



UNIVERSITI PUTRA MALAYSIA

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

LESLEY MAURICE BILUNG

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FROM LOCAL COCKLES (*ANADARA GRANOSA*) FROM TANJUNG
KARANG, KUALA SELANGOR**

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

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

By

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April 2006

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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 Tanjung 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

LESLEY MAURICE BILUNG

April 2006

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 kerintang 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:

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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
<i>V. cholera</i> O1 and O139	++++	+
<i>V. cholera</i> non-O1 / non-O139	++	++
<i>V. parahaemolyticus</i>	++++	+
<i>V. fluvialis</i>	++	-
<i>V. furnissii</i>	++	-
<i>V. hollisae</i>	++	-
<i>V. mimiscus</i>	++	+
<i>V. metschnikovii</i>	+	+
<i>V. vulnificus</i> **	+	+++
<i>V. alginolyticus</i>	-	++
<i>V. carchariae</i>	-	+
<i>V. cincinnatiensis</i>	-	+
<i>V. damsela</i>	-	+

*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), nitrofurantoin (300 µg), norfloxacin (10 µg), ofloxacin (5 µg), rifampin (5 µg), streptomycin (10 µg), sulfamethoxazole (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.

