



**UNIVERSITI PUTRA MALAYSIA**

**GENOTYPIC CHARACTERIZATION OF VIBRIO SP. ISOLATED  
FROM COCKLES OBTAINED IN PADANG, INDONESIA**

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**By**

**ZULKIFLI BIN YAAKUB**

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in  
Fulfilment of the Requirements for the Degree of Master Science**

**March 2006**



## DEDICATION

*This piece of work is dedicated to my lovely parents, who have always been by my side and given me the encouragement and support that carries me through my study period. Thanks for their endless love to me.*

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

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**Chairman: Noorjahan Banu Mohamed Alitheen, PhD**

**Faculty : Faculty of Biotechnology and Biomolecular Sciences**

*Vibrio parahaemolyticus* is a gram negative bacterium that naturally found in warm marine environments. They commonly live in shellfish, oysters and cockles and cause gastrointestinal illness in humans. Most people become infected by eating raw or undercooked shellfish. In this study, 32 isolates of *V. parahaemolyticus* were isolated from cockles obtained from Padang, Indonesia. Presumptive identification of the isolates was performed by the following analysis: antibiotic resistance, plasmid profiling, specific PCR, random amplified polymorphic DNA (RAPD)-PCR and enterobacterial repetitive intergenic consensus (ERIC)-PCR. In the antibiotic resistance test, 16 types of antibiotics were tested against this bacterium. The isolates of *V. parahaemolyticus* showed variable antibiotic resistance pattern. They are most commonly resistant to amoxicillin, bacitracin, penicillin, teicoplanin, ampicillin and carbenicillin. In the plasmid profiling analysis, 14 different profiles were determined. The most common profiles for the isolates is profile no.2 which contains 4 plasmids with 5.6, 7.2, 42.4 and

54.0 kilobase pairs respectively. For *toxR* detection, all of the isolates were positive but they gave negative results in both *tdh* and *trh* detection. This indicated that they have less potential to cause diseases. They also showed high diversity in RAPD-PCR and also ERIC-PCR analysis. They showed many differences in the polymorphism pattern and can be divided into 5 major clusters in RAPD-PCR and 4 major clusters in ERIC-PCR. Application of phenotype and genotype based methods provides information about distribution, epidemiology and pathogenesis of *V. parahaemolyticus*. The data is important to identify sources of infection of this bacterium in the case of outbreaks occurrence in humans. This study also gives us preliminary information in monitoring the potential infection risk. It also can increase our awareness about the infection potential of these bacteria.



Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan ijazah Master Sains

**PENCIRIAN SECARA GENOTIP ISOLAT *VIBRIO* SP. DI DALAM KERANG  
DIPEROLEHI DARI PADANG, INDONESIA.**

Oleh

**ZULKIFLI BIN YAAKUB**

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*Vibrio parahaemolyticus* adalah dari jenis gram negatif bacteria yang biasanya boleh di jumpai di kawasan laut yang bersuhu panas. Perumah mereka biasanya adalah haiwan berangka seperti tiram atau kerang. Bakteria ini boleh menyebabkan keracunan makanan yang melibatkan perut dan usus. Biasanya jangkitan ini disebabkan oleh pengambilan makanan laut yang mentah dan tidak masak sepenuhnya. Dalam kajian ini, sebanyak 32 strain bakteria telah dipencilkan dari kerang yang diperolehi dari Padang, Indonesia. Pencirian bakteria-bakteria ini adalah mengikut ketahanan kepada antibiotik, kehadiran plasmid, spesifik PCR, RAPD-PCR dan ERIC-PCR. Untuk ujian ketahanan antibiotik, sebanyak 16 jenis antibiotik telah diuji dan kebanyakan strain adalah tahan kepada amoxycillin, bacitracin, penicillin, teicoplanin, ampicillin and carbenicillin. Bagi ujian profile plasmid, profile yang paling kerap dijumpai ialah profile no 2 yang mengandungi 4 plasmid dengan saiz 5.6, 7.2, 42.4 and 54.0 kilobase pairs. Untuk ujian kehadiran *toxR*

gen menggunakan PCR, semua strain adalah positif manakala untuk kehadiran *tdh* dan *trh* gen semua strain adalah negatif. Ini menunjukkan strain-strain yang dikaji mempunyai potensi jangkitan yang rendah. Mereka juga menunjukkan kepelbagaian bentuk bagi ujian RAPD-PCR dan ERIC-PCR. Mereka memberikan bentuk-bentuk yang berbeza dan boleh dibahagikan kepada 5 rumpun utama bagi ujian RAPD-PCR dan 4 rumpun utama bagi ujian ERIC-PCR. Kajian terhadap fenotip dan genotip dapat memberikan maklumat tentang sebaran, salasilah genetik dan evolusi *V. parahaemolyticus*. Maklumat ini penting untuk digunakan dalam mengkaji jangkitan dan punca jangkitan bakteria ini. Ia juga dapat memberikan maklumat awal untuk mengawasi potensi jangkitannya dan sekaligus dapat meningkatkan kesedaran kita terhadap jangkitan bakteria ini.

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## TABLE OF CONTENTS

	Page
<b>DEDICATION</b>	ii
<b>ABSTRACT</b>	iii
<b>ABSTRAK</b>	v
<b>ACKNOWLEDGEMENTS</b>	vii
<b>APPROVAL</b>	ix
<b>DECLARATION</b>	xi
<b>LIST OF TABLES</b>	xv
<b>LIST OF FIGURES</b>	xvi
<b>ABBREVIATIONS</b>	xix
 <b>CHAPTER</b>	
<b>I INTRODUCTION</b>	1
Objectives	4
<b>II LITERATURE REVIEW</b>	6
<i>Vibrio parahaemolyticus</i>	6
<i>Vibrio parahaemolyticus</i>	6
Method for <i>V. parahaemolyticus</i> detection	14
Epidemiology	18
Virulence properties	22
Symptoms	25
Antibiotic	26
Plasmid	27
Polymerase chain reaction (PCR)	29
Specific-PCR	35
RAPD-PCR	35
ERIC-PCR	36
<b>III DETECTION OF <i>VIBRIO PARAHAEMOLYTICUS</i> ON (CHROMagar™ <i>Vibrio</i>), FOLLOWED BY TOXR GENE AND VIRULENCE GENES DETECTION</b>	38
Introduction	38
Material and Method	40
Source of <i>Vibrio parahaemolyticus</i>	40
Identification on ChromAgar	40
Confirmation by specific-PCR (detection of <i>toxR</i> gene)	41
Boiling cell method	41
Primer	41
PCR-protocol	42

	Virulence properties test	42
	Primer	42
	PCR-protocol	43
	Agarose gel electrophoresis	43
	Results and Discussion	44
	Conclusion	58
<b>IV</b>	<b>ANTIBIOTIC RESISTANCE</b>	<b>59</b>
	Introduction	59
	Material and Method	60
	Results and Discussion	62
	Conclusion	71
<b>V</b>	<b>PLASMID PROFILING</b>	<b>72</b>
	Introduction	72
	Material and Method	73
	Plasmid Isolation	73
	Agarose gel electrophoresis	74
	Determination of molecular weight of plasmid	75
	Results and Discussion	78
	Conclusion	83
<b>VI</b>	<b>FINGERPRINTING TEST: RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD-PCR AND ENTEROBACTERIAL REPETITIVE INTERGENIC CONSENSUS (ERIC)-PCR</b>	<b>84</b>
	Introduction	84
	Material and Method	86
	Genomic DNA extraction	86
	RAPD-PCR	87
	Primers	87
	PCR protocol	89
	ERIC-PCR	89
	Primer	89
	PCR protocol	89
	Agarose gel electrophoresis	90
	Cluster analysis of the RAPD-PCR using RAPD Distance Software	90
	Cluster analysis of the ERIC-PCR using Gel Compare Software	90
	Results and Discussion	91
	Conclusion	105

<b>VII</b>	<b>GENERAL DISCUSSION AND CONCLUSION</b>	106
	<b>REFERENCES</b>	111
	<b>APPENDICES</b>	128
	<b>BIODATA OF THE AUTHOR</b>	130

## LIST OF TABLES

<b>Table</b>	<b>Page</b>
2.1 Clinical infections associated with <i>Vibrios</i>	8
2.2 Biochemical and physical characteristic of <i>V. parahaemolyticus</i>	17
3.1 Detection of <i>V. parahaemolyticus</i> on CHROMagar™ <i>Vibrio</i>	45
3.2 Detection of the presence of <i>toxR</i> , <i>tdh</i> and <i>trh</i> genes in <i>V. parahaemolyticus</i> isolates	54
4.1 Number and percentages of resistant <i>V. parahaemolyticus</i> isolates to antibiotics	64
4.2 Antibiogram and resistotype among <i>V. parahaemolyticus</i> isolates	65
4.3 Number of resistance antibiotics versus number of isolates and their percentages	68
5.1 Sizes (in MegaDalton) of plasmids of <i>E. coli</i> V517 that were used to determine the molecular weight of plasmids in <i>V. parahaemolyticus</i> isolates	76
5.2 Plasmid profiles of <i>V. parahaemolyticus</i> isolates	81
6.1 The set of random primers used in the screening PCR	88
6.2 The RAPD-PCR and ERIC-PCR types of <i>V. parahaemolyticus</i>	97
7.1 Summary that indicates overall characteristic of all 32 <i>V. parahaemolyticus</i> isolates based on analysis and result obtained	106
AA Zone diameter (mm) for isolates <i>V. parahaemolyticus</i> on MHA when tested with 16 different antibiotics.	128

## LIST OF FIGURES

<b>Figure</b>	<b>Page</b>
2.1 <i>V. parahaemolyticus</i> shapes under microscope. This bacterium has a recognizable curved shape and single polar flagella	7
2.2 Physical map: Circular representation of the <i>V. parahaemolyticus</i> chromosome 1 (3.2 Mb) and 2 (1.9 Mb)	11
2.3 Comparison of genetic maps of two <i>V. parahaemolyticus</i> isolates: KX-V237 (KP positive) and AQ4673 (KP negative)	13
2.4 Wagatsuma Blood Agar showing hemolysin activity by Kanagawa phenomenon positive <i>Vibrio parahaemolyticus</i> strains.	24
2.5 Steps in PCR process with general parameters	31
2.6 Amplification of PCR product in number of cycles	31
3.1 (a) Plate number 48 (samples 48), (1-3) pure culture of <i>Vibrio parahemolyticus</i> isolates growth as purple colonies on CHROMagar™ <i>Vibrio</i>	46
3.1 (b) For plate 98 ( samples 28), 98 (b) and (c), showed the mixed culture of <i>Vibrio parahemolyticus</i> (purple colonies) with <i>Vibrio alginolyticus</i> (white colonies) on CHROMagar™ <i>Vibrio</i> . For samples 98 (a) no <i>Vibrio</i> species were detected.	46
3.2 (a) Detection of <i>toxR</i> gene in <i>V. parahaemolyticus</i> on 1.2% agarose gel	47
3.2 (b) Detection of <i>toxR</i> gene in <i>V. parahaemolyticus</i> on 1.2% agarose gel	47
3.2 (c) Detection of <i>toxR</i> gene in <i>V. parahaemolyticus</i> on 1.2% agarose gel	48
3.3 (a) Detection of <i>tdh</i> gene in <i>V. parahaemolyticus</i> on 1.2% agarose gel	49
3.3 (b) Detection of <i>tdh</i> gene in <i>V. parahaemolyticus</i> on 1.2% agarose gel	49
3.3 (c) Detection of <i>tdh</i> gene in <i>V. parahaemolyticus</i> on 1.2% agarose gel	50
3.4 (a) Detection of <i>trh</i> gene in <i>V. parahaemolyticus</i> on 1.2% agarose gel	51
3.4 (b) Detection of <i>trh</i> gene in <i>V. parahaemolyticus</i> on 1.2% agarose gel	51
3.4 (c) Detection of <i>trh</i> gene in <i>V. parahaemolyticus</i> on 1.2% agarose gel	52

4.1 (a)	Disc-diffusion test to determine the antibiotic resistance pattern	63
4.1 (b)	Disc-diffusion test to determine the antibiotic resistance pattern	63
4.2	Graph no. of antibiotics versus no. of isolates	66
5.1	The graphical method of relating the logarithm of the molecular weight in MegaDalton (MDa) of plasmid of <i>E.coli</i> V517	77
5.2 (a)	Agarose (0.8%) gel electrophoresis of plasmid DNA from <i>V. parahaemolyticus</i> isolates	79
5.2 (b)	Agarose (0.8%) gel electrophoresis of plasmid DNA from <i>V. parahaemolyticus</i> isolates	79
5.2 (c)	Agarose (0.8%) gel electrophoresis of plasmid DNA from <i>V. parahaemolyticus</i> isolates	80
6.1 (a)	Agarose (1.2%) gel electrophoresis of RAPD-PCR products for primer Gold Oligo OPAR3 of <i>V. parahaemolyticus</i> isolates	93
6.1 (b)	Agarose (1.2%) gel electrophoresis of RAPD-PCR products for primer Gold Oligo OPAR3 of <i>V. parahaemolyticus</i> isolates	93
6.1 (c)	Agarose (1.2%) gel electrophoresis of RAPD-PCR products for primer Gold Oligo OPAR3 of <i>V. parahaemolyticus</i> isolates	94
6.2 (a)	Agarose (1.2%) gel electrophoresis of RAPD-PCR products for primer Gold Oligo OPAR8 of <i>V. parahaemolyticus</i> isolates	95
6.2 (b)	Agarose (1.2%) gel electrophoresis of RAPD-PCR products for primer Gold Oligo OPAR8 of <i>V. parahaemolyticus</i> isolates	95
6.2 (c)	Agarose (1.2%) gel electrophoresis of RAPD-PCR products for primer Gold Oligo OPAR8 of <i>V. parahaemolyticus</i> isolates	96
6.3	RAPD-Distance cluster analysis : Gold Oligo OPAR3 and OPAR8 Primers	98
6.4 (a)	Agarose (1.2%) gel electrophoresis of ERIC-PCR products of <i>V. parahaemolyticus</i> isolates	102
6.4 (b)	Agarose (1.2%) gel electrophoresis of ERIC-PCR products of <i>V. parahaemolyticus</i> isolates	102

6.4 (c)	Agarose (1.2%) gel electrophoresis of ERIC-PCR products of <i>V. parahaemolyticus</i> isolates	103
6.5	Dendrogram showing ERIC profiles of <i>V. parahaemolyticus</i> isolates	104



**ABBREVIATIONS**

AIDS	Acquired immunodeficiency syndrome
APW	Alkaline peptone water
bp	Base pairs
CDC	Central for Disease Control and Prevention
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleoside triphosphates
ELISA	Enzyme linked immunosorbent assay
ERIC	Enterobacterial repetitive intergenic consensus
FDA	Food and Drug Administration
FEMS	Federation of European Microbiological Societies
GET	Glucose EDTA TRisHCl
GM	Genetic modified
IRUs	Intergenic repeat units
KAc	Potassium acetate
kb	Kilobase
KP	Kanagawa phenomenon
LB	Luria bertani
MAR	Multiple antibiotic resistance index
MBC	Minimum bactericidal concentration
MDa	Megadalton
MHA	Mueller-Hinton agar

<b>MIC</b>	<b>Minimum inhibitory concentration</b>
<b>NOS</b>	<b>Nitric oxide synthases</b>
<b>ORFs</b>	<b>Open reading frames</b>
<b>PCI</b>	<b>Phenol-chloroform-isoamyl</b>
<b>PCR</b>	<b>Polymerase chain reaction</b>
<b>RAPD</b>	<b>Random amplified polymorphic DNA</b>
<b>REP</b>	<b>Repetitive extragenic palindromic</b>
<b>RFLP</b>	<b>Restriction –fragment length polymorphism</b>
<b>RNA</b>	<b>Ribonucleic acid</b>
<b>rRNA</b>	<b>ribosomal ribonucleic acid</b>
<b>SDS</b>	<b>Sodium dodecyl sulphate</b>
<b>TBE</b>	<b>Tris-borate-EDTA</b>
<b>TCBS</b>	<b>Thiosulfate/citrate/bile salt/sucrose agar</b>
<i>tdh</i>	<b>Thermostable direct haemolysin</b>
<i>trh</i>	<b>TDH-related haemolysin</b>

## CHAPTER I

### INTRODUCTION

*Vibrio parahaemolyticus* is one of the most widely recognized pathogenic *Vibrio* species due to numerous outbreaks and its wide occurrence in marine environment (Joseph *et al.*, 1983; Chiou *et al.*, 1991; Mead *et al.*, 1999). This microorganism can be found in high number during summer in the United States and Europe, but all year round in Southeast Asian. It was first shown to be an enteropathogen in 1951 (in Japan). The genus *Vibrio* consists of 28 species and twelve of them are recognized as human pathogens. The major species that contributed to the pathogenesis are *V. parahaemolyticus*, *V. cholerae*, *V. vulnificus*, and *V. alginolyticus*. In recent years, increasing awareness of the infections of some other *Vibrio* spp., including *V. mimiscus*, *V. fluvialis*, *V. hollisae*, and *V. damsela* have been witnessed (Baffone *et al.*, 2000). Totally, *V. parahaemolyticus* has been implicated as a cause of at least a quarter of total food borne diseases caused by vibrios (Feldhusen, 2000).

For many Southeast Asian country including Malaysia, export of seafood is an important economic sources. Recently, our export of tiger prawn to Italy was rejected due to the detection of *V. parahaemolyticus* (European Commission, 2004) and fails to meet the bacteriological standards of importing countries. Actually, pathogenesis of *V. parahaemolyticus* strongly related to the possession of virulence genes in their genome. Thermostable direct haemolysin (TDH) and TDH-related haemolysin (TRH) are considered as important virulence factors of this organism (Honda *et al.*,



1991). But for the non pathogenic species, both genes that codes for those product were absent. The associated symptoms after infection are gastroenteritis, wound infection, septicaemia and other consequences. In addition to its role in gastroenteritis, *V. parahaemolyticus* is known to cause extraintestinal infection in humans.

Marine environments are the natural habitat of many *Vibrio* species. *V. parahaemolyticus* can be isolated from sea water or marine organism like shellfish, plankton, and oyster. Typically, it can be recovered from most biological and physical structure in marine environments where environmental temperatures and condition are compatible with its growth (Labbe and Garcia, 2001). Seafood is known to be a major source of pathogenic *vibrios* worldwide and the studies about this species are important to provide an adequate consumer protection since seafood particularly shellfish is one of the important ingredients and diet in Southeast Asian countries. Identification of the source of contamination of seafoods with *V. parahaemolyticus* is important to study epidemiology of human infection and also for controlling. The development of polymerase chain reaction (PCR) assay for detection proved to be very useful. This method is rapid, sensitive, reliable and specific for microbial screening (Kim *et al.*, 1999; Cook *et al.*, 2002). PCR method also has been used to detect the virulence properties in samples isolates and hence very useful to detect and study the pathogenesis of *V. parahaemolyticus* (Tada *et al.*, 1992).

The main objective for research groups in UPM is to contribute to the study of *V. parahaemolyticus* distribution in Southeast Asia. This project was initiated to gather



information on the levels of *V. parahaemolyticus* contamination of the seafood obtained from water sources within the ASEAN region. The information gathered was to have contributed to the development of a relevant database for epidemiological and pathogenesis comparison purposes of *V. parahaemolyticus* in the ASEAN region. This study will be focused on the cockle samples from Indonesia. The occurrence of vibrios in Indonesia is not well documented and the occurrence of vibrios in seafood is not well understood. The true incidence of *V. parahaemolyticus* in Indonesia transmitted by seafood is not known, probably due to underreporting of cases and lack of proper study on this issue. Only Lesmana *et al.*, (2001) started to conduct studies about the infection of *V. parahaemolyticus* in Indonesia and no information is reported on the distribution of *V. parahaemolyticus* in Padang, Indonesia. In Malaysia, many similar studies were conducted by Ministry of Health and universities (Son *et al.*, 1998; HUKM, 2000; Son *et al.*, 2002). Comparison of the distribution of *V. parahaemolyticus* in the nearby location can be done and the data can be used for epidemiological and pathogenesis study, but the most important thing is to see whether geographical factor may influence the data or not. Understanding the ecology of *V. parahaemolyticus* and tracing virulence isolates in seafood are important issues, since seafood is one of the important ingredients and diet in Southeast Asia.

The contribution of this study to our country is to provide preliminary information about the *V. parahaemolyticus* distribution in neighbourhood country especially Indonesia and this information can be used as a considered factor in importing process. It is also important to monitor the potential risk infection from seafood in Indonesia. Public awareness of microorganisms transmission has increased

dramatically in recent years. Bacterial characterization methods have improved our ability to detect and provided tools for tracking sources of *V. parahaemolyticus* contamination throughout food systems, especially when the sampling locations are exposed to industrial or human waste. Application of phenotype and genotype based methods also provides insight into the population genetics, epidemiology, ecology, and evolution of *V. parahaemolyticus*. The application technique of this study can be used to determine the pathogenesis of *V. parahaemolyticus* in our seafood and the information of this study can be used as a comparison to determine the source and the factor that contributed to the pathogenesis.

Characterization of this bacterium is essential to contribute preliminary information about this bacteria distribution and variation. In epidemiological study, typing method is required to provide information about an infection and also identification of sources of infection. The typing methods, either the phenotype or the genotype features, are based on the genomic and sometimes on the plasmid DNA properties. Phenotype methods that can be used are antibiotic resistance test, biotyping, serotyping and immunoblotting. Meanwhile, for genotype methods that can be used are plasmid profiling, random amplified polymorphic DNA (RAPD), Restriction-fragment-length-polymorphism (RFLP) and DNA sequencing analysis.

### **Objectives**

The objectives of this study are to determine the occurrence and characterization of *V. parahaemolyticus* in cockle samples from Padang, Indonesia. The characterization of the isolated *V. parahaemolyticus* was done by their antibiotic resistance, plasmid