UNIVERSITI PUTRA MALAYSIA

MOLECULAR CHARACTERISATION OF VIBRIO VULNIFICUS ISOLATED FROM COCKLES (ANADARA GRANOS A) AND SHRIMPS (PANAEUS INDICUS) IN MALAYSIA

TENGKU ARBRIZAL FARIZAL BT TENGKU AHMAD

FSMB 2001 12

MOLECULAR CHARACTERISATION OF *VIBRIO VULNIFICUS* ISOLATED FROM COCKLES (*ANADARA GRANOSA*) AND SHRIMPS (*PANAEUS INDICUS*) IN MALAYSIA

By

TENGKU AHBRIZAL FARIZAL BT TENGKU AHMAD

Thesis Submitted in Fulfilment of the Requirement for the Degree of Master of Science in the Faculty of Food Science and Biotechnology Universiti Putra Malaysia

June 2001

To my parents,

Tengku Ahmad Bin Tengku Salleh &

Nik Badieh Binti Nik Zaid, with love…

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

MOLECULAR CHARACTERISATION OF *VIBRIO VULNIFICUS* ISOLATED FROM COCKLES (*ANADARA GRANOSA*) AND SHRIMPS (*PANAEUS INDICUS*) IN MALAYSIA

By

TENGKU AHBRIZAL FARIZAL BT TENGKU AHMAD

June 2001

Chairman : Associate Professor Dr. Son Radu, Ph.D.

Faculty : Food Science and Biotechnology

A prevalence study was conducted to determine the presence of *Vibrio vulnificus* in cockles (*Anadara granosa*) and shrimps (*Panaeus indicus*) in Malaysia. Out of 19 samples examined from cockles and 42 samples from shrimps, 10 and 12 samples were positive for *V. vulnificus* respectively. Twenty-nine strains from cockles and 21 strains from shrimps were selected for further studies. The strains isolated from cockles and shrimps comprised both biotype 1 (17 of 29) and biotype 2 (15 of 21). All strains were tested for their susceptibility to selected antimicrobial agents and were examined for the presence of plasmid DNA. The antimicrobial susceptibility patterns indicated that most of the strains from cockles were resistant to kanamycin (96.55%), carbenicillin (86.21%) and bacitracin (82.76%). All strains from shrimps were resistant to penicillin (100%),

bacitracin (61.9%) and carbenicillin (71.4%). None of the strains were resistant to gentamicin. Twenty-six and 18 antibiotic resistance patterns were observed from cockles and shrimps, respectively. The MAR index for cockle strains was between 0.13 and 0.8, whereas, for shrimp strains, the MAR index was between 0.067 to 0.73. Thirty strains were found to contain plasmid DNA, ranging in sizes from 1.5 to 35.8 megadalton. Only five strains (strain VC9, VC26, VS6, VS18 and VS19) of *V. vulnificus* in this study harboured a large plasmid of 35.8 MDa. The conjugation study was done to investigate the transferability of the R plasmids among *V. vulnificus* strains to recipient *E. coli* K12 strain. However, no R plasmid transfer was observed. RAPD-PCR fingerprinting was used to differentiate *V. vulnificus* strains. Primers GEN 1-50-01 (5'-GTGCAATGAC3-') and GEN 1-50-08 (5'-GGAAGACAAC3-') illustrated polymorphism in most strains tested, with the DNA band sizes ranging from 0.25 to 10.0 kilobase pair. Dendrograms generated showed that primer GEN 1-50-01 illustrated 46 RAPD patterns. All strains were well separated at 100% similarity level, except strain VC16, VC23 and VS17, which were found to be clustered together. Primer GEN 1-50-08 illustrated 47 RAPD patterns and all strains were well separated into single strains at 85% similarity level. *V. vulnificus* strains from both sources were also analysed by SDS-PAGE fingerprinting to observe the protein profiles. Dendrogram generated showed that *V. vulnificus* strains illustrated 50 protein profiles, with sizes ranging between 3.4 to 212 kilodalton. The cluster analysis from both RAPD profiles and protein profiles indicates that there is a high degree of genetic diversity within *V. vulnificus* strains examined.

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

PENCIRIAN MOLIKULAR *VIBRIO VULNIFICUS* DARI KERANG (*ANADARA GRANOSA*) DAN UDANG (*PANAEUS INDICUS*) DI MALAYSIA

Oleh

TENGKU AHBRIZAL FARIZAL BT TENGKU AHMAD

Jun 2001

Pengerusi : Profesor Madya Dr. Son Radu, Ph.D.

Fakulti : Sains Makanan dan Bioteknologi

Kajian terhadap penyebaran pencilan *Vibrio vulnificus* telah dilakukan untuk menentukan kehadirannya dalam kerang (*Anadara granosa*) dan udang (*Panaeus indicus*) di Malaysia. Sembilan belas sampel kerang dan empat puluh dua sampel udang telah dikaji, dimana 10 dan 12 sampel kerang dan udang adalah positif terhadap pencilan *V. vulnificus*. Dua puluh sembilan pencilan dari kerang dan dua puluh satu pencilan dari udang telah dipilih untuk kajian seterusnya. Pencilan daripada kedua-dua sumber adalah biotip 1 dan 2, di mana kebanyakkan pencilan daripada kerang adalah biotip 1 (17 daripada 29 pencilan) dan pencilan daripada udang adalah biotip 2 (15 daripada 21 pencilan). Kesemua pencilan diuji kepekaan terhadap antibiotik dan kehadiran DNA plasmid. Hasil kajian terhadap kepekaan antibiotik mendapati pencilan daripada kerang adalah rentan terhadap kanamycin (96.55%), carbenicillin (86.21%) dan bacitracin (82.76%). Pencilan daripada udang pula menunjukkan kepekaan terhadap

penicillin (100%), bacitracin (61.9%) dan carbenicillin (71.4%). Tiada pencilan daripada udang rentan terhadap gentamicin. Sebanyak dua puluh enam dan lapan belas profil kerentangan antibiotik, masing-masing daripada kerang dan udang telah dikenalpasti. MAR index bagi pencilan daripada kerang adalah di antara 0.13 hingga 0.8, manakala MAR index bagi pencilan daripada udang adalah di antara 0.067 hingga 0.73. Tiga puluh pencilan didapati mengandungi DNA plasmid yang bersaiz di antara 1.5 hingga 35.8 megadalton. Hanya lima pencilan (pencilan VC9, VC26, VS6, VS18 dan VS19) mengandungi plasmid DNA bersaiz 35.8 MDa. Proses konjugasi telah dijalankan untuk menentukan kebolehan strain *V. vulnificus* memindahkan R plasmid kepada bakteria *E. coli* K12, tetapi hasil kajian mendapati tiada R plasmid dipindahkan. Analisis RAPD-PCR digunakan untuk mengamplikasi DNA genomik pada pencilan *V. vulnificus*. Primer GEN 1-50-01 (5'-GTGCAATGAC3-') dan GEN 1-50-08 (5'-GGAAGACAAC3-') telah menghasilkan polimofisme dalam kebanyakkan pencilan yang diuji, dengan saiz fragmen DNA di antara 0.25 hingga 10.0 kilobase. Keputusan daripada dendrogram primer GEN 1-50-01 mendapati 46 profil RAPD dihasilkan dan kesemua pencilan dapat dibezakan pada 100% kesamaan kecuali pencilan VC16, VC23 dan VS17 yang diklusterkan bersama. Manakala dendrogram primer GEN 1-50-08 menghasilkan 47 profil RAPD dan kesemua pencilan dalam dendrogram ini dapat dibezakan kepada kluster berasingan pada 85% kesamaan. Profil protein pada pencilan *V. vulnificus* juga telah dikaji dengan kaedah analisis SDS-PAGE. Dendrogram menunjukkan 50 profil protein dihasilkan dengan saiz protein di antara 3.4 hingga 212 kilodalton. Kesemua pencilan dalam dendrogram ini dapat dibezakan kepada kluster berasingan pada kesamaan 90%. Analisis kluster daripada profil RAPD dan profile protein menunjukkan darjah kepelbagaian genetik yang tinggi dalam pencilan *V. vulnificus*.

ACKNOWLEDGEMENTS

I would like to take this opportunity to thank my supervisor Assoc. Prof. Dr. Son Radu for his advice and guidance throughout the entire course of this project. Special thanks to my co-supervisors, Dr. Abdul Reezal Abdul Latif and Dr. Abdul Rahim Mutalib for their support and encouraging interest in my project.

I also like to thank all my friends in the Microbiology Laboratory and my housemates for helping me in completing this thesis.

Finally, to both of my parent and my brothers, Faisal, Fathy and Faris, thank you so much for your support, understanding and encouragement throughout my study.

I certify that an Examination Committee met on 30th June 2001 to conduct the final examination of Tengku Ahbrizal Farizal Bt. Tengku Ahmad on her Master of Science thesis entitled "Molecular Characterisation of *Vibrio vulnificus* Isolated from Cockles (*Anadara granosa*) and Shrimps (*Panaues indicus*) in Malaysia" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 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:

GULAM RUSUL RAHMAT ALI, Ph.D.
Professor
Faculty of Food Science and Biotechnology
Universiti Putra Malaysia
(Chairman)

SON RADU, Ph.D.
Associate Professor
Faculty of Food Science and Biotechnology
Universiti Putra Malaysia
(Member)

ABDUL REEZAL ABDUL LATIF, Ph.D.
Faculty of Food Science and Biotechnology
Universiti Putra Malaysia
(Member)

ABDUL RAHIM MUTALIB, Ph.D.
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

MOHD GHAZALI MOHAYIDIN, Ph.D.
Professor/ Deputy Dean of Graduate School,
Universiti Putra Malaysia

Date: 13 AUG 2001

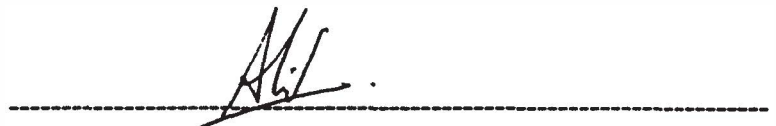
This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfilment of the requirement for the degree of Master of Science.

AINI IDERIS, Ph.D.
Professor/ Dean of Graduate School,
Universiti Putra Malaysia

Date: 09 AUG 2001

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.



TENGKU AHBRIZAL FARIZAL BT. TENGKU AHMAD

Date: 13/8/2001

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

Page

LIST OF ABBREVIATIONS

CCC	covalently closed circular
CDC	Center for Disease Control
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DH_2O	Distilled water
FDA	Food and Drug Administration
E. coli	*Escherichia coli*
g	gram
g	*gravity*
kb	kilobase
kDa	kilodalton
KIA	Kliglers iron agar
KOH	kalium hydroxide
LB	Luria-Bertani
M	molar
MDa	Megadalton
ml	milliliter
mRNA	messenger RNA
N	Newton
NaCl	Sodium chloride
PCR	polymerase chain reaction
PCI	Phenol-Chloroform-isoamyl alcohol
RAPD	random amplified polymorphic DNA
RNA	ribonucleic acid
SDS-PAGE	Sodium dodecylsulfate- polyacrylamide gel electrophoresis
Taq	*Thermus aquaticus*
tra	transfer
TCBS	Thiosulfate citrate bile salts sucrose
TSB	Trypticase soy broth
UV	ultra violet
µl	microliter
µg	microgram
V. alginolyticus	*Vibrio alginolyticus*
V. parahaemolyticus	*Vibrio parahaemolyticus*
V. vulnificus	*Vibrio vulnificus*
V	volt

CHAPTER I

INTRODUCTION

Vibrio vulnificus was first described by Riechelt *et al.* in 1976 as *Beneckea vulnifica*. However, this name was not widely used, and Farmer (1979) proposed that the species be called *Vibrio vulnificus* (Baumann *et al.*, 1980; Farmer, 1980). The discovery of this organisms began in 1964, when Special Bacteriology Section of Clinical Bacteriology Branch, Atlanta occasionally received isolates from extraintestinal sites that were first thought to be variants of *V. parahaemolyticus*. Later, these organisms were differentiated from *V. parahaemolyticus* by several biochemical reactions, including fermentation of lactose, and were referred to as the lactose-fermenting (L+) *Vibrio*. Results of deoxyribonucleic acid reassociation studies confirm that this organism to be a separate species (Blake, 1980; Twedt *et al.*, 1984).

V. vulnificus is widely distributed in aquatic environments. The presence of these organisms is not associated with pollution. These bacteria are natural marine organisms that thrive in shallow, coastal waters in the tropics and temperate climates throughout most of the world (Thampuran and Surendran, 1998). Raw seafood such as oysters, eels, shrimps and fish are the example sources of these bacteria (Lee *et al.*, 1997). *V. vulnificus* is both a human and marine animal pathogen. It can cause three types of human infections; primary septicaemia, gastroenteritis and wound infections. It is also capable of causing a rapidly fatal infection (Warner and Oliver, 1998; Moreno and Landgraf, 1998).

Almost every year, *V. vulnificus* infections cause fatilities in individuals who consumed seafood. Banatvala *et al.* (1997) reported that in 1988, a regional surveillance programme in four states along the Gulf Coast, USA found an annual rate of *V. vulnificus* infections of 0.6 per million persons and a case fatality rate of 22%. Persons with pre-existing liver disease and compromised immune system have a higher case-fatality rate (Vickery *et al.*, 2000).

The problem of drug resistance among bacteria including *V. vulnificus* has been a major problem in clinical and public health nowadays. The increased rate of antibiotic resistance could have arisen from a combination of overuse of antibiotics as well as failure to take adequate precautions to control the spread of hospital infection (Lim, 1990). Transfer of resistance factor (R-factor) has been observed *in vivo* in subjects under chemotherapy. The genes that make bacteria resistant to antibiotics are usually encoded not on their chromosomes but on smaller self-replicating companion loops of DNA called plasmids (Saunders,1984).

A variety of DNA-based typing methods have been applied to identify and characterized *V. vulnificus* species, including plasmid profiles, antibiotic susceptibility, polymerase chain reaction (PCR) analysis and protein-based typing method such as SDS-PAGE analysis. In this study, cockles and shrimps were sampled from different locations in Malaysia such as Selangor, Negeri Sembilan, Penang and Sarawak. Cockles and shrimps are popular ingredients in several types of local foods as well as in other Asian countries. These shellfish are frequently consumed in a semi-cooked condition.

The presence of this pathogen may be a hazard to consumers of raw shellfish, especially to persons most susceptible to *V. vulnificus* septicemia.

The ability to identify *V. vulnificus* quickly and reliably can be important for establishing the causes of contamination and precise determination of pathogenic strains. It seems that not all *V. vulnificus* strains are pathogenic (Doyle, 1998; Stephenson,1994). An understanding of genetic variability in *V. vulnificus* is important to differentiate the pathogenic from the non-pathogenic strains and to determine the prevalence of *V. vulnificus* in our area . The objectives of this study are to;

i) isolate and identify *V. vulnificus* from cockle (*Anadara granosa*) and shrimp (*Panaeus indicus*).

ii) determine the antibiotic susceptibility of *V. vulnificus* strains using disc-diffusion method.

iii) determine the plasmid profiles among *V. vulnificus* strains.

iv) determine the ability to transfer resistance plasmid among *V. vulnificus* strains and *E. coli* K12 strain.

v) determine the DNA fingerprint of *V. vulnificus* by random amplified polymorphic DNA (RAPD) analysis.

vi) determine the protein profiles of *V. vulnificus* strains by SDS-PAGE analysis.

CHAPTER II

LITERATURE REVIEW

2.1 *Vibrio vulnificus*

2.1.1 Taxonomy

V. vulnificus is a member of the genus *Vibrio* and is defined as gram-negative bacterium, with asporogenous rod that is straight or have a single, rigid curve. They are motile and most have a single flagellum when grown in liquid medium (Baumann *et al.*, 1980; Farmer, 1980). Some species produce unsheathed peritrichous flagella on solid medium. These bacteria produce oxidase and catalase, and ferment glucose without gas. They are facultative aerobe, best grown in alkaline medium at pH 8.0 and with the presence of 1% NaCl (Blake, 1980; Twedt *et al.*, 1984). The genus *Vibrio* is classified in the family *Vibrionaceae* according to the International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of *Vibrionaceae* (1992). There are three other genera in this family; *Photobacterium*, *Plesiomonas* and *Aeromonas* (Farmer *et al.*, 1985). Table 1 list the differentiating characteristics of all the four genera.

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

Property	*Vibrio*	*Photobacterium*	*Plesiomonas*	*Aeromonas*
Mole % Guanine + Cytosine	38 - 51	40 – 44	51	57 – 63
Sensitive to Compound O/129	+	+	+	-
D-mannitol fermentation	+	-	-	+
Na^+ ion required for growth or stimulates growth	+	+	-	-

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

The *V. vulnificus* species comprise two biotypes distinguished by certain traits and host range. Biotype 1 is an opportunistic human pathogen, which is capable of producing fatal disease after ingestion of contamined raw shellfish, or after wound infection (Amaro and Biosca, 1996). Individuals with underlying disease, such as liver cirrhosis are especially at risk of infection by this species. Biotype 1 strains can be isolated from estuarine waters and marine animals. Although this species has been present in tank water and in gills of healthy cultured eels, it is not pathogenic for eels (Coleman *et al.*, 1996). Biotype 1 is serologically heterogeneous and phenotypically similar to the type strain of the species. Meanwhile, biotype 2 is an eel pathogen that has been recovered from diseased eels but never from water or other marine animals (Biosca *et al*, 1996a). However, Son *et al*.(1998a) reported on the isolation of biotype 2 from cockles.

The biotype 2 strain is serologically homogeneous and can be differentiated from biotype 1 strains by their negative response to the indole test (Amaro and Biosca, 1996). Biotype 2 is typically recovered from diseased eel but is also reported to cause illness in humans after handling of eels. The first case of wound infection caused by this biotype 2 was reported in Denmark in 1991 and again in 1994 (Hoi *et al.*, 1998). Biotype 2 is also pathogenic for mice and is able to express the same virulence factors as those of biotype 1 such as iron uptake systems and production of exotoxins (Biosca *et al.*, 1996b; Hoi *et al.*, 1998). As reported by Amaro *et al.* (1995), this biotype was able to survive outside eels and uses water as a route of infection. All this data lead to the believe that biotype 2 is probably an opportunistic pathogen for humans.

2.1.2 Ecology of *V. vulnificus*

V. vulnificus is widely distributed in aquatic environments, especially in the coastal sea waters of the tropics and temperate climates, and contaminate filter-feeding seafood (Tacker *et al.*, 1984; Oliver *et al.*, 1995; Thampuran and Surendran, 1998). It occurs naturally and often present in clean waters, including those that are approved for the harvest of oysters and clams (Anderson *et al.*, 1995). This bacteria is called halophilic because they require a saltwater environmental for growth (Doyle, 1998; Div. of Bacterial and Mycotic Disease, 2000). The presence of this bacteria has been observed in areas with low to moderate salinity of 5 to 20% (Tison and Kelly, 1986). *V. vulnificus* is easily cultured during warm months, between April to October (Anderson *et al.* 1995; Warner and Oliver, 1998) and in cold months it is difficult to do so as the numbers of organisms are very low (Oliver *et al.*, 1995; Tamplin *et al.*, 1996).

A report by Anderson *et al*. (1995) said that the illnesses and infections associated with this bacterium are most prevalent during warm months of the year, primarily April through October. According to the reports by Roberts (1979) and Tison *et al*. (1986) this species grow well in high temperature (22 – 43°C) and they are not heat resistant. Hoi *et al*. (1999) reported that the concentration of *V. vulnificus* increased when the temperatures exceed 20°C for several weeks during warm summer. It also has been found that this organism can survive for up to 2 weeks in commercial shell stock and at least 6 days in shucked oysters under refrigeration (Kaysner *et al*., 1989). These results lead the scientists to believe there might be a correlation between the bacterium's presence with salinity and temperature of the water.

2.1.3 Disease cause by *V. vulnificus*

V. vulnificus was recognized as a human pathogen by Blake *et al.* in 1979. It has gained much attention in recent years because of its association with various disease manifestations. According to the U.S Food and Drug Administration, no major outbreak of illness have been attributed to this organism in the year 2000. However, between 1988 and 1995, the Center for Disease Control (CDC) received over 300 reports of *V. vulnificus* infections from Gulf Coast State, US (Rowland, 1999; Div. of Bactrerial and Mycotic Disease, 2000). Seventy-two cases of *V. vulnificus* infection from eating raw oyster has been reported in Florida from 1981 to 1992 and 36 (50%) patients died, making this infection the leading cause of reported deaths from foodborne illness in