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CHARACTERIZATION OF *VIBRIO PARAHAEMOLYTICUS* ISOLATED FROM LOCAL COCKLES (*ANADARA GRANOSA*) FROM TANJUNG KARANG, KUALA SELANGOR

LESLEY MAURICE BILUNG

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By

LESLEY MAURICE BILUNG

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree of Doctor of Philosophy

April 2006

DEDICATION

To :

MY LORD AND SAVIOUR AS WELL AS MY TRUE-EVER FRIEND:
LORD JESUS CHRIST

You're amazing, LORD;
Never changing
Always with me,
Enthroned within my heart,

Overflowing,
My heart
Offers the deepest honor and worship,
Amazing.....

My dad, mum, Gad, Tim, Rachel and Ding.

My grandparents : Tepu Liang n the late Tepu Sheila, Tepu Siren n Tepu Bulan as well to all my grandparents who loves n prays for me.

My aunts and uncles, n ol my lovely cousins.

My dearest friends.

My respected teachers and lecturers.

My brothers and sisters in Christ.

My Pastors and Mentors.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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April 2006

Chairman : Professor Son Radu, PhD

Faculty : Food Science and Technology

Vibrio parahaemolyticus is widespread in occurrence and has been recognized as a cause of gastroenteritis related to consumption of raw or improperly cooked seafood. Outbreaks of *V. parahaemolyticus* food poisoning are most common in Taiwan, Japan and Southeast Asia. In this study, *V. parahaemolyticus* was isolated from 62 of 100 (62%) samples of cockles (*Anadara granosa*) collected from Tanjong Karang, Kuala Selangor. A total of 62 strains were studied for the presence or absence of regulatory gene (*toxR),* virulence genes (*tdh* and *trh*), their antibiotic resistance, the occurrence of plasmids and their molecular fingerprints by Randomly Amplified Polymorphic DNA – Polymerase Chain Reaction (RAPD-PCR) and Enterobacterial Repetitive Intergenic Consensus - Polymerase Chain Reaction (ERIC-PCR) assays. All 62 strains were positive for the regulatory gene (*toxR*) of *V. parahaemolyticus.* The PCR analysis for the detection of *tdh* or *trh* genes showed two (3.2%) positive strains carrying *tdh* gene and eleven (17.7%)

strains had *trh* gene. The MPN value for all samples was more than 1100 MPN/g. This study has shown that all strains were multiple resistant to three or more of the seventeen antibiotics tested with the MAR indices ranging from 0.58-0.94. All isolates of *V. parahaemolyticus* were highly resistant towards the antibiotics tested, except one strain that was sensitive towards norfloxacin. Plasmids were found in 80% of the strains analyzed and 18 different plasmid profiles were observed. The plasmid size ranged from 2.7 to more than 54 kb. Two molecular typing methods were used in this study to examine the genetic relatedness among the *V. parahaemolyticus* strains. In the analysis by RAPD-PCR and ERIC-PCR, the size of RAPD and ERIC fragments ranged from 0.25 to 10.0 kb with an average number of ten and eight bands, respectively. Sixty-two genotypes among the 62 *V. parahaemolyticus* strains were generated using RAPD and ERIC-PCR which indicates that the strains were very diverse. Hence, this study, demonstrated that the local cockles are potential source for pathogenic *V. parahaemolyticus.*

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENCIRIAN MOLEKUL *VIBRIO PARAHAEMOLYTICUS* DIPENCIL DARIPADA KERANG TEMPATAN (*ANADARA GRANOSA*) DARI TANJUNG KARANG, KUALA SELANGOR

Oleh

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Pengerusi : Profesor Son Radu, PhD

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Penyebaran *V. parahaemolyticus* adalah meluas dan dikenalpasti sebagai punca *gastroenteritis* berkaitan dengan memakan makanan laut yang mentah atau separuh masak. Wabak keracunan makanan yang disebabkan oleh *V. parahaemolyticus* sering berlaku di Taiwan, Jepun dan Asia Tenggara. Dalam kajian ini, *V. parahaemolyticus* dipencilkan daripada 62/100 (62%) sampel kerang (*Anadara granosa*) yang diperolehi dari Tanjong Karang, Kuala Selangor. Sejumlah 62 pencilan telah dikaji untuk kehadiran *regulatory gene* (*toxR*) dan gen virulen (*tdh* dan *trh*), kerintangan antibiotik, taburan plasmid, *molecular fingerprint* dengan menggunakan teknik *Randomly Amplified Polymorphic DNA – Polymerase Chain Reaction* (*RAPD-PCR*) dan *Enterobacterial Repetitive Intergenic Consensus - Polymerase Chain Reaction* (ERIC-PCR). Kesemua pencilan adalah positif untuk kehadiran *regulatory gene* (*toxR*) *V.*

parahaemolyticus. Analisis PCR untuk kehadiran gen *tdh* dan *trh* menunjukkan dua (3.2%) pencilan membawa gen *tdh* dan sebelas (17.7%) pencilan membawa *trh* gen. Nilai MPN untuk kesemua sampel adalah melebihi 1100 MPN/g. Kajian ini menunjukkan kesemua pencilan adalah rintang terhadap tiga atau lebih antibiotik yang diuji dengan MAR indeks antara 0.58-0.94. Kesemua pencilan menunjukkan kerintangan yang tinggi terhadap antibiotik yang diuji, kecuali satu pencilan yang peka terhadap norflosaksin. Plasmid dijumpai pada 80% pencilan yang dikaji dan 18 profil plasmid yang berlainan dikesan. Saiz plasmid adalah di antara 2.7 sehingga melebihi 54 kb. Dua kaedah *molecular typing* telah digunakan dalam kajian ini untuk mengkaji perkaitan genetik di kalangan pencilan *V. parahaemolyticus.* Dalam analisis dengan menggunakan RAPD-PCR dan ERIC-PCR, ukuran fragmen RAPD dan ERIC didapati antara 0.25 to 10.0 kb dengan purata bilangan jalur masing – masing sebanyak sepuluh dan lapan. Enam puluh dua genotip di kalangan *V. parahaemolyticus* pencilan diperolehi dengan menggunakan RAPD dan ERIC-PCR yang menunjukkan pencilan adalah sangat pelbagai. Dengan itu, kajian ini, menunjukkan bahawa kerang tempatan adalah sumber yang berpotensi untuk *V. parahaemolyticus* yang patogen.

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I certify that an Examination Committee has met on 20th April 2006 to conduct the final examination of Lesley Maurice Bilung on her Doctor of Philosophy thesis entitled "Characterization of *Vibrio parahaemolyticus* Isolated from Local Cockles *(Anadara Granosa)* from Tanjung Karang, Kuala Selangor" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act of 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

_____

LESLEY MAURICE BILUNG

Date:

TABLE OF CONTENTS

		Page
DEDICATION		ii
ABSTRACT		iii
ABSTRAK		v
ACKNOWLEDGEMENTS		vii
APPROVAL		ix
DECLARATION		xi
LIST OF TABLES		xv
LIST OF FIGURES		xvii
LIST OF FLOWCHARTS		xix
CHAPTER		
I	**INTRODUCTION**	1
	The objectives of the study	9
II	**LITERATURE REVIEW**	10
	Seafood	10
	Diseases transmitted by shellfish	10
	V. parahaemolyticus	12
	Characteristics of *V. parahaemolyticus*	13
	Diseases caused by *V. parahaemolyticus*	16
	Pathogenicity of *V. parahaemolyticus*	17
	Thermostable direct hemolysin (TDH)	17
	Thermostable related hemolysin (TRH)	18
	Epidemiology of *V. parahaemolyticus* outbreaks	19
	Isolation and identification *V. parahaemolyticus*	32
	Culture method approach	32
	Enrichment media	32
	Selective enrichment media	33
	Biochemical tests	34
	Molecular approach	35
	Control measures	36
	Molecular typing of *V. parahaemolyticus*	38
	Antibiotic resistance	39
	Antibiotic resistance in *V. parahaemolyticus*	40
	Multiple Antibiotic Resistance (MAR) analysis	41
	Plasmid profiling	42
	Plasmid and Antibiotic resistance	44
	Genotyping of *V. parahaemolyticus* using RAPD-PCR and ERIC-PCR	45

Sequencing for identification or confirmation of PCR products 45

III **ISOLATION and DETECTION OF *VIBRIO PARAHAEMOLYTICUS* IN COCKLE (ANADARA GRANOSA) IN TANJONG KARANG, KUALA SELANGOR, MALAYSIA** 47

Introduction 47
Objectives of the study 51
Materials and methods 53
Sample processing, enrichment and detection of *V. parahaemolyticus* 53
Genomic DNA preparation 56
Boil cell extraction of DNA from enrichment broth and presumptive colonies of *V. parahaemolyticus* on CHROMagar™ *Vibrio* 56
Primers 57
PCR protocol 58
Agarose gel electrophoresis 60
Sequencing of *toxR* gene 63
Gel purification for DNA sequencing using DNA sequencing analysis 64
Results 65
Discussion 77

IV **ANTIBIOTIC RESISTANCE AND PLASMID PROFILING OF *VIBRIO PARAHAEMOLYTICUS* ISOLATED FROM COCKLES (*ANADARA GRANOSA*) FROM TANJONG KARANG, KUALA SELANGOR** 96

Introduction 96
Objectives of the study 98
Materials and methods 99
Bacterial isolates 99
Antibiotic susceptibility test 99
Multiple Antibiotic Resistance (MAR) indexing of Isolates 100
Plasmid DNA extraction 100
Agarose gel electrophoresis and staining 102
Detection of plasmid DNA bands 102
Results 103
Discussion 108

V MOLECULAR TYPING OF *VIBRIO PARAHAEMOLYTICUS* ISOLATES USING RANDOMLY AMPLIFIED POLYMORPHIC DNA (RAPD) FINGERPRINTING AND ENTEROBACTERIAL REPETITIVE INTERGENIC CONSENSUS (ERIC) PCR 125

Introduction 125
Materials and methods 130
Bacterial strains 130
Genomic DNA Isolation (PCI method 130
RAPD fingerprinting 131
RAPD-PCR amplification 131
Primers 131
ERIC-PCR 132
ERIC-PCR amplification 133
Primers 133
Data analysis 134
Results 135
Discussion 144

VI GENERAL DISCUSSION AND CONCLUSION 158

REFERENCES 166
BIODATA OF THE AUTHOR 191

LIST OF TABLES

Table		Page
2.1	Clinical infections associated with vibrios	12
2.2	Phenotypic characteristics of *V. parahaemolyticus*	15
2.3	The incidence of reported *V. parahaemolyticus* in Malaysia, 1999-June 2004	22
2.4	Prevalence of *V. parahaemolyticus* in seafood	25
2.5	Contribution of *V. parahaemolyticus* in foodborne diseases	28
2.6	Quantitative data for *V. parahaemolyticus* in seafood	29
2.7	Phenotypical and genotypical typing methods of pathogens	39
3.1	Primers used in this study	57
3.2	Amplification condition for PCR assay of *Vp-toxR* genes	59
3.3	Amplification condition for PCR assay of *Vp-tdh* genes	59
3.4	Amplification condition for PCR assay of *Vp-trh* genes	59
3.5	Detection of *V. parahaemolyticus* in 100 samples of cockles (*Anadara granosa*) obtained from Tanjung Karang, Kuala Selangor	66
3.6	MPN of total *V. parahaemolyticus*	69
4.1	Distribution of antimicrobial resistance of *V. parahaemolyticus*	103
4.2	The antibiotic resistance profile patterns and Multiple Antibiotic Resistance (MAR) index of *V. parahaemolyticus* from local cockles (*Anadara granosa*)	104

4.3	The plasmid profile patterns of *V. parahaemolyticus* from local cockles (*Anadara granosa*)	105
5.1	The random primers used in the screening in RAPD analysis	132
5.2	The RAPD-PCR and ERIC-PCR types of *V. parahaemolyticus*	142

LIST OF FIGURES / FLOWCHARTS

Figure		Page
3.1	Mauve colonies of *V. parahaemolyticus* grown on CHROMagar™ *Vibrio*	72
3.2	Representative of the detection of *toxR* gene in *V. parahaemolyticus* on 1.5% agarose gel	73
3.3	Representative of the detection of *toxR* gene in *V. parahaemolyticus* on 1.5% agarose gel	73
3.4	Representative of the detection of *tdh* gene in *V. parahaemolyticus* on 1.5% agarose gel	74
3.5	Representative of the detection of *tdh* gene in *V. parahaemolyticus* on 1.5% agarose gel	74
3.6	Representative of the detection of *trh* gene in *V. parahaemolyticus* on 1.5% agarose gel	75
3.7	Representative of the detection of *trh* gene in *V. parahaemolyticus* on 1.5% agarose gel	75
3.8	The comparison of partial sequence of *toxR* gene referring to *toxR* sequences in database through BLASTN program	76
4.1	Representative of the plasmid DNA profiles of *V. parahaemolyticus* on 0.8% agarose gel	107
4.2	Representative of the plasmid DNA profiles of *V. parahaemolyticus* on 0.8% agarose gel	107
5.1	Representative of the RAPD fingerprints of *V. parahaemolyticus* with primer Gen 1-50-03 on 1.5% agarose gel	136

5.2	Representative of the RAPD fingerprints of *V. parahaemolyticus* with primer Gen 1-50-08 on 1.5% agarose gel	136
5.3	Representative of the RAPD fingerprints of *V. parahaemolyticus* with primer Gen 1-50-04 on 1.5% agarose gel	137
5.4	Representative of the ERIC fingerprints of *V. parahaemolyticus* with primer ERIC on 1.5% agarose gel	137
5.5	Dendrogram showing ERIC-PCR profiles of typeable *V. parahaemoyticus* isolates	138
5.6	Dendrogram showing RAPD profiles of typeable *V. parahaemoyticus* isolates using GEN1-50-03	139
5.7	Dendrogram showing RAPD profiles of typeable *V. parahaemoyticus* isolates using GEN1-50-04	140
5.8	Dendrogram showing RAPD profiles of typeable *V. parahaemoyticus* isolates using GEN1-50-08	141

Flowcharts **Page**

3.1	Enumeration of *V. parahaemolyticus* (MPN Method)	55
3.2	Boil cell extraction	61
3.3	Specific PCR detection of *Vp-toxR* gene, *Vp-trh* gene and *Vp-tdh* gene	62

CHAPTER I

INTRODUCTION

Food borne diseases harm people in every country around the world. It is true that some of these diseases are controlled in some areas, but others are emerging or re-emerging. In spite of extensive knowledge on diseases such as salmonellosis, cholera and botulism, these diseases have not diminished and in fact they are causing increasing problems in many countries and are significantly affecting human health, as well as productivity and the international trade in food. Food borne diseases and their epidemiology involve the entire chain of production, processing and distribution of food. The level of community sanitation is important and is increasingly being recognized in both developed and developing countries. Rapid urbanization, technological advances, international movement of people, increase production of animal and food products, centralization of food processing, long chains of food distribution and changing habits have all modified the conventional approaches to the epidemiology of food borne diseases.

In recent years, vibriosis has become one of the most important bacterial diseases in marine cultures or marine organisms, affecting a large number of fish and shellfish. Interest has also increased on the role of *Vibrio* species in causing human intestinal and extra-intestinal diseases. Table 1.1 shows the

Vibrio species which are associated with human infections. The route of transmission of *Vibrio* species from the environment to humans includes consumption of undercooked or raw seafood or shellfish.

Table 1.1: *Vibrio* species which are associated with human infections

	Occurrence in human clinical specimens*	
	Intestinal	Non-intestinal
V. cholera O1 and O139	++++	+
V. cholera non-O1 / non-O139	++	++
V. parahaemolyticus	++++	+
V. fluvialis	++	-
V. furnissii	++	-
V. hollisae	++	-
V. mimiscus	++	+
V. metschnikovii	+	+
*V. vulnificus***	+	+++
V. alginolyticus	-	++
V. carchariae	-	+
V. cincinnaitiensis	-	+
V. damsela	-	+

*The symbol (+) refers to the relative frequency of each organism in clinical specimens and (-) indicated that the organism was not found.
** The ability of *V. vulnificus* to cause gastro-intestinal disease remains to be confirmed.

Source: Report of a Joint FAO/WHO Expert Consultation. 2002. Risk Assessment of *Campylobacter* spp. in broiler chicken and *Vibrio* spp. in seafood. Bangkok, Thailand.

Vibrios are most commonly enumerated either by suitable dilution and direct plating on selective medium or following enrichment as in the MPN technique (Hocking *et al.*, 1997). The MPN is usually used to calculate the numbers of organisms especially in milk and water, and for those foods whose particulate matter may interfere with accurate colony counts. Only viable organisms are enumerated by the MPN determination. The sample is prepared in such a way that bacteria are distributed randomly within it. The growth medium and conditions of incubation have been chosen so that every inoculum that contains even one viable organism will produce detectable growth. These assumptions are necessary to support the MPN method.

The progressive increase in antimicrobial resistance among pathogens in developing countries is becoming a critical area of concern (Tjaniadi *et al.*, 2003). Antimicrobial testing is an invaluable tool to screen the bacterial isolates for antibiotic resistance (Call *et al.*, 2003). A commonly used method of antimicrobial testing is performed by the disk diffusion method using guidelines established by the Bauer *et al.* (1960). The antibiotics use in the test include carbenicillin (100 μg), ceftriaxone (30 μg), cephalothin (30 μg), chloramphenicol (30 μg), clindamycin (2 μg), doxycycline (30 μg), imipenem (10 μg), nalidixic acid (30 μg), netilmicin (30 μg), nitrofurantoine (300 μg), norfloxacin (10 μg), oflaxacin (5 μg), rifampin (5 μg), streptomycin (10 μg), sulfamethixazole (25 μg), teicoplanin (30 μg) and tobramycin (10 μg). According to Krumperman (1985), Multiple Antibiotic Resistance (MAR)

index values higher than 0.2 are considered to have originated from higher risk sources of contamination like human, commercial poultry farms, swine and dairy cattle where antibiotics are often used. MAR index values lower than 0.2 are considered to have originated from animals in which antibiotics are seldom or never used. It is well known that the wide use and abuse of antibiotics in human therapy has produced MAR pathogenic microorganisms in the faeces of humans. Release of pathogenic bacteria in the faeces results in dispersal into aquatic systems where they contaminate these aquatic environments, where genetic exchange between bacteria is readily facilitated and account for a higher frequency of MAR forms. The multiple antibiotic resistance index of the isolates is defined as a/b where 'a' represents the number of antibiotics to which the particular isolate was resistant and 'b' the number of antibiotics to which the isolate was exposed to (Krumperman, 1983).

Until 1960, cholera was considered as a disease caused only by *Vibrio* species. However, since *V. parahaemolyticus* was defined as an etiologic agent of gastroenteritis, medical microbiologists have taken great interest in the relationship between the organisms and human disease. To date, it has been demonstrated that there are at least 12 *Vibrio* species, namely *V. cholera, V. parahaemolyticus, V. fluvialis, V. mimiscus, V. hollisae, V. furnissii, V. vulnificus, V. alginolyticus, V. damsela, V. cincinnatiensis, V. carchariae and V. metschnikovii. Vibrio cholera, V. parahaemolyticus* and *V. vulnificus* is universally recognized as important human pathogens, with *V. parahaemolyticus* implicated as a

cause of at least a quarter of total food borne diseases caused by vibrios (Feldhusen, 2000).

Vibrio parahaemolyticus is widely distributed in coastal and estuarine waters throughout the world. The organism has been recognized as the causative agent of gastroenteritis since its first report in 1950 (Sakazaki, 2002). Recently, with the emergence of the pandemic O3:K6 strain of *V. parahaemolyticus* causing acute gastroenteritis, this seafood-borne pathogen has assumed great significance (Matsumoto *et al.,* 2000). Sazaki *et al.* (1968) and Thompson and Vanderzant (1976) noted that the strains of *V. parahaemolyticus* associated with gastroenteritis in man produce beta-haemolysis on a high salt blood agar called Wagatsuma agar whereas only 1-2% of environmental strains show this haemolysis. This phenomenon, known as the Kanagawa phenomenon, is induced by a thermostable direct haemolysin (TDH); thus TDH has been considered the major virulence factor of *V. parahaemolyticus* (Nishibuchi and Kaper, 1985). Studies on clinical strains that were Kanagawa-negative led to the discovery of a TDH-related haemolysin (TRH), which is also considered an important virulence factor of this organism (Honda *et al.,* 1991; Honda and Iida, 1993). Although the production of TDH can be tested using Wagatsuma agar or a commercially available immunological kit, the tests are cumbersome and time consuming. Presently, there is neither a blood agar medium nor a commercially available immunological kit to detect TRH produced by this pathogen.