UNIVERSITI PUTRA MALAYSIA

GENOTYPIC CHARACTERIZATION OF VIBRIO SP. ISOLATED FROM COCKLES OBTAINED IN PADANG, INDONESIA

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DEDICATION

This piece of work is dedicated to my lovely parents, who have always been by my side and given me the encouragement and support that carries me through my study period. Thanks for their endless love to me.

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

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Chairman: Noorjahan Banu Mohamed Alitheen, PhD

Faculty : Faculty of Biotechnology and Biomolecular Sciences

Vibrio parahaemolyticus is a gram negative bacterium that naturally found in warm marine environments. They commonly live in shellfish, oysters and cockles and cause gastrointestinal illness in humans. Most people become infected by eating raw or undercooked shellfish. In this study, 32 isolates of *V. parahaemolyticus* were isolated from cockles obtained from Padang, Indonesia. Presumptive identification of the isolates was performed by the following analysis: antibiotic resistance, plasmid profiling, specific PCR, random amplified polymorphic DNA (RAPD)-PCR and enterobacterial repetitive intergenic consensus (ERIC)-PCR. In the antibiotic resistance test, 16 types of antibiotics were tested against this bacterium. The isolates of *V. parahaemolyticus* showed variable antibiotic resistance pattern. They are most commonly resistant to amoxycillin, bacitracin, penicillin, teicoplanin, ampicillin and carbenicillin. In the plasmid profiling analysis, 14 different profiles were determined. The most common profiles for the isolates is profile no.2 which contains 4 plasmids with 5.6, 7.2, 42.4 and

54.0 kilobase pairs respectively. For *toxR* detection, all of the isolates were positive but they gave negative results in both *tdh* and *trh* detection. This indicated that they have less potential to cause diseases. They also showed high diversity in RAPD-PCR and also ERIC-PCR analysis. They showed many differences in the polymorphism pattern and can be divided into 5 major clusters in RAPD-PCR and 4 major clusters in ERIC-PCR. Application of phenotype and genotype based methods provides information about distribution, epidemiology and pathogenesis of *V. parahaemolyticus*. The data is important to identify sources of infection of this bacterium in the case of outbreaks occurrence in humans. This study also gives us preliminary information in monitoring the potential infection risk. It also can increase our awareness about the infection potential of these bacteria.

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

PENCIRIAN SECARA GENOTIP ISOLAT *VIBRIO* SP. DI DALAM KERANG DIPEROLEHI DARI PADANG, INDONESIA.

Oleh

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Vibrio parahaemolyticus adalah dari jenis gram negatif bacteria yang biasanya boleh di jumpai di kawasan laut yang bersuhu panas. Perumah mereka biasanya adalah haiwan berangka seperti tiram atau kerang. Bakteria ini boleh menyebabkan keracunan makanan yang melibatkan perut dan usus. Biasanya jangkitan ini disebabkan oleh pengambilan makanan laut yang mentah dan tidak masak sepenuhnya. Dalam kajian ini, sebanyak 32 strain bakteria telah dipencilkan dari kerang yang diperolehi dari Padang, Indonesia. Pencirian bakteria-bakteria ini adalah mengikut ketahanan kepada antibiotik, kehadiran plasmid, spesifik PCR, RAPD-PCR dan ERIC-PCR. Untuk ujian ketahanan antibiotik, sebanyak 16 jenis antibiotik telah diuji dan kebanyakan strain adalah tahan kepada amoxycillin, bacitracin, penicillin, teicoplanin, ampicillin and carbenicillin. Bagi ujian profile plasmid, profile yang paling kerap dijumpai ialah profile no 2 yang mengandungi 4 plasmid dengan saiz 5.6, 7.2, 42.4 and 54.0 kilobase pairs. Untuk ujian kehadiran *toxR*

gen menggunakan PCR, semua strain adalah positif manakala untuk kehadiran *tdh* dan *trh* gen semua strain adalah negatif. Ini menunjukkan strain-strain yang dikaji mempunyai potensi jangkitan yang rendah. Mereka juga menunjukkan kepelbagaian bentuk bagi ujian RAPD-PCR dan ERIC-PCR. Mereka memberikan bentuk-bentuk yang berbeza dan boleh dibahagikan kepada 5 rumpun utama bagi ujian RAPD-PCR dan 4 rumpun utama bagi ujian ERIC-PCR. Kajian terhadap fenotip dan genotip dapat memberikan maklumat tentang sebaran, salasilah genetik dan evolusi *V. parahaemolyticus*. Maklumat ini penting untuk digunakan dalam mengkaji jangkitan dan punca jangkitan bakteria ini. Ia juga dapat memberikan maklumat awal untuk mengawasi potensi jangkitannya dan sekaligus dapat meningkatkan kesedaran kita terhadap jangkitan bakteria ini.

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ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
APW	Alkaline peptone water
bp	Base pairs
CDC	Central for Disease Control and Prevention
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleoside triphosphates
ELISA	Enzyme linked immunosorbent assay
ERIC	Enterobacterial repetitive intergenic consensus
FDA	Food and Drug Administration
FEMS	Federation of European Microbiological Societies
GET	Glucose EDTA TRisHCl
GM	Genetic modified
IRUs	Intergenic repeat units
KAc	Potassium acetate
kb	Kilobase
KP	Kanagawa phenomenon
LB	Luria bertani
MAR	Multiple antibiotic resistance index
MBC	Minimum bactericidal concentration
MDa	Megadalton
MHA	Mueller-Hinton agar

MIC	Minimum inhibitory concentration
NOS	Nitric oxide synthases
ORFs	Open reading frames
PCI	Phenol-chloroform-isoamyl
PCR	Polymerase chain reaction
RAPD	Random amplified polymorphic DNA
REP	Repetitive extragenic palindromic
RFLP	Retriction –fragment length polymorphism
RNA	Ribonucleic acid
rRNA	ribosomal ribonucleic acid
SDS	Sodium dodecyl sulphate
TBE	Tris-borate-EDTA
TCBS	Thiosulfate/citrate/bile salt/sucrose agar
tdh	Thermostable direct haemolysin
trh	TDH-related haemolysin

CHAPTER I

INTRODUCTION

Vibrio parahaemolyticus is one of the most widely recognized pathogenic *Vibrio* species due to numerous outbreaks and it is wide occurrence in marine environment (Joseph *et al.*, 1983; Chiou *et al.*, 1991; Mead *et al.,* 1999). This microorganism can be found in high number during summer in the United States and Europe, but all year round in Southeast Asian. It was first shown to be an enteropathogen in 1951 (in Japan). The genus *Vibrio* consists of 28 species and twelve of them are recognized as human pathogens. The major species that contributed to the pathogenesis are *V. parahaemolyticus, V. cholerae, V. vulnificus, and V. alginolyticus.* In recent years, increasing awareness of the infections of some other *Vibrio* spp., including *V. mimiscus, V. fluvialis, V. hollisae, and V. damsela* have been witnessed (Baffone *et al.,* 2000). Totally, *V. parahaemolyticus* has been implicated as a cause of at least a quarter of total food borne diseases caused by vibrios (Feldhusen, 2000).

For many Southeast Asian country including Malaysia, export of seafood is an important economic sources. Recently, our export of tiger prawn to Italy was rejected due to the detection of *V. parahaemolyticus* (European Commission, 2004) and fails to meet the bacteriological standards of importing countries. Actually, pathogenesis of *V. parahaemolyticus* strongly related to the possession of virulence genes in their genome. Thermostable direct haemolysin (TDH) and TDH-related haemolysin (TRH) are considered as important virulence factors of this organism (Honda *et al.,*

1991). But for the non pathogenic species, both genes that codes for those product were absent. The associated symptoms after infection are gastroenteritis, wound infection, septicaemia and other consequences. In addition to its role in gastroenteritis, *V. parahaemolyticus* is known to cause extraintestinal infection in humans.

Marine environments are the natural habitat of many *Vibrio* species. *V. parahaemolyticus* can be isolated from sea water or marine organism like shellfish, plankton, and oyster. Typically, it can be recovered from most biological and physical structure in marine environments where environmental temperatures and condition are compatible with its growth (Labbe and Garcia, 2001). Seafood is known to be a major source of pathogenic *vibrios* worldwide and the studies about this species are important to provide an adequate consumer protection since seafood particularly shellfish is one of the important ingredients and diet in Southeast Asian countries. Identification of the source of contamination of seafoods with *V. parahaemolyticus* is important to study epidemiology of human infection and also for controlling. The development of polymerase chain reaction (PCR) assay for detection proved to be very useful. This method is rapid, sensitive, reliable and specific for microbial screening (Kim *et al.*, 1999; Cook *et al.*, 2002). PCR method also has been used to detect the virulence properties in samples isolates and hence very useful to detect and study the pathogenesis of *V. parahaemolyticus* (Tada *et al.*, 1992).

The main objective for research groups in UPM is to contribute to the study of *V. parahaemolyticus* distribution in Southeast Asia. This project was initiated to gather

information on the levels of *V. parahaemolyticus* contamination of the seafood obtained from water sources within the ASEAN region. The information gathered was to have contributed to the development of a relevant database for epidemiological and pathogenesis comparison purposes of *V. parahaemolyticus* in the ASEAN region. This study will be focused on the cockle samples from Indonesia. The occurrence of vibrios in Indonesia is not well documented and the occurrence of vibrios in seafood is not well understood. The true incidence of *V. parahaemolyticus* in Indonesia transmitted by seafood is not known, probably due to underreporting of cases and lack of proper study on this issue. Only Lesmana *et al.*, (2001) started to conduct studies about the infection of *V. parahaemolyticus* in Indonesia and no information is reported on the distribution of *V. parahaemolyticus* in Padang, Indonesia. In Malaysia, many similar studies were conducted by Ministry of Health and universities (Son *et al.*, 1998; HUKM, 2000; Son *et al.*, 2002). Comparison of the distribution of *V. parahaemolyticus* in the nearby location can be done and the data can be used for epidemiological and pathogenesis study, but the most important thing is to see whether geographical factor may influence the data or not. Understanding the ecology of *V. parahaemolyticus* and tracing virulence isolates in seafood are important issues, since seafood is one of the important ingredients and diet in Southeast Asia.

The contribution of this study to our country is to provide preliminary information about the *V. parahaemolyticus* distribution in neighbourhood country especially Indonesia and this information can be used as a considered factor in importing process. It is also important to monitor the potential risk infection from seafood in Indonesia. Public awareness of microorganisms transmission has increased

dramatically in recent years. Bacterial characterization methods have improved our ability to detect and provided tools for tracking sources of *V. parahaemolyticus* contamination throughout food systems, especially when the sampling locations are expose to industrial or human waste. Application of phenotype and genotype based methods also provides insight into the population genetics, epidemiology, ecology, and evolution of *V. parahaemolyticus*. The application technique of this study can be used to determine the pathogenesis of *V. parahaemolyticus* in our seafood and the information of this study can be used as a comparison to determine the source and the factor that contributed to the pathogenesis.

Characterization of this bacterium is essential to contribute preliminary information about this bacteria distribution and variation. In epidemiological study, typing method is required to provide information about an infection and also identification of sources of infection. The typing methods, either the phenotype or the genotype features, are based on the genomic and sometimes on the plasmid DNA properties. Phenotype methods that can be used are antibiotic resistance test, biotyping, serotyping and immunoblotting. Meanwhile, for genotype methods that can be used are plasmid profiling, random amplified polymorphic DNA (RAPD), Restriction-fragment-length-polymorphism (RFLP) and DNA sequencing analysis.

Objectives

The objectives of this study are to determine the occurrence and characterization of *V. parahaemolyticus* in cockle samples from Padang, Indonesia. The characterization of the isolated *V. parahaemolyticus* was done by their antibiotic resistance, plasmid