,[5],12:i:- has been increasingly reported during recent years (Cito *et al.,* 2016; Elnekave *et al.,* 2018). The increasingly common trend in quinolone resistance among the *Salmonella* I 4,[5],12:i:- may impair the effectiveness of this antimicrobial agent in the treatment of invasive salmonellosis in human.

Multiple chromosomally-encoded efflux systems and their regulatory genes were identified in the monophasic and *S.* Typhimurium genomes. The efflux pumps mainly comprised of resistance-nodulation-division (RND), major facilitator superfamily (MFS), ATP-binding cassette (ABC), and multidrug and toxic compound extrusion (MATE) transporters. Active efflux of antimicrobial agents in bacteria contributed greatly to the development of multidrug resistance in clinically-important pathogens (Li *et al.,* 2015). Moreover, the polyspecific nature of the efflux pumps confers resistance to not only antibiotics of different classes, but also other biocidal agents such as disinfectants, antiseptics, and preservatives (Alibert *et al.,* 2016). All nine putative drug transporters that were previously identified in *S.* Typhimurium, namely AcrAB, AcrD, AcrEF, MdtABC, MdsABC, EmrAB, MdfA, MdtK, and MacAB transporters (Nishino *et al.,* 2006), were present in both STM001/70 and STM057/05. Among these, the

AcrAB, AcrD, AcrEF, MdtABC, and MdsABC are the five major efflux pumps in *S.* Typhimurium (Nishino *et al.,* 2006). The monophasic STM032/04 strain contained a similar set of efflux pumps, except for the Typhimurium-specific *mdsABC* operon and its regulatory gene *golS*. Besides contributing to the MDR phenotype of *S.* Typhimurium, these efflux pumps also play a role in the virulence of the organism. Previous study has shown that the absence of all nine efflux systems will cause *S.* Typhimurium to lose its virulence capacity (Nishino *et al.,* 2006). Moreover, the *S.* Typhimurium displays impaired ability to form biofilm when all nine efflux pumps were either lost or inhibited (Baugh *et al.,* 2012). Nonetheless, the up-regulation of efflux pumps expression often involves bacterial stress response in order to survive under stressful conditions, for example during host-pathogen interactions (Srinivasan *et al.*, 2012).

All of the routinely screened *Salmonella* virulence determinants were found in both of the *S.* Typhimurium genomes, and most of which were also identified in the monophasic genome. The five *Salmonella* pathogenicity islands (SPI-1 to 5) that are reported to have clear involvement in the virulence of *S.* Typhimurium (Fàbrega & Vila, 2013) were all present in the monophasic and *S.* Typhimurium genomes. The SPI-1 encoded type III secretion system (T3SS-1) plays an important role in *Salmonella* invasion of host cell; while SPI-2 encoded T3SS-2 is essential for intracellular survival of the pathogen (Fàbrega & Vila, 2013; Ibarra & Steele-Mortimer, 2009). The fimbrial and non-fimbrial adhesins mediate a T3SS-independent invasion of *Salmonella* (Ibarra & Steele-Mortimer, 2009; Suez *et al.,* 2013). Among the fimbrial adherence determinants identified in the *S.* Typhimurium genomes, the *bcf*, *fim*, *saf*, *stb*, *std*, *sth*, and *sti* resemble the core fimbrial gene clusters that are present in most *Salmonella* serovars, suggesting that these genes are essential for the host cell colonization of *Salmonella* (Yue *et al.,* 2012). All but one of the core fimbrial gene clusters were also

identified in the monophasic STM032/04 genome. Further analyses could be conducted to confirm the deletion of the *stb* fimbriae genomic island in the monophasic genome. Other important virulence factors such as *mgtBC*, *sodCI*, and *mig* proteins that are essential for intracellular survival were present in all three assembled genomes (Fàbrega & Vila, 2013; Ibarra & Steele-Mortimer, 2009). The major plasmid-borne virulence factors *pef* fimbriae and *spvBC* play a role in adherence, host cell cytotoxicity, and induction of pro-inflammatory response in host cells (Fàbrega & Vila, 2013). Although virulence determinants play an important role in the pathogenicity of *Salmonella*, it is important to note that the host factors are equally important in the disease manifestations (LaRock *et al.*, 2015). Nonetheless, the identification of similar virulence genes repertoire in both of the *Salmonella* I 4,[5],12:i:- and *S.* Typhimurium genomes again supported previous notion that the two serovars have comparable virulence capacity in causing human salmonellosis (Hauser *et al.*, 2010; Yang *et al.*, 2015).

## 6.6 Conclusion

Genomic deletion of the entire *fljAB* operon, *hin* and partial *iroB* genes, together with two closely-located genes STM2757 and STM2758, has resulted in the monophasic phenotype of the *S.* Typhimurium variant strain examined in this study. Highly similar genomic regions were shared between the monophasic variant and *S.* Typhimurium strains in Malaysia, except for minor variations within the prophages and the *stb* fimbriae genomic island. The monophasic strain showed a closer phylogenetic relationship with *S.* Typhimurium LT2, when compared to the two endemic *S.* Typhimurium strains. Therefore, the monophasic strain might have emerged from an LT2-like biphasic ancestor, rather than evolving from the biphasic lineage which predominated in the *S.* Typhimurium population in Malaysia. The monophasic strain might then acquires antimicrobial resistance and virulence factors through horizontal gene transfer. The Malaysian *S.* Typhimurium genomes were essentially identical,

despite the strains being isolated 35 years apart. Close phylogenetic relationship with global virulent *S.* Typhimurium strains, and the presence of multiple virulence and antimicrobial resistance determinants in the endemic strains mark the potential risk of local *S.* Typhimurium causing severe disease in humans.

# CHAPTER 7: CONCLUSION

A total of 195 viable *Salmonella enterica* strains comprised of the two major non-typhoidal *Salmonella* serovars that have been frequently isolated in Malaysia, namely the *S.* Typhimurium (n = 84) and the *S.* Enteritidis (n = 111), were examined in this study. The overall aim of the study was to characterize these two *Salmonella* serovars, both genetically and phenotypically, in order to understand the genetic association, transmission dynamics, and pathogenicity of the predominant *Salmonella* populations in Malaysia. By close examination of the *Salmonella* strains isolated from various sources and different geographical origins over a period of seven years (2002 - 2009), sufficient data was collected to elucidate the genetic relationship, antimicrobial resistance patterns and mechanisms, and the short-term evolutionary development of this endemic organism in Malaysia. Moreover, the data generated in this study could aid future studies in elucidating the epidemiology and evolution of *Salmonella*, both regionally and globally, which is important due to its epidemic nature and ability to cause severe human illnesses.

Generally, genetic homogeneity was observed in both *S.* Typhimurium and *S.* Enteritidis populations that were circulated in Malaysia. These two *Salmonella* serovars being the most commonly isolated bacteria from food, animals, and human sources in Malaysia, and the close genetic proximity among the strains have suggested a farm-to-table transmission of the pathogens. The two molecular typing tools (PFGE and MLVA) used in this study to elucidate the genetic relationship both concurred on the high genetic similarity among the strains obtained from different sources, locations, and at different time periods. Even though PFGE typing showed higher discriminatory power in the subtyping of the *Salmonella* strains, MLVA typing was able to complement PFGE by further discriminating strains that exhibited identical pulsotype. Therefore,

multiple-typing approach is recommended for future analyses of epidemiological relationship among the genetically homogeneous *S.* Typhimurium and *S.* Enteritidis populations in Malaysia. The use of whole genome sequencing as a bacterial subtyping tool can provide high resolution strain differentiation results that are essential for the epidemiology and outbreak investigations of bacterial pathogens. Moreover, the genomic sequences obtained can be used to elucidate the phylogenetic relationship among the bacterial strains, which could not be achieved by PFGE or MLVA typing. Nonetheless, the large sum of investments required for a WGS system to be set in place is inhibitory to the government-funded disease surveillance facilities in developing countries, including Malaysia. Therefore, the multiple-typing approach that includes both PFGE and MLVA remains an inexpensive bacterial strain-subtyping method that can provide sufficiently high discriminatory power for outbreak investigation and short-term epidemiological studies of *Salmonella.*

High rates of resistance towards ampicillin, sulphonamides, and tetracycline were common among both *S.* Typhimurium and *S.* Enteritidis populations in Malaysia. The *S.* Typhimurium strains also showed greater resistance towards streptomycin and cephalothin; while *S.* Enteritidis was more resistant towards nalidixic acid, trimethoprim, and sulfamethoxazole-trimethoprim. These observations showed that the two most common *Salmonella* serovars causing human infections in Malaysia were mostly resistant to the commonly-prescribed first-line drugs in the treatment of invasive salmonellosis. MDR phenotypes appeared common among the strains, albeit mainly at low-risk phase (MAR index < 0.200). Nonetheless, the presence of MDR strains poses a higher risk to public health. Moreover, the high rates of resistance in the *Salmonella* strains may result in enhanced virulence of the organism. In order to better understand the current antimicrobial resistance trend and the possible route of transmission of resistance determinants among *S.* Typhimurium and *S.* Enteritidis strains circulated in

Malaysia, a greater number of strains isolated from food and farm animals should be investigated to complement the data provided by MOH Malaysia, which is obtained from infected patients. This information could help the authorities to develop efficient strategy to curb the widespread dissemination of antimicrobial-resistant *Salmonella* strains in this country. Moreover, the data generated could contribute to the fostering of international joint-effort in reducing the disease burden of drug-resistant *Salmonella* , thereby reducing the widespread dissemination of this pathogen due to the increased frequencies of regional and cross-continents trade-and-travel activities.

Similar to most other *Salmonella* strains isolated worldwide, the most common mechanism that has led to quinolone resistance among the Malaysian *S.* Typhimurium and *S.* Enteritidis strains is via target enzyme alteration caused by mutation in the *gyrA* gene. Although strong resistance to fluoroquinolone (ciprofloxacin) was not observed, the reduced susceptibility to fluoroquinolone was noted among some of the strains. The plasmid-borne *qnrS1* gene which confers mild resistance to fluoroquinolone was detected among the quinolone-resistant strains. Two novel mutations were detected in the *parE* gene, which might have contributed to reduced susceptibility to ciprofloxacin in the strains harbouring the mutations. The increased prevalence of these quinolone and fluoroquinolone resistance mechanisms among *Salmonella* population, especially the transferrable resistance determinants found on mobile genetic elements such as plasmid, raises concern on the uncontrolled spread of these drug-resistant organisms. Therefore, steps must be taken by local authorities and international bodies in order to reduce the epidemic spread of quinolone- and fluoroquinolone-resistant *Salmonella*, to ensure the efficacy of these antibiotics in the treatment of human salmonellosis.

The HRM analysis developed in this study effectively detects point mutations in the QRDRs of the gyrase and topoisomerase IV genes in quinolone-resistant *Salmonella*.

The wild type strains and mutant strains produced distinctive melting curves when analysed. Nonetheless, as HRM analysis is only capable of detecting changes in the DNA strands, the specific base substitutions that have occurred could only be confirmed by DNA sequencing. Therefore, the HRM method is only recommended for rapid screening for mutants prior to DNA sequencing. However, as only one representative strain for each mutational profile is sequenced, the cost and effort spent are greatly reduced when a large sample pool is examined.

One monophasic variant (*Salmonella* I 4,[5],12:i:-) was identified among the *S.* Typhimurium strains examined in this study. Genomic deletion of the entire *fljAB* operon, *hin* and partial *iroB* genes, together with two closely-located genes STM2757 and STM2758, has resulted in the monophasic phenotype of the strain. Whole genome sequence analysis of the endemic *S.* Typhimurium strains showed that although isolated 35 years apart, the two genomes were essentially identical except for minor variations within the phage- and plasmid-associated regions. This finding supports previous notion on the close genetic relationship between the two *S.* Typhimurium strains, as determined by PFGE and MLVA analyses (the strains shared identical pulsotype and were SLVs). The endemic *S.* Typhimurium strains were closely clustered in the phylogenetic tree with strong bootstrap support, forming a distinct clade from the Malaysian monophasic variant which showed close phylogenetic relationship with *S.* Typhimurium LT2 instead. Therefore, the monophasic *Salmonella* I 4,[5],12:i:- in Malaysia might have emerged from an LT2-like biphasic ancestor, and subsequently obtained multiple virulence and antimicrobial resistance determinants through horizontal gene transfer. Close phylogenetic relationship with global virulent *S.* Typhimurium strains, and the presence of multiple virulence and antimicrobial resistance determinants in the endemic strains indicates that local *S.* Typhimurium is potentially hazardous to humans, posing a threat to public health in Malaysia. Whole genome sequence analysis has enabled the

comprehensive study of the genomic features and evolution of the *Salmonella* I 4,[5],12:i:- and *S.* Typhimurium in Malaysia. This study documented the first detailed genomic features of the *Salmonella* I 4,[5],12:i:-, the isolation of which has not been reported in Malaysia before, that has become one of the most important foodborne pathogens on global scale. Data generated from this study may contribute to the understanding of the genetic drive that leads to the worldwide emergence and prevalence of this monophasic organism.

## REFERENCES


Aarestrup, F. M. (2005). Veterinary drug usage and antimicrobial resistance in bacteria of animal origin. *Basic and Clinical Pharmacology and Toxicology, 96*(4), 271-281.

Abatcha, M. G., Zakaria, Z., Dhaliwal, G. K., & Thong, K. L. (2014). Occurrence of antibiotic resistant *Salmonella* isolated from dogs in Klang Valley, Malaysia. *Malaysian Journal of Microbiology, 10*(3), 219-224.

Abatcha, M. G., Zakaria, Z., Dhaliwal, K. G., & Thong, K. L. (2013). Prevalence and antimicrobial susceptibility of *Salmonella* spp. isolated from snakes in Peninsular, Malaysia. *Journal of Veterinary Advances, 3*(12), 306-312.

Abgottspon, H., Zurfluh, K., Nüesch-Inderbinen, M., Hächler, H., & Stephan, R. (2014). Quinolone resistance mechanisms in *Salmonella enterica* serovars Hadar, Kentucky, Virchow, Schwarzengrund, and 4,[5],12:i:-, isolated from humans in Switzerland, & identification of a novel *qnrD* variant, *qnrD2*, in *S.* Hadar. *Antimicrobial Agents and Chemotherapy, 58*(6), 3560-3563.

Achtman, M., Wain, J., Weill, F. X., Nair, S., Zhou, Z., Sangal, V., . . . Brisse, S. (2012). Multilocus sequence typing as a replacement for serotyping in *Salmonella enterica*. *PLoS Pathogens, 8*(6), e1002776.

Adams, D. A., Thomas, K. R., Jajosky, R. A., Foster, L., Sharp, P., Onweh, D. H., . . . Anderson, W. J. (2016). Summary of notifiable infectious diseases and conditions - United States, 2014. *MMWR Morbidity and Mortality Weekly Report, 63*, 1-152.

Adzitey, F., Gulam, R., & Nurul, H. (2012). Prevalence and antibiotic resistance of *Salmonella* serovars in ducks, duck rearing and processing environments in Penang, Malaysia. *Food Research International, 45*(2), 947-952.

Adzitey, F., Gulam, R., Nurul, H., & Rosma, A. (2013). Genetic diversity in *Salmonella* isolates from ducks and their environments in Penang, Malaysia using enterobacterial repetitive intergenic consensus. *Pakistan Veterinary Journal, 33*(3), 350-353.

Adzitey, F., Raja, A. D., & Gulam, R. (2015). Characterization of *Salmonella* Typhimurium, *Salmonella* Enteritidis and *Salmonella* Albany isolated from chickens and ducks using random amplified polymorphic DNA (RAPD)-PCR. *Journal of Microbiology, Immunology and Biotechnology, 2*, 15-21.

Agasan, A., Kornblum, J., Williams, G., Pratt, C. C., Fleckenstein, P., Wong, M., & Ramon, A. (2002). Profile of *Salmonella enterica* subsp. *enterica* (subspecies I) serotype 4,[5],12:i:- strains causing food-borne infections in New York City. *Journal of Clinical Microbiology, 40*(6), 1924-1929.

Alcaine, S. D., Soyer, Y., Warnick, L. D., Su, W. L., Sukhnanand, S., Richards, J., . . . Wiedmann, M. (2006). Multilocus sequence typing supports the hypothesis that cow- and human-associated *Salmonella* isolates represent distinct and overlapping populations. *Applied and Environmental Microbiology, 72*(12), 7575-7585.

Alexander, D. C., Fitzgerald, S. F., De Paulo, R., Kitzul, R., Daku, D., Levett, P. N., & Cameron, A. D. S. (2016). Laboratory-acquired infection with *Salmonella enterica* serovar Typhimurium exposed by whole-genome sequencing. *Journal of Clinical Microbiology, 54*(1), 190-193.

Alibert, S., N'Gompaza Diarra, J., Hernandez, J., Stutzmann, A., Fouad, M., Boyer, G., & Pages, J. M. (2016). Multidrug efflux pumps and their role in antibiotic and antiseptic resistance: A pharmacodynamic perspective. *Expert Opinion on Drug Metabolism and Toxicology*, 1-9.

Alikhan, N. F., Petty, N. K., Ben-Zakour, N. L., & Beatson, S. A. (2011). BLAST Ring Image Generator (BRIG): Simple prokaryote genome comparisons. *BMC Genomics, 12*(1), 402.

Alvarez, J., Sota, M., Vivanco, A. B., Perales, I., Cisterna, R., Rementeria, A., & Garaizar, J. (2004). Development of a multiplex PCR technique for detection and epidemiological typing of *Salmonella* in human clinical samples. *Journal of Clinical Microbiology, 42*(4), 1734-1738.

Amavisit, P., Boonyawiwat, W., & Bangtrakulnont, A. (2005). Characterization of *Salmonella enterica* serovar Typhimurium and monophasic *Salmonella* serovar 1,4,[5],12:i:- isolates in Thailand. *Journal of Clinical Microbiology, 43*(6), 2736-2740.

Andrews, W. H., & Hammack, T. S. (2001). *Salmonella.* In G. J. Jackson, R. I. Merker & R. Bandler (Eds.), *Bacteriological analytical manual (8th ed.).* Rockville, USA: US Food and Drug Administration.

Angulo, F. J., Baker, N. L., Olsen, S. J., Anderson, A., & Barrett, T. J. (2004). Antimicrobial use in agriculture: Controlling the transfer of antimicrobial resistance to humans. *Seminars in Pediatric Infectious Diseases, 15*(2), 78-85.

Angulo, F. J., Nargund, V. N., & Chiller, T. C. (2004). Evidence of an association between use of antimicrobial agents in food animals and antimicrobial resistance among bacteria isolated from humans and the human health consequences of such resistance. *Journal of Veterinary Medicine, 51*(89), 374-379.

Antunes, P., Machado, J., & Peixe, L. (2006). Characterization of antimicrobial resistance and class 1 and 2 integrons in *Salmonella enterica* isolates from different sources in Portugal. *Journal of Antimicrobial Chemotherapy, 58*(2), 297-304.

Ao, T. T., Feasey, N. A., Gordon, M. A., Keddy, K. H., Angulo, F. J., & Crump, J. A. (2015). Global burden of invasive nontyphoidal *Salmonella* disease, 2010. *Emerging Infectious Diseases, 21*(6), 941-949.

Arndt, D., Grant, J. R., Marcu, A., Sajed, T., Pon, A., Liang, Y., & Wishart, D. S. (2016). PHASTER: A better, faster version of the PHAST phage search tool. *Nucleic Acids Research, 44*(1), 16-21.

Arumugaswamy, R. K., Gulam, R., Abdul-Hamid, S. N., & Cheah, C. T. (1995). Prevalence of *Salmonella* in raw and cooked foods in Malaysia. *Food Microbiology, 12*, 3-8.

Asai, T., Sato, C., Masani, K., Usui, M., Ozawa, M., Ogino, T., . . . Watanabe, H. (2010). Epidemiology of plasmid-mediated quinolone resistance in *Salmonella enterica* serovar Typhimurium isolates from food-producing animals in Japan. *Gut Pathogens, 2*(17), 1-5.

Ashton, P. M, Peters, T., Ameh, L., McAleer, R., Petrie, S., Nair, S., . . . Dallman, T. (2014). Whole genome sequencing for the retrospective investigation of an outbreak of *Salmonella* Typhimurium DT 8. *PLoS Currents Outbreaks, 7*, 1-10.

Aziz, R. K., Bartels, D., Best, A. A., DeJongh, M., Disz, T., Edwards, R. A., . . . Zagnitko, O. (2008). The RAST Server: Rapid annotations using subsystems technology. *BMC Genomics, 9*, 75.

Baddam, R., Kumar, N., Thong, K. L., Ngoi, S. T., Teh, C. S. J., Yap, K. P., . . . Ahmed, N. (2012). Genetic fine structure of a *Salmonella enterica* serovar Typhi strain associated with the 2005 outbreak of typhoid fever in Kelantan, Malaysia. *Journal of Bacteriology, 194*(13), 3565-3566.

Bakeri, S. A., Yasin, R. M., Koh, Y. T., Puthucheary, S. D., & Thong, K. L. (2003). Genetic diversity of human isolates of *Salmonella enterica* serovar Enteritidis in Malaysia. *Journal of Applied Microbiology, 95*(4), 773-780.

Barco, L., Longo, A., Lettini, A. A., Cortini, E., Saccardin, C., Minorello, C., . . . Ricci, A. (2014). Molecular characterization of "inconsistent" variants of *Salmonella* Typhimurium isolated in Italy. *Foodborne Pathogens and Disease, 11*(6), 497-499.

Baugh, S., Ekanayaka, A. S., Piddock, L. J. V., & Webber, M. A. (2012). Loss of or inhibition of all multidrug resistance efflux pumps of *Salmonella enterica* serovar Typhimurium results in impaired ability to form a biofilm. *Journal of Antimicrobial Chemotherapy, 67*(10), 2409-2417.

Bergamini, F., Iori, A., Massi, P., & Pongolini, S. (2011). Multilocus variable-number of tandem-repeats analysis of *Salmonella enterica* serotype Gallinarum and comparison with pulsed-field gel electrophoresis genotyping. *Veterinary Microbiology, 149*(3), 430-436.

Bertels, F., Silander, O. K., Pachkov, M., Rainey, P. B., & Van Nimwegen, E. (2014). Automated reconstruction of whole-genome phylogenies from short-sequence reads. *Molecular Biology and Evolution, 31*(5), 1077-1088.

Best, E. L., Lindstedt, B. A., Cook, A., Clifton-Hadley, F. A., Threlfall, E. J., & Liebana, E. (2007). Multiple-locus variable-number tandem repeat analysis of *Salmonella enterica* subsp. *enterica* serovar Typhimurium: Comparison of isolates from pigs, poultry and cases of human gastroenteritis. *Journal of Applied Microbiology, 103*(3), 565-572.

Boerlin, P., & Reid-Smith, R. J. (2008). Antimicrobial resistance: Its emergence and transmission. *Animal Health Research Reviews, 9*(2), 115-126.

Boonmar, S., Bangtrakulnonth, A., Pornruangwong, S., Samosornsuk, S., Kaneko, K., & Ogawa, M. (1998). Significant increase in antibiotic resistance of *Salmonella* isolates from human beings and chicken meat in Thailand. *Veterinary Microbiology, 62*(1), 73-80.

Boonmar, S., Bangtrakulnonth, A., Pornrunangwong, S., Terajima, J., Watanabe, H., Kaneko, K. I., & Ogawa, M. (1998). Epidemiological analysis of *Salmonella* Enteritidis isolates from humans and broiler chickens in Thailand by phage typing and pulsed-field gel electrophoresis. *Journal of Clinical Microbiology, 36*(4), 971-974.

Bossi, L., Fuentes, J. A., Mora, G., & Figueroa-Bossi, N. (2003). Prophage contribution to bacterial population dynamics. *Journal of Bacteriology, 185*(21), 6467-6471.

Boxrud, D., Pederson-Gulrud, K., Wotton, J., Medus, C., Lyszkowicz, E., Besser, J., & Bartkus, J. M. (2007). Comparison of multiple-locus variable-number tandem repeat analysis, pulsed-field gel electrophoresis, & phage typing for subtype analysis of *Salmonella enterica* serotype Enteritidis. *Journal of Clinical Microbiology, 45*(2), 536-543.

Budiati, T., Gulam, R., Wan-Nadiah, W. A., Yahya, M. A., Rosma, A., & Thong, K. L. (2013). Prevalence, antibiotic resistance and plasmid profiling of *Salmonella* in catfish (*Clarias gariepinus*) and tilapia (*Tilapia mossambica*) obtained from wet markets and ponds in Malaysia. *Aquaculture, 372*, 127-132.

Cao, G., Meng, J., Strain, E., Stones, R., Pettengill, J., Zhao, S., . . . Allard, M. (2013). Phylogenetics and differentiation of *Salmonella* Newport lineages by whole genome sequencing. *PLoS ONE, 8*(2), e55687.

Carattoli, A. (2013). Plasmids and the spread of resistance. *International Journal of Medical Microbiology, 303*(6), 298-304.

Cardona-Castro, N., Sánchez-Jiménez, M., Lavalett, L., Múñoz, N., & Moreno, J. (2009). Development and evaluation of a multiplex polymerase chain reaction assay to identify *Salmonella* serogroups and serotypes. *Diagnostic Microbiology and Infectious Disease, 65*(3), 327-330.

Cartwright, E. J., Nguyen, T., Melluso, C., Ayers, T., Lane, C., Hodges, A., . . . Adams, J. (2016). A multistate investigation of antibiotic-resistant *Salmonella enterica* serotype I 4,[5],12:i:- infections as part of an international outbreak associated with frozen feeder rodents. *Zoonoses and Public Health, 63*(1), 62-71.

Cattoir, V., Poirel, L., Rotimi, V., Soussy, C. J., & Nordmann, P. (2007). Multiplex PCR for detection of plasmid-mediated quinolone resistance *qnr* genes in ESBL-producing enterobacterial isolates. *Journal of Antimicrobial Chemotherapy, 60*(2), 394-397.

Cavalieri, S. J., Harbeck, R. J., McCarter, Y. S., Ortez, J, H., Rankin, I. D., Sautter, R. L., … Spiegel, C. A. (2005). *Manual of antimicrobial susceptibility testing.* Washington, USA: American Society for Microbiology.

Centers for Disease Control and Prevention, CDC. (2013a). *An atlas of Salmonella in the United States, 1968-2011: Laboratory-based enteric disease surveillance.* Atlanta, Georgia, USA: US Department of Health and Human Services.

Centers for Disease Control and Prevention, CDC. (2013b). *Antibiotic resistance threats in the United States, 2013.* Atlanta, Georgia, USA: US Department of Health and Human Services.

Cesaro, A., Bettoni, R. R. D., Lascols, C., Mérens, A., Soussy, C. J., & Cambau, E. (2008). Low selection of topoisomerase mutants from strains of *Escherichia coli* harbouring plasmid-borne *qnr* genes. *Journal of Antimicrobial Chemotherapy, 61*(5), 1007-1015.

Cheah, Y. K., Lee, L. H., Noorzaleha, A. S., Radu, S., Sabrina, S., Sim, J. H., & Khoo, C. H. (2008). Characterization of multiple antimicrobial-resistant *Salmonella enterica* subsp. *enterica* isolated from indigenous vegetables and poultry in Malaysia. *Letters in Applied Microbiology, 46*(3), 318-324.

Chen, L., Yang, J., Yu, J., Yao, Z., Sun, L., Shen, Y., & Jin, Q. (2005). VFDB: A reference database for bacterial virulence factors. *Nucleic Acids Research, 33*, 325-328.

Chen, S., Cui, S., McDermott, P. F., Zhao, S., White, D. G., Paulsen, I., & Meng, J. (2007). Contribution of target gene mutations and efflux to decreased susceptibility of *Salmonella enterica* serovar Typhimurium to fluoroquinolones and other antimicrobials. *Antimicrobial Agents and Chemotherapy, 51*(2), 535-542.

Chen, X., Kong, F., Wang, Q., Li, C., Zhang, J., & Gilbert, G. L. (2011). Rapid detection of isoniazid, rifampin, and ofloxacin resistance in *Mycobacterium tuberculosis* clinical isolates using high-resolution melting analysis. *Journal of Clinical Microbiology, 49*(10), 3450-3457.

Cheng, B. Y., Wong, S. P., & Dykes, G. A. (2014). *Salmonella* associated with captive and wild lizards in Malaysia. *Herpetology Notes, 7*, 145-147.

Cheng, C. K., Cheung, M. K., Nong, W., Law, P. T. W., Qin, J., Ling, J. M. L., . . . Kwan, H. S. (2015). Next generation genome sequencing reveals phylogenetic clades with different level of virulence among *Salmonella* Typhimurium clinical human isolates in Hong Kong. *BMC Genomics, 16*(1), 688.

Chin, P. S., Yu, C. Y., A., G. Y., Yin, W.F., & Chan, K. G. (2017). Draft genome sequence of multidrug-resistant *Salmonella enterica* serovar Brancaster strain PS01 isolated from chicken meat, Malaysia. *Journal of Global Antimicrobial Resistance, 9*, 41-42.

Chiu, C. H., Su, L. H., Chu, C. H., Wang, M. H., Yeh, C. M., Weill, F. X., & Chu, C. (2006). Detection of multidrug-resistant *Salmonella enterica* serovar Typhimurium phage types DT102, DT104, and U302 by multiplex PCR. *Journal of Clinical Microbiology, 44*(7), 2354-2358.

Cho, S., Boxrud, D. J., Bartkus, J. M., Whittam, T. S., & Saeed, M. (2007). Multiple-locus variable-number tandem repeat analysis of *Salmonella* Enteritidis isolates from human and non-human sources using a single multiplex PCR. *FEMS Microbiology Letters, 275*(1), 16-23.

Choe, D. W., Hassan, L., & Loh, T. C. (2011). The prevalence of antimicrobial resistant *Salmonella* spp. and the risk factors associated with their occurrence in finisher pigs in Seberang Perai, Malaysia. *Pertanika Journal of Tropical Agricultural Science, 34*(2), 303-310.

Choo, L. C., Saleha, A. A., Wai, S. S., & Fauziah, N. (2011). Isolation of *Campylobacter* and *Salmonella* from houseflies (*Musca domestica*) in a university campus and a poultry farm in Selangor, Malaysia. *Tropical Biomedicine, 28*(1), 16-20.

Cito, F., Baldinelli, F., Calistri, P., Di Giannatale, E., Scavia, G., Orsini, M., . . . Luzzi, I. (2016). Outbreak of unusual *Salmonella enterica* serovar Typhimurium monophasic variant 1,4,[5],12:i:-, Italy, June 2013 to September 2014. *Eurosurveillance, 21*(15), 30194.

Clinical and Laboratory Standards Institute, CLSI. (2015). *Performance standards for antimicrobial susceptibility testing: Twenty-fifth informational supplement CLSI Document M100-S25*. Wayne, PA, USA: Clinical and Laboratory Standards Institute.

Coburn, B., Grassl, G. A., & Finlay, B. B. (2007). *Salmonella*, the host and disease: A brief review. *Immunology and Cell Biology, 85*(2), 112-118.

Cox, G., & Wright, G. D. (2013). Intrinsic antibiotic resistance: Mechanisms, origins, challenges and solutions. *International Journal of Medical Microbiology, 303*, 287-292.

Darling, A. E., Mau, B., & Perna, N. T. (2010). Progressive-Mauve: Multiple genome alignment with gene gain, loss and rearrangement. *PLoS ONE, 5*(6), e11147.

De la Torre, E., Zapata, D., Tello, M., Mejia, W., Frias, N., Garcia-Pena, F. J., . . . Torre, E. (2003). Several *Salmonella enterica* subsp. *enterica* serotype 4,[5],12:i:- phage types isolated from swine samples originate from serotype Typhimurium DT U302. *Journal of Clinical Microbiology, 41*(6), 2395-2400.

De Oliveira, F. A., Pasqualotto, A. P., Da Silva, W. P., & Tondo, E. C. (2010). Characterization of *Salmonella* Enteritidis isolated from human samples. *Food Research International, 45*, 1000-1003.

Deng, X., Shariat, N., Driebe, E. M., Roe, C. C., Tolar, B., Trees, E., . . . Fields, P. I. (2015). Comparative analysis of subtyping methods against a whole-genome-sequencing standard for *Salmonella enterica* serotype Enteritidis. *Journal of Clinical Microbiology, 53*(1), 212-218.

Dhanoa, A., & Fatt, Q. K. (2009). Non-typhoidal *Salmonella* bacteraemia: Epidemiology, clinical characteristics and its' association with severe immunosuppression. *Annals of Clinical Microbiology and Antimicrobials, 8*(1), 15-22.

Diana, J. E., Pui, C. F., & Radu, S. (2010). Enumeration of *Salmonella* spp., *Salmonella* Typhi and *Salmonella* Typhimurium in fruit juices. *International Food Research Journal, 19*(1), 51-56.

Douadi, B., Thong, K. L., Watanabe, H., & Puthucheary, S. D. (2010). Characterization of drug-resistant *Salmonella enterica* serotype Typhimurium by antibiograms, plasmids, integrons, resistance genes, and PFGE. *Journal of Microbiology and Biotechnology, 20*(6), 1042-1052.

Dyall-Smith, M. L., Liu, Y., & Billman-Jacobe, H. (2017). Genome sequence of an Australian monophasic *Salmonella enterica* subsp. *enterica* Typhimurium isolate (TW-Stm6) carrying a large plasmid with multiple antimicrobial resistance genes. *Genome Announcements, 5*(33), e00793-17.

Eaves, D. J., Randall, L., Gray, D. T., Buckley, A., Woodward, M. J., White, A. P., & Piddock, L. J. V. (2004). Prevalence of mutations within the quinolone resistance-determining region of *gyrA, gyrB, parC,* and *parE* and association with antibiotic resistance in quinolone-resistant *Salmonella enterica*. *Antimicrobial Agents and Chemotherapy, 48*(10), 4012-4015.

Echeita, M. A., Aladuena, A., Cruchaga, S., & Usera, M. A. (1999). Emergence and spread of an atypical *Salmonella enterica* subsp. *enterica* serotype 4,[5],12:i:- strain in Spain. *Journal of Clinical Microbiology, 37*(10), 3425.

Economou, V., & Gousia, P. (2015). Agriculture and food animals as a source of antimicrobial-resistant bacteria. *Infection and Drug Resistance, 8*, 49-61.

Efron, B., Halloran, E., & Holmes, S. (1996). Bootstrap confidence levels for phylogenetic trees. *Proceedings of the National Academy of Sciences, 93*(23), 13429-13429.

Elemfareji, O. I., & Thong, K. L. (2013). Comparative virulotyping of *Salmonella* Typhi and *Salmonella* Enteritidis. *Indian Journal of Microbiology, 53*(4), 410-417.

Elnekave, E., Hong, S., Mather, A. E., Boxrud, D., Taylor, A. J., Lappi, V., . . . Alvarez, J. (2018). *Salmonella enterica* serotype 4,[5],12:i:- in swine in the United States Midwest: an emerging multidrug resistant clade. *Clinical Infectious Diseases, 66*(6), 877-885.

Ethelberg, S., Lisby, M., Torpdahl, M., Sorensen, G., Neimann, J., Rasmussen, P., . . . Helms, M. (2004). Prolonged restaurant-associated outbreak of multidrug-resistant *Salmonella* Typhimurium among patients from several European countries. *Clinical Microbiology and Infection, 10*(10), 904-910.

Fàbrega, A., & Vila, J. (2013). *Salmonella enterica* serovar Typhimurium skills to succeed in the host: Virulence and regulation. *Clinical Microbiology Reviews, 26*(2), 308-341.

Fàbrega, A., Du Merle, L., Le Bouguenec, C., De Anta, M. T. J., & Vila, J. (2009). Repression of invasion genes and decreased invasion in a high-level fluoroquinolone-resistant *Salmonella* Typhimurium mutant. *PLoS ONE, 4*(11), e8029.

Fàbrega, A., Sánchez-Céspedes, J., Soto, S., & Vila, J. (2008). Quinolone resistance in the food chain. *International Journal of Antimicrobial Agents, 31*(4), 307-315.

Fàbrega, A., Soto, S. M., Ballesté-Delpierre, C., Fernández-Orth, D., De Anta, M. T. J., & Vila, J. (2014). Impact of quinolone-resistance acquisition on biofilm production and fitness in *Salmonella enterica*. *Journal of Antimicrobial Chemotherapy, 65*(7), 1815-1824.

Fakhr, M. K., Nolan, L. K., & Logue, C. M. (2005). Multilocus sequence typing lacks the discriminatory ability of pulsed-field gel electrophoresis for typing *Salmonella enterica* serovar Typhimurium. *Journal of Clinical Microbiology, 43*(5), 2215-2219.

Falagas, M. E., & Bliziotis, I. A. (2007). Pandrug-resistant Gram-negative bacteria: The dawn of the post-antibiotic era? *International Journal of Antimicrobial Agents, 29*(6), 630-636.

Fashae, K., Ogunsola, F., Aarestrup, F. M., & Hendriksen, R. S. (2010). Antimicrobial susceptibility and serovars of *Salmonella* from chickens and humans in Ibadan, Nigeria. *The Journal of Infection in Developing Countries, 4*(8), 484-494.

Feasey, N. A., & Gordon, M. A. (2014). *Salmonella* infections. In J. Farrar, P. J. Hotez, T. Junghanss, G. Kang, D. Lalloo & N. J. White (Eds.), *Manson's tropical infectious diseases (23rd ed.)* (pp. 337-348). London, United Kingdom: W. B. Saunders.

Foley, S. L., & Lynne, A. M. (2008). Food animal-associated *Salmonella* challenges: Pathogenicity and antimicrobial resistance. *Journal of Animal Science, 86*(14), 173-187.

Foley, S. L., White, D. G., McDermott, P. F., Walker, R. D., Rhodes, B., Fedorka-Cray, P. J., . . . Zhao, S. (2006). Comparison of subtyping methods for differentiating *Salmonella enterica* serovar Typhimurium isolates obtained from food animal sources. *Journal of Clinical Microbiology*, *44*(10), 3569-3577.

Gallati, C., Stephan, R., Hächler, H., Malorny, B., Schroeter, A., & Nüesch-Inderbinen, M. (2013). Characterization of *Salmonella enterica* subsp. *enterica* serovar 4,[5],12:i:- clones isolated from human and other sources in Switzerland between 2007 and 2011. *Foodborne Pathogens and Disease, 10*(6), 549-554.

García, P., Guerra, B., Bances, M., Mendoza, M. C., & Rodicio, M. R. (2011). IncA/C plasmids mediate antimicrobial resistance linked to virulence genes in the Spanish clone of the emerging *Salmonella enterica* serotype 4,[5],12:i:-. *Journal of Antimicrobial Chemotherapy, 66*(3), 543-549.

García, P., Hopkins, K. L., García, V., Beutlich, J., Mendoza, M. C., Threlfall, J., . . . Guerra, B. (2014). Diversity of plasmids encoding virulence and resistance functions in *Salmonella enterica* subsp. *enterica* serovar Typhimurium monophasic variant 4,[5],12:i:- strains circulating in Europe. *PLoS ONE, 9*(2), e89635.

García, P., Malorny, B., Hauser, E., Mendoza, M. C., & Rodicio, M. R. (2013). Genetic types, gene repertoire, and evolution of isolates of the *Salmonella enterica* serovar 4,[5],12:i:- Spanish clone assigned to different phage types. *Journal of Clinical Microbiology, 51*(3), 973-978.

García, P., Malorny, B., Rodicio, M. R., Stephan, R., Hächler, H., Guerra, B., & Lucarelli, C. (2016). Horizontal acquisition of a multidrug-resistance module (R-type ASSuT) is responsible for the monophasic phenotype in a widespread clone of *Salmonella* serovar 4,[5],12:i:-. *Frontiers in Microbiology, 7*, 680.

Garcia-Russell, N., Elrod, B., & Dominguez, K. (2009). Stress-induced prophage DNA replication in *Salmonella enterica* serovar Typhimurium. *Infection, Genetics and Evolution, 9*(5), 889-895.

Gelband, H., Miller-Petrie, M., Pant, S., Gandra, S., Levinson, J., Barter, D., . . . Laxminarayan, R. (2015). The state of the world's antibiotics 2015. *Wound Healing Southern Africa, 8*(2), 30-34.

Gilbert, G. L. (2008). Using MLVA to type strains of *Salmonella* Typhimurium in New South Wales. *New South Wales Public Health Bulletin, 19*, 29-31.

Goh, Y. L., Yasin, R., Puthucheary, S. D., Koh, Y. T., Lim, V. K. E., Taib, Z., & Thong, K. L. (2003). DNA fingerprinting of human isolates of *Salmonella enterica* serotype Paratyphi B in Malaysia. *Journal of Applied Microbiology, 95*(5), 1134-1142.

Grimont, P. A. D., & Weill, F. X. (2007). *Antigenic formulae of the Salmonella serovars*. Paris, France: Institute Pasteur.

Guan, X., Xue, X., Liu, Y., Wang, J., Wang, Y., Wang, J., . . . Yang, B. (2013). Plasmid-mediated quinolone resistance: Current knowledge and future perspectives. *Journal of International Medical Research, 41*(1), 20-30.

Guerra, B., Laconcha, I., Soto, S. M., González-Hevia, M. Á., & Mendoza, M. C. (2000). Molecular characterisation of emergent multiresistant *Salmonella enterica* serotype 4,[5],12:i:- organisms causing human salmonellosis. *FEMS Microbiology Letters, 190*(2), 341-347.

Guerra, B., Soto, S. M., Arguelles, J. M., & Mendoza, M. C. (2001). Multidrug resistance is mediated by large plasmids carrying a class 1 integron in the emergent *Salmonella enterica* serotype 4,[5],12:i:-. *Antimicrobial Agents and Chemotherapy, 45*(4), 1305-1308.

Gulam, R., Jamal, K., Radu, S., Cheah, C. T., & Yassin, R. M. (1996). Prevalence of *Salmonella* in broilers at retail outlets, processing plants and farms in Malaysia. *International Journal of Food Microbiology, 33*(2), 183-194.

Harbottle, H., White, D. G., McDermott, P. F., Walker, R. D., & Zhao, S. (2006). Comparison of multilocus sequence typing, pulsed-field gel electrophoresis, & antimicrobial susceptibility typing for characterization of *Salmonella enterica* serotype Newport isolates. *Journal of Clinical Microbiology, 44*(7), 2449-2457.

Hauser, E., Tietze, E., Helmuth, R., Junker, E., Blank, K., Prager, R., . . . Malorny, B. (2010). Pork contaminated with *Salmonella enterica* serovar 4,[5],12:i:-, an emerging health risk for humans. *Applied and Environmental Microbiology, 76*(14), 4601-4610.

Heir, E., Lindstedt, B. A., Nygård, I., Vardund, T., Hasseltvedt, V., & Kapperud, G. (2002). Molecular epidemiology of *Salmonella* Typhimurium isolates from human sporadic and outbreak cases. *Epidemiology and Infection, 128*(3), 373-382.

Hendriksen, R. S., Hyytia-Trees, E., Pulsrikarn, C., Pornruangmong, S., Chaichana, P., Svendsen, C. A., . . . Mikoleit, M. (2012). Characterization of *Salmonella enterica* serovar Enteritidis isolates recovered from blood and stool specimens in Thailand. *BMC Microbiology, 12*(1), 92.

Hendriksen, R. S., Vieira, A. R., Karlsmose, S., Lo-Fo-Wong, D. M. A., Jensen, A. B., Wegener, H. C., & Aarestrup, F. M. (2011). Global monitoring of *Salmonella* serovar distribution from the World Health Organization Global Foodborne Infections Network Country Data Bank: Results of quality assured laboratories from 2001 to 2007. *Foodborne Pathogens and Disease, 8*(8), 887-900.

Hermans, A. P. H. M., Beuling, A. M., Van Hoek, A. H. A. M., Aarts, H. J. M., Abee, T., & Zwietering, M. H. (2006). Distribution of prophages and SGI-1 antibiotic-resistance genes among different *Salmonella enterica* serovar Typhimurium isolates. *Microbiology, 152*(7), 2137-2147.

Herrero-Fresno, A., Leekitcharoenphon, P., Hendriksen, R. S., Olsen, J. E., & Aarestrup, F. M. (2014). Analysis of the contribution of bacteriophage ST64B to *in vitro* virulence traits of *Salmonella enterica* serovar Typhimurium. *Journal of Medical Microbiology, 63*(3), 331-342.

Hoffmann, M., Zhao, S., Pettengill, J., Luo, Y., Monday, S. R., Abbott, J., . . . Li, C. (2014). Comparative genomic analysis and virulence differences in closely related *Salmonella enterica* serotype Heidelberg isolates from humans, retail meats, and animals. *Genome Biology and Evolution, 6*(5), 1046-1068.

Hoorfar, J., Baggesen, D. L., & Porting, P. H. (1999). A PCR-based strategy for simple and rapid identification of rough presumptive *Salmonella* isolates. *Journal of Microbiological Methods, 35*(1), 77-84.

Hooton, S. P. T., Timms, A. R., Cummings, N. J., Moreton, J., Wilson, R., & Connerton, I. F. (2014). The complete plasmid sequences of *Salmonella enterica* serovar Typhimurium U288. *Plasmid, 76*, 32-39.

Hooton, S. P. T., Timms, A. R., Moreton, J., Wilson, R., & Connerton, I. F. (2013). Complete genome sequence of *Salmonella enterica* serovar Typhimurium U288. *Genome Announcements, 1*(4), e00467-13.

Hopkins, K. L., Davies, R. H., & Threlfall, E. J. (2005). Mechanisms of quinolone resistance in *Escherichia coli* and *Salmonella*: Recent developments. *International Journal of Antimicrobial Agents, 25*(5), 358-373.

Hopkins, K. L., De Pinna, E., & Wain, J. (2012). Prevalence of *Salmonella enterica* serovar 4,[5],12:i:- in England and Wales, 2010. *Eurosurveillance, 17*(37), 20275.

Hopkins, K. L., Kirchner, M., Guerra, B., Granier, S. A., Lucarelli, C., Porrero, M. C., . . . Mevius, D. J. (2010). Multiresistant *Salmonella enterica* serovar 4,[5],12:i:- in Europe: A new pandemic strain? *Eurosurveillance, 15*(22), 19580.

Hopkins, K. L., Lawson, A. J., Connell, S., Peters, T. M., & De Pinna, E. (2011). A novel real-time polymerase chain reaction for identification of *Salmonella enterica* subspecies *enterica*. *Diagnostic Microbiology and Infectious Disease, 70*(2), 278-280.

Hopkins, K. L., Maguire, C., Best, E., Liebana, E., & Threlfall, E. J. (2007). Stability of multiple-locus variable-number tandem repeats in *Salmonella enterica* serovar Typhimurium. *Journal of Clinical Microbiology, 45*(9), 3058-3061.

Hopkins, K. L., Peters, T. M., De Pinna, E., & Wain, J. (2011). Standardisation of multilocus variable-number tandem-repeat analysis (MLVA) for subtyping of *Salmonella enterica* serovar Enteritidis. *Eurosurveillance, 16*(32), 19942.

Hunter, P. R., & Gaston, M. A. (1988). Numerical index of the discriminatory ability of typing systems: An application of Simpson's index of diversity. *Journal of Clinical Microbiology, 26*(11), 2465-2466.

Huoy, L., Pornruangwong, S., Pulsrikarn, C., & Chaturongakul, S. (2014). Molecular characterization of Thai *Salmonella enterica* serotype Typhimurium and serotype 4,[5],12:i:- reveals distinct genetic deletion patterns. *Foodborne Pathogens and Disease, 11*(8), 589-592.

Ibarra, J. A., & Steele-Mortimer, O. (2009). *Salmonella* - the ultimate insider. *Salmonella* virulence factors that modulate intracellular survival. *Cellular Microbiology, 11*(11), 1579-1586.

Ido, N., Lee, K., Iwabuchi, K., Izumiya, H., Uchida, I., Kusumoto, M., . . . Akiba, M. (2014). Characteristics of *Salmonella enterica* serovar 4,[5],12:i:- as a monophasic variant of serovar Typhimurium. *PLoS ONE, 9*(8), e104380.

Intan, H. I., Zubaidah, C. D., Norazah, A., & Norlijah, O. (2008). Subdural collections due to non-Typhi *Salmonella* infections in two Malaysian children. *Singapore Medical Journal, 49*(7), 186-189.

Issenhuth-Jeanjean, S., Roggentin, P., Mikoleit, M., Guibourdenche, M., De Pinna, E., Nair, S., . . . Weill, F. X. (2014). Supplement 2008–2010 (no. 48) to the White–Kauffmann–Le Minor scheme. *Research in Microbiology, 165*(7), 526-530.

Jackson, B. R., Griffin, P. M., Cole, D., Walsh, K. A., & Chai, S. J. (2013). Outbreak-associated *Salmonella enterica* serotypes and food commodities, United States, 1998–2008. *Emerging Infectious Diseases, 19*(8), 1239-1244.

Jacoby, G., Cattoir, V., Hooper, D., Mart ínez-Mart ínez, L., Nordmann, P., Pascual, A., . . . Wang, M. (2008). *qnr* Gene nomenclature. *Antimicrobial Agents and Chemotherapy, 52*(7), 2297-2299.

Jegathesan, M. (1983). Phage types of *Salmonella* Typhi isolated in Malaysia over the 10-year period 1970-1979. *The Journal of Hygiene, 90*(1), 91-97.

Jegathesan, M. (1984). *Salmonella* serotypes isolated from man in Malaysia over the 10-year period 1973-1982. *Epidemiology and Infection, 92*(3), 395-399.

Joseph, P. G., Sivanandan, S. P., & Yee, H. T. (1988). Animal *Salmonella* surveillance in Peninsular Malaysia, 1981–1985. *Epidemiology and Infection, 100*(3), 351-359.

Karunakaran, R., Tay, S. T., Fairuz, F. R., Lim, B. B., & Puthucheary, S. D. (2014). Molecular analysis of ciprofloxacin resistance among non-typhoidal *Salmonella* with reduced susceptibility to ciprofloxacin isolated from patients at a tertiary care hospital in Kuala Lumpur, Malaysia. *Japanese Journal of Infectious Diseases, 67*(3), 157-162.

Karunakaran, R., Tay, S. T., Fairuz, F. R., Lim, B. B., Sam, J. I. C., Maria, K. B., . . . Puthucheary, S. D. (2012). Ceftriaxone resistance and genes encoding extended-spectrum beta-lactamase among non-typhoidal *Salmonella* species from a tertiary care hospital in Kuala Lumpur, Malaysia. *Japanese Journal of Infectious Diseases, 65*(5), 433-435.

Kehrenberg, C., De Jong, A., Friederichs, S., Cloeckaert, A., & Schwarz, S. (2007). Molecular mechanisms of decreased susceptibility to fluoroquinolones in avian *Salmonella* serovars and their mutants selected during the determination of mutant prevention concentrations. *Journal of Antimicrobial Chemotherapy, 59*(5), 886-892.

Kérouanton, A., Marault, M., Lailler, R., Weill, F. X., Feurer, C., Espié, E., & Brisabois, A. (2007). Pulsed-field gel electrophoresis subtyping database for foodborne *Salmonella enterica* serotype discrimination. *Foodborne Pathogens and Disease, 4*(3), 293-303.

Khoo, C. H., Cheah, Y. K., Lee, L. H., Sim, J. H., Noorzaleha, A. S., Shiran, M. S., . . . Sabrina, S. (2009). Virulotyping of *Salmonella enterica* subsp. *enterica* isolated from indigenous vegetables and poultry meat in Malaysia using multiplex-PCR. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology, 96*(4), 441-457.

Khoo, E., Roseliza, R., Khoo, L. L., Nafizah, M., Saifu, N. R., Hasnah, Y., . . . Ramlan, M. (2015). Antimicrobial resistance of *Salmonella enterica* serovar Typhimurium from various meats received in VRI. *Malaysian Journal of Veterinary Research, 6*(1), 61-65.

Kim, K. Y., Park, J. H., Kwak, H. S., & Woo, G. J. (2011). Characterization of the quinolone resistance mechanism in foodborne *Salmonella* isolates with high nalidixic acid resistance. *International Journal of Food Microbiology, 146*(1), 52-56.

Kingsley, R. A., Msefula, C. L., Thomson, N. R., Kariuki, S., Holt, K. E., Gordon, M. A., . . . Dougan, G. (2009). Epidemic multiple drug resistant *Salmonella* Typhimurium causing invasive disease in sub-Saharan Africa have a distinct genotype. *Genome Research, 19*(12), 2279-2287.

Klochko, A., & Wallace, M. R. (2014). Salmonellosis treatment and management. *Medscape Reference: Drugs, Diseases and Procedures*. Retrieved from http://emedicine.medscape.com/article/228174-treatment

Klumpp, J., & Fuchs, T. M. (2007). Identification of novel genes in genomic islands that contribute to *Salmonella* Typhimurium replication in macrophages. *Microbiology, 153*(4), 1207-1220.

Kosman, E., & Leonard, K. J. (2005). Similarity coefficients for molecular markers in studies of genetic relationships between individuals for haploid, diploid, and polyploid species. *Molecular Ecology, 14*(2), 415-424.

Krumperman, P. H. (1983). Multiple antibiotic resistance indexing of *Escherichia coli* to identify high-risk sources of fecal contamination of foods. *Applied and Environmental Microbiology, 46*(1), 165-170.

Kruy, S. L., Van Cuyck, H., & Koeck, J. L. (2011). Multilocus variable number tandem repeat analysis for *Salmonella enterica* subspecies. *European Journal of Clinical Microbiology and Infectious Diseases, 30*, 465-473.

Laconcha, I., López-Molina, N., Rementeria, A., Audicana, A., Perales, I., & Garaizar, J. (1998). Phage typing combined with pulsed-field gel electrophoresis and random amplified polymorphic DNA increases discrimination in the epidemiological analysis of *Salmonella* Enteritidis strains. *International Journal of Food Microbiology, 40*(1), 27-34.

Langridge, G. C., Fookes, M., Connor, T. R., Feltwell, T., Feasey, N., Parsons, B. N., . . . Thomson, N. R. (2015). Patterns of genome evolution that have accompanied host adaptation in *Salmonella*. *Proceedings of the National Academy of Sciences, 112*(3), 863-868.

Laorden, L., Herrera-León, S., Martínez, I., Sanchez, A., Kromidas, L., Bikandi, J., . . . Garaizar, J. (2010). Genetic evolution of the Spanish multidrug-resistant *Salmonella enterica* 4,[5],12:i:- monophasic variant. *Journal of Clinical Microbiology, 48*(12), 4563-4566.

LaRock, D. L., Chaudhary, A., & Miller, S. I. (2015). Salmonellae interactions with host processes. *Nature Reviews Microbiology, 13*(4), 191-205.

Lee, A. S. G., Ong, D. C. T, Wong, J. C. L., Siu, G. K. H., & Yam, W. C. (2012). High-resolution melting analysis for the rapid detection of fluoroquinolone and streptomycin resistance in *Mycobacterium tuberculosis*. *PLoS ONE, 7*(2), e31934.

Lee, L. H., Cheah, Y. K., Noorzaleha, A. S., Sabrina, S., Sim, J. H., Khoo, C. H., & Radu, S. (2008). Analysis of *Salmonella* Agona and *Salmonella* Weltevreden in Malaysia by PCR fingerprinting and antibiotic resistance profiling. *Antonie van Leeuwenhoek, 94*(3), 377-387.

Lee, S. W., & Wee, W. (2011). Antibiogram and heavy metal resistance pattern of *Salmonella* spp. isolated from wild Asian sea bass (*Lates calcarifer*) from Tok Bali, Kelantan, Malaysia. *Jordan Journal of Biological Sciences, 4*(3), 125-128.

Lee, W. S., Hafeez, A., Hassan, H., Raja, N. S., & Puthucheary, S. D. (2005). Focal non-typhoidal *Salmonella* infections from a single center in Malaysia. *Southeast Asian Journal of Tropical Medicine and Public Health, 36*(3), 678-682.

Lee, W. S., Puthucheary, S. D., & Boey, C. C. M. (1998). Non-typhoid *Salmonella* gastroenteritis. *Journal of Paediatrics and Child Health, 34*(4), 387-390.

Lee, W. S., Puthucheary, S. D., Parasakthi, N., & Choo, K. E. (2003). Antimicrobial susceptibility and distribution of non-typhoidal *Salmonella* serovars isolated in Malaysian children. *Journal of Tropical Pediatrics, 49*(1), 37-41.

Leekitcharoenphon, P., Lukjancenko, O., Friis, C., Aarestrup, F. M., & Ussery, D. W. (2012). Genomic variation in *Salmonella enterica* core genes for epidemiological typing. *BMC Genomics, 13*, 88.

Leekitcharoenphon, P., Nielsen, E. M., Kaas, R. S., Lund, O., & Aarestrup, F. M. (2014). Evaluation of whole genome sequencing for outbreak detection of *Salmonella enterica*. *PLoS ONE, 9*(2), e87991.

Li, L. M., Grassly, N. C., & Fraser, C. (2014). Genomic analysis of emerging pathogens: methods, application and future trends. *Genome Biology, 15*(11), 541.

Li, X. Z., Plésiat, P., & Nikaido, H. (2015). The challenge of efflux-mediated antibiotic resistance in Gram-negative bacteria. *Clinical Microbiology Reviews, 28*(2), 337-418.

Liebana, E. (2002). Molecular tools for epidemiological investigations of *S. enterica* subspecies *enterica* infections. *Research in Veterinary Science, 72*(3), 169-175.

Liebisch, B., & Schwarz, S. (1996). Evaluation and comparison of molecular techniques for epidemiological typing of *Salmonella enterica* subsp. *enterica* serovar Dublin. *Journal of Clinical Microbiology, 34*(3), 641-646.

Liew, M., Pryor, R., Palais, R., Meadows, C., Erali, M., Lyon, E., & Wittwer, C. (2004). Genotyping of single-nucleotide polymorphisms by high-resolution melting of small amplicons. *Clinical Chemistry, 50*(7), 1156-1164.

Lim, B. K., & Thong, K. L. (2009). Application of PCR-based serogrouping of selected *Salmonella* serotypes in Malaysia. *Journal of Infection in Developing Countries, 3*(6), 420-428.

Lindgren, M. M., Kotilainen, P., Huovinen, P., Hurme, S., Lukinmaa, S., Webber, M. A., . . . Hakanen, A. J. (2009). Reduced fluoroquinolone susceptibility in *Salmonella enterica* isolates from travelers, Finland. *Emerging Infectious Diseases, 15*(5), 809-812.

Lindstedt, B. A., Heir, E., Gjernes, E., & Kapperud, G. (2003). DNA fingerprinting of *Salmonella enterica* subsp. *enterica* serovar Typhimurium with emphasis on phage type DT104 based on variable number of tandem repeat loci. *Journal of Clinical Microbiology, 41*(4), 1469-1479.

Lindstedt, B. A., Torpdahl, M., Nielsen, E. M., Vardund, T., Aas, L., & Kapperud, G. (2007). Harmonization of the multiple-locus variable-number tandem repeat analysis method between Denmark and Norway for typing *Salmonella* Typhimurium isolates and closer examination of the VNTR loci. *Journal of Applied Microbiology, 102*(3), 728-735.

Lindstedt, B. A., Vardund, T., Aas, L., & Kapperud, G. (2004). Multiple-locus variable-number tandem-repeats analysis of *Salmonella enterica* subsp. *enterica* serovar Typhimurium using PCR multiplexing and multicolor capillary electrophoresis. *Journal of Microbiological Methods, 59*(2), 163-172.

Ling, J. M., Chan, E. W., Lam, A. W., & Cheng, A. F. (2003). Mutations in topoisomerase genes of fluoroquinolone-resistant salmonellae in Hong Kong. *Antimicrobial Agents and Chemotherapy, 47*(11), 3567-3573.

Ling, M. L., & Wang, G. C. Y. (2001). Epidemiological analysis of *Salmonella* Enteritidis isolates in Singapore. *Journal of Infection, 43*(3), 169-172.

Liu, B., Zhang, L., Zhu, X., Shi, C., Chen, J., Liu, W., . . . Shi, X. (2011). PCR identification of *Salmonella* serogroups based on specific targets obtained by comparative genomics. *International Jounal of Food Microbiology, 144*, 511-518.

Loman, N. J., Misra, R. V., Dallman, T. J., Constantinidou, C., Gharbia, S. E., Wain, J., & Pallen, M. J. (2012). Performance comparison of benchtop high-throughput sequencing platforms. *Nature Biotechnology, 30*, 434-439.

Loveless, B. M., Yermakova, A., Christensen, D. R., Kondig, J. P., Heine III, H. S., Wasieloski, L. P., & Kulesh, D. A. (2010). Identification of ciprofloxacin resistance by SimpleProbe™, high resolution melt and Pyrosequencing™ nucleic acid analysis in biothreat agents: *Bacillus anthracis*, *Yersinia pestis* and *Francisella tularensis*. *Molecular and Cellular Probes, 24*(3), 154-160.

Lucarelli, C., Dionisi, A. M., Filetici, E., Owczarek, S., Luzzi, I., & Villa, L. (2012). Nucleotide sequence of the chromosomal region conferring multidrug resistance (R-type ASSuT) in *Salmonella* Typhimurium and monophasic *Salmonella* Typhimurium strains. *Journal of Antimicrobial Chemotherapy, 67*(1), 111-114.

Lukinmaa, S., Nakari, U., Eklund, M., & Siitonen, A. (2004). Application of molecular genetic methods in diagnostics and epidemiology of food-borne bacterial pathogens. *Apmis, 112*(11), 908-929.

Lunn, A. D., Fàbrega, A., Sánchez-Céspedes, J., & Vila, J. (2010). Prevalence of mechanisms decreasing quinolone-susceptibility among *Salmonella* spp. clinical isolates. *International Microbiology, 13*(1), 15-20.

Luo, Y., Kong, Q., Yang, J., Golden, G., Wanda, S. Y., Jensen, R. V., . . . Curtiss III, R. (2011). Complete genome sequence of the universal killer *Salmonella enterica* serovar Typhimurium UK-1 (ATCC 68169). *Journal of Bacteriology, 193*(15), 4035-4036.

Maiden, M. C. J., Bygraves, J. A., Feil, E., Morelli, G., Russell, J. E., Urwin, R., . . . Caugant, D. A. (1998). Multilocus sequence typing: A portable approach to the identification of clones within populations of pathogenic microorganisms. *Proceedings of the National Academy of Sciences, 95*(6), 3140-3145.

Majowicz, S. E., Musto, J., Scallan, E., Angulo, F. J., Kirk, M., O'Brien, S. J., . . . Hoekstra, R. M. (2010). The global burden of non-typhoidal *Salmonella* gastroenteritis. *Clinical Infectious Diseases, 50*(6), 882-889.

Malorny, B., Junker, E., & Helmuth, R. (2008). Multi-locus variable-number tandem repeat analysis for outbreak studies of *Salmonella enterica* serotype Enteritidis. *BMC Microbiology, 8*(1), 84.

Martínez-Martínez, L., Pascual, A., & Jacoby, G. A. (1998). Quinolone resistance from a transferable plasmid. *The Lancet, 351*(9105), 797-799.

Mather, A. E., Reid, S. W., Maskell, D. J., Parkhill, J., Fookes, M. C., Harris, S. R., . . . Thomson, N. R. (2013). Distinguishable epidemics of multidrug-resistant *Salmonella* Typhimurium DT104 in different hosts. *Science, 341*(6153), 1514-1517.

McArthur, A. G., Waglechner, N., Nizam, F., Yan, A., Azad, M. A., Baylay, A. J., . . . Wright, G. D. (2013). The comprehensive antibiotic resistance database. *Antimicrobial Agents and Chemotherapy, 57*(7), 3348-3357.

McClelland, M., Sanderson, K. E., Spieth, J., Clifton, S. W., Latreille, P., Courtney, L., . . . Du, F. (2001). Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. *Nature, 413*(6858), 852-856.

Michael, G. B., Butaye, P., Cloeckaert, A., & Schwarz, S. (2006). Genes and mutations conferring antimicrobial resistance in *Salmonella*: An update. *Microbes and Infection, 8*(7), 1898-1914.

Modarressi, S., & Thong, K. L. (2010). Isolation and molecular subtyping of *Salmonella enterica* from chicken, beef and street foods in Malaysia. *Scientific Research and Essays, 5*(18), 2713-2720.

Moreno-Switt, A. I., Soyer, Y., Warnick, L. D., & Wiedmann, M. (2009). Emergence, distribution, and molecular and phenotypic characteristics of *Salmonella enterica* serotype 4,[5],12:i:-. *Foodborne Pathogens and Disease, 6*(4), 407-415.

Morozova, O., & Marra, M. A. (2008). Applications of next-generation sequencing technologies in functional genomics. *Genomics, 92*(5), 255-264.

Mossong, J., Marques, P., Ragimbeau, C., Huberty-Krau, P., Losch, S., Meyer, G., . . . Schneider, F. (2007). Outbreaks of monophasic *Salmonella enterica* serovar 4,[5],12:i:- in Luxembourg, 2006. *Eurosurveillance, 12*(6), 719.

Mulvey, M. R., Finley, R., Allen, V., Ang, L., Bekal, S., El Bailey, S., . . . Gilmour, M. (2013). Emergence of multidrug-resistant *Salmonella enterica* serotype 4,[5],12:i:- involving human cases in Canada: Results from the Canadian Integrated Program on Antimicrobial Resistance Surveillance (CIPARS), 2003–10. *Journal of Antimicrobial Chemotherapy, 68*(9), 1982-1986.

Nei, M. (1987). *Molecular evolutionary genetics*. New York, USA: Columbia University Press.

Ngoi, S. T., & Thong, K. L. (2013). Molecular characterization showed limited genetic diversity among *Salmonella* Enteritidis isolated from humans and animals in Malaysia. *Diagnostic Microbiology and Infectious Disease, 77*(4), 304-311.

Ngoi, S. T., Lindstedt, B. A., Watanabe, H., & Thong, K. L. (2013). Molecular characterization of *Salmonella enterica* serovar Typhimurium isolated from human, food, & animal sources in Malaysia. *Japanese Journal of Infectious Diseases, 66*(3), 180-188.

Nishino, K., Latifi, T., & Groisman, E. A. (2006). Virulence and drug resistance roles of multidrug efflux systems of *Salmonella enterica* serovar Typhimurium. *Molecular Microbiology, 59*(1), 126-141.

Noorzaleha, A. S., Gulam, R., Zaiton, H., Abdul, R., Siti, I., Nishibuchi, M., & Radu, S. (2003). Incidence of *Salmonella* spp. in raw vegetables in Selangor, Malaysia. *Food Control, 14*(7), 475-479.

Norazah, A., Hii, S. Y. F., Rohaidah, H., & Rahizan, I. (2017). Draft genome sequence of *Salmonella enterica* serovar Typhi IMR_TP298/15, a strain with intermediate susceptibility to ciprofloxacin, isolated from a typhoid outbreak. *Genome Announcements, 5*(9), e01740-16.

Norazah, A., Tang, S. G. H., Ghani, M. K. A., & Koh, Y. T. (2012). The discrimination of d-tartrate positive and d-tartrate negative *S. enterica* subsp. *enterica* serovar Paratyphi B isolated in Malaysia by phenotypic and genotypic methods. *The Malaysian Journal of Pathology, 34*(1), 35-39.

Nor-Faiza, S., Saleha, A. A., Jalila, A., & Fauziah, N. (2013). Occurrence of *Campylobacter* and *Salmonella* in ducks and duck eggs in Selangor, Malaysia. *Tropical Biomedicine, 30*(1), 155-158.

Nori, E. M., & Thong, K. L. (2010). Differentiation of *Salmonella enterica* based on PCR detection of selected somatic and flagellar antigen. *African Journal of Microbiology Research, 4*(9), 871-879.

Nur-Eliana, S., Julia, A., Zaidah, R., Azura, H., Asma, I., & Maizan, M. (2014). Establishment of an in-house Loop-Mediated Isothermal Amplification (LAMP) for a rapid detection of *Salmonella* Typhi and *Salmonella* Paratyphi A at low-resource settings. *Journal of Food Safety, 34*(1), 69-75.

Octavia, S., & Lan, R. (2009). Multiple locus variable number of tandem repeat analysis of *Salmonella enterica* serovar Typhi. *Journal of Clinical Microbiology, 47*(8), 2369-2376.

Oliva, M., Monno, R., D'Addabbo, P., Pesole, G., Dionisi, A. M., Scrascia, M., . . . Pazzani, C. (2017). A novel group of IncQ1 plasmids conferring multidrug resistance. *Plasmid, 89*, 22-26.

Ong, L. P., Muniandy, K., How, S. P., Yip, L. S., & Lim, B. K. (2014). *Salmonella* isolation from poultry farms in Malaysia from 2011 to 2013. *Malaysian Journal of Veterinary Research, 5*(1), 180-181.

Onwuezobe, I. A., Oshun, P. O., & Odigwe, C. C. (2012). Antimicrobials for treating symptomatic non-typhoidal *Salmonella* infection. *Cochrane Database of Systematic Reviews, 11*, CD001167.

Overbeek, R., Olson, R., Pusch, G. D., Olsen, G. J., Davis, J. J., Disz, T., . . . Stevens, R. (2014). The SEED and the rapid annotation of microbial genomes using subsystems technology (RAST). *Nucleic Acids Research, 42*(1), 206-214.

Padungtod, P., & Kaneene, J. B. (2006). *Salmonella* in food animals and humans in northern Thailand. *International Journal of Food Microbiology, 108*(3), 346-354.

Pang, J. C., Chiu, T. H., Helmuth, R., Schroeter, A., Guerra, B., & Tsen, H. Y. (2007). A pulsed field gel electrophoresis (PFGE) study that suggests a major world-wide clone of *Salmonella enterica* serovar Enteritidis. *International Journal of Food Microbiology, 116*(3), 305-312.

Park, S. H., Aydin, M., Khatiwara, A., Dolan, M. C., Gilmore, D. F., Bouldin, J. L., . . . Ricke, S. C. (2014). Current and emerging technologies for rapid detection and characterization of *Salmonella* in poultry and poultry products. *Food Microbiology, 38*, 250-262.

Patterson, S. K., Borewicz, K., Johnson, T., Xu, W., & Isaacson, R. E. (2012). Characterization and differential gene expression between two phenotypic phase variants in *Salmonella enterica* serovar Typhimurium. *PLoS ONE, 7*(8), e43592.

Petrovska, L., Mather, A. E., AbuOun, M., Branchu, P., Harris, S. R., Connor, T., . . . Page, A. (2016). Microevolution during the emergence of a monophasic *Salmonella* Typhimurium epidemic in the United Kingdom. *Emerging Infectious Diseases, 22*(4), 150531.

Pietzka, A. T., Stoger, A., Huhulescu, S., Allerberger, F., & Ruppitsch, W. (2011). Gene scanning of an internalin b gene fragment using high-resolution melting curve analysis as a tool for rapid typing of *Listeria monocytogenes*. *The Journal of Molecular Diagnostics, 13*(1), 57-63.

Poirel, L., Cattoir, V., & Nordmann, P. (2012). Plasmid-mediated quinolone resistance: Interactions between human, animal, and environmental ecologies. *Frontiers in Microbiology, 3*(24), 1-7.

Preisler, A., Mraheil, M. A., & Heisig, P. (2006). Role of novel *gyrA* mutations in the suppression of the fluoroquinolone resistance genotype of vaccine strain *Salmonella* Typhimurium *vacT* (*gyrA* D87G). *Journal of Antimicrobial Chemotherapy, 57*(3), 430-436.

Preziosi, M. J., Kandel, S. M., Guiney, D. G., & Browne, S. H. (2012). Microbiological analysis of non-typhoidal *Salmonella* strains causing distinct syndromes of bacteremia or enteritis in HIV/AIDS patients in San Diego, California. *Journal of Clinical Microbiology, 50*(11), 3598-3603.

Price, M. N., Dehal, P. S., & Arkin, A. P. (2010). FastTree 2 – approximately maximum-likelihood trees for large alignments. *PLoS ONE, 5*(3), e9490.

Pui, C. F., Wong, W. C., Chai, L. C., Lee, H. Y., Ahmad, N., Tuan-Chilek, T. Z., . . . Radu, S. (2011). Multiplex PCR for the concurrent detection and differentiation of *Salmonella* spp., *Salmonella* Typhi and *Salmonella* Typhimurium. *Tropical Medicine and Health, 39*(1), 9-15.

Pui, C. F., Wong, W. C., Chai, L. C., Nillian, E., Farinazleen, M. G., Cheah, Y. K., . . . Radu, S. (2011). Simultaneous detection of *Salmonella* spp., *Salmonella* Typhi and *Salmonella* Typhimurium in sliced fruits using multiplex PCR. *Food Control, 22*(2), 337-342.

Puthucheary, S. D., Ng, K. P., Asma, H., Nadeem, R., & Hamimah, H. (2004). Salmonellosis in persons infected with human immunodeficiency virus: A report of seven cases from Malaysia. *Southeast Asian Journal of Tropical Medicine and Public Health, 35*(2), 361-365.

Radu, S., Ansary, A., Salmah, I., & Maznah, A. (1995). Survey of plasmids and resistance factors among veterinary isolates of *Salmonella* Enteritidis in Malaysia. *World Journal of Microbiology and Biotechnology, 11*(3), 315-318.

Raja, A. D., & Gulam, R. (2014). Tracing possible cross contamination of *Salmonella* serovars in culled layers and eggs using RAPD-PCR. *Malaysian Journal of Veterinary Research, 5*(1), 248-252.

Rambaut, A. (2016). *FigTree v1.4.3*. Retrieved from http://tree.bio.ed.ac.uk/software/figtree/

Ramisse, V., Houssu, P., Hernandez, E., Denoeud, F., Hilaire, V., Lisanti, O., . . . Vergnaud, G. (2004). Variable number of tandem repeats in *Salmonella enterica* subsp. *enterica* for typing purposes. *Journal of Clinical Microbiology, 42*(12), 5722-5730.

Reed, G. H., & Wittwer, C. T. (2004). Sensitivity and specificity of single-nucleotide polymorphism scanning by high-resolution melting analysis. *Clinical Chemistry, 50*(10), 1748-1754.

Revez, J., Espinosa, L., Albiger, B., Leitmeyer, K. C., Struelens, M. J., & ECDC National Microbiology Focal Points and Experts Group. (2017). Survey on the use of whole-genome sequencing for infectious diseases surveillance: Rapid expansion of European national capacities, 2015–2016. *Frontiers in Public Health, 5*, 347.

Ribot, E. M., Fair, M. A., Gautom, R., Cameron, D. N., Hunter, S. B., Swaminathan, B., & Barrett, T. J. (2006). Standardization of pulsed-field gel electrophoresis protocols for the subtyping of *Escherichia coli* O157:H7, *Salmonella*, and *Shigella* for PulseNet. *Foodborne Pathogens and Disease, 3*(1), 59-67.

Ricke, S. C., & Calo, J. R. (2015). Antibiotic resistance in pathogenic *Salmonella.* In X. Yan & C. R. Jackson (Eds.), *Antimicrobial resistance and food safety* (pp. 37-53). San Diego, USA: Academic Press.

Roseliza, R., Maswati, M. A., Hasnah, Y., & Ramlan, M. (2011). Identification of *Salmonella* serotypes isolated from meat samples in Malaysia. *Malaysian Journal of Veterinary Research, 2*(1), 59-64.

Ruiz, J. (2003). Mechanisms of resistance to quinolones: Target alterations, decreased accumulation and DNA gyrase protection. *Journal of Antimicrobial Chemotherapy, 51*(5), 1109-1117.

Ruiz, J., Pons, M. J., & Gomes, C. (2012). Transferable mechanisms of quinolone resistance. *International Journal of Antimicrobial Agents, 40*(3), 196-203.

Rychlik, I., Gregorova, D., & Hradecka, H. (2006). Distribution and function of plasmids in *Salmonella enterica*. *Veterinary Microbiology, 112*, 1-10.

Salipante, S. J., SenGupta, D. J., Cummings, L. A., Land, T. A., Hoogestraat, D. R., & Cookson, B. T. (2015). Application of whole-genome sequencing for bacterial strain typing in molecular epidemiology. *Journal of Clinical Microbiology, 53*(4), 1072-1079.

Salwani, M. H., Nuramin, A. M., Fauziah, M. N., Hani, M. H., Wan-Mansor, H., Phua, K. K., . . . Ismail, A. (2014). Genetic relationship and correspondence of *Salmonella* Typhi isolated from water samples in typhoid outbreak localities with food handlers and contact by using pulsed-field gel electrophoresis (PFGE). *Asian Pacific Journal of Tropical Disease, 4*(3), 242.

Salwani, M. H., Sim, K. S., Fauziah, M. N., Hani, M. H., Wan-Mansor, H., Nazalan, N., & Ismail, A. (2015). Complete genome sequence of *Salmonella enterica* subsp. *enterica* serovar Typhi isolate B/SF/13/03/195 associated with a typhoid carrier in Pasir Mas, Kelantan, Malaysia. *Genome Announcements, 3*(6), e01285-15.

Sanpong, P., Theeragool, G., Wajjwalku, W., & Amavisit, P. (2010). Characterization of multiple-antimicrobial resistant *Salmonella* isolated from pig farms in Thailand. *Kasetsart Journal, 44*, 643-651.

Seixas, R., Santos, T. R., Machado, J., Tavares, L., Bernardo, F., Semedo-Lemsaddek, T., & Oliveira, M. (2016). Phenotypic and molecular characterization of *Salmonella* 1,4,[5],12:i:- R-Type ASSuT isolates from humans, animals, and environment in Portugal, 2006–2011. *Foodborne Pathogens and Disease, 13*(11), 633-641.

Silva, C., Calva, E., & Maloy, S. (2014). One health and food-borne disease: *Salmonella* transmission between humans, animals, and plants. *Microbiology Spectrum, 2*(1), OH-0020-2013.

Silva, C., Calva, E., Calva, J. J., Wiesner, M., Fernández-Mora, M., Puente, J. L., & Vinuesa, P. (2015). Complete genome sequence of a human-invasive *Salmonella enterica* serovar Typhimurium strain of the emerging sequence type 213 harboring a multidrug resistance IncA/C plasmid and a blaCMY-2-carrying IncF plasmid. *Genome Announcements, 3*(6), e01323-15.

Silva, C., Calva, E., Puente, J. L., Zaidi, M. B., & Vinuesa, P. (2016). Complete genome sequence of *Salmonella enterica* serovar Typhimurium strain SO2 (Sequence Type 302) isolated from an asymptomatic child in Mexico. *Genome Announcements, 4*(2), e00253-16.

Sim, K. Y., Chye, F. Y., & Fan, H. Y. (2012). Microbiological quality and the impact of hygienic practices on the raw milk obtained from the small-scale dairy farmers in Sabah, Malaysia. *International Journal of Agricultural and Food Science, 2*(2), 55-59.

Singh, A., Goering, R. V., Simjee, S., Foley, S. L., & Zervos, M. J. (2006). Application of molecular techniques to the study of hospital infection. *Clinical Microbiology Reviews, 19*(3), 512-530.

Siti, S. M., Saleha, A. A., & Jalila, A. (2014). Occurrence of antibiotic resistant *Salmonella* and *Campylobacter* in wild birds. *Malaysian Veterinary Journal, 26*(2), 17-19.

Sjölund-Karlsson, M., Folster, J. P., Pecic, G., Joyce, K., Medalla, F., Rickert, R., & Whichard, J. M. (2009). Emergence of plasmid-mediated quinolone resistance among non-Typhi *Salmonella enterica* isolates from humans in the United States. *Antimicrobial Agents and Chemotherapy, 53*(5), 2142-2144.

Slinger, R., Bellfoy, D., Desjardins, M., & Chan, F. (2007). High-resolution melting assay for the detection of *gyrA* mutations causing quinolone resistance in *Salmonella enterica* serovars Typhi and Paratyphi. *Diagnostic Microbiology and Infectious Disease, 57*(4), 455-458.

Song, Y., Roumagnac, P., Weill, F. X., Wain, J., Dolecek, C., Mazzoni, C. J., . . . Achtman, M. (2010). A multiplex single nucleotide polymorphism typing assay for detecting mutations that result in decreased fluoroquinolone susceptibility in *Salmonella enterica* serovars Typhi and Paratyphi A. *Journal of Antimicrobial Chemotherapy, 65*(8), 1631-1641.

Soon, J. M., Singh, H., & Baines, R. (2011). Foodborne diseases in Malaysia: A review. *Food Control, 22*, 823-830.

Soyer, Y., Switt, A. M., Davis, M. A., Maurer, J., McDonough, P. L., Schoonmaker-Bopp, D. J., . . . Gröhn, Y. T. (2009). *Salmonella enterica* serotype 4,[5],12:i:-, an emerging *Salmonella* serotype that represents multiple distinct clones. *Journal of Clinical Microbiology, 47*(11), 3546-3556.

Srinivasan, V. B., Vaidyanathan, V., Mondal, A., & Rajamohan, G. (2012). Role of the two component signal transduction system CpxAR in conferring cefepime and chloramphenicol resistance in *Klebsiella pneumoniae* NTUH-K2044. *PLoS ONE, 7*(4), e33777.

Strahilevitz, J., Jacoby, G. A., Hooper, D. C., & Robicsek, A. (2009). Plasmid-mediated quinolone resistance: A multifaceted threat. *Clinical Microbiology Reviews, 22*(4), 664-689.

Struelens, M. J., Bauernfeind, A., Van Belkum, A., Blanc, D., Cookson, B. D., Dijkshoorn, L., . . . Gerner-Smidt, P. (1996). Consensus guidelines for appropriate use and evaluation of microbial epidemiologic typing systems. *Clinical Microbiology and Infection, 2*(5), 2-11.

Suez, J., Porwollik, S., Dagan, A., Marzel, A., Schorr, Y. I., Desai, P. T., . . . Gal-Mor, O. (2013). Virulence gene profiling and pathogenicity characterization of non-typhoidal *Salmonella* accounted for invasive disease in humans. *PLoS ONE, 8*(3), e58449.

Sullivan, M. J., Petty, N. K., & Beatson, S. A. (2011). Easyfig: A genome comparison visualizer. *Bioinformatics, 27*(7), 1009-1010.

Swaminathan, B., Barrett, T. J., & Hunter, S. B. (2001). PulseNet: The molecular subtyping network for foodborne bacterial disease surveillance, United States. *Emerging Infectious Diseases, 7*(3), 382-389.

Taguchi, M., Kawahara, R., Seto, K., Inoue, K., Hayashi, A., Yamagata, N., . . . Kashiwagi, E. (2009). Plasmid-mediated quinolone resistance in *Salmonella* isolated from patients with overseas travelers' diarrhea in Japan. *Japanese Journal of Infectious Diseases, 62*(4), 312-314.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., & Kumar, S. (2011). MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution, 28*(10), 2731-2739.

Tang, P., & Gardy, J. L. (2014). Stopping outbreaks with real-time genomic epidemiology. *Genome Medicine, 6*(11), 104.

Tavechio, A. T., Ghilardi, A. C. R., & Fernandes, S. A. (2004). Multiplex PCR identification of the atypical and monophasic *Salmonella enterica* subsp. *enterica* serotype 1,4,[5],12:i:- in São Paulo State, Brazil: Frequency and antibiotic resistance patterns. *Revista do Instituto de Medicina Tropical de São Paulo, 46*(2), 115-117.

Taylor, A. J., Lappi, V., Wolfgang, W. J., Lapierre, P., Palumbo, M. J., Medus, C., & Boxrud, D. (2015). Characterization of foodborne outbreaks of *Salmonella enterica* serovar Enteritidis with whole-genome sequencing single nucleotide polymorphism-based analysis for surveillance and outbreak detection. *Journal of Clinical Microbiology, 53*(10), 3334-3340.

Terabayashi, Y., Juan, A., Tamotsu, H., Ashimine, N., Nakano, K., Shimoji, M., . . . Hirano, T. (2014). First complete genome sequence of *Salmonella enterica* subsp. *enterica* serovar Typhimurium strain ATCC13311 (NCTC74), a reference strain of multidrug resistance, as achieved by use of PacBio Single-Molecule Real-Time Technology. *Genome Announcements, 2*(5), e00986-14.

Thong, K. L., & Ang, C. P. (2011). Genotypic and phenotypic differentiation of *Salmonella enterica* serovar Paratyphi B in Malaysia. *Southeast Asian Journal of Tropical Medicine and Public Health, 42*(5), 1178-1189.

Thong, K. L., & Modarressi, S. (2011). Antimicrobial resistant genes associated with *Salmonella* from retail meats and street foods. *Food Research International, 44*(9), 2641-2646.

Thong, K. L., Cheong, Y. M., Puthucheary, S. D., Koh, C. L., & Pang, T. (1994). Epidemiologic analysis of sporadic *Salmonella* Typhi isolates and those from outbreaks by pulsed-field gel electrophoresis. *Journal of Clinical Microbiology, 32*(5), 1135-1141.

Thong, K. L., Cordano, A. M., Rohani, Y., & Pang, T. (1996). Molecular analysis of environmental and human isolates of *Salmonella* Typhi. *Applied and Environmental Microbiology, 62*(1), 271-274.

Thong, K. L., Goh, Y. L., Radu, S., Noorzaleha, S., Yasin, R., Koh, Y. T., . . . Puthucheary, S. D. (2002). Genetic diversity of clinical and environmental strains of *Salmonella enterica* serotype Weltevreden isolated in Malaysia. *Journal of Clinical Microbiology, 40*(7), 2498-2503.

Thong, K. L., Lai, K. S., & Goh, Y. L. (2003). Technical improvement in DNA preparation for pulsed-field gel electrophoresis. *Malaysian Journal of Science, 22*(2), 55-59.

Thong, K. L., Lai, W. L., & Dhanoa, A. (2011). Antimicrobial susceptibility and pulsed-field gel electrophoretic analysis of *Salmonella* in a tertiary hospital in northern Malaysia. *Journal of Infection and Public Health 4*(2), 65-72.

Thong, K. L., Ngeow, Y. F., Altwegg, M., Navaratnam, P., & Pang, T. (1995). Molecular analysis of *Salmonella* Enteritidis by pulsed-field gel electrophoresis and ribotyping. *Journal of Clinical Microbiology, 33*(5), 1070-1074.

Thong, K. L., Ngoi, S. T., Chai, L. C., & Teh, C. S. J. (2016). Quinolone resistance mechanisms among *Salmonella enterica* in Malaysia. *Microbial Drug Resistance, 22*(4), 259-272.

Thong, K. L., Puthucheary, S. D., & Pang, T. (1998). Outbreak of *Salmonella* Enteritidis gastroenteritis: investigation by pulsed-field gel electrophoresis. *International Journal of Infectious Diseases, 2*(3), 159-163.

Thong, K. L., Puthucheary, S. D., Rohani, Y., Pratiwi, S., Maria, P., Eddy, S., . . . Pang, T. (1995). Analysis of *Salmonella* Typhi isolates from Southeast Asia by pulsed-field gel electrophoresis. *Journal of Clinical Microbiology, 33*(7), 1938-1941.

Tien, Y. Y., Wang, Y. W., Tung, S. K., Liang, S. Y., & Chiou, C. S. (2011). Comparison of multilocus variable-number tandem repeat analysis and pulsed-field gel electrophoresis in molecular subtyping of *Salmonella enterica* serovars Paratyphi A. *Diagnostic Microbiology and Infectious Disease, 69*(1), 1-6.

Tindall, E. A., Petersen, D. C., Woodbridge, P., Schipany, K., & Hayes, V. M. (2009). Assessing high-resolution melt curve analysis for accurate detection of gene variants in complex DNA fragments. *Human Mutation, 30*(6), 876-883.

Tiong, V., Thong, K. L., Yasim, Y., Yasmin, A. H., Sam, J. I. C., & Hamimah, H. (2010). Macrorestriction analysis and antimicrobial susceptibility profiling of *Salmonella enterica* at a university teaching hospital, Kuala Lumpur. *Japanese Journal of Infectious Diseases, 63*, 317-322.

Todar, K. (2008). *Todar's online textbook of bacteriology: Salmonella and salmonellosis.* Retrieved from http://www.textbookofbacteriology.net/index.html

Trüpschuch, S., Laverde Gomez, J. A., Ediberidze, I., Flieger, A., & Rabsch, W. (2010). Characterisation of multidrug-resistant *Salmonella* Typhimurium 4,[5],12:i:-DT193 strains carrying a novel genomic island adjacent to the *thrW* tRNA locus. *International Journal of Medical Microbiology, 300*(5), 279-288.

Tunung, R., Chai, L. C., Usha, M. R., Lee, H. Y., Fatimah, A. B., Farinazleen, M. G., & Radu, S. (2007). Characterization of *Salmonella enterica* isolated from street food and clinical samples in Malaysia. *ASEAN Food Journal, 14*(3), 161-173.

Turner, A. K., Nair, S., & Wain, J. (2006). The acquisition of full fluoroquinolone resistance in *Salmonella* Typhi by accumulation of point mutations in the topoisomerase targets. *Journal of Antimicrobial Chemotherapy, 58*(4), 733-740.

Ueda, S., & Kuwabara, Y. (2009). The rapid detection of *Salmonella* from food samples by loop-mediated isothermal amplification (LAMP). *Biocontrol Science, 14*(2), 73-76.

Van Belkum, A. (2003). High-throughput epidemiologic typing in clinical microbiology. *Clinical Microbiology and Infection, 9*(2), 86-100.

Van Belkum, A. (2007). Tracing isolates of bacterial species by multilocus variable number of tandem repeat analysis (MLVA). *FEMS Immunology and Medical Microbiology, 49*(1), 22-27.

Van Cuyck, H., Farbos-Granger, A., Leroy, P., Yith, V., Guillard, B., Sarthou, J., . . . Kruy, S. (2011). MLVA polymorphism of *Salmonella enterica* subspecies isolated from humans, animals, and food in Cambodia. *BMC Research Notes, 4*, 306.

Van, T. T. H., Nguyen, H. N. K., Smooker, P. M., & Coloe, P. J. (2012). The antibiotic resistance characteristics of non-typhoidal *Salmonella enterica* isolated from food-producing animals, retail meat and humans in Southeast Asia. *International Journal of Food Microbiology, 154*(3), 98-106.

Veldman, K., Cavaco, L. M., Mevius, D., Battisti, A., Franco, A., Botteldoorn, N., . . . Aarestrup, F. M. (2011). International collaborative study on the occurrence of plasmid-mediated quinolone resistance in *Salmonella enterica* and *Escherichia coli* isolated from animals, humans, food and the environment in 13 European countries. *Journal of Antimicrobial Chemotherapy, 66*(6), 1278-1286.

Wallace, D. L. (1983). A method for comparing two hierarchical clusterings: Comment. *Journal of the American Statistical Association, 78*(383), 569-576.

Wattiau, P., Boland, C., & Bertrand, S. (2011). Methodologies for *Salmonella enterica* subsp. *enterica* subtyping: gold standards and alternatives. *Applied and Environmental Microbiology, 77*(22), 7877-7885.

Weill, F. X., Bertrand, S., Guesnier, F., Baucheron, S., Cloeckaert, A., & Grimont, P. A. (2006). Ciprofloxacin-resistant *Salmonella* Kentucky in travelers. *Emerging Infectious Diseases, 12*(10), 1611-1612.

White, H., & Potts, G. (2006). Mutation scanning by high resolution melt analysis. Evaluation of rotor-gene 6000 (Corbett Life Science), HR-1 and 384-well lightscanner (Idaho Technology). Wessex, United Kingdom: National Genetics Reference Laboratory.

Wittwer, C. T., Reed, G. H., Gundry, C. N., Vandersteen, J. G., & Pryor, R. J. (2003). High-resolution genotyping by amplicon melting analysis using LCGreen. *Clinical Chemistry, 49*(6), 853-860.

World Health Organization, WHO. (2014). *Antimicrobial resistance: Global report on surveillance 2014*. Geneva, Switzerland: WHO Document Production Services.

Yang, X., Wu, Q., Zhang, J., Huang, J., Guo, W., & Cai, S. (2015). Prevalence and characterization of monophasic *Salmonella* serovar 1,4,[5],12:i:- of food origin in China. *PLoS ONE, 10*(9), e0137967.

Yap, K. P., Gan, H. M., Teh, C. S. J., Baddam, R., Chai, L. C., Kumar, N., . . . Thong, K. L. (2012a). Genome sequence and comparative pathogenomics analysis of a *Salmonella enterica* serovar Typhi strain associated with a typhoid carrier in Malaysia. *Journal of Bacteriology, 194*(21), 5970-5971.

Yap, K. P., Teh, C. S. J., Baddam, R., Chai, L. C., Kumar, N., Avasthi, T. S., . . . Thong, K. L. (2012b). Insights from the genome sequence of a *Salmonella enterica* serovar Typhi strain associated with a sporadic case of typhoid fever in Malaysia. *Journal of Bacteriology, 194*(18), 5124-5125.

Yap, K. P., Gan, H. M., Teh, C. S. J., Chai, L. C., & Thong, K. L. (2014). Comparative genomics of closely related *Salmonella enterica* serovar Typhi strains reveals genome dynamics and the acquisition of novel pathogenic elements. *BMC Genomics, 15*(1), 1007.

Yaqub, A. G., Zakaria, Z., Saleha, A. A., Siti, K. B., Jalila, A., & Sharina, O. (2012). High prevalence of multi-drug resistant bacteria in selected poultry farms in Selangor, Malaysia. *Asian Journal of Animal and Veterinary Advances, 7*(9), 891-897.

Yasin, R. M., Jegathesan, M., & Tiew, C. C. (1997). *Salmonella* serotypes isolated in Malaysia over the ten-year period 1983-1992. *Asia-Pacific Journal of Public Health, 9*(1), 1-5.

Yasin, R. M., Tiew, C. C., & Jegathesan, M. (1995). Human salmonellosis in Malaysia for the period 1989-July 1994. *Southeast Asian Journal of Tropical Medicine and Public Health, 26*(3), 457-460.

Yue, M., Rankin, S. C., Blanchet, R. T., Nulton, J. D., Edwards, R. A., & Schifferli, D. M. (2012). Diversification of the *Salmonella* fimbriae: a model of macro- and microevolution. *PLoS ONE, 7*(6), e38596.

Zamperini, K., Soni, V., Waltman, D., Sanchez, S., Theriault, E. C., Bray, J., & Maurer, J. J. (2007). Molecular characterization reveals *Salmonella enterica* serovar 4,[5],12:i:- from poultry is a variant Typhimurium serovar. *Avian Diseases, 51*(4), 958-964.

Zhang, S., Yin, Y., Jones, M. B., Zhang, Z., Kaiser, B. L. D., Dinsmore, B. A, . . . Deng, X. (2015). *Salmonella* serotype determination utilizing high-throughput genome sequencing data. *Journal of Clinical Microbiology, 53*(5), 1685-1692.

Zhou, Y., Liang, Y., Lynch, K. H., Dennis, J. J., & Wishart, D. S. (2011). PHAST: A fast phage search tool. *Nucleic Acids Research, 39*, 347-352.

## LIST OF PUBLICATIONS AND PAPERS PRESENTED

(a) ***Publications (in reverse chronological order)***

1. Ngoi, S. T., Yap, K. P., & Thong, K. L. (2018). Genomic characterization of endemic *Salmonella enterica* serovar Typhimurium and *Salmonella enterica* serovar I 4,[5],12:i:- isolated in Malaysia. *Infection, Genetics and Evolution, 62*, 109-121.

2. Thong, K. L., Ngoi, S. T., Chai, L. C., & Teh, C. S. J. (2016). Quinolone resistance mechanisms among *Salmonella enterica* in Malaysia. *Microbial Drug Resistance, 22*(4), 259-272.

3. Ngoi, S. T., Teh, C. S. J., Chai, L. C., & Thong, K. L. (2015). Overview of molecular typing tools for the characterization of *Salmonella enterica* in Malaysia. *Biomedical and Environmental Sciences, 28*(10), 751-764.

4. Ngoi, S. T., & Thong, K. L. (2014). High resolution melting analysis for rapid mutation screening in gyrase and topoisomerase IV genes in quinolone-resistant *Salmonella enterica*. *BioMed Research International Journal, 2014*, 718084.

5. Ngoi, S. T., & Thong, K. L. (2013). Molecular characterization showed limited genetic diversity among *Salmonella* Enteritidis isolated from humans and animals in Malaysia. *Diagnostic Microbiology and Infectious Disease, 77*, 304-311.

6. Ngoi, S. T., Lindstedt, B. A., Watanabe, H., & Thong, K. L. (2013). Molecular characterization of *Salmonella enterica* serovar Typhimurium isolated from human, food and animal sources in Malaysia. *Japanese Journal of Infectious Diseases, 66*, 180-188.

7. Ngoi, S. T., & Thong, K. L. (2012). Antimicrobial susceptibility and genotypic characterization of clinical *Salmonella* Enteritidis strains isolated from a tertiary hospital in Malaysia by using multilocus variable number of tandem repeat analysis and pulsed-field gel electrophoresis. *International Journal of Infectious Diseases, 16*(1), 234-235.

(b) ***Papers presented (in reverse chronological order)***

1. Thong, K. L., & Ngoi, S. T. (2015, Apr). *Comparative genomics and variations in carbohydrate utilization between monophasic and biphasic Salmonella enterica serovar Typhimurium*. Paper presented at the International Conference on Typhoid and Invasive NTS Disease, Bali, Indonesia.

2. Ngoi, S. T., & Thong, K. L. (2014, Sep). *Genomic variations in a monophasic variant of Salmonella enterica serovar Typhimurium isolated in Malaysia*. Paper presented at the National Postgraduate Seminar, Universiti Putra Malaysia, Serdang, Malaysia.

3. Thong, K. L., & Ngoi, S. T. (2013, Oct). *High resolution melt curve analysis for rapid mutation screening in quinolone-resistant Salmonella*. Paper presented at the ASM Conference on *Salmonella*: The Bacterium, the Host and the Environment, Boston, Massachusetts, USA.

4. Ngoi, S. T., & Thong, K. L. (2013, Jun). *High resolution melt curve analysis for mutation detection in quinolone-resistant determining region of DNA topoisomerase IV genes in Salmonella*. Paper presented at the MSMBB Scientific Meeting, Kuala Lumpur, Malaysia.

5. Ngoi, S. T., & Thong, K. L. (2013, Mar). *High resolution melt curve analysis to detect mutations in quinolone-resistant determining region of gyr genes in Salmonella*. Paper presented at the International Symposium on Antimicrobial Agents and Resistance, Kuala Lumpur, Malaysia.

6. Ngoi, S. T., & Thong, K. L. (2012, Jul). *Multi-drug resistance and genetic relatedness among Malaysian Salmonella enterica serovar Typhimurium isolated from food and animals*. Paper presented at the National Postgraduate Seminar, Kuala Lumpur, Malaysia.

7. Ngoi, S. T., & Thong, K. L. (2012, Jun). *Antimicrobial susceptibility and genotypic characterization of clinical Salmonella Enteritidis strains isolated from a tertiary hospital in Malaysia by using multilocus variable number of tandem repeat analysis and pulsed-field gel electrophoresis*. Paper presented at the International Congress on Infectious Disease, Bangkok, Thailand.

8. Ngoi, S. T., & Thong, K. L. (2011, Dec). *Optimisation of multilocus variable-number tandem repeat analysis for Salmonella Typhimurium and the effect of dilution factor for capillary electrophoretic analysis*. Paper presented at the International Congress of Malaysian Society for Microbiology, Penang, Malaysia.

9. Ngoi, S. T., & Thong, K. L. (2010, Oct). *Evaluation of multilocus variable number tandem repeat analysis (MLVA) in determining genetic diversity of Salmonella enterica serovar Typhimurium*. Paper presented at the My1Bio Conference, Kuala Lumpur, Malaysia.

10. Ngoi, S. T., & Thong, K. L. (2009, Dec). *Development of multiple-locus variable-number tandem repeat analysis (MLVA) for subtyping of Salmonella enterica subsp. enterica serovar Typhimurium*. Paper presented at the International Congress of Malaysian Society for Microbiology, Penang, Malaysia.