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Preliminary Study on Genetic Distance of *Vibrio parahaemolyticus* Isolates from Diseased Fish and Shrimp Brackishwater Ponds by Random Amplified Polymorphic DNA (RAPD) in Malaysia

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Abstract

This study was conducted to determine the genetic distance of *V. parahaemolyticus* isolates from diseased fish (clinical) and shrimp brackishwater ponds (environmental) in Malaysia;four clinical isolates (numbered F1 to F4) and six environmental isolates (W1 to W6) from shrimp brackishwater ponds. The isolates were identified using conventional biochemical tests in combination with BBL Crystal Kit ™. Two published 10-mer arbitrary primers (Gen1-50-01 and Gen1-50-02) were used for detecting DNA polymorphisms. RAPDistance program (version 1.04) was used to analyze the RAPD patterns. Dendrogram was then constructed from the combined results of two primers using Neighbour-Joining Tree (NJTREE) algorithm. The primers detected DNA polymorphisms in all isolates except W1 and W2, which were DNA monomorphic. Each primer generated fingerprints of up to 20 bands, ranging from 100 to 6000 bp. Dendrogram elucidated a tree of two main clusters (A and B) where a close genetic distance between the clinical isolates (F2, F3 and F4) except isolate F1 was observed to be closer to environmental isolates W3 and W5. Isolates W1, W2 and W4 were found to be genetically far from any clinical isolates.

Introduction

Vibrio spp are the natural inhabitants of estuarine and marine environments, well known for causing vibriosis in fish worldwide. Several species are known to be pathogenic to aquatic animals as well as humans. Marine fish reared at high stocking density in water of high organic load can easily succumb to vibriosis. Acute vibriosis usually results in hemorrhagic septicemia

and mortality. Fish survived the acute infection show focal hemorrhagic ulcers or focal necrotic lesions on the mouth or skin surface, and along the edge of fins. Sub-epidermal lesion manifests as 'blackening" on skin, which could lead to shallow but intensive hemorrhagic ulcer with a white dermal collagenous rim and a black pigmentary halo (Inglis 1993).

The rapid growth of brackishwater and marine cultures has seen the increment of vibriosis in seabass *Lates calcarifer* (Arulampalam, 1995). Vibriosis has resulted in millions of losses to the industries. For instance, in Pulau Ketam alone (Selangor, Malaysia), seabass culture lost RM48 million to vibriosis in 1990 (Chan, 1997). Many studies on vibriosis in fish were mainly focused on *V. anguillarum* especially in the 1970s and 1980s as it caused tremendous economic losses to the fish industry in the temperate countries. Vibrios like *V. alginolyticus* (Colwell and Grimes, 1984) and *V. parahaemolyticus* (Schäperclaus, 1986) were not regarded as fish pathogens as the former was not widely discovered in causing primary disease in fish, while the latter mostly caused disease in humans in Japan. Thus they were considered to have minor importance to marine fish production. Nevertheless, in Malaysia, *V. parahaemolyticus* and *V. alginolyticus* were identified as the important bacterial pathogen in causing disease in fish (Leong, 1992). Unfortunately, there are limited published reports on their virulence and pathogenicity in Malaysia. Hence, this study was undertaken to examine the genetic distance of the clinical and environmental *V. parahaemolyticus* isolates in Malaysia by RAPD.

Materials and Methods

Bacterial isolation and identification

Samplings were carried out in fish cultures and shrimp ponds in Malaysia. The bacterial isolate from moribund fish was considered as a clinical isolate while bacterial isolate from shrimp pond was considered as an environmental isolate. The fish kidneys were streaked with sterile wire loop onto thiosulfate citrate bile salts sucrose agar (TCBS, Oxoid® UK) and incubated for 18 to 24 h at 37ºC. As for the environmental isolates, brackishwater samples were collected from different shrimp farms. The samples were overlaid onto TCBS agar using spread plate method and incubated as above (Table 1). Suspected green colonies were picked and maintained on tryptone soya agar (TSA, Oxoid® UK) incorporated with 2% NaCl. Isolate identification was done using biochemical tests (MacFaddin, 1980) in combination with BBL Crystal Kit™ (Becton Dickinson Microbiology Systems, USA). The species was determined based on the Bergey's manual (Holt et al., 1994) and BBL Crystal Enteric/NF 4.0 electronic codebook.

Random amplified polymorphic DNA (RAPD) amplification

Prior to amplification, genomic DNA of *V. parahaemolyticus* was extracted using Wizard® Genomic DNA purification Kit (Promega®, USA). Two pub-

lished 10-mer arbitrary primers Gen 1-50-01 (5'GTG CAA TGA 3') and Gen 1-50-02 (5' CAA TGC GTC T 3') (Son et al., 1998) were used for amplifications. The amplifications were performed in a mixture of 25 µl consisting of 1X reaction buffer, 2.5 mM $MgCl_2$, 0.2 mM nucleotide mix, 0.5 µM primer, 1U Platinum® *Taq* DNA polymerase (Gibco, USA) and 1 µl of genomic DNA. A negative control without DNA was included in each run to monitor for contamination. Amplifications were carried out in a thermal cycler, GeneAMP PCR System 2400 (PE Applied Biosystems, USA). The amplification started with 1 cycle of pre-denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 2 min, annealing at 36°C for 1 min, and elongation at 72°C for 5 min. The amplification ended with a final elongation at 72°C for 5 min. Ten microlitres of the RAPD products were analyzed on 1% agarose gel in 1X TBE buffer at 100V in parallel with 100-bp and 1-kb DNA ladders (Promega®, USA) and visualized by ethidium bromide.

RAPD profile analysis

The RAPD banding profiles generated by each primer were recorded as binary data of "1" or "0" (presence or absence of bands). The binary data were encoded and analyzed with a RAPD pattern analysis program, RAPDistance version 1.04 (Armstrong *et al*., 1998). The encoded data were used for distance calculation using Nei and Li's distance matrix (1979). Dendrogram was constructed from the combined distance matrices of 2 primers using a tree-building program, Neighbour-Joining Tree (NJTREE) algorithm (Li and Ferguson 1998).

Results and Discussion

Bacterial isolation and identification

A total of three clinical *V. parahaemolyticus* isolates were obtained from diseased seabass *Lates calcarifer* in an angling farm in Ulu Klang while one

Table 1. Strains no., sources and sites of *Vibrio parahaemolyticus* isolates obtained from various places in Malaysia

Strain No	Sample	Site
Fl/C	Diseased seabass	Ulu Klang
F2/C	Diseased seabass	Ulu Klang
F3/C	Diseased seabass	Ulu Klang
F4/C	Diseased grouper	Penang
W1/E	Shrimp pond	Kuala Selangor
W2/E	Shrimp pond	Lukut
W3/E	Shrimp pond	Lukut
W4/E	Shrimp pond	Kuala Selangor
W5/E	Shrimp pond	Kuala Selangor
W6/E	Shrimp pond	Kuala Selangor

Keys: F: fish, W: water, C; clinical and E: environmental

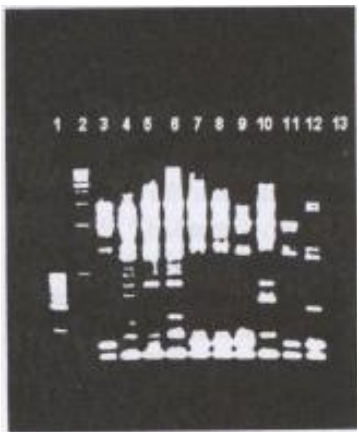

Fig. 1. RAPD banding profiles of *V. parahemolyticus* isolates obtained with primer Gen1-50-01. Lane 1: 100-bp DNA molecular mass marker; lane 2: 1-kb DNA molecular mass marker; lane 3: isolate F1; lane 4: isolate F2; lane 5: isolate F3; lane 6: isolate F4; lane 7: isolate W1; lane 8: isolate W2; lane 9; isolate W3; lane 10: isolate W4; lane 11: isolate W5; lane 12; isolate W6 and lane 13: negative control

Fig. 2. RAPD banding profiles of *V. parahemolyticus* isolates obtained with primer Gen1-50-02. Lane 1: 100-bp DNA molecular mass marker; lane 2: 1-kb DNA molecular mass marker; lane 3: isolate F1; lane 4: isolate F2; lane 5: isolate F3; lane 6: isolate F4; lane 7: isolate W1; lane 8: isolate W2; lane 9; isolate W3; lane 10: isolate W4; lane 11: isolate W5; lane 12; isolate W6 and lane 13: negative control

isolate was obtained from a grouper *Epinephelus tauvina* hatchery in Penang. As for the environmental isolates, four isolates were obtained from Kuala Selangor and two isolates from Lukut (Table 1).

RAPD amplification and analysis

In all 10 isolates, two primers successfully generated scorable and reproducible RAPD multibanding profiles ranging from 100 to 6,000 basepair (bp). (Figs. 1 and 2). The profiles indicated polymorphism in all *V. parahaemolyticus* isolates except isolates W1 and W2 that shared an identical RAPD profile. When analyzed by Gen1-50-02, isolates W1 and W2 were also monomorphic but the profile was different from that generated by primer Gen-50-01 (Figs. 1 and 2).

Dendrogram constructed using the combined RAPD results elucidated a tree of two main clusters, A and B (Fig. 3). The latter was subdivided into B1, B2 and B3. Isolates F1, W3 and W5 were clustered in A, isolates W1 and W2 in B1, isolates F2, F3, F4 and W6 in B2, and isolates W4 in B3. The isolates from diseased seabass and grouper as well as shrimp ponds were observed grouping together either in Cluster A and Cluster B2. Cluster A was consisted of isolates from Ulu Klang, Kuala Selangor and Lukut. This showed that the clinical isolate (F1) from seabass was genetically closer to environmental isolates of brackishwater shrimp with genetic distance values of 0.259, 0.074 and 0.210 respectively as compared to other three clinical isolates (F2, F3 and F4) which also infected the seabass and grouper. Cluster B indicated that B1 and B2 were closer in terms of genetic distance, which bear the value of 0.083 and 0.056 as compared to Cluster B3 that had genetic distance of 0.23. Cluster B1 was consisted of the environmental (shrimp ponds) isolates from Kuala Selangor and Lukut. This showed that in these two ponds, they possessed similar *V. parahaemolyticus* strains in spite of different geographical areas. Cluster B2 was most com-

plicated as it contained the isolates from two different species of fishes (seabass and grouper) that originated two distinct locations, Ulu Klang and Penang, respectively. Although isolates F2 and F3 were both isolated from the seabass in Ulu Klang, they were separated into sub intraclusters of genetic distance value of 0.064 which were further subdivided into genetic distance values of 0.151 and 0.034. Environmental isolates W6 of Kuala Selangor, was situated farthest in this Cluster B2 whereby, it cultivated genetic distance value of 0.143. Isolate W4 possessed furthest genetic distance value in the Cluster B viz. 0.23 although it originated in Kuala Selangor brackishwater shrimp ponds as isolates W1, W4, W5 and W6. This showed that the genomic profiles exhibited by *V. parahaemolyticus* isolates from shrimp ponds varied.

In recent years, molecular techniques have been used for analysis of intraspecies genetic diversity among *Vibrio* spp., for examples, lipopolysaccarides typing, total protein profiling (sodium dodecyl-sulfate-polyacrinamide gel electrophoresis), DNA sequencing, plasmid profiling, pulsed field gel electrophoresis, amplified-fragment length polymorphisms (AFLP) and randomly amplified polymorphic DNA (RAPD). Of these methods, RAPD has been widely used for typing both Gram-positive and Gram-negative bacteria (Mazurier et al., 1992; Brousseau et al., 1993; Lawrence et al., 1993, Linton et al., 1995;), and more specifically *Vibrio* spp. (Høi et al., 1997, Arias et al., 1998). RAPD analysis on bacterial species and strains is of epidemiological and clinical importance (Son *et al*., 1998). Epidemiologically, RAPD is useful for tracking in outbreak and pinpointing strains involved. The present study revealed DNA polymorphism in 8 out of 10 isolates, suggesting genetic heterogenecities of *V. parahaemolyticus*. No RAPD profile was representative of neither clinical nor environmental isolates. Furthermore, there was no correlation between genetic and geographical distances of isolates. Isolates of distant geographical locations appeared close in their genetic distance and vice versa. The monomorphic genomic profiles of isolates W1 and W2 indicated a close genetic relationship between them in spite of a geographical distance of about 50 km. This is supported by Wong et al., (1999) who found that geographic origins of *V. parahaemolyticus* strains obtained from patients involved in food poisoning outbreaks in Taiwan did not have any significant effect on the clustering of the RAPD types.

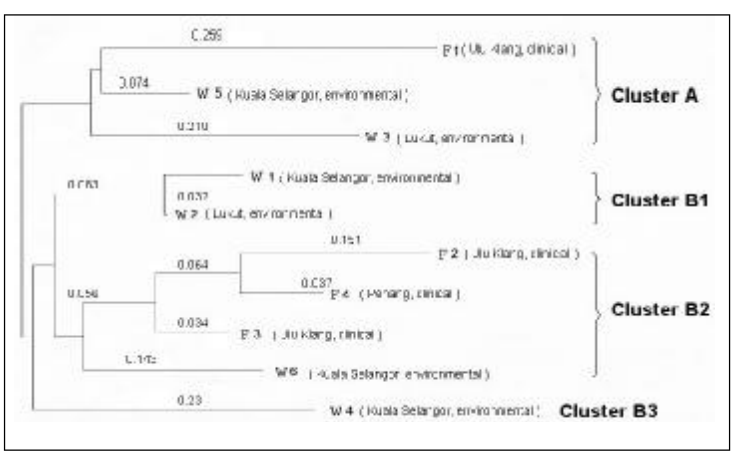

Fig. 3. Dendrogram based on RAPD profiles of 10 *Vibrio parahaemolyticus* isolates revealed by primers Gen 1-50-01 and Gen 1-50-09. Branch length represents the genetic distance between isolates in each cluster. Genetic distances are indicated on each arm of the tree.

Conclusion

The present study revealed DNA polymorphism suggesting genetic heterogenecities in *V. parahaemolyticus* isolates in Malaysia. However, RAPD profile was representative neither of clinical nor environmental isolates. There was also no correlation between genetic and geographical distances of isolates. Therefore detailed studies on more isolates should be conducted in the future.

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