UNIVERSITI PUTRA MALAYSIA

MOLECULAR CHARACTERIZATION OF VIBRIO SPECIES ISOLATED FROM SEAWATER

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By

YUHERMAN

Thesis Submitted in Fulfilment of the Requirement for the degree of Doctor of Philosophy in the Faculty of Food Science and Biotechnology Universiti Putra Malaysia

July 2001

DEDICATION

TO BOTH MY PARENTS

H. MOCHAMMAD DIN BIN BURHAN

AND

HJ. MARDIANA BINTI SALEH

TO MY SON

FACHRUL FARIZAN

TO MY UNCLE

Drs. ASNOL AMRI ***AND*** FAMILY

FOR THEIR MORAL SUPPORT AND ENCOURAGEMENT

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

MOLECULAR CHARACTERIZATION OF *VIBRIO* SPECIES ISOLATED FROM SEAWATER

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YUHERMAN

July 2001

Chairman: **Associate Professor Dr. Son Radu**

Faculty: **Food Science and Biotechnology**

A study was conducted to determine the prevalence of *Vibrio* species in seawater samples obtained from the coast of Malacca, Penang (Batu Feringgi, George Town), and Terengganu, respectively.

Four *Vibrio cholerae* O139, 10 *V. cholerae* non-O1, 7 *V. cholerae* O1, 160 *Vibrio parahaemolyticus*, and 57 *Vibrio vulnificus* strains were isolated from 240 seawater samples 4/32 (0.13%), 3/32 (0 09%), 2/32 (0.06%), and 24/32 (0.75%) seawater samples obtained from Malacca were positive for *V. cholerae* O139, *V. cholerae* non-O1, *V. cholerae* O1, and *V. parahaemolyticus* strains, respectively. 6/30 and 7/30 seawater samples obtained from Batu Feringgi and George Town beaches were positive for *V. parahaemolyticus* strains, respectively. All the *V. parahaemolyticus* strains were Kanagawa-negative. Fifty seven *V. vulnificus* strains of biotype 1 were isolated from 11 (18.33%) of 60 seawater samples

obtained around Marang beach (Terengganu). The antibiotic resistance patterns of all *Vibrio* species strains tested showed that all were resistant to one or more of the antibiotics tested. Of 15 antibiotics tested against all the *Vibrio* species strains, *V. cholerae* O139 serogroup, clinical *V. cholerae* and environmental *V. cholerae* have Antibiotic Resistance Index (ARI) values of 0.55, 0.56, and 0.57, respectivcely. 89, 18, 4, 8, and 4 strains of *V. parahaemolyticus*, *V. vulnificus*, *V. cholerae* O1, *V. cholerae* non-O1, and *V. cholerae* O139 serogroups harboured plasmid(s) with sizes ranging from 1.3 to 16 MDa, respectively. 85/120 and 29/40 isolates of *V. parahaemolyticus* isolated from Malacca, and Penang were positive for the presence of *toxR* gene, respectively, and none the strains were positive for the *tdh*, *trh*, and *ctx* genes. All clinical strains of *V. cholerae* O1 and non-O1 and environmental isolates of *V. cholerae* O139 were positive for the *ctx* gene. The Randomly Amplified Polymorphic DNA – Polymerase Chain Reaction assay of environmental and clinical *V. cholerae* O1 and non-O1 strains generated 15, 15, 7, and 9 RAPD-types with primers Gen 15003, Gen 15005, Gen 15007, and Gen 15008, respectively. All *V. cholerae* strains generated a total of 30 RAPD-types based on primers used, whereas 48 RAPD-types were observed among the 57 *V. vulnificus* strains. The RAPD-PCR of *V. parahaemolyticus* generated 133 and 137 RAPD-types obtained with primers Gen 15001 and Gen 15002, respectively. Combination of both these primers generated 154 types for 160 strains of *V. parahaemolyticus*. 28 distinct *V. parahaemolyticus* clusters and 20 single isolates were observed at similarity level of approximately 70% based on ERI consensus. 6 major clusters and 2 single isolates were observed at the same

similarity level among clinical and environmental isolates of *V. cholerae*. Multiple PFGE banding patterns were observed among 28/160 representative strains of *V. parahaemolyticus* with sizes ranging <48 to 340 kb. Seven different PFGE patterns were identified among the 14 strains of clinical *V. cholerae*. Twelve PFGE patterns were observed among 14 representative of 57 strains of *V. vulnificus*. ERIC-PCR and RAPD-PCR methods gave higher resolution among the three molecular methods that were used in characterization of the *Vibrio* species isolated from seawater. ERIC-PCR appeared to have the best discriminatory power, followed closely by RAPD-PCR and Pulsed-Field Gel Electrophoresis.

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

PENCIRIAN MOLIKULAR SPESIS *VIBRIO* YANG DIASINGKAN DARIPADA AIR LAUT

Oleh

YUHERMAN

Julai 2001

Pengerusi: **Profesor Madya Dr. Son Radu**

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Suatu kajian telah dijalankan untuk mencirikan spesis *Vibrio* yang diasingkan daripada air laut dipantai Melaka, Penang (Batu Feringgi, George Town) dan Terengganu. Daripada 240 sampel air laut yang diuji, 4 *Vibrio cholerae* O139, 10 *V. cholerae* bukan-O1, 7 *V. cholerae* O1, 160 *Vibrio parahaemolyticus*, 57 *Vibrio vulnificus* diasingkan.

Kewujudan spesis *Vibrio* dalam sampel air laut daripada Melaka adalah sebanyak 4/32 (0.13%), 3/32 (0.09%), 2/32 (0.06%), 24/32 (0.75%) yang merupakan positif kepada kehadiran spesis *V. cholerae* O139, *V. cholerae* bukan-O1, *V. cholerae* O1 dan *V. parahaemolyticus*. Enam daripada 30 sampel air laut daripada pantai Batu Feringgi dan 7 daripada 30 sampel air laut daripada pantai George Town adalah positif dengan 16 dan 24 kultur *V. parahaemolyticus* dapat diasingkan. Kesemua

kultur *V. parahaemolyticus* adalah negatif-Kanagawa. Lima puluh tujuh kultur daripada biotip 1 untuk *V. vulnificus* diasingkan daripada 11 (18.33%) daripada 60 sampel air laut yang diperolehi daripada pantai Marang (Terengganu). Kesemua spesis *Vibrio* yang diuji untuk corak ketahanan antibiotik menunjukkan ketahanan terhadap satu atau lebih antibiotik. Daripada 15 antibiotik yang diuji terhadap spesis *Vibrio*, *V. cholerae* kumpulan sero O139, *V. cholerae* klinikal dan *V. cholerae* persekitaran menunjukkan nilai index ketahanan antibiotik sebanyak 0.55, 0.56 dan 0.57. Lapan puluh sembilan *V. parahaemolyticus*, 18 *V. vulnificus*, 4 *V. cholerae* O1, 8 *V. cholerae* bukan-Ol dan 4 *V. cholerae* kumpulan sero O139 mempunyai plasmid yang mempunyai julat saiz daripada 1.3 hingga 16 MegaDalton (MDa).

Penemuan gen virulen melalui pengeseian PCR untuk *V. parahaemolyticus* menunjukkan 75% (85/120) dan 73% (29/40) kultur *V. parahaemolyticus* daripada Melaka dan Penang memberikan bacaan positif kepada kehadiran gen *toxR*. Namun demikian, tiada bacaan positif diperolehi untuk 160 kultur *V. parahaemolyticus* untuk gen *tdh*, *trh* dan *ctx*. Kultur klinikal *V. cholerae* O1 dan bukan-O1 serta asingan daripada persekitaran untuk *V. cholerae* O139 adalah positif untuk gen *ctx*. Pengeseian RAPD-PCR untuk kultur klinikal dan persekitaran *V. cholerae* O1 dan bukan-O1 menghasilkan 15, 15, 7 dan 9 jenis RAPD dengan menggunakan primer Gen 15003, Gen 15005, Gen 15007 dan Gen 15008. Kesemua kultur *V. cholerae* menghasilkan 30 jenis RAPD untuk primer yang digunakan manakala 48 jenis RAPD diperhatikan dalam 57 kultur *V. vulnificus*. Keputusan RAPD-PCR untuk *V. parahaemolyticus* menghasilkan 133 dan 137 jenis RAPD dengan primer Gen

15001 dan Gen 15002. Gabungan kedua primer ini menghasilkan 154 jenis untuk 160 kultur *V. parahaemolyticus.*

Analisa corak ERIC-PCR dengan menggunakan dendrogram yang dihasilkan daripada perisian Gelcompar versi 4.1 menunjukkan 28 koloni *V. parahaemolyticus* yang spesifik dan 20 asingan diperhatikan pada peringkat yang sama sebanyak 70%. Dalam kultur *V. cholerae* yang diasingkan daripada sampel persekitaran dan klinikal, 6 koloni dan 2 asingan diperhatikan pada peringkat yang sama. Analisa corak ERIC-PCR dalam kultur *V. vulnificus* menghasilkan 12 kelompok yang utama dan 3 asingan pada peringkat yang sama.

Corak jaluran PFGE diperhatikan dalam cap jari yang dihasilkan daripada 28 wakil daripada 160 kultur *V. parahaemolyticus* dengan julat saiz lebih daripada 48 hingga 340 kilobasa. Tujuh corak PFGE telah dikenalpasti daripada 14 kultur klinikal *V. cholerae.* Dua belas corak PFGE telah dipastikan daripada 14 wakil daripada 57 kultur *V. vulnificus.*

Kaedah ERIC-PCR dan RAPD-PCR menghasilkan resolusi yang lebih tinggi diantara tiga kaedah molikular yang digunakan untuk pencirian spesis *Vibrio* yang diasingkan dari air laut. ERIC-PCR lebih tinggi kuasa diskriminasi dan diikuti oleh RAPD-PCR dan PFGE.

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I certify that an Examination Committee met on 30th July 2001 to conduct the final examination of Yuherman on his Doctor of Philosophy thesis entitled "Molecular Characterization of *Vibrio* Species Isolated from Seawater" 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:

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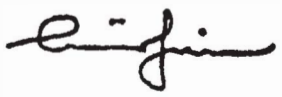
This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy.



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08 NOV 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.

YUHERMAN
Name of Candidate

Date: 22-08-2001

LIST OF TABLES

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LIST OF ABBREVIATIONS

APW	Alkaline Peptone Water
ARI	Antibiotic Resistance Index
cAMP	Cyclyc Adenosine MonoPhosphate
CDC	Center of Disease Control and Prevention
CFU	Colony-Forming Unit
CT	Cholera Toxin
CVC	Clinical *Vibrio cholerae*
DNA	Deoxyribo Nucleic Acid
EDTA	Ethylene Diamine Tetra Acetic
ERIC	Enterobacterial Repetitive Intergenic Consensus
EVC	Environmental *Vibrio cholerae*
GET	Glucose-EDTA-Tris base
GS	Gelatin Salt
IMR	Institute for Medical Research
KIA	Kligler Iron Agar
KP	Kanagawa Phenomenon
LIA	Lysine Iron Agar
MDa	Mega Dalton
min	minute(s)
mCPC	modified Cellobiose-Polymyxin B-colistin
NCCLS	National Committee for Clinical Laboratory Standard
O/F	Oxidase / Fermentation
ONPG	O-Nitrophenyl-β-Galactopyranoside
PCR	Polymerase Chain Reaction
PFGE	Pulsed-Field Gel Electrophoresis
RAPD	Randomly Amplified Polymorphic DNA
rpm	round per minute
SDS	Sodium Dodecyl Sulfate
sec	second(s)
SOD	Superoxidase Dismutase
tdh	Thermostable Direct Hemolysin
trh	Thermostable Direct Hemolysin-Related Hemolysin
TCBS	Thiosulfate Citrate Bile salts Sucrose
TCI	Thiosulfate Chloride Iodide
TSA	Trypticase Soy Agar
TSI	Triple Sugar Iron
TTGA	Taurocholate Tellurite Gerlatin Agar
VV	*Vibrio vulnificus*
VPP	*Vibrio parahaemolyticus* Penang
VPM	*Vibrio parahaemolyticus* Malacca
µg	Micro-gram
µl	Micro-liter

CHAPTER I

INTRODUCTION

Bacteria belonging to the genus *Vibrio* are widely distributed in the aquatic environment and are considered to be autochthonous bacteria in marine and estuarine waters (Nishibuchi and Kaper, 1985). There is considerable evidence that surface waters, especially estuary and lagoon systems with elevated salinity are natural habitats of *Vibrio* (Bockemuhl *et al*, 1986). The *Vibrio* from seawater have attracted increasing attention since one of them is an important cause of food poisoning in Japan, where numerous outbreaks have followed consumption of raw fish dishes. In 1951, Japanese researchers discovered that *Vibrios* are a common cause of illness among people who eat fish caught in bacterially contaminated water. Since then, it has been found to cause about a quarter of all reported cases of diarrhea in Japan, where fish in often eaten raw. Outbreaks have also been reported around the world, including almost all coastal states of the United States, and epidemics have occurred in Latin America and Southeast Asia. In the nineteenth century, pandemic of Asiatic cholera occurred from the Far East to Africa, other parts of Asia, Europe, and North America. During the present century, the disease appears to have been more or less limited to India and surrounding areas although epidemics have occurred in other parts of the world, including Egypt, Indonesia, Korea and the Philippines (Wistreich and Lachtman, 1988). Today, cholera infection had been reported from India, Bangladesh, Nepal, Burma, Thailand, Malaysia, Saudia Arabia, China and Pakistan, Italia, Spain, Japan, Australia (Venkateswaran *et al.*, 1989, Amaro *et al.*, 1990, Albert, 1994,

Desmarchelier *et al.*, 1995, Barbieri *et al.*, 1999, Radu *et al.*, 1999). However, the geographic locations of cholera outbreaks have not changed dramatically for hundreds of years. In the USA, cholera was a severe epidemic disease, and the pattern was very similar from 1800 to 1900, until the introduction of safe drinking water. Where cholera has occurred, as in Bangladesh, the pattern even in 1998 has not changed much.

There have been seven pandemics of cholera in recorded history. Even though the etiological agents of the first four pandemics are not known since they occurred in the time before such agents could be recognized, the last three pandemics are known to be due to *Vibrio cholerae* serogroup O1. The current global epidemics of cholera are part of the tail of the seventh pandemic, which began in the 1960s (Colwell and Huq, 1999) Since 1883 when the causative agent, the Cholera Vibrio, was discovered by Robert Koch in epidemic in Egypt and India (Davis *et al.*, 1973), the disease continues to spread in other parts of the world. The latest case, large epidemic of cholera-like disease occurs in Bangladesh caused by *Vibrio cholerae* O139 synonim Bengal. In this case, the causative agent or this organism was included into *Vibrio cholerae* non-O1. According to the Cholerae Working Group Report (1994), epidemics of cholerae caused by *Vibrio cholerae* O1 occur regularly in Bangladesh, however, until lately *Vibrio cholerae* non-O1 has been associated with sporadic cases of diarrhoeal disease in many parts of the world. This epidemic began in December 1992 in Southern Bangladesh and spread throughout the country. By the end of March 107,297 cases of diarrhea and 1473 death had been reported (Albert, 1994). This disease has also a close relationship with major public health problem confronting developing countries,