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Molecular confirmation and characterization of *Vibrio parahaemolyticus* from retailed fish

*Vincent, M., Chan, C. S. W. and Apun, K.

Resource Biotechnology Programme, Faculty of Resource Science and Technology
University Malaysia Sarawak, 94300 Kota Samarahan, UNIMAS

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Abstract

The present study was conducted to assess the rapid molecular identification and characterization of 45 *Vibrio parahaemolyticus* isolates from 15 samples of 3 different types of fish (Kembung, Bawal and Sangeh) in the Kuching-Samarahan district. Polymerase chain reaction (PCR) based confirmation was done targeting the 450 bp fragment of the thermolabile *(tl)* gene, while DNA fingerprinting was performed using Randomly Amplified Polymorphic DNA (RAPD) PCR with the primer GEN15008. All the 45 *V. parahaemolyticus* isolates were positive for the tl gene, however, only 34 were typable via RAPD-PCR with bands sizes ranging from slightly over 250 bp to 2.5 kbp. The degree of diversity was then determined via the Simpson Index which showed a value of 0.891, indicating high diversity among the isolates. Data from the RAPD-PCR fingerprints were later used to construct a dendrogram for clustal analysis. From the dendrogram, the 34 isolates were grouped into 2 major clusters containing 26 and 8 isolates, respectively. Further analyses of the dendrogram also indicated that the 34 isolated were clustered according to the period of sampling. This is an interesting observation as it shows the high discriminatory capability of RAPD-PCR to be used as molecular epidemiological tool to study the temporal distribution of *V. parahaemolyticus.*

Introduction

Vibrio parahaemolyticus is a bacterium from the family *Vibrionaceae*. It is naturally found in coastal marine waters and seafood throughout the world (Quiroz-Guzmán *et al*., 2013; Micky *et al*., 2014). This halophilic Gram-negative bacterium is normally isolated from brackish water and marine environment (Lesley *et al*., 2011). *V. parahaemolyticus* is present in higher concentrations during summer when salinity is higher in the aquatic environment (Zulkifli *et al*., 2009).

When ingested, *V. parahaemolyticus* causes diarrhea, often with abdominal cramping, nausea, vomiting fever and chills as described by Sakata *et al*. (2012) and Micky *et al.* (2014). Usually these symptoms occur within 48 hours of ingestion. Most individuals become infected when they consume raw and undercooked fish or shellfish that is contaminated with this bacterium due to its ability to multiply rapidly in seafood (Nelapati *et al.*, 2012). Less commonly, this organism can also cause an infection of the skin when an open wound is exposed to warm seawater. This usually happens when a person with a cut or abrasion swims in seawater containing a high number of these bacteria (Bej *et al*., 1999). According to Daniels *et al*. (2000), treatment with antibiotic is not necessary in most cases of *V. parahaemolyticus* infections as the illness is usually self-limiting. Severe symptoms are rare and occur more commonly in individuals with weakened immune systems. Most people can recover from this illness without any further treatment. However, in severe or prolonged illnesses, antibiotics such as tetracycline, ampicillin or ciprofloxicin may be administered (Lesley *et al.*, 2011).

Globally, *V. parahaemolyticus* is a common cause of foodborne disease and there have been numerous reports of *V. parahaemolyticus* outbreaks in the last few decades. In the United States, *V. parahaemolyticus* was reported to be the causal pathogen in an outbreak involving residents of Connecticut, New Jersey and New York during July-September 1998 due to the consumption of contaminated oysters and clams harvested from Long Island. This was the first reported outbreak of *V. parahaemolyticus* linked to consumption of shellfish harvested from New York waters (Velazquez-Roman *et al*., 2014). The largest reported outbreak in North America involving *V. parahaemolyticus* infections occurred when 209 individuals were admitted after eating raw oysters harvested from California, Oregon and Washington in the United States and from British Columbia in Canada, during July-August 1997 (Centers for

*Corresponding author.
Email: *vmicky@frst.unimas.my*

Disease Control and Prevention, 1998). In Japan and Taiwan, outbreaks of food poisoning caused by *V. parahaemolyticus* have been reported as results of contaminated Sashimi and Sushi consumptions (Novotny *et al*., 2004).

Currently, the standard method for the detection and identification of *V. parahaemolyticus* is by using microbiological media enrichment such as Alkaline Peptone Water (APW), Thiosulfate-citrate-bile salts-sucrose (TCBS) agar and a range of biochemical tests that may take up to a week long (Sakata *et al*., 2012). These procedures are not only lengthy, but their reproducibilities are also low (Nelapati *et al*., 2012). Furthermore, extra time is also needed for media preparation and incubation periods which may result in unwanted contaminations (Chan *et al*., 2014; Micky *et al*., 2014). Therefore, this study was conducted to apply Polymerase Chain Reaction (PCR) to rapidly identify *V. parahaemolyticus* targeting the thermolabile (tl) gene (Micky *et al.*, 2014). Randomly Amplified Polymorphic DNA (RAPD) PCR was also performed to fingerprint and characterize all the *V. parahaemolyticus* isolates. RAPD-PCR is a common method to perform genotyping among *Vibrio* spp. as this procedure is known to be able to reveal a high level of DNA diversity within common bacterial isolates, and thus is useful in discriminating the isolates (Radu *et al.*, 2002; Nanvazadeh *et al*., 2013). Furthermore, this method can be accomplished within a short span of time and limited budget (Skorić *et al*., 2012).

Materials and Methods

Bacterial isolation and sample processing

A total of 45 *V. parahaemolyticus* isolates were revived from a collection of *V. parahaemolyticus* cultures isolated from 15 samples of 3 different fish types (Kembung, Bawal and Sangeh) in the Kuching-Samarahan district between March 2003 and July 2003. The bacterial isolation was performed by standard methods as described by Lesley *et al.* (2011). Firstly, the samples were enriched by homogenizing 25 g of each sample with 225 ml Alkaline Peptone Water (APW, pH 8.6) in a Stomacher bag (1:10 dilution). The homogenates were then incubated for 8-16 hours at 37°C. After the incubation period, the upper layer of the APW enrichment broth was then aseptically streaked onto Thiosulfate-citrate-bile salts-sucrose (TCBS) agar (Oxoid, UK). The plates were then incubated at 37°C for another 8-16 hours, upon which, 3 suspected well-isolated green colonies were plated onto CHROMagar (Chromagar, France) for *V. parahaemolyticus* pre-confirmation. Presumptive *V. parahaemolyticus* isolates form mauve colonies on the chromogenic medium. The isolates were then stored in Luria broth (BBL, USA) supplemented with 3% (w/v) sodium chloride (Fluka, Switzerland) and 20% (v/v) glycerol (Fluka, Switzerland).

DNA preparation

Chromosomal DNA used as the template DNA for PCR amplification was prepared as described by Ausubel *et al.* (1990). The process of DNA extraction was started by transferring 1.5 ml of overnight culture into sterile Eppendorf tubes and was centrifuged at 10, 000 rpm for 1 minute. The supernatant was then discarded and 700 µl of TE buffer was added followed by a short vortexing. Then, 5 µl of Proteinase K (25 mg/ml) and 10 µl of 10% SDS were added and the solution was mixed gently and incubated at 60ºC for about 1 hour. 500 µl of PCI mixture were then added to the incubated solution and centrifuged at 12, 000 rpm for another 1 minute. After centrifugation, 200 µl of the upper layer of the supernatant was transferred into a new tube and added with 200 µl 3 M KAc and 400 µl of isopropanol followed by a thorough mixing. The solution was then left at room temperature for 5 minutes prior to centrifugation at 12, 000 rpm for 7 minutes. The supernatant was discarded and the pellet was then washed with 500 µl of 70% ethanol and centrifuged at 12, 000 rpm for 5 minutes. After the centrifugation, the supernatant was discarded and the pellet was air-dried at room temperature for about 30 minutes before dissolving in 50 µl of sdH_2O. The DNA was stored at -20ºC until further use.

Specific PCR Amplification

Oligonucleolides to amplify a 450 bp fragment of the species-specific *V. parahaemolyticus* thermolabile (tl) gene (F-tl: 5'- AAA GCG GAT TAT GCA GAA GCA CTG-3' and R-tl: 5'- GCT ACT TTC TAG CAT TTT CTC TGC-3') were synthesized by MWG (Germany). The PCR amplification for the *V. parahaemolyticus* isolates was carried out based on Zulkifli *et al.* (2009) with slight modifications in 25 µl PCR mixtures containing 2.5 µl of 10X PCR buffer, 2.0 µl of 25 mM $MgCl_2$, 0.5 µl of 10 mM dNTPs, 0.6 µl of each 5 pmol primer, 0.3 µl of 2.5 U Taq DNA polymerase, 1.0 µl of DNA template and sterile distilled water. *V. parahaemolyticus* strain VP28 was used as the positive control.

Amplifications were performed using a thermal cycler (Eppendorf Mastercyler PCR System, Perkin Elmer, US) that was programmed with an initial denaturation step of 3 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 58°C, and 2 min at 72°C; and a final extension of 5 min at 72°C.

The PCR amplification products were visualized by running 10 µl products in a 1.0% agarose gel in 1X TAE buffer. A 1 kb DNA ladder (Promega, USA) was used to detect DNA size. The gel was electrophoresed at 70 V for one hour which was then stained with ethidium bromide (Sigma, Germany), visualized using UV transilluminator and captured using a Polaroid camera.

RAPD-PCR amplifications

Prior to RAPD-PCR amplification, a set of four 10-mer RAPD primers were screened to obtain the primer with the best amplification. Out of the set of four, GEN 15008 was chosen. GEN 15008 with the sequence of 5'-GGAAGACAAC-3' was also synthesized by MWG. The RAPD-PCR cocktail mixtures consisted of 2.5 µl of 10X PCR buffer, 2.0 µl of 25 mM $MgCl_2$, 0.5 µl of 10 mM dNTPs, 0.6 µl of each 5 pmol primer, 0.3 µl of 2.5 U DNA polymerase, 1.0 µl of DNA template and sterile distilled water.

RAPD-PCR amplifications were performed with an initial denaturation step of 3 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 33°C, and 2 min at 72°C; and a final extension of 5 min at 72°C. RAPD-PCR amplifications of each isolates were performed twice for confirmation of accuracy and reproducibility of the products. The PCR amplification products were visualized by running 10 µl products in a 1.0% agarose gel in 1X TAE buffer. A 1 kb DNA ladder was used to determine DNA size with each gel electrophoresis set at 70 V for one and half hour. Upon staining with ethidium bromide, the gels were visualized using a UV transilluminator and the image captured.

RAPD-PCR Data Analyses

The RAPD-PCR gels obtained were digitalized, scored and analyzed using the DendroUPGMA online software (http://genomes.urv.cat/UPGMA/). From the DNA fragments scores, similarities among the isolates were determined based on the DICE coefficient logarithm and translated into a dendrogram. The RAPD-PCR bands of the 34 isolates were also used to calculate clonal polymorphism using the Simpson Diversity Index.

Results and Discussion

In this study, all 15 fish samples were positive for Vibrio spp. when tested on TCBS agar and CHROMagar. On TCBS, *V. parahaemolyticus* produced green colonies while on CHROMagar, purple colonies were formed. From these colonies, 45 presumptive *V. parahaemolyticus* isolates were identified after initial standard biochemical testing (Nelapati *et al.*, 2012).To further confirm their identities, PCR were performed on all the presumptive *V. parahaemolyticus* isolates, targeting the species-specific thermolabile *(tl)* gene (450 bp). All 45 isolates were positive for tl gene, as shown by the representative isolates in Figure 1. Specific PCR, such as the one performed in this study, has high discriminatory power. Besides, it is also low cost, and can reduce the time to obtained results compared to full biochemical assays which require one week to

Table 1. RAPD-PCR patterns using primer GEN 15008 for 38 *V. parahaemolyticus strains* from 3 different types of fish

Sampling Month	Isolate designation	Type of Fish	RAPD Pattern
March 2003	S3-1	Sangeh	D
	S3-2	Sangeh	C
	S3-3	Sangeh	B
	K3-1	Kembung	B
	K3-2	Kembung	A
	K3-3	Kembung	B
	B3-1	Bawal	A
	B3-2	Bawal	B
	B3-3	Bawal	B
April 2003	S4-1	Sangeh	E
	S4-2	Sangeh	B
	S4-3	Sangeh	E
	K4-1	Kembung	B
	K4-2	Kembung	B
	K4-3	Kembung	B
	B4-1	Bawal	E
	B4-2	Bawal	B
	B4-3	Bawal	*UT
May 2003	S5-1	Sangeh	*UT
	S5-2	Sangeh	A
	S5-3	Sangeh	*UT
	K5-1	Kembung	*UT
	K5-2	Kembung	*UT
	K5-3	Kembung	*UT
	B5-1	Bawal	*UT
	B5-2	Bawal	*UT
	B5-3	Bawal	*UT
June 2003	S6-1	Sangeh	F
	S6-2	Sangeh	H
	S6-3	Sangeh	G
	K6-1	Kembung	L
	K6-2	Kembung	L
	K6-3	Kembung	K
	B6-1	Bawal	I
	B6-2	Bawal	J
	B6-3	Bawal	M
July 2003	S7-1	Sangeh	*UT
	S7-2	Sangeh	*UT
	S7-3	Sangeh	O
	K7-1	Kembung	N
	K7-2	Kembung	N
	K7-3	Kembung	J
	B7-1	Bawal	J
	B7-2	Bawal	J
	B7-3	Bawal	N

*UT - Untypable

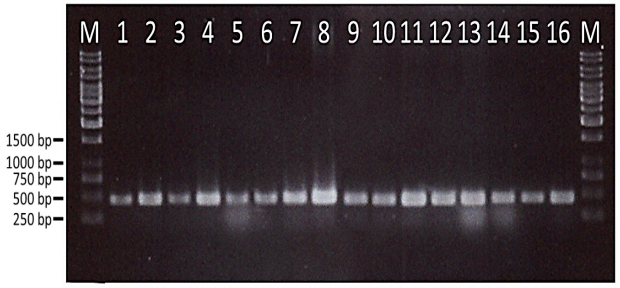

Figure 1. (c) Agarose (1.0%) gel electrophoresis of the PCR product for the *tl* gene (450 bp) of the *V. parahaemolyticus* isolates. Lane M - 1 kb ladder. Lanes 2 to 15 represent isolates S3-1, B3-2, K3-3, S4-1, B4-2, K4-3, S5-1, B5-2, K5-3, S6-1, S-3, B6-2, K6-3, S7-1, B-2. Lane 16 - VP28 (positive control).

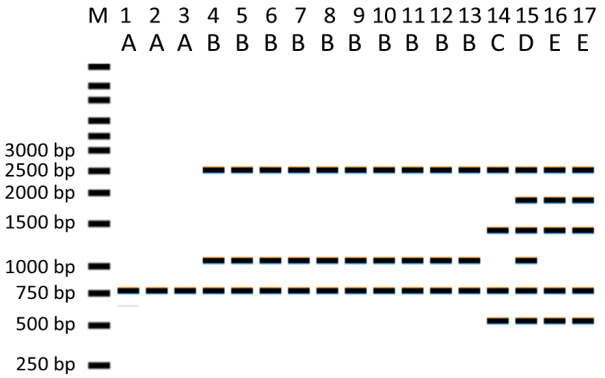

Figure 2. Digitalized RAPD patterns obtained using primer Gen15008 for 19 *V. parahaemolyticus* isolates. Lane M - 1 kb ladder. Lanes 1 to 17 represent isolates B3-1, K3-2, S5-2, B3-2, B3-3, B4-2, K3-1, K3-3, K4-1, K4-2, K4-3, S3-3, S4-2, S3-2, S3-1, B4-1, S4-1. Letters across the top indicates the RAPD patterns.

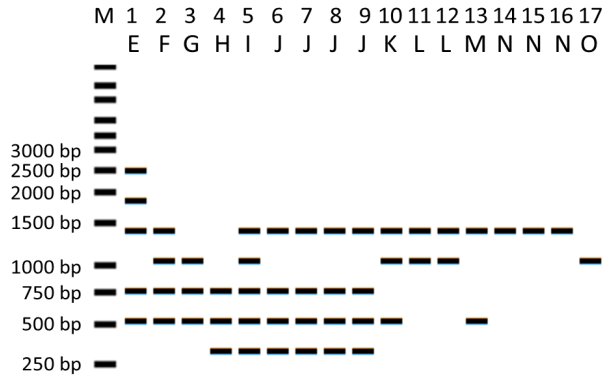

Figure 3. Digitalized RAPD patterns obtained using primer Gen15008 for 19 *V. parahaemolyticus* isolates. Lane M - 1 kb ladder. Lanes 1 to 17 represent isolates S4-3, S6-1, S6-3, S6-2, B6-1, B6-2, B7-1, B7-2, K7-3, K6-3, K6-1, K6-2, B6-3, B7-3, K7-1, K7-2, S7-3. Letters across the top indicates the RAPD patterns

conduct.

Next, RAPD-PCR was performed to generate DNA fingerprints from the 45 *V. parahaemolyticus* isolates. One important factor in performing RAPD-PCR is the selection of appropriate primers as primers differ in their discriminatory powers (Zulkifli *et al*., 2009). Therefore, it is necessary to screen the selection of primers available. In our study, after an initial screening of four RAPD primers, only primer GEN10008 was satisfactory in generating clear, usable and reproducible patterns.

From the 45 isolates, only 34 were typable via RAPD-PCR (Table 1). From the digitalized RAPD patterns (Figure 2 and 3), 15 types of patterns were generated with bands sizes ranging from slightly over 250 bp to 2.5 kbp. Eleven isolates failed to produce any products using GEN15008 primer, and, these isolates are referred as untypable (Zulkifli *et al*., 2009). The interesting observation of these eleven untypable cultures is that most of the isolates were isolated in the month of May, 2003. The isolate with the most number of bands was S3-1 (n=6) while the isolates with the least number of bands (n=1) were B3-1, K3-2, S5-2, B7-3, K7-1, K7-2 and S7-3.

The results in this study showed a high diversity of polymorphism between *V. parahaemolyticus* isolates as determined via RAPD-PCR and mathematical conversion of the results using Simpson's Index of Diversity. As described by Beals *et al.* (1999), Simpson Index of Diversity was used for measuring species diversity in a community. In this study, the application of this calculation was used to measure the clonal diversity. The Simpson Index of Diversity for all the 34 isolates based on RAPD-PCR results is 0.891, indicating a high index of diversity among the isolates. The high diversity index among the isolates suggests that the *V. parahaemolyticus* isolated from the 3 different types of fish were of different clonal origin.

Phylogenetic dendrogram was constructed and analyzed using the DendroUPGMA online software to determine the relationship between the isolates. The dendrogram in Figure 4 shows that all typable isolates were divided into 2 major clusters, namely Cluster 1 and Cluster 2 (Figure 2). All isolates in Cluster 1(A) – 3 isolates (B3-1, K3-2, S5-2), Cluster 1(B) – 10 isolates (B3-2, B3-3, B4-2, K3-1, K3-3, K4-1, K4-2, K4-3, S3-3, S4-2), Cluster 1(C) – 5 isolates (S3-2, S3-1, B4-1, S4-1, S4-3), Cluster 1(D) – 8 isolates (S6-1, S6-3, S6-2, B6-1, B6-2, B7-1, B7-2, K7-3) and Cluster 2 – 8 isolates (K6-3, K6-1, K6-2, B6-3, B7-3, K7-1, K7-2, S7-3) showed similarity between 0% to 90.9%. The clustering in the dendrogram showed that the isolates from the 3 different types of fish were mostly clustered based on the month it was collected. For example, isolates S4-1, S4-2, B4-1, B4-3, K4-1, K4-2 and K4-3 from June are clustered in a same major cluster. But the second major cluster was divided into 2 sub-clusters which contains 14 and 17 isolates, respectively. The sub-clusters mostly consist of those isolates from the month of March, May and July with exceptions for B4-2 and S4-3.

Due to the sensitivity of *V. parahaemolyticus* toward low temperature, abundance of the organisms were detected during summer in temperate zone (Khan *et al.*, 2002). In tropical countries such as Malaysia, the lowest and highest numbers of V. parahaemolyticus are during rainy and dry months respectively (Nelapati *et al*., 2012).

Epidemiological studies of *V. parahaemolyticus* have garnered increased interest in the last few decades as this bacterium is reported to be causing more infections worldwide (Zulkifli *et al*., 2009,

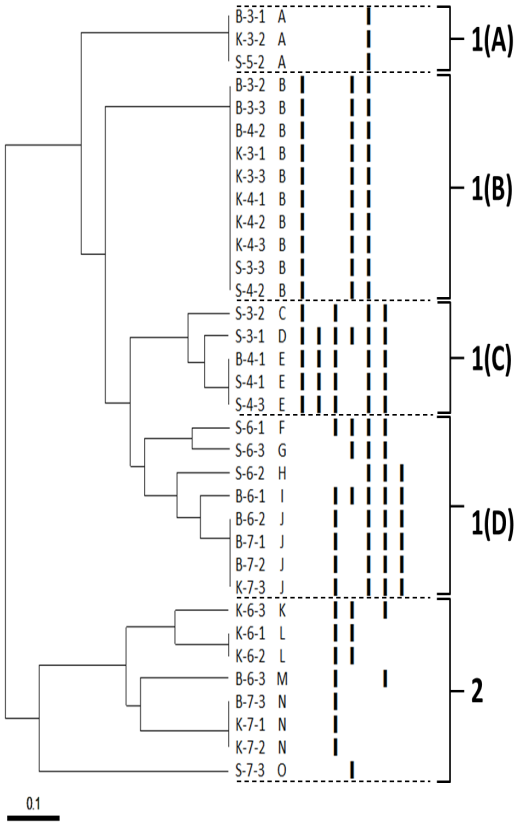

Figure 4. Dendrogram of 34 isolates of *V. parahaemolyticus* based on the RAPD-PCR fingerprints

Micky *et al.*, 2014). To achieve this, RAPD-PCR is an ideal procedure as DNA fingerprints can be generated with very minute amount of template DNA, using unlimited number of primers. Furthermore, this procedure is rapid, easy to perform, less laborious and relative cheaper when compared to other molecular fingerprinting method such as oligonucleotide-microarray (Nanvazadeh *et al.*, 2013). By using RAPD-PCR, no prior knowledge of the DNA sequences is needed but at the same time, distinct patterns for each sample may be generated and analyzed to determine the relationship between the isolates of interest.

Conclusion

In conclusion, the results of this study indicated RAPD-PCR is effective in detecting polymorphism and estimating genetic distance among different isolates of *V. parahaemolyticus*. From the RAPD-PCR result, the study found that *V. parahaemolyticus* isolated from 3 different types of fish (sangeh, kembung and bawal) were of different clonal origin, with some exceptions. The diversity of the isolates showed an index of 0.891 by using the Simpson Index of Diversity calculation. In addition, there is a strong correlation of the data that suggest the potential application of RAPD-PCR for temporal population studies.

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