UNIVERSITI PUTRA MALAYSIA

ISOLATION AND MOLECULAR CHARACTERISATION OF VIBRIO VULNIFICUS AND VIBRIO PARAHAEMOLYTICUS FROM COCKLES (ANADARA GRANOSA) IN MALAYSIA

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ISOLATION AND MOLECULAR CHARACTERISATION OF *VIBRIO VULNIFICUS* AND *VIBRIO PARAHAEMOLYTICUS* FROM COCKLES (*ANADARA GRANOSA*) IN MALAYSIA

By

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Thesis Submitted in Fulfilment of the Requirements for the
Degree of Master of Science in the Faculty
of Food Science and Biotechnology
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TO

my *parents*

my *wife* Najat

and

my son Taj

for their love and affection

who has elevated my

ambition

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February 1999

Chairman: Dr. Zaiton Hassan

Faculty: Food Science and Biotechnology

Antibiotic susceptibility, plasmid profiles and random amplification of polymorphic DNA were used to study strains of *Vibrio vulnificus* and *Vibrio parahaemolyticus* isolated from cockles (*Anadara granosa*). 36 strains of *V. vulnificus* isolates were examined. The prevalent biotype was biotype 1(72.2% of the isolates) and 2 (27.8%). Twenty one strains of biotypes 1 and 2 harboured plasmid DNA ranging in size from 1.4 to 9.7 megaDalton. No particular plasmid profile was predictive of a particular pattern of antibiotic susceptibility. Two primers demonstrated polymorphisms in all strains tested, producing bands ranging from 0.25 to 2.7 kb, indicating a high variability among both biotypes 1 and 2 of the *V. vulnificus* strains investigated. RAPD identity across biotypes was also observed among the *V. vulnificus* strains. 35 *Vibrio parahaemolyticus* Kanagawa-negative strains were isolated. Twenty six strains of *V. parahaemolyticus* were carried small plasmid(s) of 1.3 to 9.7 MegaDalton that enabled

the *V. parahaemolyticus* to be grouped into eight plasmid patterns. The RAPD fingerprinting using three primers demonstrated polymorphisms in all thirty-five strains of *V. parahaemolyticus* tested, producing bands ranging from 0.25 to 3.9 kb. The RAPD profiles revealed a high level of DNA sequence diversity within the *V. parahaemolyticus* strains tested, and that cockles in the study area are populated by genetically polymorphic strains of *V. parahaemolyticus*.

Abstrack tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
Sebagai memenuhi keperluan Ijazah Master Sains

PEMENCILAN DAN PENCIRIAN MOLEKUL *VIBRIO VULNIFICUS* DAN *VIBRIO PARAHAEMOLYTICUS* DARI KERANG (*ANADARA GRANOSA*) DI MALASIA

OLEH

NASRELDIN ELHADI HUSSEIN

Febuari 1999

Pengerusi : Dr. Zaiton Hassan

Fakulti : Sains Makanan dan Bioteknologi

Kerentanan antibiotik, profil plasmid dan amplifikasi secara rawak DNA polimofik digunakan untuk mengkaji pencilan *Vibrio vulnificus* dan *V. parahaemolyticus* yang dipencilkan dari kerang. Tiga puluh enam pencilan *V. vulnificus* dikaji. Biotip yang prevalen ialah 1 (72.2% daripada pencilan) dan 2 (27.8%). Di antara mereka, 21 pencilan dari biotip 1 dan 2 pencilan dari biotip 2 mempunyai plasmid DNA bersaiz dari 1.4 hingga 9.7 megadalton. Tidak ada profil plasmid yang bersesuaian dengan mana-mana corak kerentanan antibiotik. Dua primer menghasilkan polimofisma dalam semua pencilan yang diuji, menunjukkan jalur bersaiz dari 0.25 hingga 2.7 kb, menunjukkan perbezaan yang tinggi dikalangan kedua-dua biotip 1 dan 2 *V. vulnificus* yang dikaji. Untuk *V. parahaemolyticus* 35 pencilan kanagawa-negatif dipencilkan. Dua puluh enam pencilan membawa plasmid kecil dari 1.3 hingga 30 mDa yang membolehkan *V.*

parahaemolyticus dikumpulkan kepada lapan corak plasmid. Amplifikasi corak DNA polimofik (RAPD) yang didapati dari tiga primer yang diuji, menghasilkan tiga puluh lima polimorpisma pencilan *V. parahaemolyticus* dengan julat 0.25 sehingga 3.9 kb. Profil RAPD menunjukan diversiti jujukan DNA yang tinggi di kalangan pencilan *V. parahaemolyticus* yang diuji, dan kerang dikawasan yang dikaji mempunyai pencilan *V. parahaemolyticus* yang polimofik dari segi genetik.

CHAPTER I

GENERAL INTRODUCTION

Vibrio vulnificus and *Vibrio parahaemolyticus* are marine and estuarine bacterial species. *Vibrio vulnificus* comprises two biotypes distinguished by certain phenotypic traits and host range (Tison *et al.*, 1982). Biotype 1 is an opportunistic human pathogen resulting from the consumption of raw shellfish and wound infections after exposure to marine environments (Amaro *et al.*, 1992), whereas biotype 2 is an eel pathogen that has been recovered from diseased eels but never from water or other marine animals (Biosca *et al.*, 1991). Though biotype 2 is considered as obligate eel pathogen, it has now been reported to be cause septicemia through direct contact of open wounds with infected eels (Jan-Veenstra *et al.*, 1992). To better determine the health risk associated with exposure to *V. vulnificus*, epidemiological tracking of strains is required. This may be achieved by the use of DNA fingerprinting, which allows rapid and sensitive differentiation between *V. vulnificus* strains (Huys *et al.*, 1996). The pathogenecity of *V. parahaemolyticus* is believed to be associated with a lethal toxin (Sarkar *et al.*, 1987), a vascular permeability factor (Honda *et al.*, 1976), and thermostable direct hemolysin and related hemolysins (Taniguchi *et al.*, 1990). Human infections with *V. parahaemolyticus* are usually linked to the consumption of raw or mishandled seafood or through a wound (Johnson *et al.*, 1984) and is an important agent of human gastroenteritis. Despite the ubiquity of *V. parahaemolyticus* in marine and estuarine environments, and in shellfish, there is great variability in the incidence and distribution in different regions depending on the seasons, fecal pollution, sample type and experimental variables (Depaola *et al.*, 1990). Hence, due to the fact that most strains of environmental and seafood isolates are likely to be

avirulent, it may prove difficult to correlate the presence of *V. parahaemolyticus* in shellfish with the development of disease in humans. Randomly amplified polymorphic DNA (RAPD) PCR is a genotyping analysis method, which has increasingly been used to compare strains of numerous bacterial species because of the generic capabilities of the PCR system. In addition, the determination of plasmid profiles can aid in the differentiation of isolates in epidemiological investigation. The present study characterized 36 isolates of *V. vulnificus* and 35 isolates of *V. parahaemolyticus* isolated from cockles (*Anadara granosa*) by antimicrobial resistance, plasmid profiles and random amplification of polymorphic DNA analysis.

Objectives

The prevalence of *V. vulnificus* and *V. parahaemolyticus* in Malaysia is not well documented. Less light has been thrown on the sources of infection transmission.

1- To determine the presence of *V. vulnificus* and *V. parahaemolyticus* in cockles (*Anadara granosa*), and to identify if cockles has any potential for transmission of the pathogen.

2- To compare the antibiotic susceptibility patterns and plasmid profiles among *V. vulnificus* and *V. parahaemolyticus* isolates.

3- To use RAPD-PCR technique to differentiate isolates of *V. vulnificus* and *V. parahaemolyticus* from cockles (*Anadara granosa*).

CHAPTER II

LITERATURE REVIEW

Introduction

Vibrio parahaemolyticus and *Vibrio vulnificus* are marine bacteria, an inhabitant of estuarine waters. *V. parahaemolyticus* is a causative agent of human gastroenteritis through consumption of contaminated seafoods (Barker *et al.,* 1974; Nolan *et al.,* 1984; Twedet *et al.,* 1989). The organism is naturally present in coastal and estuarine environments on both coasts of the United States and other worldwide locations (Kaysner *et al.,* 1990). *V. vulnificus* causes three types of human infections; primary septicemia, gastroenteristis and wound infections (Klontz *et al.*, 1988, Wright *et al.*, 1996). *Vibrio vulnificus* is phenotypically similar to *V. parahaemolyticus* and has been recognized as a highly virulent pathogen (Oliver *et al.,* 1989, Martin *et al.*, 1991).

V. vulnificus and *V. parahaemolyticus* are recognised of the most pathogenic *Vibrio* species which can cause life threatening human infections when involved in wound infections, septicemia and foodborne gastroenteritis (Johnston *et al.,* 1986; Morris *et al.,* 1985, Hagen *et al.*, 1994). These organisms contaminate filter-feeding seafood, such as oysters and clams (Tacket *et al.,* 1984; Oliver *et al.,* 1985, Austin *et al.*, 1987). The incidence of these pathogens in shellfish is higher during the summer, and *V. vulnificus* has been found to survive for up to 2 weeks in commercial shell stock and at least 6 days in sucked oysters under refrigeration (Kaysner *et al.,* 1989).

V. parahaemolyticus is an enteric pathogens transmitted to humans primarily through a wound infection through consumption of raw or mishandled seafoods, or through a wound, and this pathogen have been a source of disease outbreaks in Taiwan, Japan and other coastal regions (Joseph *et al.*, 1982, Johnson *et al.*, 1984, Janda *et al.*, 1988, Chiou *et al.*, 1991). Though the exact mechanism of its pathogenic effect is still not clearly understood, epidemiological studies associate it with a lethal toxin (Sarkar *et al.*, 1987), a vascular permeability factor (Honda *et al.*, 1976), and thermostable direct haemolysin (TDH) and related haemolysins (Takeda *et al.*, 1983, Nishibuchi *et al.*, 1989, Taniguchi *et al.*, 1990, Honda *et al.*, 1991,).

Taxonomy

Vibrio vulnificus and *V. parahaemolyticus* are classified in the family *Vibrionaceae* according to the International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of *Vibrionaceae* (1992). It is differentiated from the family *Pseudomonaceae* in that its members will grow anaerobically and from the family *Enterobacteriaceae* in that its members are sensitive to the Vibriostatic compound O/129 (Sundaram *et al.*, 1993), and are oxidase positive (Janda *et al.*, 1988, Kelly *et al.*, 1991, Farmer *et al.*, 1992). The family *Vibrionaceae* contains three other genera. (Table 1) lists the differentiating characteristics of all the four genera. Members of the genus *Vibrio* are characterized as gram-negative rods that are straight or have a single rigid curve, they are motile with a single polar flagellum, they produce oxidase (with the exception of *Vibrio metchnikovii*) and catalase, and they ferment glucose with no gas production (with the

exception of *Vibrio fluvialis*). The genus currently contains 28 species, at least 10 of which may cause illness in humans (Bode *et al.,l* 1986; Farmer *et al.,* 1985).

Table 1. Differentiation of the four genera forming the Family *Vibrionaceae*a*

Property	*Vibrio*	*Photobacterium*	*Plesiomonas*	*Aeromonas*
Mole % Guanine + Cytosine	38- 51	40 - 44	51	57-63
Sensitive to compound O129	+	+	+	-
D-mannitol fermentation	+	-	-	+
Na^+ ion required for growth or stimulates growth	+	+	-	-

+, genus positive for property.
-, genus negative for property.
* (Farmer *et al.,* 1985).

Isolation and Identification of *V. vulnificus* and *V. parahaemolyticus*

V. vulnificus and *V. parahaemolyticus* can be isolated by growing the suspension samples on Thiosulfate citrate bile salts sucrose agar (TCBS). The TCBS agar is known to be an excellent medium for selective isolation of vibrios (Tamplin *et al.*, 1981., Lotz *et al.*, 1983, Tamplin *et al.*, 1988, Elliot *et al.*, 1992). The colonies of both species growing on TCBS medium are green colour due to the inability to ferment sucrose

(Wright *et al.*, 1993). A number of biochemical tests are used to distinguished *V. vulnificus* from *V. parahaemolyticus* as shown in (Table 2). *V. vulnificus* further coprises two biotypes (Biosca *et al.*, 1991). Biotype 1 and biotype 2 which can be distinguished by the biochemical tests described by (Biosca *et al.*, 1996) as shown in (Table 3).

Table 2 : Differentiation of the Argnine-Negative, Lysine-postive Species *V. alginolyticus*, *V. parahaemolyticus*, and *V. vulnificus*

Property	Reaction of		
	V. alginolyticus	*V. parahaemolyticus*	*V. vulnificus*
Fermentation of			
cellobiose	-	-	-
lactose	-	-	[+]
salicin	-	-	+
Growth in			
8% NaCl	+	+	-
10 % NaCl	V	[+]	-
Voges - Proskauer	+	-	-
Sucrose fermentation	+	-	[-]
L- Arabinose fermentation	-	[+]	-

Symbols: + almost all strains positive, usually 90% or more; [+], most strains positive, usually 75-89; V, strain to strain variation, 26-74% positive, [-], few strains positive, usually 11-25%; -, all most no strain positive, usually0-10%.
Source: Farmer *et al.*, 1985, Kelly *et al.*, 1992.

Table 3: Biochemical characteristics differentiating biotypes 1 and 2 of *V. vulnificus*[a]

Characteristics	Biotype 1	Biotype 2
O/129 sensitivity		
10 µg	d	-
150 µg	d	-
Ornithine decarboxylase	+	d
Growth at 42^0C	d	-
Acid from D-mannitol	d	-
Production of Indole	+	-
Utilization of:		
D-Mannitol	d	-
Lactose	d	-

[a]Source Biosca *et al.*, (1996).

Biochemical Characteristics of *V. parahaemolyticus*

V.parahaemolyticus is a facultative anaerobe capable of both respiratory and fermentative metabolism (Baumann, *et al.*, 1984). Molecular oxygen is a universal electron acceptor for the *Vibrio* species. They neither denitrify nor fix molecular nitrogen. *V. parahaemolyticus*, like all *Vibrio* species, is a chemo-organotroph and can grow in minimal medium containing D-glucose and NH_4Cl.The species. ferments D-glucose with

the production of acid and no gas. It multiplies over a wide temperature range from less than 20 to 40^0C. Sodium ions stimulate the growth of all *Vibrio* species. and are required for most by these species (Varnam *et al.*, 1991).*V. parahaemolyticus* can multiply in substrates with salinity ranging from 1% to 8% of NaCl. The organism grows best in media with a 2-3% NaCl or sea water base. It is therefore not surprising that *V. parahaemolyticus* is primarily an inhabitant of aquatic environments with a wide range of salinities and is commonly found on the surface and in the gut of marine and estuarine animals (Chan *et al.*, 1989).

V. vulnificus, *V. parahaemolyticus* and *V. alginolyticus* can be differentiated (Farmer *et al.,* 1985) according to key reactions shown in (Table 2). *V. parahaemolyticus* can be differentiated from *V. vulnificus* by its growth in 8% NaCl, its ability to ferment arabinose, and its inability to ferment cellobiose, lactose, and salicin. *V. parahaemolyticus* fails to grow in 10% salt, to produce acetoin, and to ferment sucrose,.*V. alginolyticus* is positive in all three tests (Mercedes and Blanch *et al.*, 1994).

Kanagawa Test

In 1968, an observation that was important for the biochemical characterization of *V. parahaemolyticus* and ultimately critical to the distinction of pathogenic strains was made by Miyamoto *et al.* It was found that isolates from clinical cases of gasteroenteritis were haemolytic, whereas those recovered from seawater and seafish were non-haemolytic on a special medium (Wagatsuma agar, Wagatsuma, *et al.,* 1968) containing

human red blood cells. The thermostable extracellular haemolysin responsible for this difference (Sarkar *et al.,* 1987, Terai *et al.*, 1991, Yoh *et al.*, 1991, Suzuki *et al.*, 1994) was designated the Kanagawa phenomenon to distinguish it from other hemolytic factors present in *Vibrio* species (Kita *et al.,* 1993), regardless of their source. The results of an extensive survey revealed that 96% of the 2,720 strains isolated from patients with diarrhea were positive when tested for Kanagawa hemolysin, whereas only 1% of the 650 strains from seafish were Kanagawa positive (Sakazaki *et al.,* 1968, Wagatsuma *et al.*, 1974).

Growth and survival characteristics of *V. vulnificus*

V. vulnificus, like most vibrios, is not fastidious and is easily cultured in variety of media (Hsu *et al.*, 1998). Optimal NaCl concentrations appear to be between 1% and 3%, although 0.5% NaCl present in many routine laboratory media provides very good growth. Kelly *et al.,* (1982) reported similar results, with no growth at less than 0.1% or greater than 5% NaCl, and optimal growth in 1-2% NaCl. The optimal temperature for growth of *V. vulnificus* is 37^0C (Kelly *et al.,* 1982).

Growth is luxurient in unsupplemented heart infusion or brain heart infusion (BHI) broth. The presence of glucose in BHI medium represses the production of the hemolysin cytotoxin produced by *V. vulnificus* (Kreger *et al.*, 1981) and they found that growth in BHI totally represses production of the albuminase normally produced by *V.*

vulnificus (Oliver *et al.*, 1986) . Epidemiological data indicate that *V. vulnificus* infections occur only during warm months and this species is rarely isolated from cold waters (David and Ruple *et al.*, 1992). *V. vulnificus* is sensitive to cold and experiences metabolic damage at low temperatures, which may explain the organisms seasonal occurrence. *V. vulnificus* can be isolated from the marine environment only during those month when water temperatures are warm (Kaysner *et al.*, 1987 and O' Neill *et al.*, 1990). A similar inability to culture estuarine vibrios when water column temperatures are low has been reported for *V. parahaemolyticus* (Kelly *et al.*, 1988), *V. mimicus* (Chowdhury *et al.*, 1989), and *Vibrio cholerae* (Brayton *et al.*, 1987).

Characteristics of *V. vulnificus* Disease

Primary Septicemia

V. vulnificus is unusual in its ability to produce disease by two different portals of entry. Following ingestion of the bacterium, a primary septicemia is produced which carries a high fatality rate (Kelly *et al.*, 1981, Janda *et al.*, 1988, Rippey *et al.*, 1992, Jackson *et al.*, 1997) . Alternatively, the bacterium may enter through a skin lesion as simple as an insect bite. A summary of 57 cases of primary septicemia and 54 cases of wound infections produced by *V. vulnificus* (Blake *et al.*, 1979, Bonner *et al* 1983, Tacket *et al.*, 1984, Johnston *et al.*, 1985, Howard *et al.*, 1986, Bantavala *et al.*, 1997) is shown in (Table 4).

Tabe 4

A summary of 57 cases of primary septicemia and 57 cases of wound infections produced by *V. vulnificus*

	Primary septicemia (n=57)	Wound infections (n=54)
Age (yr)	53	63
Males	82%	79%
Symptoms		
Fever	94	85
Chills	86	86
Hypotension	43	19
Nausea	60	37.5
Vomiting	35	30
Diarrhea	30	7
Abdominal pain	44	0
Secondary lesions	69	6
Chronic disease	94	57
Liver disease	76	21
Diabetes	9	15
Cancer	3	13
Raw oyster consumption	85	11
Sea water / shellfish exposure	19	89
Median incubation time (h)	26	16
Amputation/debridement/grafting	38	58
Fatal	56	22

Source: Bonner *et al*., (1983).

The primary reservoir of *V. vulnificus* in nature is sea water, and case studies of persons developing *V. vulnificus* septicemia have consistently implicated raw sea food, especially oysters, in the epidemiology of this disease. About 85% of the patients summarized in (Table 4) had a recent history of raw oyster consumption. Environmental data indicating significant numbers of *V. vulnificus* cells in these oysters correlated well