MOLECULAR CHARACTERIZATION OF *Vibrio alginolyticus* ISOLATED FROM *Orbicularia orbiculata* AND *Corbiculla moktiana*

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By

NOOR RASYILA MOHAMED NOOR

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

September 2007

DEDICATION

This piece of work is a token of dedication to my beloved parents, who have always be a good friend, a good critic and a good supporter and also encouraged me a lot throughout my study. Thank you for their endless love and always be by my side.

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

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Chairman: Zaiton binti Hassan, PhD

Faculty: Food Science and Technology

Bivalves are prone to bacterial contamination and can be health risk to the consumers. Antibiotic resistance, plasmid profiling and randomly amplified polymorphic DNA analysis (RAPD-PCR) were used to characterize and to detect the clonal relatedness of a total of 60 strains of *Vibrio alginolyticus* isolated from bivalve (*Orbicularia orbiculata*) obtained from Tanjung Karang, Selangor, Malaysia and bivalve (*Corbiculla moktiana*) from Lake Sangkarak, Padang, Indonesia. Antibiotic susceptibility testing to 13 antibiotics showed that all (60) strains were 100% susceptible to gentamicin. All *V. alginolyticus* isolated from *O. orbiculata* were 100% resistant to penicillin G. In contrast, there was variability of resistance for *C. moktiana* isolates towards antibiotic tested. The common resistant patterns for the isolates were resistant to three antibiotics but they did not share the same type of multiple antibiotic resistance. 46.7% and 76.7% of the plasmid occurrence rates were observed among *V.*

alginolyticus isolated from bivalve *O. orbiculata* (Malaysia) and *C. moktiana* (Indonesia), respectively. The plasmid sizes ranged between 3.4 to 27.8 kbp with 18 plasmid patterns. A large number of strains (38.3%) were devoid of plasmid but were resistant to penicillin G, nalidixic acid and chloramphenicol. No correlation could be established between the presence of plasmids and antibiotic resistant because many resistant strains were without plasmid. For RAPD-PCR analysis, ten 10-mer primers were screened for both samples and only one primer – primer 3 (Gold - Oligo OPAR 3- 5' GTGAGGCGCA 3') showed reproducible and clear banding patterns. Gold-Oligo OPAR 3 produced 59 RAPD patterns. Dendrogram was constructed and analyzed. Four clusters with four untypeable strains were generated. There were similar clones isolated from *O. Orbiculata* (Malaysia) and *C. moktiana* (Indonesia) and also clones specific to either *O. Orbiculata* or *C. moktiana*. The high level of genomic diversity among these isolates irrespective of geographic origin was confirmed by this technique. It was suggested that strains of *V. alginolyticus* from both bivalve species were closely related to each other although they were isolated from different geographical areas. The aquatic environment including the bivalves is implicated as the reservoirs for *V. alginolyticus* and consequently they are responsible for transmission to human. The findings stressed the importance of this bacteria to food safety, public health an even economic.

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

PENCIRIAN SECARA MOLEKULAR *Vibrio alginolyticus* YANG DIPENCILKAN DARI *Orbicularia orbiculata* DAN *Corbiculla moktiana*

Oleh

NOOR RASYILA MOHAMED NOOR

September 2007

Pengerusi: Zaiton binti Hassan, PhD

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SIput cenderung kepada kontaminasi bakteria dan boleh mendatangkan risiko kepada kesihatan pengguna. Ujian kerintangan antibiotik, pencirian plasmid dan analisis *randomly amplified polymorphic DNA analysis* (RAPD-PCR) digunakan untuk mencirikan dan mengesan perhubungan klonal sejumlah 60 strain *V. alginolyticus* dipencilkan daripada siput (*Orbicularia orbiculata*) yang diperolehi dari Tanjung Karang, Selangor, Malaysia dan siput (*Corbiculla moktiana*) dari Tasik Sangkarak, Padang, Indonesia. Ujian pengaruh antibiotik terhadap 13 antibiotik menunjukkan semua (60) strain 100% mudah terpengaruh kepada gentamicin. Semua *V. alginolyticus* yang dipencilkan daripada *O. orbiculata* adalah 100% rintang terhadap penicillin G. Sebaliknya, terdapat kepelbagaian dalam kerintangan pencilan *C. moktiana* terhadap antibiotik-antibiotik yang diuji. Corak kerintangan yang lazim untuk pencilan ini adalah rintang terhadap tiga antibiotik tetapi mereka tidak berkongsi kerintangan antibiotik pelbagai yang sama. 46.7% dan 76.7% kadar kewujudan plasmid diperhatikan dikalangan pencilan *V. alginolyticus* masing-masing dari siput *O. orbiculata* (Malaysia) dan *C. moktiana* (Indonesia). Saiz plasmid dianggarkan antara 3.4 to 27.8 kbp dengan 18 corak plasmid. Sebilangan besar strain yang rintang (38.3%) tidak mempunyai plasmid tetapi rintang terhadap penicillin G, asid nalidisik dan kloramfernicol. Tiada perkaitan dapat diterbitkan antara kehadiran plasmid dan kerintangan antibiotiklkerana kebanyakan strain tiada plasmid. Dalam analisis RAPD-PCR, sepuluh primer 10-mer disaring untuk kedua-dua sample dan hanya satu primer – primer 3 (Gold - Oligo OPAR 3- 5’ GTGAGGCGCA 3’) menunjukkan terbitan dan corak jaluran yang jelas. Gold-Oligo OPAR 3 menerbitkan 59 corak RAPD. Dendrogram dibina dan dianalisa. Empat kelompok beserta empat strain kekal tidak dapat dikelaskan dapat dijana. Terdapat klon yang serupa seperti klon yang dipencilkan daripada *O. Orbiculata* (Malaysia) dan *C. moktiana* (Indonesia) dan juga klon yang spesifik terhadap sama ada *O. Orbiculata* atau *C. moktiana*. Kepelbagaian genomik yang tinggi dikalangan pencilan ini dapat dipastikan melalui teknik ini tanpa mengira geografi asal. Dicadangkan strain *V. alginolyticus* dari kedua-dua species siput ini adalah berkait rapat antara satu sama lain walaupun mereka dipencilkan dari kawasan geografi yang berbeza. Persekitaran akuatik termasuk siput dianggap sebagai takungan *V. alginolyticus* dan bertanggungjawab dalam transmisi kepada manusia. Kajian ini menekankan kepentingan bakteria ini terhadap keselamatan dalam makanan, orang awam dan juga ekonomi.

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Last but not least, a grateful thanks to my loving parents, my beloved husband and both of my brothers and sisters for their love, support and motivation that gives me strength to work hard to fulfill my goal.

May Allah bless all of you.

I certify that an Examination Committee met on **19 September 2007** to conduct the final examination on **Noor Rasyila Binti Mohamed Noor** on her **Master of Science** thesis entitle **Molecular Characterization of *Vibrio alginolyticus* Isolated From *Orbicularia orbiculata* And *Corbiculla moktiana*** in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulation 1981. The Committee recommends that the candidate be awarded the Master of Science.

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DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at UPM or at any other institution.

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NOOR RASYILA MOHAMED NOOR

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CHAPTER 1

INTRODUCTION

Bivalve is a traditionally popular part of diet and economically important factor in South East Asia especially Malaysia and Indonesia, but there are no recent studies conducted on bivalve with *V. alginolyticus* obtained from these regions. Sea food, especially the bivalve can readily be contaminated with pathogenic microorganisms because of the texture of their flesh and also their living habits in the microbe laden habitat they inhabit. In the case of being contaminated with pathogenic bacteria, bivalve poses a serious threat for public health. Fear of contracting disease from eating bivalve is believed to have also contributed to decline in the consumption and production of bivalve (Ong and Rabihah, 1989).

Vibrio spp. are normal inhabitant of the aquatic environment (Tison and Kelly, 1984) and this genus now includes a large number of species that are ubiquitous in many environments which can be isolated from water, sediments and seafood, such as fish, oyster and, in particular bivalve (Jakšić *et al.,* 2002). In shrimp hatcheries, some *Vibrio* species have been associated with elevated mortality of shrimp, such as *V. campbellii* on *Penaeus indicus*, *Penaeus monodon* and *Penaeus semisulcatus* and *V. harveyi* and *V. parahaemolyticus* on *P. monodon* and *Litopenaeus vannamei* (Gabriel *et al*., 2002). The presence of *V. vulnificus* and *V. parahaemolyticus* in other hatcheries such as the cockles

Anadara granosa may be hazardous to consumers (Son *et al,*. 1998). *V. alginolyticus* is part of the normal marine flora and during warm periods, this bacterium can reach concentrations in bivalve sufficient to cause diseases in humans and also was the most common *Vibrio* isolated from mussels and seawater.

A research made on the presence of *Vibrio* spp., in fresh and frozen seafood of different origin, revealed that *V. alginolyticus* was the most representative species with an incidence of 90% in the case of fresh seafood harvested in a costal area of Ancona (Serratore *et al.*, 1999). *V. alginolyticus* has been reported to be the most common species in Europe and North America and also the most common species with the frequency of 34/47 among the *Vibrio* isolates identified (Fatma *et al.,* 2005). Among these 47 *Vibrio* isolates, *V. cholerae* is the important cause of bacterial diarrhea and there is still endemic presence of this pathogen in some Asian countries (Hedayat *et al.*, 2003). *V. parahaemolyticus* generally results in a self-limiting gastroenteritis also is a common pathogen in Asia, causing 197 outbreaks and 8976 cases in Taiwan during 1986-1995 as well as numerous outbreaks in Japan and some South East Asian countries (Ellin, 1998). Besides *V. cholerae* and *V. paraheamolyticus*, several *Vibrio* species are capable of producing gastrointestinal disorder in humans. *V. alginolyticus* has been reported as associated with gastrointestinal infection upon exposure to contaminated seafood or seawater but the exact role as a pathogen is unclear (French *et al.*, 1989). The role of other *Vibrio* spp., such as *V. fluvialis, V. furnissi, V. hollisae, V. mimicus, V. metschnikovii* and *V. damsela* has not been firmly established although they are implicated as responsible for a smaller, but relevant number of cases (Morris, 1999) in some of which the infection source was attributed to contaminated food (Abbott and Janda, 1994). Therefore they are found worldwide in marine and estuarine environments and consequently are commonly recovered from seafood (Ripabelli *et al.*, 2003).

Infectious diarrhea is a major cause of morbidity and economic losses in many developed and developing countries (Yam *et al.,* 1999). It is estimated that 1 in 2000 meals of raw bivalve results in disease, making these bivalve one of the most hazardous foods (Rosa and Loredana, 2002). Seafood eaten cooked or raw, and/or seawater, is clearly important vehicles of transmission for non-01 *V. cholerae, V. parahaemolyticus, V. vulnificus* and *V. alginolyticus* (Powell, 1999). In coastal environments, a close relation between the consumption of raw seafood and occurrence of episodes of human intestinal and extraintestinal infection attributed to some species has been observed (Rosa and Loredana, 2002). However, only a few studies have been conducted on the characterization of *V. alginolyticus* strains isolated from bivalve, cultured fish or marine water (Zorilla *et al.*, 2000).

The biochemical determination and identification of environmental *Vibrio* species has been problematic, time consuming and very often confusing because of their great diversity (Alsina and Blanch, 1994; Sudheesh *et al*., 2001). *V. parahaemolyticus* and *V. alginolyticus* isolates are very closely related (Wachsmuth, 1980) so it is important to identify bacterial species and strains accurately with respect to treatment purposes. Rapid development in the field of molecular biology has resulted in an alternative DNA based technique for the detection of genetic polymorphism. There is a wide range of molecular technique available, each with its own advantages and disadvantages. RAPD-PCR is a rapid method to characterize genetic differences and is used to fingerprint a variety of bacterial species (Ripabelli *et al.*, 2003). Majority of organisms could be differentiated between its strains based on the polymorhism in the genomic fingerprint produced by the RAPD-PCR. Plasmid profiling is also a relatively quick and easy characterization method, but carriage can be unstable (especially during *in vitro* cultivation), and may also be poor discriminatory tools (Farber, 1996). Elucidation of the antimicrobial susceptibility of potential pathogenic *Vibrios* is important for prophylaxis and treatment of *Vibrio* infections in human being and in cultured marine organisms. In some cases, plasmid involvement in resistance to many antibiotics has been proven (Molina-Aja *et al.*, 2002). Without the plasmid profiles, one would have thought that *V. alginolyticus* isolates with similar antibiotic resistance patterns were derived from the same ancestral strain. In this case, plasmid analysis helped to recognize the presence of more than a single clone and reflects the additional and diverse microbial contamination that can and usually does occur during distribution and the natural surrounding in the study area. The value of plasmid analysis in ecological studies is self-evident.

The main target for this research is to contribute in the study of *V. alginolyticus* distribution which is focused on bivalve samples from Malaysia and Indonesia. It was initiated to collect information on the molecular characterization of *V. alginolyticus* contaminated in bivalve obtained from water sources within these two regions. In Malaysia (Son *et al.*, 1998a; Son *et al.*, 1998b; Najiah *et al.*, 2003; Elhadi *et al.*, 2004; Gwendelynne *et al.*, 2005) and Indonesia (Molitoris *et al.*, 1985; Lesmana *et al.*, 2001; Tjaniadi *et al.*, 2003) similar studies were conducted by Ministry of Health and universities regarding the infection of other *Vibrios* but not much information was reported on the distribution of *V. alginolyticus* in both countries. The occurrence of *V. alginolyticus* in Malaysia and Indonesia are not well documented and the occurrence of this *Vibrio* in seafood is not well understood. The true incidence of *V. alginolyticus* infections for both countries transmitted by seafood is not known probably due to underreporting of cases and lack of proper study on this issue. Since these organisms are distributed in aquatic environments, it is essential to gain a more detailed knowledge about the ecology of the organisms. Comparison of the distribution of *V. alginolyticus* in Malaysia and Indonesia can be done and the data collected can be beneficial for epidemiological and pathogenical study with the consideration whether geographical factor may influence the data or not.

The contribution of this research to our country is to provide preliminary information about the *V. alginolyticus* distribution a part of Malaysia and Indonesia. The information collected contributed to the development of a relevant database for epidemiological and pathogenicity comparison purposes of *V. alginolyticus*. This information can be used as a guide in importing process and also to monitor the potential risk of infection from seafood in both countries. The interest about molecular characterization of *V. alginolyticus* in this study was to observe on the distribution and relationship in terms of similarities among them although they were isolated from bivalve *Orbicularia orbiculata* in Malaysia and *Corbiculla moktiana* in Indonesia.

Objectives

The objectives of this study were

1. To isolate *V. alginolyticus* from bivalve *Orbicularia orbiculata* in Malaysia and *Corbiculla moktiana* in Indonesia.
2. To characterize *V. alginolyticus* using antibiotic susceptibility test and plasmid profiling.
3. Detecting clonal relatedness of strains by using Randomly Amplified Polymorphic DNA Analysis

CHAPTER 2

LITERATURE REVIEW

Detection of *Vibrio alginolyticus* from *Orbicularia orbiculata* and *Corbiculla moktiana*

The Scenario of Bivalve

Mollusc production in Malaysia includes production of cephalopods, gastropods and bivalves from capture fisheries based on natural stocks and production of bivalves from aquaculture systems based almost exclusively on natural spats. Bivalve mollusc in Malaysia consists of a great variety of species. A total of 185 species from 44 families have been listed and the major species of commercially importance have been reported more recently (Ong and Rabihah, 1989). The bivalve species that are landed and of commercial importance at present including are as in Table 1. For Indonesia, mollusc production also obtained from natural stock collection. Some of the coastal areas are rich in bivalve resources including cockles, mussels and oysters, which are traditionally collected and utilized as food (Untong and Muhammad, 1989).

Table 1. Major bivalve species in Malaysia that are commercially important

Bivalve species	Scientific name
Cockles	*Anadara granosa, A. antiquate*
Carpet / Venus clam	*Paphia undulate, Meretrix meretrix*
Mussels	*Perna viridis, Molodiolus philippinarium*
Oysters	*Ostrea folium, saccostrea cucullata, Crassostrea belcheri, C. iredalei, Pinctada sp.*
Razor clams	*Solen brevis, Cultellus lacteus, Pharella acuminata*
Fan shells	*Pinna bicolor, P. incurve*
Giant clam	*Tridacna spp.* (especially in Sabah)
Others	*Orbicularia orbiculata, Geloina erosa, Placuna placenta, Pholas orientalis, Donax faba, Pecten sp.*

Figure 1 shows the maps of the areas where the samples in this study were obtained. Tanjung Karang is an old fishing village with cheap seafood restaurants and paddy growing town in Selangor, Malaysia. It is loosely translated into English as the "Cape of Corals". It is located in the district of Kuala Selangor about 15 kilometers away from the town of Kuala Selangor. Tanjung Karang is separated by Selangor River and also a part of Strait of Malacca which famous for their tourist attraction places such as the bird and firefly watching (Kampung Tanjung Karang Travel Guide).

Another ideal place for recreation, water sport and fishing village is Lake Singkarak. It is located between the cities of Padang Panjang and Solok. It has an area of 107.8 km², being approximately 21 km long and 7 km wide. Lake Singkarak is the widest lake in West Sumatra and the second widest lake in Sumatra Island after Toba Lake. The natural outlet for excess water is the Ombilin River which flows eastward to the Strait of Malacca. Lake Singkarak is located in 362.5 m height from the sea level, has special species of fish that only live in this lake and the only in the world. The locals called it as Bilih Fish *(Mystacoleuseus Padangensis)*. Uniquely, this fish cannot live in other habitat, both in an aquarium and in a pool. The people around are often selling this fish and the visitors can enjoy the specific taste of this fish in the restaurants that located around the lake (Singkarak Lake Tourism).

Figure 1. Map of the areas from which the samples described in this study were obtained.

While the potential for increasing bivalve and generally all aquaculture production in Malaysia is considered to be good, several constraints have been identified in the development. These include the unreliable of spat supply because bivalve production for food in Malaysia is still totally dependent on natural seeds which sometimes these bivalves were collected in insufficient quantities. There is a great diversity of bivalve species in Malaysia, existing knowledge on the biology of the more important species which is necessary for management of the natural stocks and on the technology of artificial propagation is still inadequate. Bivalve production is generally promoted on account of its being able to produce good animal protein at relatively low cost, the low price can also act as constraint to the economic production of some species. Although geographical, climatic and environmental conditions are generally very conducive for aquaculture development in Malaysia, the site or environment are still problematic. With the increasing urban and industrial development, often requiring land-fill and reclamation of possible culture sites, aquatic pollution due to domestic and industrial effluent and increased sediment load arising mining and land development schemes, certain areas have been rendered less suitable or even totally unsuitable for the culture of bivalve or other species (Ong and Rabihah, 1989). So far, bivalve culture in Indonesia has not developed mainly due to health hazards and consumer preferences. No sanitary control is imposed on collected bivalves. Although the great majority of capture and potential culture sites are pollution-free it is felt that some means of