MOLECULAR EPIDEMIOLOGY OF *Salmonella enterica* serovar Typhi ISOLATES FROM KELANTAN, MALAYSIA USING VARIABLE NUMBER TANDEM REPEAT (VNTR)

C.W.SALMA BINTI C.W.ZALATI

UNIVERSITI SAINS MALAYSIA

2016

MOLECULAR EPIDEMIOLOGY OF *Salmonella enterica* serovar Typhi ISOLATES FROM KELANTAN, MALAYSIA USING VARIABLE NUMBER TANDEM REPEAT (VNTR)

by

C.W.SALMA BINTI C.W.ZALATI

Thesis submitted in fulfillment of the requirements for the degree of Master of Science

August 2016

ACKNOWLEDGEMENT

All praises to Allah SWT and blessings upon His Prophet SAW for giving me the strength, guidance, patience and comfort in completing this project and the dissertation write up.

I wish to express my sincerest gratitude and appreciation to my main supervisor, Assoc. Prof. Dr. Maizan Mohamed for her guidance, help and encouragement throughout my study and without her, this thesis would not have been completed or written. I am also grateful to both of my co-supervisors, Assoc. Prof. Dr. Phua Kia Kien and Assoc. Prof. Dr. Zaidah Abdul Rahman for their guidance throughout my whole study.

Special thanks to the laboratory staffs especially Jaafar, Haslizai, Hafiza, Amin and Zafri for their help and encouragement that have helped me through numerous obstacles during my study. I would like to extend my warm and sincere thanks to my friends; Julia, Eliana, Fadhilah, Faizul, Goay and Aziana who helped me with my work and provided the emotional support to get through the difficult times during my masters. I am also grateful to all lecturers and administrative staffs of INFORMM for helping me in every possible way they could and continuously giving moral support during my candidature.

Finally, I would like to thank my beloved family members, C.W.Zalati, Rohayati, C.W.Sharmila, C.W.Rusydi, C.W.Shamrin, C.W.Razlan, C.W.Syahirah, C.W.Aleeya

Syafika and all other family members for their encouragement, support, patience, tolerance and understanding to allow me to pursue my study. My humble thanks also to the people who had been helping me directly or indirectly towards completion of my study.

Special thanks goes to the MyMaster and USM Graduate Assistant Scheme (GA) for providing the financial support throughout my study, and to the Research University Grant (Grant No: 1001/CIPPM/812059) for providing funding to support the research.

TABLE OF CONTENTS

ACKNOWLEDGEMENT ....

TABLE OF CONTENTS iv

LIST OF TABLE viii

LIST OF FIGURE ix

LIST OF SYMBOL AND ABBREVIATION xi

ABSTRAK xiii

ABSTRACT xv

CHAPTER 1: INTRODUCTION 1

1.1 General Background on *Salmonella* 1

1.2 *Salmonella enterica* subspecies *enterica* serovar Typhi 3

1.3 Typhoid Fever 4

1.4 Typhoid Carrier 6

1.5 Epidemiology of Typhoid Fever 6

1.6 Laboratory Diagnosis of Typhoid Fever 11

1.6.1 Culture Method 11

1.6.2 Serological Methods 12

1.6.2 (a) Widal Agglutination Test 12

1.6.2 (b) TUBEX® 14

1.6.2 (c) Typhidot® 14

1.6.3 Polymerase Chain Reaction (PCR) 15

1.7 Molecular Epidemiology 16

1.8 Types of Typing Methods 18

1.9 Pulsed-Field Gel Electrophoresis (PFGE) 20

1.10 Variable Number Tandem Repeat (VNTR) 23

1.11 Rationale of the study 29

1.12 Objectives of the study 30

1.12.1 Main Objective 30

1.12.2 Specific Objective 30

CHAPTER 2: METHODOLOGY 31

2.1 Materials 31

2.1.1 Bacterial isolates 31

2.1.2 Media and Chemicals 32
2.1.3 Media Preparation for Culture Purposes 34
2.1.3 (a) Nutrient Broth 34
2.1.3 (b) Nutrient Agar 34
2.1.3 (c) Triple Sugar Iron (TSI) Agar 34
2.1.3 (d) Urea Solution 30% (w/v) 35
2.1.3 (e) Urea Agar Base 35
2.1.3 (f) Simmons Citrate Agar 35
2.1.3 (g) Methyl Red Vogus-Proskauer (MRVP) Medium 36
2.1.3 (h) Sulphate Indole Motility (SIM) Medium 36
2.1.4 Reagents and Buffer Preparation for VNTR Purposes 37
2.1.4 (a) Sodium Hydroxide (NaOH) (3.0 M) 37
2.1.4 (b) Ethylenediaminetetraacetic Acid (EDTA)(0.5 M) 37
2.1.4 (c) Tris-Borate EDTA Buffer (5x) 37
2.1.4 (d) Agarose (w/v) 38
2.2 Methods 39
2.2.1 Identification of *S. enterica* ser. Typhi 39
2.2.2 Serotyping Procedure 39
2.2.3 Bacterial DNA Extraction 40
2.2.4 Determination of Concentration and Purity of Extracted DNA 41
2.2.5 Selection of VNTR loci and PCR primers 41
2.2.6 Preparation of Primer Stock Solution 42
2.2.7 Preparation of Primer Working Solution 43
2.2.8 Screening for Length Polymorphism by PCR of Individual VNTR Loci 43
2.2.9 Optimisation of Monoplex and Multiplex PCR Assays 44
2.2.9 (a) Initial Monoplex and Multiplex PCR Assay 44
2.2.9 (b) Annealing Temperature 44
2.2.9 (c) Primer Concentration 45
2.2.9 (d) $MgCl_2$ Concentration 45
2.2.9 (e) Primer Concentration 45
2.2.9 (f) Taq DNA PolymeraseConcetration 45
2.2.10 Evaluation of Monoplex and Multiplex PCR Assays 46
2.2.11 Specificity of Multiplex PCR Assays 46
2.2.12 Gel Electrophoresis of PCR Products 46

2.2.13 Gene Sequencing of Monoplex PCR Products 47
2.2.14 Computer Analysis of VNTR Patterns 47
2.2.15 Interpretation of VNTR Patterns 48
2.2.15 (a) Definitions Used When Interpreting VNTR Patterns 48
2.2.15 (b) Definitions Used for Computer Analysis of VNTR Patterns 49
CHAPTER 3: RESULTS 50
3.1 Identification of *S. enterica* ser. Typhi Isolates from Kelantan, Malaysia 50
3.2 VNTR analysis of *S. enterica* ser. Typhi Isolates from Kelantan, Malaysia 51
3.2.1 Screening for Length Polymorphism by PCR of Individual VNTR Loci 51
3.2.2 Optimisation and Evaluation of Monoplex PCR Assay 54
3.2.2 (a) Optimisation of Monoplex PCR Assay 54
3.2.2 (ai) Optimisation of Annealing Temperature 54
3.2.2.(aii) Optimisation of $MgCl_2$ Concentration 54
3.2.2 (aiii) Optimisation dNTPs Concentration 54
3.2.2 (aiv) Optimisation of *Taq* DNA Polymerase Concentration 57
3.2.2 (av) Optimisation of Primer Concentration 57
3.2.2 (b) Evaluation of Monoplex PCR Assay 57
3.2.3 Optimisation, Specificity and Evaluation of Multiplex PCR Assay 61
3.2.3 (a) Optimisation of Multiplex PCR Assay 61
3.2.3 (ai) Optimisation of Annealing Temperature 61
3.2.3.(aii) Optimisation of Primer Concentration 61
3.2.3 (aiii) Optimisation $MgCl_2$ Concentration 61
3.2.3 (aiv) Optimisation of dNTPs Concentration 64
3.2.3 (av) Optimisation of *Taq* DNA Polymerase Concentration 64
3.2.3 (b) Specificity of Multiplex PCR Assay 64
3.2.3 (c) Evaluation of Multiplex PCR Assays 67
3.2.4 Distribution of *S. enterica* ser. Typhi Strains in Kelantan, Malaysia 73
3.2.5 Genomic Comparison of *S. enterica* ser. Typhi Isolates from Acute and Carrier Isolates 82
3.2.6 Genomic Comparison of *S. enterica* ser. Typhi Isolates from Outbreak Cases of Typhoid Fever 87
3.2.7 Genomic Comparison of *S. enterica* ser. Typhi Isolates from Sporadic Cases of Typhoid Fever 93

3.2.8 Analysis of *S. enterica* ser. Typhi Isolates from Households in Kelantan, Malaysia 98

3.2.9 Analysis of *S. enterica* ser. Typhi Isolates from Human and Environmental Samples 100

3.3 Sequence Analysis of Individual VNTR Alleles 102

3.4 Comparison between VNTR and PFGE Methods for the Same *S. enterica* ser. Typhi Isolates 116

CHAPTER 4: DISCUSSION 124

4.1 Screening for Length Polymorphism by PCR of Individual VNTR Loci 124

4.2 Multiplex PCR-based VNTR Analysis 126

4.3 Distribution of *S. enterica* ser. Typhi Strains in Kelantan, Malaysia 129

4.4 Genomic Comparison of *S. enterica* ser. Typhi Isolates from Acute and Carrier Isolates 132

4.5 Genomic Comparison *S. enterica* ser. Typhi Isolates from Outbreak Cases of Typhoid Fever 134

4.6 Genomic Comparison of *S. enterica* ser. Typhi Isolates from Sporadic Cases of Typhoid Fever 136

4.7 Analysis of *S. enterica* ser. Typhi Isolates from Households in Kelantan, Malaysia 138

4.8 Sequence Analysis of Individual VNTR Alleles 140

4.9 Comparison between VNTR and PFGE Methods for the Same *S. enterica* ser. Typhi Isolates 142

CHAPTER 5: CONCLUSION AND FUTURE RECOMMENDATION 145

5.1 Conclusion 145

5.2 Future Recommendation 147

REFERENCES 148

APPENDICES 158

Appendix A 158

Appendix B 159

LIST OF PUBLICATION AND PRESENTATIONS 161

LIST OF TABLE

Table 1.1 Biochemical tests for differentiation of *S. enterica* ser. Typhi from related subspecies (Adapted from WHO, 2003)..........................3

Table 1.2 Incidence rate of typhoid fever in Malaysia by states from the year 2002 to 2012 (Departmemt of Statistics, Malaysia, 2013).........10

Table 2.1 List of media, reagents and chemicals used in this study...................33

Table 2.2 List of published PCR primers flanking the five VNTR loci selected from the *S. enterica* ser. Typhi CT18 genome 42

Table 3.1 Biochemical test results for *S. enterica* ser. Typhi isolates................50

Table 3.2 Summary of the distribution of *S. enterica* ser. Typhi strains from sporadic cases of typhoid fever in different districts in Kelantan, Malaysia..95 - 97

Table 3.3 DNA sequences of *S. enterica* ser. Typhi strains with VNTR copy number for TR1 locus...104 - 106

Table 3.4 DNA sequences of *S. enterica* ser. Typhi strains with VNTR copy number for TR2 locus..107 - 114

Table 3.5 DNA sequences of *S. enterica* ser. Typhi strains with VNTR copy number for TR3 locus..115

Table 3.6 List of VNTR and PFGE patterns of 200 *S. enterica* ser. Typhi isolates between the year 2002 to 2012 ....118 - 119

Table A List of other *Salmonella* species..158

LIST OF FIGURE

Figure 1.1 Nomenclature of *Salmonella* according to the Kauffman-White scheme (Adapted from CDC, USA, 2002) 2

Figure 1.2 Global distribution of typhoid fever in the year 2002 (Adapted from Crump *et al.*, 2004) 9

Figure 1.3 Incidence rate of typhoid fever in Kelantan from the year 2002 to 2012 (Adapted from Kelantan State Health Department, 2012) 9

Figure 1.4 General principle of PFGE method 22

Figure 1.5 Variation in number of tandem repeats in a tandem repeat locus for 4 different strains 24

Figure 1.6(a) Decreased number of repeats in tandem repeat region by forward replication slippage 24

Figure 1.6(b) Increased number of repeats in tandem repeat region by backward replication slippage 25

Figure 1.7 Summary of VNTR method 28

Figure 3.1 Gel electrophoresis of TR1, TR2, TR3, TR4 and TR5 VNTR loci of 38 *S. enterica* ser. Typhi isolates using TR1, TR2, TR3, TR4 and TR5 primer sets 53

Figure 3.2 Optimisation of annealing temperature for monoplex PCR assay for TR1 and TR2 loci 55

Figure 3.3 Optimisation of $MgCl_2$ concentration for monoplex PCR assay for TR1 and TR2 loci 55

Figure 3.4 Optimisation of dNTPs concentration for monoplex PCR assay for TR1 and TR2 loci 56

Figure 3.5 Optimisation of *Taq* DNA Polymerase concentration for monoplex PCR assay for TR1 and TR2 loci 58

Figure 3.6 Optimisation of primer concentration for monoplex PCR assay for TR1 and TR2 loci 58

Figure 3.7 Monoplex PCR products of TR1 locus 59

Figure 3.8 Monoplex PCR products of TR2 locus 60

Figure 3.9 Optimisation of annealing temperature for multiplex PCR assay 62

Figure 3.10 Optimisation of primer concentration for multiplex PCR assay 62

Figure 3.11 Optimisation of $MgCl_2$ concentration for multiplex PCR assay 63

Figure 3.12 Optimisation of dNTPs concentration for multiplex PCR assay 65

Figure 3.13 Optimisation of *Taq* DNA Polymerase concentration for multiplex PCR assay 65

Figure 3.14 Specificity of the multiplex PCR assay for strain typing *S. enterica* ser. Typhi isolates 66

Figure 3.15 The multiplex PCR products of *S. enterica* ser. Typhi isolates from Kelantan, Malaysia .... 68 - 69

Figure 3.16 Database of VNTR Bank that recorded all the VNTR patterns and details of *S. enterica* ser. Typhi isolates 70

Figure 3.17 VNTR analysis performed on 2 *S. enterica* ser. Typhi isolates for stability testing through serial subcultures process 71

Figure 3.18 VNTR analysis performed on 4 *S. enterica* ser. Typhi isolates for reproducibility testing 72

Figure 3.19 38 VNTR strains of 200 *S. enterica* ser. Typhi isolates from Kelantan, Malaysia .... 74 - 77

Figure 3.20 Distribution of *S. enterica* ser. Typhi strains (n=200) based on VNTR patterns 78

Figure 3.21 Distribution of *S. enterica* ser. Typhi strains between the year 2002 to 2012 80

Figure 3.22 Distribution of *S. enterica* ser. Tphi strains in different districts in Kelantan, Malaysia 81

Figure 3.23 Dendrogram of *S. enterica* ser. Typhi strains for carrier isolates .... 83 - 86

Figure 3.24 Dendrogram of *S. enterica* ser. Typhi strains for outbreak isolates .... 89 - 92

Figure 3.25 Dendrogram of household transmission among household members 99

Figure 3.26 Dendrogram of *S. enterica* ser. Typhi strains for environmental and human samples 101

Figure 3.27 Dendrogram showing 38 *S. enterica* ser. Typhi strains with number of VNTR repeats for TR1 and TR2 loci 103

Figure 3.28 Dendrogram of VNTR and PFGE patterns for 200 *S. enterica* ser. Typhi isolates .... 120 - 123

Figure B1 Stability testing for PFGE method (Fadhilah, 2010) 159

Figure B2 Reproducibility testing for PFGE method (Fadhilah, 2010) 160

LIST OF SYMBOL AND ABBREVIATION

Symbols / Abbreviations / Acronyms	Definitions
+	Positive
-	Negative or minus
>	Greater than
≥	Greater than or equal to
<	Less than
≤	Less than or equal to
%	Percentage
°	Degree
°C	Degree Celsius
1×	1 time
cm	Centimeter
dH_20	Distilled water
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphate
g	Gram
H_2S	Hydrogen sulphide
IgG	Immunoglobulin G
IgM	Immunoglobulin M
I	Intermediate
kb	Kilo base
kDa	Kilo Dalton
L	Liter
M	Molar
mM	millimolar
mg	Milligram
mg/ml	Milligram per milliliter
$MgCl_2$	Magnesium chloride
ml	Milliliter
mm	Millimeter
MW	Molecular weight
NA	Nutrient agar
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NB	Nutrient broth
ng	Nanogram
ng/µl	Nanogram per microliter
nm	Nanometer
OD	Optical density
PBR	Phosphate buffer saline
PCR	Polymerase Chain Reaction
pH	Potential hydrogen
R	Resistant
RE	Restriction enzyme
rpm	Revolutions per minute

Symbols / Abbreviations / Acronyms	Definitions
rcf	Relative centrifugal force
sec	Second
ser.	Serovar
sp.	Species
S	Sensitive
TIFF	Tagged Image File Format
TSI	Triple Sugar Iron
U	Unit
UV	Ultraviolet
USA	United States of America
V	Volt
w/v	Weight per Volume
v/v	Volume per Volume
µg	Microgram
µg/ml	Microgram per milliliter
µM	Micromolar
µm	Micrometer
µl	Microliter
WHO	World Health Organisation

EPIDEMIOLOGI MOLEKULAR ISOLAT *Salmonella enterica* serovar Typhi DARI KELANTAN, MALAYSIA MENGGUNAKAN VARIABLE NUMBER TANDEM REPEAT (VNTR)

ABSTRAK

Keupayaan untuk mencirikan isolat bakteria merupakan alatan kritikal dalam penyiasatan epidemiologi untuk mengawal penyebarannya. Beberapa jenis kaedah perincian telah dibangunkan untuk *Salmonella enterica* serovar Typhi (*S. enterica* ser. Typhi) seperti ribotiping, Pulsed-Field Gel Electrophoresis (PFGE), Random Amplification of Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP) dan Variable Number Tandem Repeat (VNTR). Kaedah PFGE telah dianggap sebagai kaedah standard emas untuk perincian *Salmonella* dengan kuasa diskriminasi yang hebat. Namun, kaedah ini mempunyai beberapa limitasi termasuklah tenaga kerja yang banyak, masa yang panjang dan memerlukan kakitangan yang berpengalaman serta peralatan yang mahal. Maka, dalam kajian ini, kaedah multiplex Polymerase Chain Reaction (mPCR) yang menggunakan primer yang berada di hujung loki VNTR telah dibangunkan untuk mencirikan 200 isolat *S. enterica* ser. Typhi dari Kelantan, Malaysia. Profil VNTR yang dihasilkan boleh dianalisa dengan mudah melalui pemerhatian visual selepas gel electrophoresis konvesional. Analisa jujukan asid nukleik dan penentuan bilangan salinan VNTR untuk setiap unit ulangan telah dilakukan dengan menggunakan perisian ChromasPro dan Tandem Repeat Finder. Analisa kluster menggunakan Perisian Fingerprinting Quest Versi 5.10 (Bio-Rad, USA) telah dilakukan untuk mengkaji hubungan di antara kesemua isolat *S. enterica* ser. Typhi. Kajian ini telah mendedahkan potensi dua loki

VNTR, TR1 dan TR2, untuk digunakan sebagai penanda molekul bagi mencirikan isolat *S. enterica* ser. Typhi dari Kelantan. Sebanyak 200 isolat telah dicirikan kepada 38 corak VNTR (VNTR01-VNTR38) di mana setiap corak telah dianggap sebagai strain *S. enterica* ser. Typhi yang berbeza. Strain *S. enterica* ser. Typhi VNTR14 telah dikenalpasti sebagai strain utama yang predominan dalam negeri ini. Sumber infeksi mungkin berpunca daripada pembawa memandangkan strain yang dijumpai pada isolat pembawa turut dijumpai pada isolat akut. Pelbagai strain telah dikenalpasti daripada kes wabak dan sporadik bagi demam kepialu yang menunjukkan bahawa kes-kes tersebut adalah disebabkan oleh sumber infeksi yang berbeza. Infeksi kepialu yang berlaku di setiap rumah berkongsi corak VNTR yang sama yang menunjukkan bahawa mereka telah dijangkiti daripada strain yang sama. Kesemua 38 strain *S. enterica* ser. Typhi mempunyai bilangan salinan VNTR yang berbeza bagi setiap loki di mana unit ulangan 7-bp yang terdiri daripada 5 hingga 19 salinan dan unit ulangan 8-bp yang terdiri daripada 7 hingga 42 salinan telah diperhatikan pada loki TR1 dan TR2. Sebagai kesimpulannya, kaedah VNTR yang digunakan dalam kajian ini menyediakan satu kaedah yang mudah, cepat dan murah serta kuasa diskriminasi yang tinggi untuk mencirikan *S. enterica* ser. Typhi.

MOLECULAR EPIDEMIOLOGY OF *Salmonella enterica* serovar Typhi ISOLATES FROM KELANTAN, MALAYSIA USING VARIABLE NUMBER TANDEM REPEAT (VNTR)

ABSTRACT

The capability to strain type bacterial isolates is a critical tool in epidemiological investigation in order to control their dissemination. A number of strain typing methods have been developed for *Salmonella enterica* serovar Typhi (S. *enterica* ser. Typhi) such as ribotyping, Pulsed-Field Gel Electrophoresis (PFGE), Random Amplification of Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP) and Variable Number Tandem Repeat (VNTR). The PFGE method has been considered as the gold standard method for *Salmonella* typing with excellent discriminatory power. However, this method has some limitations including labour intensive, time consuming and requires skilled technicians as well as expensive equipment. Therefore, in this study, a multiplex Polymerase Chain Reaction (mPCR) method incorporating primers flanking to the VNTR loci was developed for molecular typing of 200 *S. enterica* ser. Typhi isolates from Kelantan, Malaysia. The VNTR profiles produced could be easily analysed by visual inspection after conventional gel electrophoresis. The analysis of DNA sequence and determination of VNTR copy number of the respective repeat were performed by using ChromasPro and Tandem Repeat Finder Software. Cluster analysis was performed by using Fingerprinting Quest Software version 5.10 (Bio-Rad, USA) to study the relationship between all *S. enterica* ser. Typhi isolates. This study revealed that only two potential VNTR loci, TR1 and TR2, can be used as molecular markers for strain typing of *S. enterica* ser. Typhi

isolates from Kelantan. A total of 200 isolates were able to be segregated into 38 VNTR patterns (VNTR01-VNTR38) where each pattern was considered as a distinct strain of *S. enterica* ser. Typhi. The *S. enterica* ser. Typhi strain VNTR14 has been identified as the predominant strain that predominate this state. The source of infection most probably came from typhoid carriers since strains found in carrier isolates were also found in acute isolates. Multiple strains were identified from outbreak and sporadic cases of typhoid fever which suggests that these cases were due to different sources of infection. The typhoid infection that occurred in each household shared the same VNTR pattern which suggests that they were being infected from the same strain. All of the 38 *S. enterica* ser. Typhi strains have different VNTR copy numbers for each locus in which 7-bp repeat unit ranging from 5 to 19 copies and 8-bp repeat unit ranging from 7 to 42 copies were observed in TR1 and TR2 loci. As the conclusion, the VNTR method established in this study provides a simple, rapid, cost-effective and high discriminatory power for typing the *S. enterica* ser. Typhi.

CHAPTER 1

INTRODUCTION

1.1 General Background on *Salmonella*

Genus *Salmonella* was named after the American veterinary pathologist, Daniel Elmer Salmon in 1884. He was the first pathologist who isolated *S. enterica* ser. Choleraesuis from porcine intestine which revealed that it was a gram negative rod-shaped bacterium, motile, facultative anaerobic and belonged to the family *Enterobacteriaceae* (Black, 2005). Most of the bacteria from this genus are pathogenic and can cause diseases in both human and animals (Tirado & Schmidt, 2001). *Salmonella* nomenclature is quite complex where different systems were used by the scientists and the current usage of this nomenclature often combines several nomenclature systems that might cause confusion.

Nowadays, the initial nomenclature system proposed by Kauffman has evolved from the initial one serotype-one species to other complicated nomenclature systems. Under the initial scheme, serological identification of O (somatic) and H (flagella) antigens of the bacteria were analysed where each of the serotype was considered as a separate species. A total of 2463 species of *Salmonella* was found under this scheme (Kauffmann, 1966; Brenner *et al*., 2000). Another nomenclature demonstrated by DNA-DNA hybridisation was proposed by Crosa *et al*. in 1973. They suggested that all serotypes and subgenera I (*S. enterica* subsp. *enterica*), II (*S. enterica* subsp. *salamae*) and IV (*S. enterica* subsp. *houtenae*) were related at species level and belonged to a single species while subspecies V was classified under species

Salmonella bongori (Crosa *et al*., 1973). Other nomenclature was also proposed by Le Minor & Popoff (1987) where seven subgenera of *Salmonella* were referred as subspecies (subspecies I,II, IIIa, IIIb, IV, V and VI).

According to the nomenclature system recommended by the Centres for Disease Control and Prevention (CDC), two species of genus *Salmonella* which were *Salmonella enterica* and *Salmonella bongori* belonged to the *Enterobacteriaceae* family (Black, 2004). The *S. enterica* was further subdivided into six subspecies; *S. enterica* subsp. *enterica*, *S. enterica* subsp. *salamae, S. enterica* subsp. *arizonae, S. enterica* subsp. *diarizonae, S. enterica* subsp. *houtenae and S. enterica* subsp. *indica* in which they were differentiated biochemically and by genetic relatedness (Brenner *et al*., 2000). Figure 1.1 shows the nomenclature of *Salmonella* recommended by CDC.

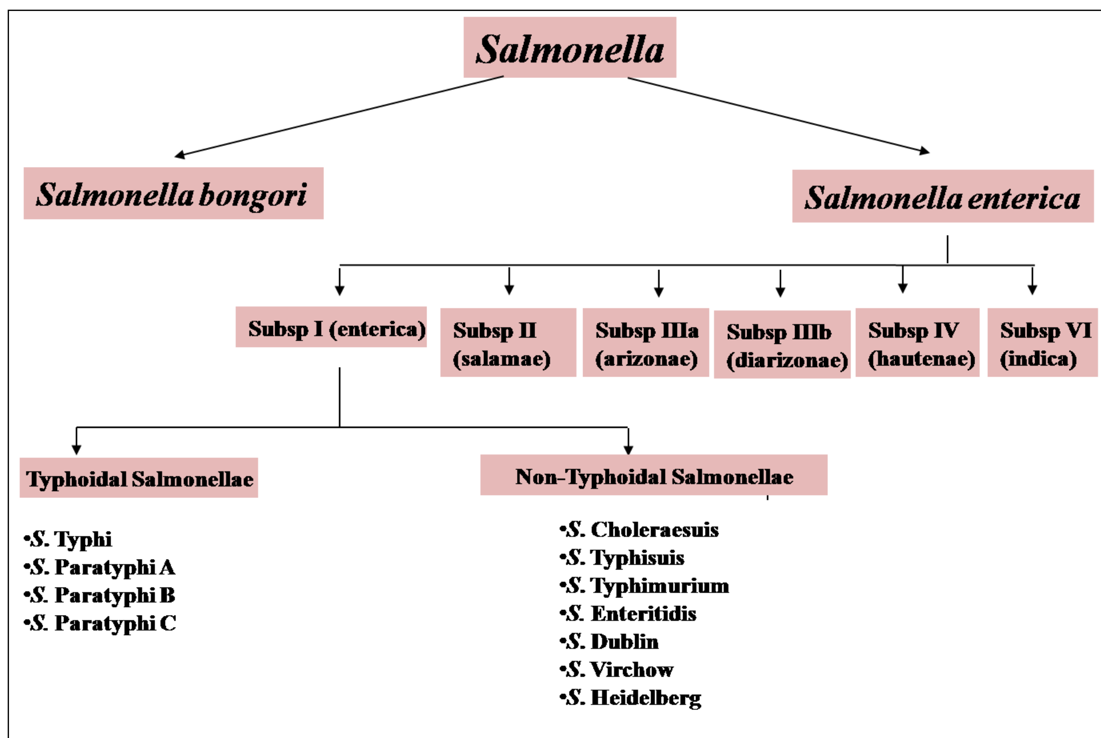

Figure 1.1 Nomenclature of *Salmonella* according to the Kauffman-White scheme (Adapted from CDC, USA, 2002)

1.2 *Salmonella enterica* subspecies *enterica* serovar Typhi

S. enterica ser. Typhi is a gram negative bacterium, rod-shaped bacillus with a length of 2 to 3 µm and a diameter of 0.4 to 0.5 µm. It is also known as a facultative anaerobic bacterium since aerobic metabolism is carried out when oxygen is present but later shift to anaerobic metabolism when oxygen is absent (Black, 2004). This bacterium has peritrichous flagella projected from all directions to be used for mobility. It is also a non-spore forming bacteria that cannot survive in environment for a long period of time and requires human as host (Black, 2005). *S. enterica* ser. Typhi is transmitted by faecal-oral route where the bacteria are excreted out from the host through faeces and enters new host through oral route by ingestion of contaminated food and water (Crum, 2003). This bacterium can be distinguished from other *Salmonella* serovars by its biochemical properties as described in Table 1.1.

Table 1.1 Biochemical tests for differentiation of *S. enterica* ser. Typhi from related subspecies (Adapted from WHO, 2003)

Organism	**Kliger's Iron Agar Test**				**Motility, Indole, Urea Tests**			**Citrate Test**
	Slant	Butt	H_2S	Gas	Motility	Indole	Urea	-
S. enterica ser. Typhi	Alkaline	Acid	Wk+	-	+	-	-	-
S. enterica ser. Paratyphi A	Alkaline	Acid	-	+	+	-	-	-
Other *Salmonella* sp.	Alkaline	Acid	V	V	+	-	-	V

'+' = Positive; '-' = Negative; 'Wk +' = Weak positive; V = Variable result; H_2S = Hydrogen sulphide

1.3 Typhoid Fever

Typhoid fever is a systemic disease caused by bacteria *S. enterica* ser. Typhi. This disease was endemic in most of the European countries and North America in the late 19th and early 20th centuries due to the urbanisation of populations during industrial revolution and poorly planned public water system. Fortunately, the incidence of typhoid fever in the Western countries was found to be drastically reduced since water chlorination and sand filtration were introduced to the populations (Kingsley & Dougan, 2009). This disease, however, is still endemic and commonly occurred in both underdeveloped and developing countries such as India, Southeast Asia, Africa, Central and South America (Hirose *et al*., 2002).

Typhoid fever often occurs in the regions with poor sanitation, untreated water and sewage as well as lack of clean drinking water. The etiological agent of this disease is transmitted through faecal-oral route by ingestion of contaminated food and water. Food supply can be contaminated not only by infected stool but also due to the poor sanitation and hygiene practices. Untreated sewage and water supply can also cause this disease when a large number of people use the same water sources (Mirza *et al*., 2000).

The etiological agent of typhoid fever which is *S. enterica* ser. Typhi invaded human body through ingestion of contaminated food and water (Bhan *et al*., 2005). After ingestion, the bacteria passed through pylorus and went to the small intestine which later penetrated the mucosa of terminal ileum through specialised antigen-sampling cells, M-cells and eventually became invasive infection (Talaro, 2008). The bacteria

lypopolysaccharide induced the secretion of cytokines from the epithelial cells and lead to infiltration of peripheral blood leucocytes into lamina propria (Bhan *et al*., 2005). The bacteria were later taken up by macrophages without killing and draining them into mesenteric nodes, thoracic duct and general circulation before releasing them into bloodstream (WHO, 2003). These bacteria, within twenty four hours of ingestion, reached the reticuloendothelial system such as liver, spleen as well as bone marrow and started to replicate inside the organs. Later, the infected person shows the symptoms of typhoid fever when the bacteria were shed back into the bloodstream (House *et al*., 2008).

Incubation period can be defined as the period starting from ingestion of contaminated food and water until the first symptom appears. For typhoid fever, the incubation period may vary from six to thirty days where the onset of the disease is nonspecific and insidious. Patients frequently came to the hospital with fever that runs as high as 38 to 40 ^{o}C with initial signs and symptoms such as malaise, loss of appetite, constipation in adults or diarrhoea in children, relative bradycardia and presence of rose spots on abdomen and chest (Crum, 2003; Levine, 2009).

Almost 10% of the untreated typhoid patients may develop serious complications during the third and fourth weeks of infection. They may experience a continuation of high fever along with severe constipation or diarrhoea, tachycardia, hypertension and uncomfortable distended abdomen. About 10 to 15% of the untreated patients may also develop severe diseases that are caused by a range of complications including gastrointestinal hemorrhage, gastrointestinal perforation as well as a variety of

neuropsychotic manifestations such as psychosis and confusion (Kingsley & Dougan, 2009).

1.4 Typhoid Carrier

A typhoid carrier is generally meant as a person carries the bacteria *S. enterica* ser. Typhi without showing any clinical signs and symptoms. A chronic carrier is recognised as a patient that is recovered from the disease but still continually excrete the bacteria intermittently through faeces or urine after a year of infection (Parry *et al.*, 2002; WHO, 2003). WHO estimated that about 95% of typhoid patients completely recover from this disease, while the remaining 5% becoming carriers of the disease. According to Nath *et al.* (2010), the gall bladder and liver have been suggested as the main niches for these bacteria in chronic typhoid carriers. The fact that these carriers are asymptomatic made them unaware that they could transmit the disease to other people. Fortunately, these carriers can be treated by antimicrobial therapy in which the first-line drugs such as ampicillin and trimethoprim are recommended for the treatment.

1.5 Epidemiology of Typhoid Fever

It is very difficult to determine the true incidence rate of typhoid fever since active surveillance and microbiological facilities are expensive and not widely available in the region where the disease is endemic (Kingsley & Dougan, 2009). According to Crump *et al.* (2004), the incidence of typhoid fever was over 22 million cases with 220, 000 deaths every year worldwide. They estimated that the incidence rate was far greatest in Southern Africa, South Central Asia and South East Asia with more than

100 cases per 100,000 populations while fewer cases involving 10 to 100 cases per 100,000 populations were reported in the rest of Asia, Latin America and Oceania (Figure 1.2) (Crump *et al*., 2004). The latest incidence rate of typhoid fever worldwide has been reported in 2010 in which the incidence rate was estimated to be around 13.5 million cases (Buckle *et al*., 2012).

The incidence rate of typhoid fever in Malaysia was reported below 5 per 100,000 populations from the year 1995 until 2012. This country is classified as a low endemic region for typhoid fever since less than 10 cases per 100,000 populations were reported every year (Crump *et al*., 2004). Currently, the occurrence of typhoid fever in Malaysia is sporadic with occasional outbreaks in the areas where the sanitation systems are poor (Anita *et al*., 2009). In comparison with all states in Malaysia, Kelantan showed the highest number of typhoid cases as shown in Table 1.2. This North-eastern state in Peninsular Malaysia with coordinates $5^{o}\ 25'\ 0''$ north and $101^{o}\ 55'\ 0''$ east had reported the highest number of typhoid cases in the year 2005 with the incidence rate of 56.7 per 100,000 populations.

The huge increment in the incidence rate was due to the typhoid outbreak where the flood risk areas were reported to be the major contributors of the outbreak (Shah *et al*., 2012). Majority of the rural communities in Kelantan still depend on well water supply. Most of these wells were contaminated with sewage overflow during the flood season which further contributed to the increment of the cases (Aziah, 2009). Other contributing factors that lead to the outbreak were poor hygiene and practice of food handlers as well as the presence of chronic carriers (Shah *et al*., 2012). However, a significant improvement was achieved in reducing the incidence of typhoid fever in

Kelantan from 14.7 cases per 100,000 populations in 2000 to 1.8 cases per 100,000 populations in 2012 (Figure 1.3) (Kelantan State Health Department, 2012).

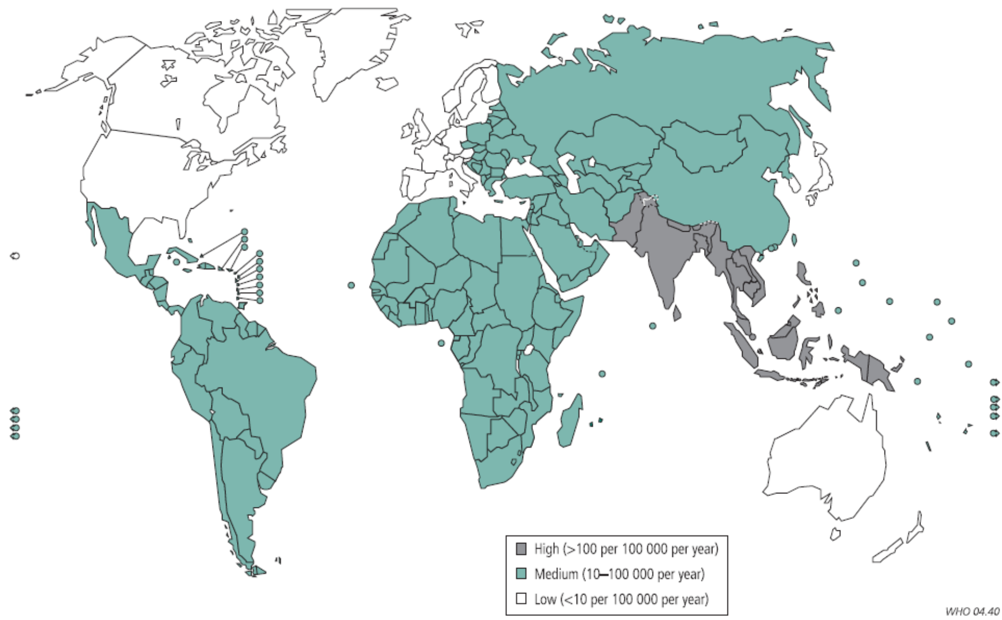

Figure 1.2 Global distribution of typhoid fever in the year 2002 (Adapted from Crump *et al.*, 2004)

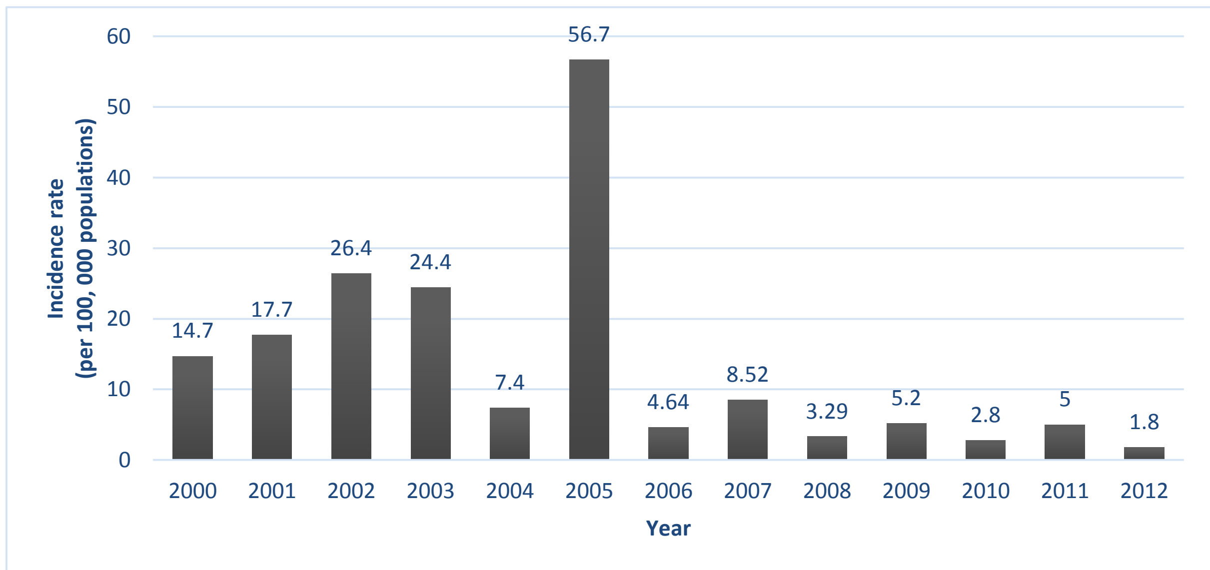

Figure 1.3 Incidence rate of typhoid fever in Kelantan from the year 2002 to 2012 (Adapted from Kelantan State Health Department, 2012)

Table 1.2 Incidence rate of typhoid fever in Malaysia by states from the year 2002 to 2012 (Departmemt of Statistics, Malaysia, 2013)

State	2005	2006	2007	2008	2009	2010	2011	2012
Malaysia	4.1	0.8	1.2	0.7	1.1	0.7	0.8	0.8
Johor	0.4	0.4	1.4	0.2	0.4	0.4	0.2	0.2
Kedah	0.7	0.4	0.5	0.3	0.9	0.1	0.4	0.2
Kelantan	56.7	4.64	8.52	3.29	5.2	2.8	5.0	1.8
Melaka	0.1	0.3	1.0	0.1	0.4	0.0	0.0	0.0
Negeri Sembilan	0.7	0.0	0.1	0.2	0.5	0.2	0.1	0.2
Pahang	0.8	0.1	1.2	0.5	0.3	0.2	2.0	0.4
Perak	0.8	0.6	0.9	0.4	0.5	0.2	0.3	0.7
Perlis	0.0	0.4	0.0	0.4	0.0	0.9	0.0	0.0
Pulau Pinang	0.6	0.2	1.1	0.2	0.0	0.1	0.0	0.1
Sabah	2.1	1.3	1.1	1.8	1.6	2.5	1.5	1.1
Sarawak	0.7	0.7	0.3	0.2	0.3	0.2	0.3	1.4
Selangor	0.6	0.4	0.4	0.4	1.7	0.6	0.7	0.6
Terengganu	0.8	0.9	0.3	0.2	0.5	0.4	0.0	0.3
W.P. Kuala Lumpur	0.1	0.1	0.4	0.4	0.4	0.7	0.4	2.0

1.6 Laboratory Diagnosis of Typhoid Fever

1.6.1 Culture Method

Most of the symptoms for typhoid fever are nonspecific and may mimic the symptoms of dengue, malaria, hepatitis and scrub typhus. Therefore, it is very important to perform laboratory analysis in order to confirm the clinical diagnosis of typhoid fever especially in typhoid endemic regions. One of the laboratory diagnosis for typhoid fever is culture method. The current gold standard method for confirming a case of typhoid fever is by performing culture method from the bone marrow. Bone marrow aspirate culture has 80 to 95% sensitivity compared to the other culture of specimens. However, this method is rarely used since the specimen is difficult to be obtained and the procedure is relatively invasive (Bhutta, 2006). Therefore, due to this reason, blood culture is used for the diagnosis especially during the acute stage of infection.

The blood sample collected from suspected patient is inoculated into blood culture bottle (BACTEC) and incubated at 37 ^{o}C. The sample is then subcultured onto a few selective media which are Deoxycholate Citrate agar, Xylose Lysine Deoxycholate agar or MacConkey agar followed by incubation at 37 ^{o}C for 24 hours. Non-lactose fermenting colonies with black centres on Deoxycholate Citrate agar, transparent red colonies with black centres on Xylose Lysine Deoxycholate agar and non-lactose fermenting smooth colonies on MacConkey agar can be observed indicating the positive result of *S. enterica* ser. Typhi.

Unfortunately, by performing blood culture method, a relatively large volume of blood approximately 10 to 15 mL is required and false negative result may be obtained for

the patients that already received antibiotic treatment (WHO, 2003). Even though blood culture is more practical, it is still less sensitive compared to bone marrow culture since only 60 to 80% of patients showed positive result during early stage of infection. The blood sample is not always available and even if it is available, it may take about two to three days to perform the analysis. As a result, the diagnosis may be delayed and inappropriate antimicrobial treatment might be given to the patient without the disease (Archibald & Reller, 2001; Wain & Hosoglu, 2008).

Stool and urine cultures can also be performed even though the sensitivity of both cultures is quite low. About 60 to 70% of stool cultures usually showed negative results during the first week of infection, however, the result may change to positive during the third week of infection in untreated patient (Lesser & Miller, 2001).

1.6.2 Serological Methods

Several serological methods have been developed over the past years in order to increase the rapidity and to simplify the diagnosis of typhoid fever. This methods may include Widal agglutination test, TUBEX® and Typhidot ®.

1.6.2 (a) Widal Agglutination Test

Widal agglutination test was developed by F Widal in 1896 to aid in typhoid fever diagnosis. It is performed by measuring agglutinating antibodies in the serum of the infected patient against H (flagellar) and O (somatic) antigens of *S. enterica* ser. Typhi. Positive result of the test was demonstrated by the agglutination during acute and

convalescent period of infection collected at least ten days apart with the evidence of four-fold rise of antibody titre (WHO, 2003; Parry *et al*., 2011).

Widal agglutination test can be performed in two formats which are slide test and tube test. The first format can be used for rapid screening where O and H antigens suspension are mixed with patient's serum and the positive results showing agglutination will later be serially diluted to determine the concentration of antibody present. The second format is performed in order to confirm the result and to clarify the inconsistent agglutination observed in the first format. The tube test format also provides better result since the serially diluted samples are incubated at 37 ^{o}C up to 20 hours to allow for better agglutination (Olopoenia & King, 2000).

This serological test has been largely abandoned in many developed countries due to several reasons including low prevalence of typhoid fever, improved sanitation and sewage system as well as proper hygiene. However, in developing countries where the culture method is unavailable, it is the only test that is available for typhoid fever diagnosis in suspected patients. Unfortunately, this serological test was reported to have some shortcomings. The results for the test were varied not only between the laboratories but also within the same laboratory when the antigens are prepared from different sources (Keddy *et al*., 2011). False positive results may also be obtained since the test has cross-reactivity with other infectious agents, resulting in over-diagnosis of typhoid fever (Olopoenia & King, 2000).

1.6.2 (b) TUBEX®

TUBEX® is a simple, rapid and user-friendly test used to detect the presence of anti-O9 antibodies in patient's serum by assessing their ability to inhibit the reaction between antigen-coated brown and antibody-coated blue reagents. The separation is enabled by magnetic force and the result is read based on the resultant colour of the supernatant. In principle, when the O9 antibodies are present in patient's serum, the binding between antibody-coated indicator particle and the antigen-coated magnetic particles in the solution will be inhibited resulting in visible blue colour solution (Naheed *et al.*, 2008).

1.6.2 (c) Typhidot®

Typhidot® is an enzyme-linked immunosorbent assay (ELISA)-based immunodot test developed by Malaysia Bio-Diagnostic Research Company. It is developed to detect the presence of IgM and IgG in the patient's sera by antigen-antibody reaction against the outer membrane protein of *S. enterica* ser. Typhi. Two dots of antigens labelled as G and M which corresponds to IgG and IgM are impregnated onto the membrane. The result is considered positive when both of the dots appeared as darker to or more than the dot of positive control (Ismail *et al.*, 1991).

The patient is considered to have an acute infection when the test is positive for IgG and IgM. However, the IgG can be present in the patient for more than 2 years which results in difficulty of interpretation in differentiating between acute and convalescence cases. Patient who being reinfected with the disease may not only cause

the IgG to be boosted but the IgM can also be masked. Therefore, to solve this problem, the subsequent test kit known as Typhidot-M® is developed. This method inactivates the total IgG in the serum sample to allow the binding of IgM with the antigen on the nitrocellulose membrane (Ismail *et al*., 1998).

1.6.3 Polymerase Chain Reaction (PCR)

The conventional methods for detection of *S. enterica* ser. Typhi such as culture and serological methods have several limitations that include time-consuming, expensive, poor sensitivity and specificity which result in poor identification (Elizaquivel & Aznar, 2008). Therefore, other method that is quick and sensitive in bacterial detection has been developed. Polymerase Chain Reaction (PCR)-based method has been successfully used to detect bacterial pathogen not only in clinical samples but also in aquatic environments as well as food products (Moganedi *et al*., 2007). It has been proven that this method provides a few advantages including rapid procedure, high specificity and sensitivity as well as shorter analysis time (Germini *et al*., 2009).

The Polymerase Chain Reaction developed by Kary Mullis in 1983 was used to amplify a specific region of DNA strand. A typical amplification reaction comprises of template DNA, a set of primers, thermostable DNA polymerase, deoxyribonucleotide triphosphates (dNTPs), buffer solution and magnesium. Each PCR cycle consists of three steps which are denaturation, annealing and extension. Denaturation step is the step where template DNA is denatured and become a single stranded to provide a room for the primers and polymerase enzyme to attach. The second step is the annealing of the primers to the single stranded DNA template while

the third step is the extension step where the polymerase enzyme adds the complementary nucleotides to the template DNA to produce a new double stranded DNA. The same cycle is repeated on the newly synthesized DNA fragments and in the end, million copies of target DNA are produced. There are several PCR-based methods that have been developed for the detection of *S. enterica* ser. Typhi such as nested PCR (Kumar *et al.*, 2002; Hashimoto *et al.*, 1995), multiplex PCR (Hirose *et al.*, 2002; Ngan *et al.*, 2010) and real-time PCR (Farrell *et al*., 2005).

1.7 Molecular Epidemiology

Molecular epidemiology is a discipline combining both 'molecular', the use of molecular biology techniques to characterise nucleic acid- or amino acid-based content and 'epidemiology', the study of determinants of disease occurrence and its distribution in human populations (Foxman & Riley, 2001). Previously, structured questionnaires were used to obtain epidemiological data in monitoring the transmission pattern of a disease (Bruisten & Schouls, 2009). These questionnaires were filled either by patients themselves or skilled nurses and public health workers. However, the data for those questionnaires maybe incomplete or the information is incorrect.

Later on, molecular techniques have been widely applied in controlling the infectious diseases for both pathogen detection and typing. PCR and other nucleic acid amplification techniques are used for the detection, while molecular typing is performed to identify the routes of transmission within and among populations (Bruisten & Schouls, 2009). These techniques are actually not a substitute for

conventional methods but they were applied to solve many epidemiological problems that cannot be approached. These techniques improve the data by providing more sensitive and specific measurements which facilitates epidemiological activities such as identifying the patterns of transmission, outbreak investigation and disease surveillance. They also help to provide clues for possible infectious causes of cancer and other chronic diseases as well as providing better understanding of the disease pathogenesis at molecular level. Despite those advantages, they however, are more expensive, time consuming and labour intensive compared to the conventional methods (Foxman & Riley, 2001).

Strain typing is applied in the epidemiological setting for several purposes; i) aiding in source tracing to elucidate whether the transmission come from patient to patient or from an environmental source to patient (Grundmann *et al*., 2005; Bruisten & Schouls, 2009), ii) revealing the phenotypical properties of the pathogen in terms of infectivity, virulence or drug resistance (Al-Sanouri *et al*., 2008; Bruisten & Schouls, 2009), iii) assessing the effectiveness of treatment whether patients experience relapse of the pathogen or possibility of being re-infected by another strain (Maharjan *et al*., 2005; Bruisten & Schouls, 2009), iv) monitoring the geographical spread of endemic virulent strains as well as recognising emergence of new strains of pathogen of certain disease (Sitchenko *et al*., 2009) and v) identifying sampling and laboratory errors (Bruisten & Schouls, 2009).

1.8 Types of Typing Methods

Nowadays, several typing methods are available for typing of the pathogens. Typing methods can be classified into phenotypic and genotypic methods. Regardless of both classifications, several criteria need to be considered when evaluating them. The criteria include the typeability, reproducibility and discriminatory power. The ability of a technique to obtain positive results for each isolates analysed is referred as typeability. The second criterion, reproducibility is the ability of the technique to obtain the same result when a particular strain is repeatedly tested (Van Leeuwen *et al.*, 2009). The method used must also has high discriminatory power in which it is able to differentiate between two unrelated strains and at the same time it is able to demonstrate the relationships of all the organisms isolated from the same sources (Van Belkum *et al.*, 2007; Van Leeuwen *et al.*, 2009).

The phenotypic methods are those methods that were used to differentiate the strains by characterising the products of gene expression (Van Leeuwen *et al.*, 2009). These methods include biotyping, serotyping, antimicrobial susceptibility patterns, plasmid typing and phage typing (Bruisten & Schouls, 2010). Unfortunely, since these methods involve the expression of gene products, there might be a chance that the properties of gene expression will be varied, resulting in lack of typeability, poor reproducibility and discriminatory power (Van Leeuwen *et al.*, 2009).

More recently, the second classification of typing method which is genotypic methods has been widely used in molecular epidemiology discipline. These methods are those that are based on the direct analysis of genetic structure of organism using molecular-

based typing methods that allow them to be differentiated at strain level (Tenover *et al.*, 2006; Van Belkum *et al.*, 2007 & Georing, 2010). The genotypic methods can be further subdivided into three main categories which are DNA banding pattern-, DNA sequencing-, and DNA hybridisation-based methods (Li *et al.*, 2009).

The DNA banding pattern-based methods discriminate the strains based on the size differences of DNA fragments. These fragments can be generated by DNA digestion using restriction enzymes, DNA amplification or by a combination of both. The restriction enzymes recognise and cut the target DNA precisely at defined sequences while the DNA amplification amplifies billion copies of DNA fragments (Blakesley, 1987). The examples for these methods are Pulsed-Field Gel Electrophoresis (PFGE), Restriction Fragment Length Polymorphism (RFLP), Repetitive sequencing-based PCR (REP-PCR), Multilocus Variable Number Tandem Repeat Analysis (MLVA) and Amplified Fragment Length Polymorphism (AFLP) (Li *et al.*, 2009). Both PFGE and VNTR will be further discussed in the next chapter.

DNA sequencing-based methods are methods that generate the original sequence of nucleotides and identify the polymorphisms in their DNA. These methods have high reproducibility compared to the first classification since the DNA sequences can be easily stored in online database and compared among laboratories (Li *et al.*, 2009). The examples of these methods include Multilocus Sequence Typing (MLST), Whole genome sequencing and Single Nucleotide Polymorphisms (SNP) genotyping (Li *et al.*, 2009; Van Leeuwen *et al.*, 2009). For the third category of genotypic methods, DNA hybridisation-based methods are methods that involve the analysis of DNA hybridisation to the probes of known sequences in order to discriminate the strains.

The examples of these methods are spoligotyping and microarrays (Li *et al*., 2009; Van Leeuwen *et al*., 2009).

1.9 Pulsed-Field Gel Electrophoresis (PFGE)

Pulsed-Field Gel Electrophoresis (PFGE) was first introduced by David C. Schwartz and Charles Cantor in 1984 (Schwartz & Cantor, 1984). It is an electrophoretic technique used to separate larger DNA molecules ranging from 10 kb to 10 Mb. When the DNA molecules with the size of more than 20 kb were run in the conventional constant electric field, they usually showed the same mobility which makes them impossible to be differentiated. Therefore, to solve this problem, the electrical field that periodically changes direction is applied in the PFGE so that the larger DNA molecules can be separated (Figure 1.4) (Schwartz & Cantor, 1984). This technique has been considered as a gold standard method among molecular typing methods and has been applied to type various bacteria including *Salmonella* (Noble *et al*., 2012), *Shigella* (Ribot *et al*., 2006) and *Campylobactor jejuni* (Graves & Swaminathan, 2001).

The choice of restriction enzyme used in PFGE is an important factor to determine the banding pattern since the cleavage sites of each restriction enzyme is unique. Chen *et al*. (2005) reported that higher resolution in PFGE can be achieved when the restriction enzyme with long and infrequent occurring recognition motifs were used (Chen *et al*., 2005). Besides restriction enzyme, there are several factors that affect the limit of resolution of PFGE including the uniformity of the two electric fields, the electric pulses' duration, the ratio of the pulses time for each of the alternating electric fields, the angles of the two electric fields to the gel and the strength ratio of the electric fields

(Maiden & Pollard, 2002). Therefore, the use of PFGE methods to generate, store and compare the patterns might be limited for the epidemiological studies if it is not properly standardised (Singh *et al*., 1999).

PFGE has been widely used in many disciplines including epidemiology and microbiology. Currently, many online databases have been developed for global comparison of PFGE patterns. One of them, PulseNet, is the largest PFGE database that is used to track the foodborne bacteria such as *Escherichia coli* O157:H7, nontyphoidal *Salmonella*, *Shigella* and *Listeria monocytogenes* (Ransom & Kaplan, 1998; Swaminathan *et al*., 2006). Molecular epidemiology study conducted in Hong Kong between periods of 2000 to 2004 found 66 different PFGE strains of *S. enterica* ser. Typhi (Kam *et al.*, 2007). The characterisation of *S. enterica* ser. Typhi isolates using PFGE method in Colombia and Argentina found 83 strains of *S. enterica* ser. Typhi that were circulating in these countries (Salve *et al.*, 2006). Other study done by Thong *et al*. (1995) to strain type the *S. enterica* ser. Typhi from different part of Malaysia using PFGE method found 48 strains from 60 isolates tested. Even though this technique produces consistent pattern within and between the laboratories as well as excellent discriminatory power, it still suffer from several limitations such as time consuming, labour-intensive and it also requires skilled technician and sophisticated equipment to perform the analysis (Li *et al*., 2009).

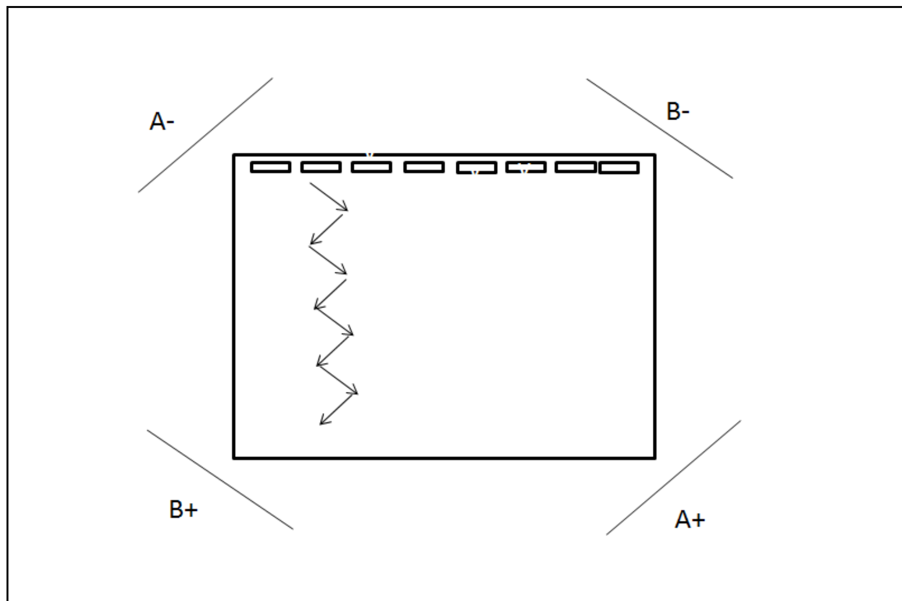

Figure 1.4 General principle of PFGE method

An agarose gel is represented by the box; a series of short horizontal rectangles indicate the wells into which the DNA plugs are loaded. A and B represent two sets of electrodes. When the A electrodes are activated, the DNA is driven anodically downward and to the right as indicated by the first arrow. When the electrodes are turned off, the B electrodes are immediately activated. The DNA now moves downward and to the left. The path of the DNA molecules is subjected to continue alternation of field directions as shown by the arrows (Sambrook & Russell, 2000).

1.10 Variable Number Tandem Repeat (VNTR)

Variable Number Tandem Repeat (VNTR) analysis is one of the DNA banding pattern-based methods used to type the microorganisms. This method provides data in a simple and clear format based on the number of repetitive sequences (Ramazanzadeh & McNerney, 2007). It employs the naturally occurring variation in number of tandem repeated DNA sequences that are found to be widely dispersed in both noncoding regions and genes of bacterial genome (Vergnaud & Denoeud, 2000). The number of tandem repeats in a particular locus may differ between strains as shown in figure 1.5 and due to this variation; such loci are known as Variable Number Tandem Repeat (VNTR) loci.

Tandem repeats are formed because of the DNA polymerase error that occurs during replication slippage in which it incorrectly copies the repeat regions and as a result, they are either duplicated or deleted (Bruisten & Schouls, 2010). Two types of replication slippage can occurred which are forward and backward replication slippage. The forward replication slippage often occurs when tandem repeat region contains many repeats. This repeat region in single stranded DNA often forming loop and the DNA polymerase may accidentally skip the loop region resulting in replicated strand with a decreased number of tandem repeats. During backward replication slippage, the DNA polymerase sometimes stutters in the areas where tandem repeats are located and as a result, the numbers of repeats are increasing. Figure 1.6(a) and 1.6(b) showed the processes of forward and backward replication slippage.

GATACAAACTGGGAAACTTGGAAACTGGGTCCACAG

Tandem Repeats

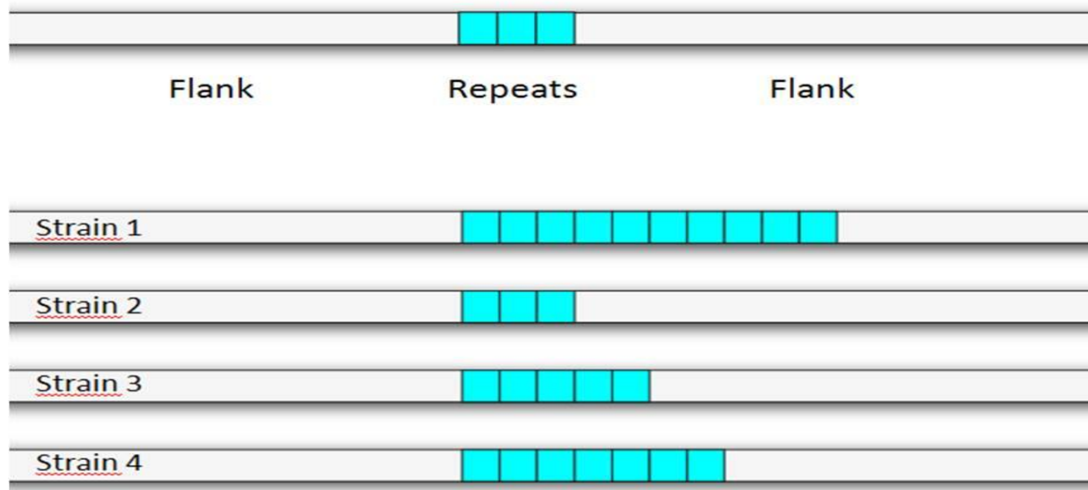

Figure 1.5 Variation in number of tandem repeats in a tandem repeat locus for 4 different strains

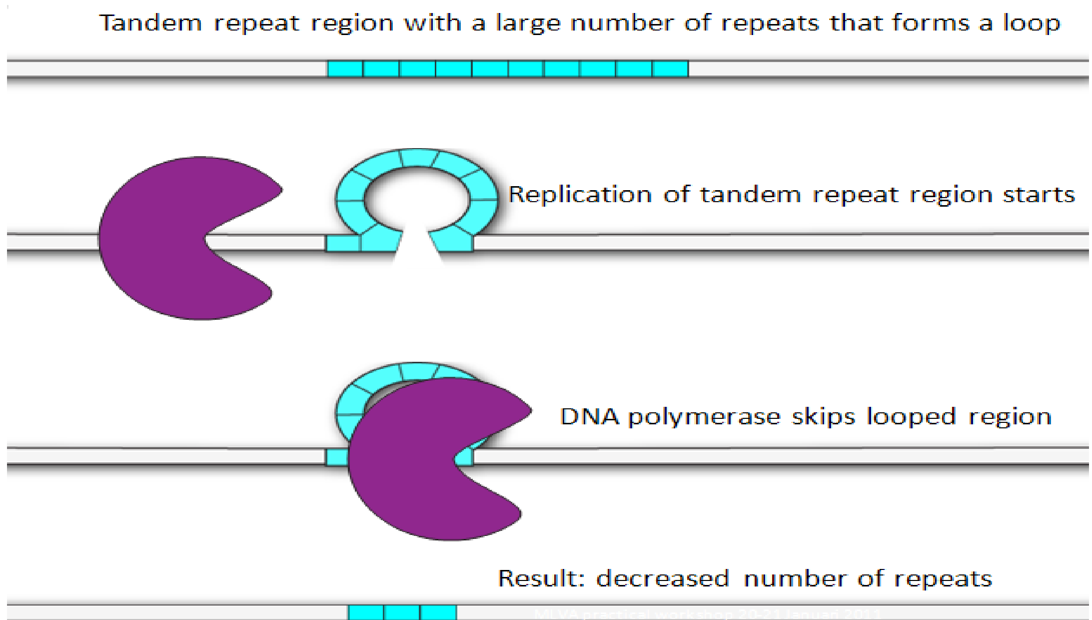

Figure 1.6(a) Decreased number of repeats in tandem repeat region by forward replication slippage