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Molecular characterisation of *Vibrio parahaemolyticus* carrying *tdh* and *trh* genes using ERIC-, RAPD- and BOX-PCR on local Malaysia bloody clam and Lala

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Abstract

Molecular typing methods have been widely applied for many purposes. In this study, such methods were adopted as DNA fingerprinting tools to determine the origin and divergence of virulent *Vibrio parahaemolyticus* strains found in local seafood. Although not all strain carry virulent *tdh* and *trh* gene, increasing prevalence demands an effective fingerprinting scheme which can constantly monitor and trace the sources of such emerging food pathogens. By using ERIC-, RAPD-, and BOX-PCR methods, 33 *Vibrio parahaemolyticus* isolates from local Malaysia bloody clam (*Anadara granosa*) and Lala (*Orbicularia orbiculata*) with confirmed presence of *tdh* and *trh* gene were characterised, followed by determination of clonal relatedness among virulent strains using cluster analysis and discriminatory index. This study also involved application of Immunomagnetic Separation (IMS) Method which significantly improved the specificity of strain isolation. Cluster analysis using Unweighted Pair Group Mathematical Averaging (UPGMA) and Dice Coefficient shown clustering according to isolation food source, IMS level and haemolysin gene possessed. Nevertheless, different DNA fingerprinting methods generated different clustering at different similarity cut-off percentage, regardless as individual or as composite dendrograms. ERIC- and RAPD-PCR composite fingerprinting relatively shown the highest discriminatory index at following similarity cut-off percentage: 0.68 at 50%; 0.83 at 65%; and 0.93 at 75%. Discriminatory power increased with similarity cut-off percentage. However, result also suggested that BOX-PCR might be an effective fingerprinting tool, as it generated three clusters with no single-colony isolate at 70% similarity cut-off. This study not only achieved its objective to determine clonal relatedness among virulent strains from local seafood via characterisation, but also speculated the best possible combination of molecular typing methods to effectively do so.

Introduction

Gastroenteritic pathogen from seafood has evolved and emerged sporadically across different regions, causing diarrhoea, vomiting, fever and other illnesses. One pathogen of global attention is none other than the *Vibrio parahaemolyticus* (*V. parahaemolyticus*), which is the focus of this study. It is a Gram-negative, halophilic, asporogenous rod shaped bacterium that is straight or has a single rigid curve. It has a single polar flagellum and is motile when grown in liquid medium (Baumann and Schubert, 1984). This marine bacterium can be found in a wide range of consumable seafood such as sardine, codfish, mackerel, clam, scallop, oyster, crab, lobster, shrimp and octopus (Liston, 1990). Since *V. parahaemolyticus* infects via faecal-oral route, consumption of undercooked and *V. parahaemolyticus* contaminated seafood may cause acute gastroenteritic symptoms such as vomiting, diarrhoea, headache, nausea, abdominal cramps and low fever (Kaysner and DePaola, 2001). Apart from its prevalence on diverse seafood, *V. parahaemolyticus* can also be found in different regions of the world. Reported *V. parahaemolyticus* associated outbreaks can be found in the United States (Molenda *et al*., 1972; Daniels *et al*., 2000), European countries (Pasquier, 1816; Molero *et al*., 1989; Martinez-Urtaza *et al*., 2005) and Northeast Asian countries particularly Japan (Okuda *et al*., 1997; Chiou *et al*., 2000; Vuddhakul *et al*., 2000). Although not all *V. parahaemolyticus* strains are virulent (Shirai *et al*., 1990) and there has yet to be any reported outbreaks in Malaysia, virulent *V. parahaemolyticus* strains carrying *tdh* and

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trh gene can be isolated from coastal seawater (Tanil *et al*., 2005; Noorlis *et al*., 2011) and local cockles (Bilung *et al*., 2005) in Peninsular Malaysia. In order to address this profound threat to food sanitation and safety in Malaysia, an effective fingerprinting scheme is required to constantly monitor and trace the sources of such emerging food pathogens.

In this study, various DNA fingerprinting methods were used, such as Enterobacterial Repetitive Intergenic Consensus (ERIC-), Random Amplification of Polymorphic DNA (RAPD-) and BOX-Polymerase Chain Reaction (PCR). With application of these fingerprinting methods complemented by enhanced isolation of Immunomagnetic Separation (IMS) technology, this study's main objective was to characterise isolated *V. parahaemolyticus* carrying *tdh* and *trh* gene using DNA fingerprinting methods, thus determine their clonal relatedness. This study hypothesised that there is specific clonal relatedness among *V. parahaemolyticus* carrying *tdh* and *trh* gene, with respect to ERIC-, RAPD- and BOX-PCR fingerprinting.

Materials and Methods

Sample collection and isolation

Sampling for two bivalves of interest: bloody clam (*Anadara granosa*) and Lala (*Orbicularia orbiculata*) were performed according to stomaching procedure as described by Sharpe and Jackson (1972). Bacterial suspensions containing released mix of marine bacteria, including *V. parahaemolyticus* were filtered. The suspension was incubated at 37ºC for 6 hours in 0.1% Alkaline Peptone Water (APW) without shaking, then further incubated at 37ºC for 6 to 8 hours in Salt Polymyxin Broth (SPB) as a second enrichment step for *Vibrio* bacteria. Isolation of *V. parahaemolyticus* out of the *Vibrio* bacteria pool was performed using Immunomagnetic Separation (IMS), followed by purification of isolate using CHROMAgar™ Vibrio plates (Kalnauwakul *et al*., 2007).

DNA extraction and toxin gene detection

Once bacterial DNA is extracted from *V. parahaemolyticus* using Phenol: Chloroform: Isoamyl Alcohol (PCI) extraction method, 33 virulent *V. parahaemolyticus* strains carrying *tdh* and *trh* gene were identified and confirmed using specific-PCR targeting the species-specific *toxR* region found in *V. parahaemolyticus* (Kim *et al*., 1999; Dileep *et al.*, 2003). Purity of DNA extract was also assessed using Biophotometer (Eppendorf, Germany).

DNA fingerprinting protocols

ERIC-PCR was performed as described by Hulton *et al.* (1991). RAPD-PCR was performed as described by Son *et al.* (1998). BOX-PCR was performed as described by Versalovic *et al*. (1994). All amplified products were resolved by electrophoresis in 1.5% agarose gel and documented under ultraviolet transillumination using Gel Documentation System (Alpha Imager, Alpha Innotech, USA), after ethidium bromide staining.

Fingerprinting data analysis

Gel documentation acquired was analysed using BioNumerics version 6.0 software (Applied Maths, Kortrijk, Belgium), for cluster analysis via Unweighted Pair Group Mathematical Averaging (UPGMA) and Dice Coefficient. Dendrograms generated from different DNA fingerprinting methods were evaluated as single and as composite dendrograms, to find the most meaningful clustering. Quantitative Discriminatory Index was also calculated using the following formula as described by Hunter and Gaston, 1988.

$$D = 1 - \left(\frac{1}{N(N-1)}\right)\sum_{j=1}^{s} n_j(n_j - 1)$$

Where
D is the discriminatory index (DI)
N is the total number of colonies in the sample population
s is the total number of clusters described
n_j is the number of colonies belonging to the j^{th} cluster.

Result

Gathered data and observation made at different steps were analysed to confirm clonal relatedness among virulent *V. parahaemolyticus* strains. At the isolation step, mauve-coloured single colonies on CHROMAgar™ Vibrio were confirmation for positive *V. parahaemolyticus* colonies whereas toxin gene detection step, primers as described by Kim *et al.* (1999) and Dileep *et al.* (2003) were used to confirm possession of toxin regulatory gene in 33 isolated colonies namely from 2 distinct samples, which were bloody clam and Lala.

In DNA fingerprinting, gel documentations from ERIC-, RAPD- and BOX-PCR were observed using Gel Documentation System (Alpha Imager, Alpha Innotech, USA). Figure 1 shows the representative gel documentation, which is the BOX-PCR fingerprint. At fingerprint data analysis step, observations in terms of banding size and number of banding for each gel documentation obtained from every DNA fingerprinting method, were tabulated in Table 1.

Table 1. Summary for observation made on banding size and number of banding for various DNA fingerprinting method

Fingerprinting Method	Average No. of Bands	Smallest No. of Bands	Largest No. of Bands	Smallest Band Size	Heaviest Band Size
ERIC-PCR	9	5	11	250 bp	1500 bp
RAPD-PCR	7	3	12	500 bp	> 2500 bp
BOX-PCR	16	13	18	< 250 bp	2500 bp

Table 2. Summary for discriminatory index derived from different fingerprinting at different cut-off percentage

Fingerprinting method	Similarity % cut-off range	No. of major clusters at certain percentage (%) *	Discriminatory Index at different cut-off percentage (%)		
			50%	65%	75%
ERIC	70 – 90 %	6 cluster (75%)	0.549	0.775	0.883
RAPD	34 – 54 %	4 clusters (50%)	0.604	0.856	0.902
BOX	48 – 72 %	3 clusters (70%)	0.220	0.220	0.714
ERIC+RAPD	**40 – 60 %**	**5 clusters (60%)**	**0.678**	**0.830**	**0.928**
BOX+RAPD	37 – 60 %	4 clusters (60%)	0.511	0.604	0.902
ERIC+BOX	48 – 55 %	2 clusters (50%)	0.366	0.572	0.782
ERIC+BOX+RAPD	40 – 65 %	5 clusters (65%)	0.576	0.822	0.900

* Major clusters do not include single isolates formed at said cut-off percentage. The cut-off percentage (in bracket) is determined upon formation of most meaningful clustering for each dendrogram.

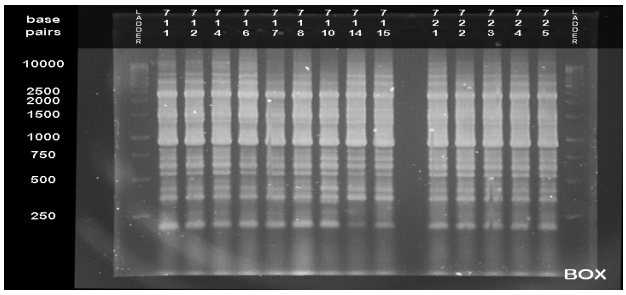

Figure 1. Representative of BOX-PCR fingerprint of *V. parahaemolyticus* isolates with primer BOX. Ladder represents 1kb DNA ladder. The number indicates the sample number (top), IMS number (middle) and colony number (bottom).

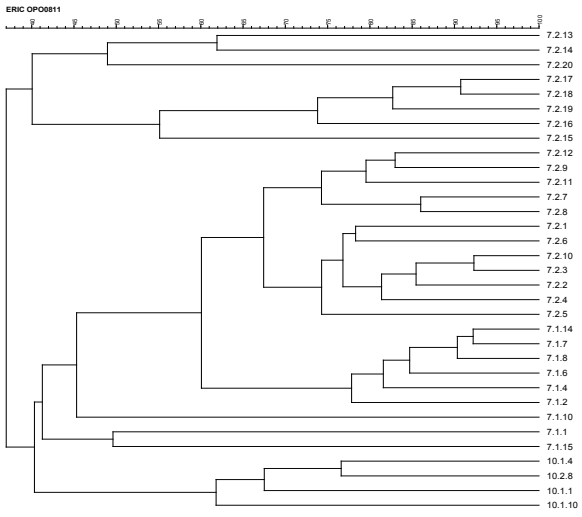

Figure 2. Representative dendrogram showing similarity of 33 *V. parahaemolyticus* colonies based on the combination of ERIC- and RAPD-PCR gel documentation using UPGMA method.

Prior discriminatory index calculation, cluster analysis had to be performed using Unweighted Pair Group Mathematical Averaging (UPGMA) Method and Dice Coefficient in BioNumerics Software. UPGMA is a common hierarchical clustering method used to generate rooted tree, known as the dendrogram, while Dice Coefficient is a similarity measure that revealed extremely close related colonies, because it exclude negative co-occurrences (Dalirsefat *et al*., 2009). Figure 2 shows the representative dendrogram, out of the nine single and composite dendrograms generated. Discriminatory Index at three different similarity cut-off percentage as shown in Figure 2 were calculated for each dendrogram generated. Discriminatory Index at fixed similarity cut-off percentage across all dendrogram generated were tabulated in Table 2.

Discussion

Effective molecular characterisation via different PCR methods

With the advancement in DNA fingerprinting methods, molecular characterisation of virulent *V. parahaemolyticus* strains are now possible using different PCR methods, namely ERIC-, RAPD- and BOX-PCR. This study shows that there is specific clonal relatedness among *V. parahaemolyticus* carrying *tdh* and *trh* gene, based on clustering that group together colonies of same isolation source, same IMS level or same toxin gene possessed. Therefore, the hypothesis for this study is proven.

All dendrograms derived from different DNA fingerprinting demonstrated strong clustering based on isolation source, IMS level and toxin gene possessed. Reversely, this further validates the effectiveness of all mentioned DNA fingerprinting methods in detecting and clustering related colonies from similar or diverse sources. This fingerprinting to be used as characterisation method would be highly applicable in determining origins and divergence of virulent strains, as mentioned previously. But more importantly, we can speculate the best fingerprinting scheme to achieve this characterisation.

Strain-specific DNA fingerprinting via genetic sequence detection

DNA fingerprinting methods used in this study are some of the many molecular typing methods used in characterising bacterial strains. Other methods include Loop-Mediated Isothermal Amplification (LAMP) (Nemoto *et al.*, 2008); Multiplex real-time PCR (Okura *et al*., 2003); Rapid PCR (Khan *et al*., 2002); Pulse Field Gel Electrophoresis (PFGE) (Wong *et al.*, 2000); and Plasmid Profiling (Baba *et al*., 1991). Recent molecular typing approaches have enabled researchers to distinguish genotypic distinctions directly at prokaryotes and eukaryotes.

This study has taken few different fingerprinting methods for comparison. Firstly, this study compares

the different banding formed between random arbitrary sequence and specific repetitive sequence amplification. Non degenerative oligonucleotides of random, arbitrary sequences have been used as primers in RAPD-PCR, while coding sequences of repetitive genes have been used as primers in ERIC-, BOX-PCR respectively for generating strain-specific bacterial DNA fingerprinting (Zulkifli *et al*., 2009). ERIC primer synthesises DNA sequences outward from inverted repeats, while BOX primer anneals on box-A subunit of BOX elements. On another comparison, RAPD-PCR targets random gene sequences that are complementary to its short PCR primer which is 10-mers in length. Meanwhile, ERIC- and BOX-PCR focus on specific consensus gene sequences about 21-mers in length, which are repetitive and interspersed across the bacterial genome. As a result, banding pattern as shown in ERIC- and BOX-PCR (Figure 1) are in greater uniformity as compared to RAPD, due to its primer's specificity.

Secondly, this study compares the different banding formed between conventional and non-conventional fingerprinting method for *V. parahaemolyticus.* ERIC- and RAPD-PCR are more widely adopted as the standard fingerprinting tool for *V. parahaemolyticus*. Meanwhile, BOX-PCR is rarely used for V. parahaemolyticus although theoretically it works similarly as ERIC-PCR to effectively detect conservative gene sequences in Gram-negative enteric bacteria (Versalovic *et al*., 1994). As a result, BOX-PCR generates banding of sufficient complexity with average of more than 10 bands per colony, thus enables distinction and clustering of all colonies via accurate characterisation with minimal single colony grouping. The decreased in complexity of generated banding for ERIC- and RAPD-PCR with average of less than 10 bands per colony, makes it more difficult to make fine distinctions between closely related colonies. In other instances, composite analysis of RAPD and ERIC-PCR promises discrimination of bacteria isolates according to sources of isolation (Yoke-Kqueen *et al*., 2008).

From the two major comparisons made, gene detection as strain-specific DNA fingerprinting has been proven effective for more extensive analysis, which include but not limited to microbe diversity study, bacteria classification and molecular epidemiological study on pathogens. However, more importantly, different fingerprinting methods have its pros and cons respectively. It is important that researchers understand the limitations and the advantages of each fingerprinting method used, in order to deduce a meaningful clustering.

This study has extended beyond proving the clonal relatedness of virulent *V. parahaemolyticus* strains, and venture deeper into identifying the best fingerprinting scheme for molecular characterisation of *V. parahaemolyticus.* In order to recommend the most effective fingerprinting schemes in a more quantitative manner, single and composite dendrogram analysis are performed and Discriminatory Index for each generated dendrogram is calculated to quantitatively compare discriminatory power among different fingerprinting.

Table 3. Summary on clustering pattern in different fingerprinting methods

Fingerprinting method	Similarity cut-off percentage	No. of major clusters	No. of ungrouped single colony produced
ERIC	75%	6	5
RAPD	50%	4	2
BOX	**70%**	**3**	**0**
ERIC+RAPD	60%	5	5
BOX+RAPD	60%	4	1
ERIC+BOX	50%	2	2
ERIC+BOX+RAPD	65%	5	5

Discriminatory index as indicator of effective fingerprinting scheme

Discriminatory Index is a quantitative measure on probability of two unrelated strains being characterized as the different type. In other words, the higher the discriminatory index, the greater the probability of discriminating two unrelated strains, thus the greater the effectiveness of a particular fingerprinting method to discriminate different strains. Despite being mutually exclusive, an increasing trend in discriminatory index can be observed with the increase in similarity cut-off percentage. Logically, the greater the similarity, the more closely related those given strains are and less likely other unrelated strains are to be clustered together. This results to greater discrimination among strains.

BOX-PCR as complementary DNA fingerprinting for V. parahaemolyticus

The effectiveness of a DNA fingerprinting method is not solely determined by its ability to discriminate unrelated strains, but also its ability to form meaningful clustering. In other words, BOX-PCR fingerprinting is able to form meaningful clustering based on isolation source, IMS level and toxin gene possessed, at low similarity cut-off percentage with no ungrouped single colony. Table 3 shows the clustering pattern in different fingerprinting methods, with number of ungrouped single colony produced.

Improved V. parahaemolyticus selection using immunomagnetic separation

Due to the specificity of antibody binding to antigen found only in *V. parahaemolyticus*, the IMS

beads can effectively isolate *V. parahaemolyticus* from a pool of bacteria. Therefore, DNA extraction that followed after this crucial step would be containing high percentage of pure *V. parahaemolyticus* DNA extract, in which its purity can be proven via DNA purity assessment. This step ultimately improved the clustering process, as it reduces the chances of unwanted amplification from non *V. parahaemolyticus* DNA fragments.

Conclusion

V. parahaemolyticus carrying *tdh* and *trh* gene was accurately isolated, identified, and characterised to trace clonal relatedness among virulent *V. parahaemolyticus* strains, thus determine the origin and divergence of such strains found in local seafood. This study shown the presence of a wide heterogeneity within bivalves shellfish strains of *V. parahaemolyticus*. Furthermore, there is a proven correlation between clonal relatedness and sampling source in which *V. parahaemolyticus* colonies were isolated.

Since *V. parahaemolyticus* is an enteric pathogen transmitted via faecal-oral route, and shellfish contaminated with this pathogen have been reported as sources of disease outbreaks across the world, surveillance on contamination in food samples from food stock and retail seafood is important to reduce potential *V. parahaemolyticus* outbreak in Malaysia.

In conclusion, ERIC-RAPD composite fingerprinting proves to be most effective in terms of discriminatory power, regardless of similarity cut-off percentage. Moreover, BOX-PCR based fingerprinting proves to be an effective complementary approach for V. parahaemolyticus as it produces meaningful clustering based on isolation source, Immunomagnetic Separation (IMS) level and toxin gene possessed with minimal number of ungrouped single colony. In addition, IMS method has significantly improved the results of this study.

Nevertheless, the findings in this study may be slightly biased since there were only two major samples confirmed with virulent gene. However, the proven conclusion from this study as mentioned above has shown that composite analysis of various DNA fingerprinting can accurately identify molecular pattern in the mass of genetic polymorphism, for the use of molecular marker gene detection in the epidemiology study of *V. parahaemolyticus*.

Considering the limitation of this study, increased sampling from various food sources may be continued, with usage of BOX primer as an unconventional approach to *V. parahaemolyticus* DNA fingerprinting. Apart from amplification-based DNA fingerprinting, plasmid profiling should also be included in future study, thus identify new correlation between amplification and non-amplification DNA fingerprinting banding pattern if have any.

Other recommendations for the future of this study includes typing using pulse field gel electrophoresis, restriction enzyme analysis, Next-Generation DNA sequencing, toxin protein structural conformation and 3D visualisation.

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