

## ABSTRACT

In this study, *Salmonella enterica* serovar Typhi (*S. Typhi*) strains collected from outbreak and sporadic cases of typhoid fever during the years 1998 to 2007 in Malaysia were characterized using three PCR-based methods, i.e., restricted arbitrarily primed PCR (resAP-PCR), repetitive element PCR (rep-PCR) and variable-number tandem repeat (VNTR) analysis. The objective of this study was to explore the utility of VNTR and resAP-PCR as newer molecular methods in comparison with the commonly used method of rep-PCR to subtype *S. Typhi*. ResAP-PCR analysis was conducted using arbitrary primers STHae8 and STAlu9 following endonuclease digestion of *S. Typhi* DNA with *HaeIII* and *AluI* enzymes. Rep-PCR was performed by using REP-F and REP-R primers, corresponding to the highly conserved REP repeated DNA elements. VNTR analysis was performed by multiplex approach using four primers (TR1, TR2, TR3, TR5) flanking VNTR loci in the CT18 strain of *S. Typhi*.

The *S. Typhi* strains were typeable by three methods in assigning a defined type to each strain tested. Restricted AP-PCR amplification of *HaeIII* and *AluI* digested DNA using STHae8 and STAlu9 primers was not able to discriminate *S. Typhi* strains tested. In addition, resAP-PCR analysis using STHae8 primer demonstrated poor reproducibility. Similarly, rep-PCR with the primers REP-F and REP-R was not useful as a subtyping tool for *S. Typhi* as all strains shared the same predominant banding profile. In contrast, multiplex VNTR assay displayed diversity based on four VNTR loci studied, with total allele numbers ranging from 2 to 19 and Nei's diversity ( $D$ ) values ranging from 0.04 to 0.93. Sequence analysis of individual alleles confirmed the presence of VNTRs among the strains and identified 42 VNTR

profiles. The results revealed that the copy number of repeats in CT18 strain of *S. Typhi* was highly correlated with the *D* value ( $R^2 = 0.57$ ) and number of alleles ( $R^2 = 0.87$ ) observed across diverse strains. The VNTR analysis showed that significant genetic heterogeneity exists at TR1, TR2 and TR3 loci of *S. Typhi* strains in Malaysia. Analysis of the VNTR patterns indicated that *S. Typhi* strains obtained from sporadic cases were much more heterogeneous than those obtained during outbreaks of typhoid fever. The findings in this study demonstrate that VNTR is able to provide a rapid, highly discriminative, and reproducible typing method for epidemiological surveillance and outbreak investigation of *S. Typhi* strains.

## ABSTRAK

*Salmonella enterica* serovar Typhi yang dikaji terdiri dari strain bakteria yang dikumpul dari kes demam typhoid di Malaysia dari tahun 1998 hingga 2007. Strain bakteria ini dikaji dengan menggunakan tiga kaedah PCR iaitu, 'restricted arbitrary primed PCR' (resAP-PCR), 'repetitive element PCR' (rep-PCR), dan 'variable-number tandem repeats' (VNTR). Objektif kajian ini adalah untuk meneroka kegunaan VNTR dan resAP-PCR sebagai kaedah molekul yang baru berbanding dengan kaedah rep-PCR untuk menjeniskan *S. Typhi*. Analisa resAP-PCR dijalankan dengan menggunakan arbitrary primers STHae8 dan STAlu9 dan penghadaman endonuclease ampikon dengan menggunakan enzim *HaeIII* dan *AluI*. Rep-PCR dijalankan dengan menggunakan primer REP-F dan REP-R. Analisa VNTR dijalankan dengan menggunakan pendekatan multiplex mengandungi empat primer iaitu (TR1, TR2, TR3, dan TR5) flanking VNTR loci di strain *S. Typhi* CT18.

Strain-strain *S. Typhi* boleh ditaip dengan 3 kaedah. Amplifikasi AP-PCR pada *HaeIII* dan *AluI* digested DNA dengan menggunakan primer STHae8 dan STAlu9 tidak dapat mendiskriminasi *S. Typhi* yang dikaji. Selain itu, keputusan resAP-PCR ini dengan menggunakan primer STHae8 tidak dapat dihasilkan semula. Analisa rep-PCR dengan menggunakan primer REP-F dan REP-R adalah tidak berguna memandangkan keputusan yang sama diperolehi. Multiplex assay VNTR dapat menunjukkan diversiti *S. Typhi* melalui 4 VNTR loci yang dikaji dengan jumlah allel dari 2-19 dan indeks diversiti Nei's ( $D$ ) dari 0.04-0.93. Analisa sequence pada allel individu mengesahkan kehadiran 42 profil VNTR. Keputusan ini menunjukkan bahawa salinan ulangan nombor di strain *S. Typhi* CT18 mempunyai hubungan yang tinggi dengan nilai  $D$  ( $R^2 =$

0.57) dan nombor allele ( $R^2 = 0.87$ ) didapati pada strain. Analisa VNTR menunjukkan bahawa strain-strain *S. Typhi* yang didapati dari kes sporadik adalah amat berbeza berbanding dengan kes outbreak demam typhoid. Kajian ini menunjukkan kaedah VNTR adalah cepat, mempunyai diskriminasi yang tinggi dan boleh ulang semula serta berguna dalam surveillance epidemiologi dan siasatan outbreak *S. Typhi*.

## ACKNOWLEDGEMENT

First and foremost, I would like to express my sincere and deep appreciation to my supervisor Prof. Thong Kwai Lin for all her patience guidance and advice throughout this project study. Indeed, I would not have completed my research without her guidance, help and support.

I wish to dedicate this project to my beloved parents, Shahin and Nezam for their unflagging love, generosity, and guidance through all my years. My special thanks go to my sisters, Sahar and Sara, and my aunt, Mahin for their undying devotion and endless support during my life. I would have failed completing this project without their love and support.

My gratitude is also extended to my lab mates and friends in particular Ms. Cindy Teh, Ms. King Ting, and Ms. Shabnam for their generous help, advice and support throughout my research. Their kindness is always remembered.

Finally, I would like to thank to all who have inspired and helped me out completing this research project.

# TABLE OF CONTENTS

<b>ABSTRACT</b>	<b>ii</b>
<b>ABSTRAK</b>	<b>iv</b>
<b>ACKNOWLEDGEMENT</b>	<b>vi</b>
<b>TABLE OF CONTENTS</b>	<b>vii</b>
<b>LIST OF TABLES</b>	<b>xi</b>
<b>LIST OF FIGURES</b>	<b>xii</b>
<b>LIST OF SYMBOLS AND ABBREVIATIONS</b>	<b>xiii</b>
<b>CHAPTER 1.0: INTRODUCTION</b>	<b>1</b>
1.1 Background of Study	2
1.2 Objectives of Study	4
<b>CHAPTER 2.0: LITERATURE REVIEW</b>	<b>5</b>
2.1 The Genus <i>Salmonella</i> : Nomenclature and Classification	6
2.2 Overview of <i>Salmonella enterica</i> serovar Typhi	7
2.2.1 Historical Overview	7
2.2.2 Morphology and Characteristics	8
2.2.3 Genomic Characteristics	9
2.2.4 Epidemiology of Typhoid Fever	11
2.2.5 Pathogenesis and Clinical Features	13
2.2.6 Diagnosis and Detection of Typhoid Fever	15
2.2.7 Treatment and Prevention of Typhoid Fever	17
2.3 Methods of Bacterial Typing	18

2.3.1	Phenotypic Methods	20
2.3.2	Genotypic Methods	21
2.3.3	Polymerase Chain Reaction (PCR)	22
2.3.4	Restricted Arbitrary Primed PCR (resAP- PCR)	24
2.3.5	Repetitive Elements PCR (rep-PCR)	25
2.3.6	Variable-Number Tandem Repeat (VNTR)	27
<b>CHAPTER 3.0:</b>	<b>MATERIALS AND METHODS</b>	<b>30</b>
3.1	Preparation of Common Media, Buffers and Solutions	31
3.1.1	Luria-Bertani (LB) Agar	31
3.1.2	Luria-Bertani (LB) Broth	31
3.1.3	Bismuth Sulphite Agar (BSA)	31
3.1.4	10X Tris-borate EDTA (TBE) buffer, pH 8.3	32
3.1.5	0.5X Tris-borate EDTA (TBE) buffer	32
3.1.6	1 M Tris, pH 8.0	32
3.1.7	0.5 M EDTA, pH 8.0	33
3.1.8	Tris-EDTA (TE) buffer, pH 8.0	33
3.1.9	2.5% Agarose Gel for PCR	33
3.1.10	Phosphate Buffered Saline (PBS) Buffer, pH 7.3	34
3.1.11	80% Ethanol	34
3.1.12	50% Glycerol	34
3.1.13	Ethidium Bromide (EtBr)	34
3.2	Bacterial Strains and Purity Check	35
3.3	Confirmation of <i>S. Typhi</i> Strains by PCR	37
3.3.1	DNA Template Preparation	37

3.3.2	PCR Amplification	37
3.3.3	PCR Products Analysis	38
3.4	Restricted Arbitrary Primed PCR (resAP-PCR)	39
3.4.1	Genomic DNA Extraction	39
3.4.2	Restriction Endonuclease Digestion	41
3.4.3	Restricted AP-PCR Amplification	41
3.4.4	PCR Products Analysis	43
3.5	Repetitive Element PCR (Rep-PCR)	43
3.5.1	DNA Template Preparation	43
3.5.2	Rep-PCR Amplification	43
3.5.3	PCR Products Analysis	44
3.6	Variable Number Tandem Repeat (VNTR)	44
3.6.1	DNA Template Preparation	44
3.6.2	VNTR Loci and Oligonucleotide Primers	45
3.6.3	PCR Amplification of VNTR Loci	46
3.6.4	PCR Products Analysis	46
3.6.5	DNA Sequence Analysis	46
3.6.6	Data Analysis	47
<b>CHAPTER 4.0:</b>	<b>RESULTS</b>	<b>48</b>
4.1	Bacterial Strains and Purity Check	49
4.2	Confirmation of <i>S. Typhi</i> Strains by PCR	49
4.3	Restricted AP-PCR (resAP-PCR) Analysis	51
4.4	Repetitive Element PCR (Rep-PCR) Analysis	53
4.5	Variable-Number Tandem Repeat (VNTR) Analysis	55

4.5.1	PCR Analysis of VNTR Loci	55
4.5.2	Sequence Analysis of Potential VNTR Loci	57
4.5.3	Allelic Profiles and VNTR Profile Designations	59
4.5.4	Allelic Diversity of VNTR Loci	62
<b>CHAPTER 5.0:</b>	<b>DISCUSSION</b>	<b>63</b>
5.1	Comparison of ResAP-PCR, Rep-PCR, and VNTR	64
5.2	Variable-Number Tandem Repeat (VNTR) Analysis	67
5.2.1	PCR and Sequence Analysis of VNTR Loci	67
5.2.2	Allelic Diversity of VNTR Loci	69
5.2.3	Epidemiological Analysis and Genetic Diversity	70
<b>CHAPTER 6.0:</b>	<b>CONCLUSION</b>	<b>74</b>
	<b>BIBLIOGRAPHY</b>	<b>76</b>
	<b>APPENDIX</b>	<b>93</b>

## LIST OF FIGURES

<b>Figure 3.1:</b> Bacterial strains and purity check.	36
<b>Figure 3.2:</b> Genomic DNA extraction steps.	39
<b>Figure 4.1:</b> Multiplex PCR confirmation of some of <i>S. Typhi</i> strains by using three types of primers Hil A (789 bp), ST (332 bp), and SPA (496bp).	50
<b>Figure 4.2:</b> Representative resAP-PCR fingerprint patterns for some of <i>S. Typhi</i> strains; (a) <i>Hae</i> III digested DNA by using STHae8 primer set, (b) <i>Alu</i> I digested DNA by using STAlu9 primer set.	52
<b>Figure 4.3:</b> Representative Rep-PCR fingerprint patterns for some of <i>S. Typhi</i> strains.	54
<b>Figure 4.4:</b> Representative VNTR banding profiles of multiplex PCR at TR1, TR2, TR3, and TR5 loci for some of <i>S. Typhi</i> strains.	56
<b>Figure 4.5:</b> Nucleotide sequence of the VNTR loci.	58
<b>Figure 5.1:</b> Correlation between repeat copy number and <i>D</i> values.	70

## LIST OF TABLES

<b>Table 3.1:</b>	The components of digestion reaction.	41
<b>Table 3.2:</b>	The primers used for resAP-PCR amplification.	42
<b>Table 3.3:</b>	The primers used for rep-PCR amplification.	44
<b>Table 3.4:</b>	The primers used for PCR amplification of selected VNTR loci from the CT18 strain of <i>S. Typhi</i> .	45
<b>Table 4.1:</b>	The allelic profiles and VNTR profile designations based on the copy number of the respective repeats at each VNTR locus.	60
<b>Table 4.2:</b>	Characteristics of VNTR loci studied.	62
<b>Table 5.1:</b>	Comparison of 10 selected <i>S. Typhi</i> strains for the patterns produced by resAP-PCR, rep-PCR, and VNTR.	66
<b>Appendix 1:</b>	Background data for <i>S. Typhi</i> strains used in this study.	94

## LIST OF SYMBOLS AND ABBREVIATIONS

°	Degree
°C	Degree Celsius
µl	Microlitre
µg	Microgram
%	Percent
AFLP	Amplified Fragment Length Polymorphism
AP-PCR	Arbitrarily Primed PCR
bp	base pair
BSA	Bismuth Sulphite Agar
<i>D</i>	Nei's diversity index
ddH <sub>2</sub> O	Deionised distilled water
dH <sub>2</sub> O	Distilled water
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleotide Triphosphates
EDTA	Ethylene Diamine Tetraacetic Acid
EtBr	Ethidium Bromide
g	gram
h	hour
HCl	Hydrogen chloride
Kb	Kilobase pair
LB	Luria-Bertani
LPS	Lipopolysaccharide Analysis
M	Molar

MDR	Multidrug-resistant
MgCl <sub>2</sub>	Magnesium chloride
MLEE	Multilocus Enzyme Electrophoresis
mM	millimolar
mg	miligram
ml	millilitre
mRNA	Messenger Ribonucleic Acid
NaOH	Natrium/ Sodium Hydroxide
No.	Number
OD	Optical density
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
PFGE	Pulsed-Field Gel Electrophoresis
pH	per hydrogen
RAPD	Random Amplified Polymorphic DNA
REP	Repetitive element sequence-based
Rep-PCR	Repetitive Element PCR
ResAP-PCR	Restricted Arbitrarily Primed PCR
RNase	Ribonuclease
rpm	Revolutions per minutes
s	seconds
<i>S. Typhi</i>	<i>Salmonella enterica</i> serovar Typhi
TBE	Tris-Borate-EDTA
TE	Tris-EDTA
Tris	Tris (Hydroxymethyl) methylamine

UV	Ultraviolet
VNTR	Variable-Number Tandem Repeat
w/v	Weight per unit volume