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RANDOM AMPLIFICATION POLYMORPHIC DNA-PCR (RAPD) ANALYSIS OF *Vibrio alginolyticus* STRAINS ISOLATED FROM GREEN MUSSELS (*Perna viridis*) IN MARUDU BAY, SABAH

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Abstract

Vibrio alginolyticus infection is common in aquatic species. The species has been isolated from infected green mussels (*Perna viridis*) cultivated at Marudu Bay, Sabah. The infection of *V. alginolyticus* was prevalent since 2009 and caused more than 60% mortality of the mollusc. This study was conducted on *V. alginolyticus isolated* from clinically infected *P. viridis* at cultivation area in Marudu Bay from 2013. Twenty isolates were randomly selected and subjected to RAPD-PCR analysis using 20 sets of RAPD primers. Results showed that the most suitable primers were OPA 3, OPA 5, OPA 9, OPA 10 and OPA 12 where the significant bands ranging from 100-2500 bp. Genetic variability among the isolates as revealed by these primers indicated that RAPD-PCR method has good discriminative ability and can be used as a rapid typing method of differentiating *V. alginolyticus* strains isolated from infected green mussels for epidemiological investigation.

Keywords: Vibrio alginolyticus, green mussels (*Perna viridis*), random amplified polymorphic DNA (RAPD)

1.0 INTRODUCTION

The Asian green mussels, *Perna viridis* is native to the tropical waters and is widely distributed in the Indo-Pacific region of Asia [7]. Currently, they are being extensively cultured in many Asian countries; largely because of their value as a low cost of animal protein for human consumption [10]. In Malaysia, mussel is widely distributed along the Straits of Malacca and, to some extent, in certain parts of Sabah, particularly on Borneo Island and the east coast of peninsular Malaysia [6]. In Sabah, *P. viridis* have been extensively cultivated in Marudu Bay which is situated in the northern region of Sabah. There are several problems that hinder the sustainable production of *P. viridis* in Marudu Bay such as the climate changes, presence of heavy metals from environment and pathogens. In recent years, an increased number of bacterial infections in green mussels (*P. viridis*) have been reported. *V. alginolyticus* has been identified as the primary pathogen isolated from hemolymph, adductor muscles, gills and gastrointestinal track of clinically infected mussels.

The Gram-negative bacterium is of marine origin that associated with several diseases of marine animals including fish, crustaceans, molluscs and coral reefs [13, 14, 15, 12, 18]. Considered as a halophilic bacteria, *V. alginolyticus* is resistance to high

concentrations of salt and water in order to survive in different environments of seawater and brackish water [17]. These abundance of bacterial pathogens in *P. viridis* leads to vibriosis; a diseases that frequently affecting the fishes, molluscs and crustaceans [11]. This disease can actually affecting both humans and the marine organisms. In human, vibriosis commonly presents as acute diarrhea, wound infections, ears infection and gastroenteritis while in organisms itself, vibriosis is responsible in mortality of *P. viridis* due to the bacterial infection. Thus, causing the significant loss to the aquaculture industry [13].

Typing of microbial pathogens or identifying bacteria at the strain level is particularly important for diagnosis, treatment, and epidemiological surveillance of bacterial infections [8]. The random amplified polymorphic DNA (RAPD) assay is a powerful tool for genetic studies [4] and it has been used for typing and differentiation of bacteria for the study of genetic relationships between strains and species of microorganisms, plants and animals [5]. RAPD analysis is useful as a screening genotyping method [16]. Thus, this study was aimed to characterize the molecular typing of the strains of *V. alginolyticus* isolated from green mussels, *P. viridis* in Marudu Bay, Sabah by using Random Amplified Polymorphism DNA (RAPD-PCR) in order to provide a basis for epidemiological study of this species.

Table 1 Selected primers use in RAPD of *Vibrio alginolyticus*

No	Primers	Primer sequence
1	OPA 3	5'- AGTCAGCCAC -3'
2	OPA 5	5'- AGGGGTCTTG -3'
3	OPA 9	5'- GGGTAACGCC -3'
4	OPA 10	5'- GTGATCGCAG -3'
5	OPA 12	5'- TCGGCGATAG -3'

2.0 EXPERIMENTAL

2.1 Mussel Samples Collection

27 clinically infected green mussels (*P. viridis*) were collected from Marudu Bay, Sabah between January to May 2014. The mussels were dissected using sterile dissecting tools to obtain the gills, muscles, mantels, foot and gastrointestinal tract.

2.2 Isolation and Identification of *Vibrio alginolyticus*

V. alginolyticus strains were isolated from organs of green mussels using Thiosulfate Citrate Bile Salts Sucrose (TCBS) agar. Bacteria that grew on TCBS agar were subcultured until single colonies were obtained. The bacteria were identified using the Vitek2 System bioMérieux SA, USA (biochemical identification).

2.3 Extraction of DNA

Genomic DNA from the bacteria was extracted by using microwave method. 1 mL fresh culture of bacteria in Tryptic Soy Broth (TSB) were centrifuged at 6000 rpm for 15 minutes. The pellet were heated in microwave for 10 seconds for 3 times. About 50 µL of deionized water were added into the pellet. Genomic DNA were stored in 4°C and can be kept for 6 months until further analysis.

2.4 Screening of Random Amplification Polymorphic DNA (RAPD) Primers

Twenty random primers were obtained from FirstBASE Laboratories (OPA1-OPA20). A single individual of bacteria isolate was screened for every primers and the primers that gave clear and polymorphic profiles were chosen for further study (D'amato, M. E. and D. Corach, 1997).

2.5 Amplification of Random Amplification Polymorphic DNA (RAPD)–PCR

Five sets of primers namely OPA 3, OPA 5, OPA 9, OPA10 and OPA 12 (Table 1) were selected based on the sharpness and clearness of the bands. The best primers will produce more than three clear fragments. The total reactions of 25 µL was used with the final concentration containing 1 µL of genomic DNA, 12 µL of MyTaq™ Mix, 1 µL of 20 µM primer and deionized water. The reactions were carried out in Thermal cycler with Dual Alpha Unit (BIO RAD). Standard amplification conditions were programmed for 30 seconds of denaturation at 94°C, 30 seconds of annealing temperature at 36°C, 1 minute of primers extension at 72°C and the reaction were completed with 2 minutes of final extension at 72°C. PCR products were separated using 1.2% agarose gel in 1x TBE buffer, 110V for 90 min.

2.6 RAPD Data Analysis

Bands of DNA fragments were scored (1 for band visible, 0 for no band visible). Only clear and reproducible bands were taken into consideration to access phylogenetic distance by using NTSYSpc 2.10j (Numerical Taxonomy and Multivariate Analysis System version 2.1j) software package.

3.0 RESULTS AND DISCUSSION

The selected primers that used to generate the RAPD pattern of *V. alginolynoticus* isolates were OPA 3,

OPA 5, OPA 9, OPA 10 and OPA 12. The selection of primers were made based on the clearness and reproducible bands produced. The reproducibility of bands generated by these five primers were evaluated by amplifying the genomic DNA of 20 bacterial isolates three times and the results were compared. The results showed that these primers (OPA 3, OPA 5, OPA 9, OPA 10 and OPA 12) produced consistent and repeatable band patterns. Thus, selected to be used for further analysis.

By applying five selected primers (OPA 3, OPA 5, OPA 9, OPA 10 and OPA12), the results revealed that the bands were ranged from 100 bp to 2500 bp. From Figure 2, different primers generated different fragment numbers of DNA amplification products of twenty isolates of *V. alginolyticus*. For each primer, almost similar pattern was observed for some of the isolates but the differences can be detected by the presence and absence of faint band.

The phylogenetic tree (Figure 3) of *V. alginolyticus* strains in Marudu Bay, Sabah was constructed. The dendogram revealed that 20 strains of *V. alginolyticus* were separated into 2 distinct clusters which differentiating the two closely related group of bacteria. The first cluster consisted of all strains except S19 and the second cluster consist of only S19. These two clusters were joined together at about 0.56 genetic distance level. The second cluster showed that only S19 isolate was genetically different compared to other *V. alginolyticus* isolates. Other isolates were genetically similar as they were in the same cluster at cut-off value of 0.6667. However, *V. alginolyticus* in the cluster 1 were slightly different to each other even though they were in the same cluster due to the presence of multiple subgroups in that cluster. Overall, genetic distance levels of *V. alginolyticus* isolates in green mussels (*P.viridis*) at Marudu Bay ranged from 0.56 to 1.00.

The similarity index among *V. alginolyticus* isolated from *P. viridis* in Marudu Bay is shown by the software. The higher similarity index suggest that the individuals in the population have closer genetic relation among them [9]. Meanwhile, the lower similarity index suggest that the individuals in the population have farther genetics relation [9]. The similarity index within *V. alginolyticus* ranged from 0.0000 to 1.0000. Thus, shown that the individuals of *V. alginolyticus* isolated in *P. viridis* in Marudu Bay have closer relationship between each other.

RAPD technique has been used widely in genetic study as a useful and powerful method for identification of genetic variation between strains and species of microorganisms, plants and animals [5]. It is important to identify and characterize the pathogenic bacteria at the strain level for diagnosis, treatment and epidemiological surveillance of bacterial infection especially for bacteria exhibiting high levels of antibiotic resistance or virulence [8]. The utilization of RAPD method has been effectively applied to characterize several organisms including shrimp [3], fish [1] and cockles [2]. However, there is still lack of study for genetic population of *V.alginolyticus* in green mussels, *P. viridis* at Marudu Bay, Sabah.

V. alginolyticus, V. parahaemolyticus, *V. cholera* and other *vibrios* species in marine organism such as Study on different green mussel samples collected from Marudu Bay revealed that there is close relationship between the populations of mussel-associated *V. alginolyticus*. As suggested by Hasan and friends (2009), the genetic similarity of the isolates is slightly correlated to their geographic locations. One factor that may contribute to the similarities between this species is due to the location of rafting area of mussels that situated in the same circulated water in Marudu Bay where it is engulfed by Kudat and Bengkoka Peninsulas, in northern Sabah (Figure 1).

The same cultivation site for clams at this area was also contributed to the multiplication of *Vibrio sp*. since they are known as the host for bacteria and other pathogens. The bay also possess narrow entrance with large volume of water entering the bay with slow flowing out rate. From the topographical of Marudu Bay area during the sampling, it is obvious that the water movement into the area did not circulate well to the open sea. Since the circulation and water movement are not that effective, all these conditions worked in concert to affect the condition of the mussels. When the water quality deteriorated especially during the flood enters the cultivation area, the mussels were stressed by the changes in the environment and made it prone to infection.

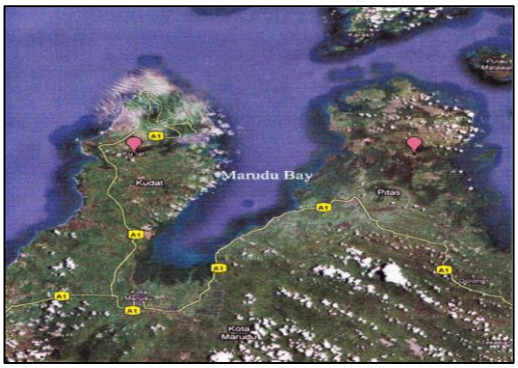

Figure 1 Sampling site at Marudu Bay (Courtesy by Google Maps)

4.0 CONCLUSION

As conclusion, results from this study showed that *V. alginolyticus* strains in Marudu Bay exhibit close relationship within each other except for one isolate. This method has shown to be rapid, sensitive, discriminative and cost effective in typing of the *V. alginolyticus* from green mussels in Marudu Bay, Sabah.

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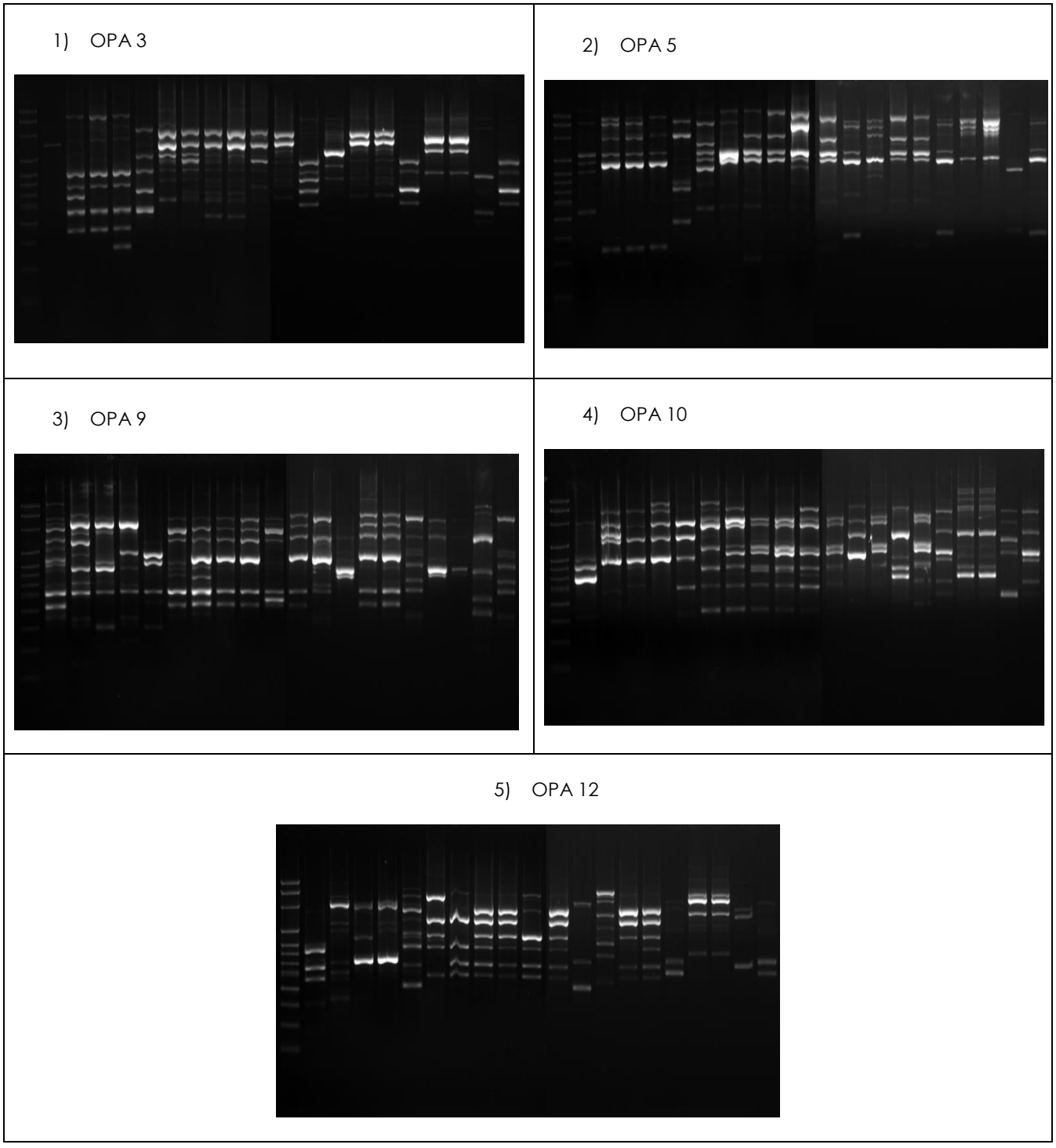

Figure 2 RAPD banding patterns of *V. alginolyticus* from green mussels, *P. viridis* in Marudu Bay, Sabah. 100 bp DNA ladder was used as a standard

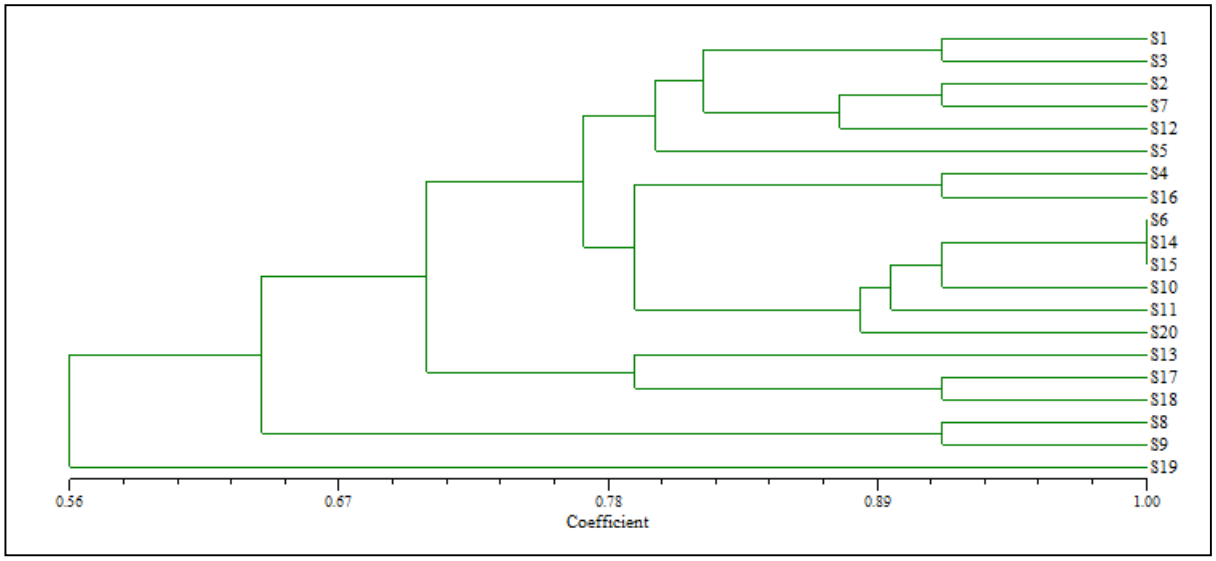

Figure 3 Dendogram of *V. alginolyticus* from green mussels*, P. viridis* in Marudu Bay, Sabah. Isolates are considered as a cluster at cut-off value of 0.6667

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