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Biofilm assessment of *Vibrio parahaemolyticus* from seafood using Random Amplified Polymorphism DNA-PCR

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Abstract

Pathogenic *Vibrio parahaemolyticus* is one of the leading causes of bacterial gastroenteritis in many countries. Among the strains examined, 36 RAPD-types were found when amplified with primers OPA8 and OPA10. The analysis shows the majority of *V. parahaemolyticus* isolates originated from seafood were branched into four major clusters at 18.2%, 20.7% 34% and 3.4% similarity levels. This suggests that there is potential for a single strain to be distributed widely within a population and there also potential for multiple contaminating strains of different clonal lineages to be present within the same population. Optimum temperature (37ºC) was the highest and stable formation of biofilm. The total percentage of biofilm formation at 37ºC was 33.33% for each of weak, moderate and strong biofilm producers. Room temperature produces 61.1% of weak biofilm producer, while 13. 89% for moderate biofilm producers and produce 25% of strong biofilm. While a total of 91.67% weak biofilm producers at 4ºC and 8:33% for room temperature and no growth of strong biofilm. Upon analysis, strong biofilm was tracked from the largest group at 37°C and room temperature which produce 27.27% of strong biofilm producer respectively. Interestingly, they are derived from cockles.

Introduction

Pathogenic *V. parahaemolyticus* is one of the leading causes of bacterial gastroenteritis derived from seafood in many Asian countries. Due to the nature and source of marine halophilic *V. parahaemolyticus*, raw seafood contaminated naturally and is a leading cause of infection (Desmarchelier *et al*., 2003). Clinical syndrome such as gastroenteritis with watery diarrhea, abdominal cramps, myalgias, nausea, vomiting, headache, low-grade fever, and rarely but sudden cardiac arrhythmia is the most common infection by *V. parahaemolyticus* (Oliver *et al*., 1997; McLaughlin *et al*., 2005).

These days, various types of DNA typing methods are used to determine the relationship between bacterial isolates. Rapid amplification of polymorphic DNA (RAPD) and PCR has been used in various scientific studies to generate DNA profiles via computer software. RAPD assay involves the use of short primers (usually of 8 to 12 nucleotides) of arbitrary sequence to amplify genomic DNA, expecting DNA fragments. Besides that, RAPD assays use a single primer that is able to anneal and prime to multiple locations randomly distributed throughout the whole genome, thus producing a spectrum of amplified products characteristic of the template DNA.

Kaneko and Colwell (1995) reported about *V. parahaemolyticus* can exist planktonically or attached to submerged, inert and animate surfaces, including suspended particulate matter, zooplankton, fish and shellfish. This organism is recognized as leading cause gastroenteritis worldwide, primarily in areas of the world where seafood consumption is high such as Southeast Asia (Joseph *et al.*, 2005). In fact, it is an emerging pathogen in 1997, when there is large outbreak of *V. parahaemolyticus* food poisoning, associated to raw oyster consumption which occurred along the Pacific coast (CDC, 1996). A biofilm is a structured community of microorganisms encapsulated within a self-developed polymeric matrix and adherent to a living or inert surface. Biofilms have been found not only on food production surfaces but on food products themselves, including sprouts, spinach and lettuce (Fett, 2000).

This raw seafood should be refrigerated as soon as possible and held in the refrigeratoras the the generation time at optimum conditions was 9-10 minutes. This means that the low levels of this bacteria present on food can increase from 10^2 to 10^5 cfu/g within 2 - 3 hours at 20 - 35°C. However, cooking temperature to an internal temperature of greater than 65°C is effective in eliminating contamination

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but the mortality of *V. parahaemolyticus* occurred slowly at 47°C. When introduced to freezing mode, the numbers of bacteria will initially decline and then remain stable for long periods in seafood (ICMSF, 1995). Therefore, the main objective for this study is to fingerprinting the *V. parahaemolyticus* isolates from seafood and to investigate the capabilities to form biofilm in difference temperature.

Materials and Methods

V. parahaemolyticus isolates

A total of 36 isolates of *V. parahaemolyticus* were recovered from seafood samples cockles (*Anadara granosa*), clams (*Mya arenaria*), shrimps (*Penaeous* spp.) and squids (*Loligo opalescens*) obtained from hypermarkets and wet markets. A control of *V. parahaemolyticus* (ATCC 17802) from the American Type Culture Collection (Rockville, MD, USA) was selected as positive control. These strains have been confirmed by using specific-PCR targeting the toxr regulator gene in *V. parahaemolyticus* as mentioned by (Kim *et al.*, 1999; Lesley *et al*., 2005). Isolates were revived from glycerol stocks using TSB (Bacto™, France) with 3% NaCl (Merck, Germany) and were incubated at 37°C for 18 to 24 hours in an orbital shaker.

Genomic DNA isolation for Randomly Amplified Polymorphic DNA (RAPD)

Prior to amplification, the 36 isolates of *V. parahaemolyticus* strains were grown overnight and their genomic DNA were extracted by boiled cells method. A 1 ml portion of each broth was subjected to centrifugation at 13, 400 × *g* for 1 min and the pellet was resuspended in 500 µl of sterile distilled water. The mixture was boiled for 10 min and then immediately cooled at -20°C for 10 min before it was centrifuged at 13, 400 × *g* for 3 min. The supernatant was kept for use in RAPD fingerprinting.

RAPD-PCR reaction

The RAPD-PCR was performed in a 25 µl volume containing 1X PCR buffer, 6.0 mM of $MgCl_2$, 0.4 mM of dNTPs, 4 µM of primer, 2.5 units of *Taq* DNA polymerase, and 3 µl of DNA template. All reagents were purchased from Promega (Madison, WI, USA) while the primer was synthesized by 1st BASE. Amplifications were carried out in a VeritiTM 96 wells thermal cycler (Applied Biosystems, USA) with the following reaction conditions: pre-denaturation at 95°C for 1 min; 35 cycles of denaturation at 95°C for 1 min, annealing at 36°C for 1 min, extension at 72°C for 2 min; and a final extension at 72°C for 10 min. The PCR products were stored at 4°C. Amplification products were fractionated by electrophoresis through 1.5% agarose gel with 0.5X TBE, using 80 Volts, and detected by staining with ethidium bromide and 1kb DNA ladder (Promega) was used as DNA size marker. The fragments were visualized under UV transilluminator (SynGene Gel Documentation System). The ladder consists of 13 fragments that range in size from 500 to 10,000 base pairs. For the interpretation of the fingerprints, the GelCompare 4.2 software package (Applied Maths, Belgium) was used. Computer based normalization and interpolation of the DNA profiles, and numerical analysis using the Pearson product moment correlation coefficient, with 1% position tolerance, were performed. Dendrograms were constructed using the unweighted pair group linkage analysis method (UPGMA). For convenience, the correlation level was expressed as percentage similarity.

V. parahaemolyticus in vitro biofilm formation in difference temperature

Total of 36 strains of *V. parahaemolyticus* positive strains and *V. parahaemolyticus* ATCC 17802 were assessed for the *in vitro* biofilm formation in the wells of commercially available, presterilized, polystyrene flat-bottomed 96-well microtiter plates. As proposed by Sandoe *et al.* (2006) with standard dilution 1.0×10^6 CFU/ml, the wells of microtiter plates were filled with 100 µl of Tryptic soy broth (TSB) with 3% NaCl medium. Column 2 served as positive control (*V. parahaemolyticus* ATCC 17802) and column 1 was the negative control (Fresh medium). Biofims were generated by pipetting 100 µl of the standard inoculums into selected wells of the microtiter plates. The plates was covered and sealed with parafilm. Then, plate was incubated at different temperature at 4°C, 37°C and room temperature without agitation for 24 h. After incubation, the medium was discarded, and nonadherent cells were removed by thoroughly washing the biofilm three times with sterile phosphate buffered saline (PBS). Finally, plates were inverted and drained by blotting them with paper towels to remove any residual medium. Biofilms were then ready to be assessed for their biofilm-forming capacity.

Quantification of Vibrio parahaemolyticus biofilm

Biofilm formation was quantified by a crystal violet assay recently described Djordjevic *et al.* (2002). Briefly, the biofilm-coated wells of microtitre plates as described above for biofilm formation and treatment were vigorously shaken in order to remove all nonadherent bacteria. The remaining attached

bacteria were washed twice with 200 µl of 50 mmol PBS (pH 7) and air-dried for 45 min. Then, each of the washed wells was stained with 110 µl of 0.4% aqueous crystal violet solution for 45 min. Afterward, each well was washed twice with 350 µl of sterile distilled water and immediately de-stained with 200 µl of 95% ethanol. After 45 min of de-staining, 100 µl of de-staining solution was transferred to a new well and the amount of the crystal violet stain in the de-staining solution was measured with atonable microplate reader (VERSAMAX, Sunnyvale, CA, USA) at OD 650 nm.

Statistical analysis

All measurements were carried out in three duplicate and reported as the mean ± of independent trials. Terms with higher p-value ($p > 0.05$) were statistically considered non-significant on the response variable. All data was analyzing using Microsoft Office Excel 2007.

Results and Discussion

Seafood can make an important contribution to the nutrient requirement for all consumers, especially among the growing children and the senior citizen. Malaysia is the second largest exporter of seafood after European countries as its main market. Yet, the European have discovered environmental and hygiene standards in the Malaysian industry to be wanting. The rapid growth in aquaculture production has made it is essential to the economy of the developing countries and, in the some cases of traded aquatic product, this sector has become one of the important source of supplies or the main suppliers. Hence, the contamination of seafood by microorganism will affect the export values toward Malaysia economy.

V. parahaemolyticus is a beneficial microorganism can be a model for review the adaption to the growth of bacteria on the surface because it featured a robust swarming motility and biofilm production. When growing in liquid culture, the *V. parahaemolyticus* cell is a short rod with a single sheathed polar flagellum that is used for swimming motility. Generally, biofilms is described as an accumulation of microorganisms in multicellular structures, adhered to each other and the surface of the matrix of exopolymers (O'Toole *et al*., 2000). In our initial characterization of biofilm development, a hypothesis based on differences in their ability to form a difference in temperature will have very different capacities for biofilm formation. Microtitre plates with 24-hour incubation in the temperature difference in this study indicate that biofilm production was influenced by temperature.

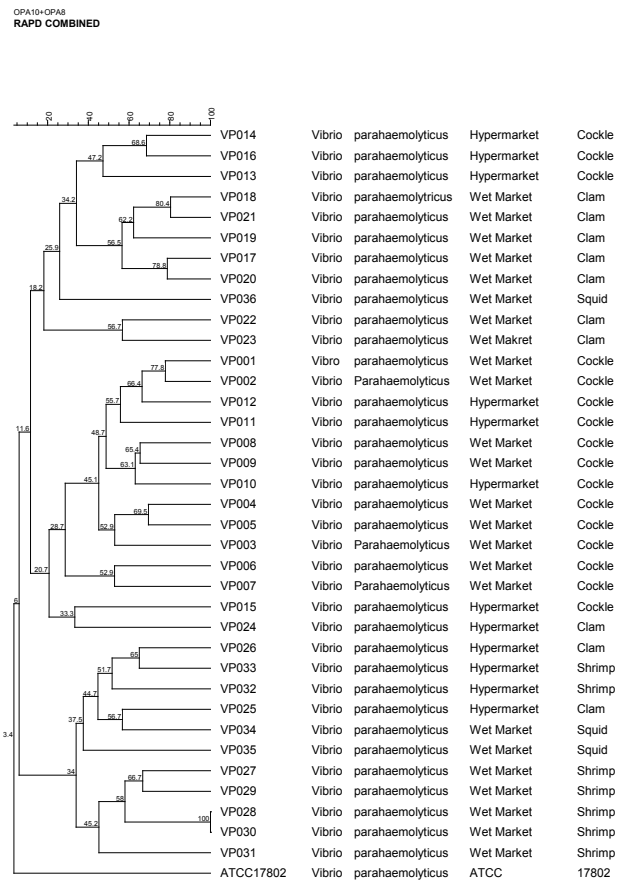

Figure 1. Dendrogram of RAPD profiles of *V. parahaemolyticus* strains using OPA8 and OPA10

According to Harvey *et al.* (2007) strains were arbitrarily designated as weak (< 0.6), moderate (0.6 - 1.2) or strong (> 1.2) biofilm formers according CV-OD 650 value with difference temperature. No significant correlation was observed between biofilm production and planktonic growth of *V. parahaemolyticus.* The measurement of turbidity (OD) at 650 nm from microplate reader (VERSAMAX, Sunnyvale, CA, USA) showed the stronger the biofilm formation of *V. parahaemolyticus*, the potential to be infected by *V. parahaemolyticus* is getting higher as well. Figures 2, 3 and 4 show the capabilities of *V. parahaemolyticus* isolates to form biofilm in different temperature. From the observation, 37ºC was the highest and stable formation of biofilm of *V. parahaemolyticus* compares than two other temperature. The total percentage of biofilm formation in 37ºC was 33.33% for each of weak, moderate and strong biofilm producers as referred to Figure 5.

The design capacity of the biofilm was measured at room temperature and at the end it has 61.1% weak biofilm producers, 13.89% moderate biofilm producer and total of 25% strong biofilm producers. When the seafood product was contaminated with *V. parahaemoyticus* and the surrounding temperature is in ambient condition, *V. parahaemolyticus* is a strong

Table 1. RAPD types and subtypes of the 36 typable *V. parahaemolyticus* strains

							Biofilm producers (<0.6 = Weak, 0.6-1.2 = Moderate, >1.2 = Strong		
Type	No. of Strains (%)	Subtype	No. of Strains (%)	Strain No.	Location	Type of Drinks	37 °C	RT	4°C
A	11 (30.56)	A1	1 (2.78) (1/36)	VP014	Hypermarket	Cockle	W	W	W
		A2	1 (2.78)	VP016	Hypermarket	Cockle	W	W	W
		A3	1 (2.78)	VP013	Hypermarket	Cockle	S	S	W
		A4	1 (2.78)	VP018	Wet market	Clam	W	W	W
		A5	1 (2.78)	VP021	Wet market	Clam	M	M	W
		A6	1 (2.78)	VP019	Wet market	Clam	S	S	W
		A7	1 (2.78)	VP017	Wet market	Clam	S	S	W
		A8	1 (2.78)	VP020	Wet market	Clam	M	W	W
		A9	1 (2.78)	VP036	Wet market	Clam	W	W	W
		A10	1 (2.78)	VP022	Wet market	Clam	S	W	W
		A11	1 (2.78)	VP023	Wet market	Clam	S	W	W
B	14 (38.89)	B11	1 (2.78)	VP001	Wet market	Cockle	S	W	W
		B12	1 (2.78)	VP002	Wet market	Cockle	W	S	W
		B13	1 (2.78)	VP012	Hypermarket	Cockle	M	M	W
		B14	1 (2.78)	VP011	Hypermarket	Cockle	S	S	W
		B15	1 (2.78)	VP008	Wet market	Cockle	W	W	W
		B16	1 (2.78)	VP009	Wet market	Cockle	S	M	W
		B17	1 (2.78)	VP010	Hypermarket	Cockle	W	M	W
		B18	1 (2.78)	VP004	Wet market	Cockle	W	W	W
		B19	1 (2.78)	VP005	Wet market	Cockle	W	W	W
		B20	1 (2.78)	VP003	Wet market	Cockle	M	W	W
		B21	1 (2.78)	VP006	Wet market	Cockle	M	W	W
		B22	1 (2.78)	VP007	Wet market	Cockle	W	W	W
		B23	1 (2.78)	VP015	Hypermarket	Cockle	M	W	W
		B24	1 (2.78)	VP024	Hypermarket	Clam	M	S	W
C	11(30.56)	C1	1 (2.78)	VP026	Hypermarket	Clam	S	S	W
		C2	1 (2.78)	VP033	Hypermarket	Shrimp	M	W	W
		C3	1 (2.78)	VP032	Hypermarket	Shrimp	M	W	W
		C4	1 (2.78)	VP025	Hypermarket	Clam	M	S	W
		C5	1 (2.78)	VP034	Wet market	Squid	M	W	W
		C6	1 (2.78)	VP035	Wet market	Squid	M	W	W
		C7	1 (2.78)	VP027	Wet market	Shrimp	S	S	W
		C8	1 (2.78)	VP029	Wet market	Shrimp	S	S	W
		C9	2(5.56)	VP028	Wet market	Shrimp	W	W	W
				VP030	Wet market	Shrimp	S	W	W
		C10	1 (2.78)	VP031	Wet market	Shrimp	W	W	W
D	1(2.78)	D1	1 (2.78)	ATCC	17802	ATCC17802	W	W	W

• RT ; room temperature

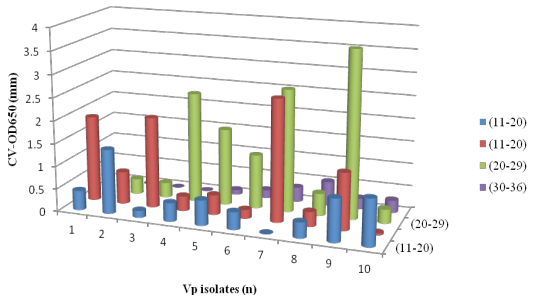

Figure 2. *V. parahemolyticus* biofilm formation with room temperature

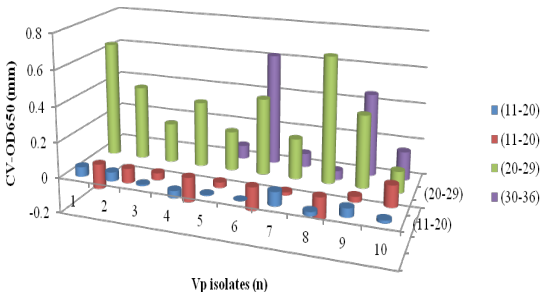

Figure 3. *V. parahaemolyticus* biofilm formation with 4 °C

biofilm producer. In addition, one of the factors accelerate the rapid growth of *V. parahaemolyticus* detection in wet market is the environment temperature (27 - 30°C) which can induces growth of this *V. parahaemolyticus*. However, when introduced into low temperature at 4ºC, *V. parahaemolyticus* can form a total of 91.67% weak biofilm producers and 8:33% in room temperature and no growth of strong

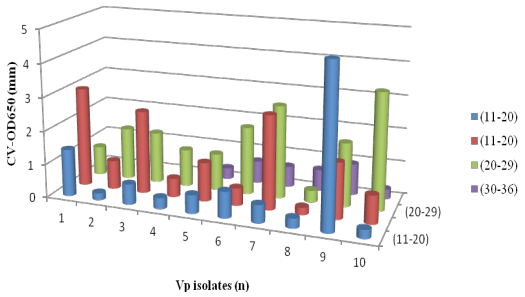

Figure 4. *V. parahaemolyticus* biofilm formation in 37°C

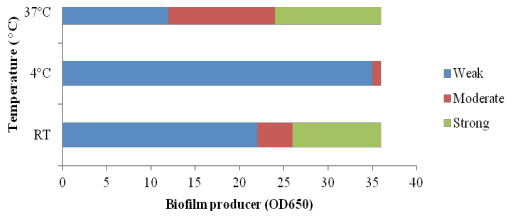

Figure 5. Number of biofilm producer of *V. parahaemolytiucus* with different temperature (Weak biofilm : < 0.6, Moderate : 0.6-1.2, > Strong : < 1.2) (Harvey *et al*., 2007)

biofilm producer.

The data and result from this RAPD assay were analyzed via GelCompare 4.2 software; hence, the outcome of analysis is presented as dendograms tree (Figure 1) and interpreted into Table 1. The dendrogram demonstrated the relationship of the isolates based on RAPD banding patterns. The analysis showed that majority of the *V. parahaemolyticus* isolates originated from seafood were branched into four major clusters (Type A,

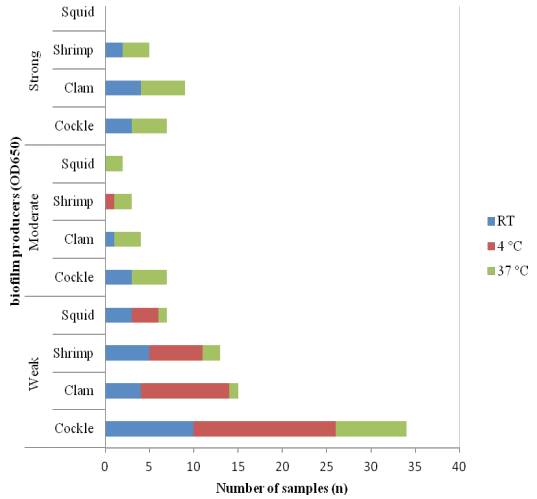

Figure 6. Number of biofilm producer of *V. parahaemolytiucus* with difference temperature according to the positive isolates

Type B, Type C and Type D) at 18.2%, 20.7% 34% and 3.4% similarity levels. All the clusters were related at 6% similarity. The dendrogram indicates the clustering of majority of the isolates originated from wet market 72.73% (8/11) into cluster Type A; while cluster Type B was member by isolates from wet market 64.29% (9/14) as well and cluster Type C was predominant by isolates from wet market as well with 63.64% (7/11). From observation based on the seafood types, only some seafood of the same types was in the same group (Shrimp from cluster Type C from wet market). Interestingly, our study has found out that isolates VP028 and VP030 has 100% similarities and this result demonstrated that strains of the sampling location were clustered into the same group and both clusters were related at 44.7% similarities. The similarities value for both location and sources were 47.2%. There was an evidence of both repeated isolation of similar strains and the isolation of multiple genotypically distinct strains. This suggests that while there is potential for a single strain to be distributed widely within a population, there is also potential for multiple contaminating strains of different clonal lineages to be present within the same population. The single cluster was ATCC 17802 originated from Shirasu food poisoning, Japan (Sakazaki *et al*., 2008) with 3.4% similarity value with others group.

The RAPD fingerprinting in this study demonstrate the producers of biofilm in each of the isolates from seafood in every cluster. Based in Table 1, the combination analysis of RAPD profiles with biofilm formation abilities was obtained. Upon analysis, Type B was the largest group comprises of 38.89% of the typable strains. A total of 92.86% (13/14) isolates in this group originated from cockles while only one single strain (VP024) derived from clam. From this group, a total of 21.43% (3/14) produce strong biofilm producer derived from cockle in 37ºC and room temperature respectively. While Cluster Type A tracked an amount of 45.45% of the isolates can produce strong biofilm which are majority derived from clam and 27.27% able to produce strong biofilm at room temperature. Cluster Type C stated a total of 36.36% produced strong biofilm at 37ºC and room temperature respectively. The combination of the RAPD with biofilm formation abilities to form is useful in tracking the source of the contamination and potential samples that will contribute to the high risk formation of biofilm can cause infection.

The optimum temperature for *V. parahaemolyticus* can form stable biofilm meeting with the agreement in International Commission on Microbiological Specifications for Foods (1990) stated that the optimum temperature for *V. parahaemolyticus* can growth is at 37°C. Thus, with the data obtained, *V. parahaemolyticus* not only can produce a stable biofilm growth due to the appropriate temperature but it have a potential to form in human body when orally consumed. As study from Bonaventura *et al*. (2000), stated the *in vitro* ability of microorganism to produce biofilm is generally tested at 37ºC, the inner temperature of human body. As related to the infectious dose for healthy individuals, recorded in outbreaks, an amount of 10^5 – 10^9 viable cells and results in an acute illness, following a short incubation period of between 4 – 30 hours.

Low temperature not only can inhibit the growth of planktonic cells of *V. parahaemolytcus* but its formation of biofilm as lower at 4°C. These results are compatible with Xiuping and Tu (1996) which states that the stress of nutrient deprivation and low temperature cause the induction of viable non culturable in some pathogenic bacteria. The level of storage temperature to stored seafood especially during transport for imports and exports can be below 4°C. The growth density of *V. parahaemolyticus* influenced by the temperature during the transportation and post-harvest processing, causing bacteria to grow to hazardous levels which could be dangerous if it do not immediately refrigerated (Cook *et al*., 1990).

Unfortunately, according to Cook *et al.* (1990) cooling and freezing could not completely destroy, but mishandling of seafood in the retail markets and food-service establishments can cause the rapid growth of *V. parahaemolyticus* as well (Deepanjali *et al*., 2005). This could be hazardous if the location of the biofilm occurs in food processing environments such after sloughing. The bacteria can incorporate into biofilm which can contaminate other surfaces, as well as food products. Therefore, introduction

to low temperature can be one of the treatment and more studies are needed to validate the efficacy of low temperature depuration in reducing naturally accumulated *V. parahaemolyticus* in a commercial scale. In striving to enable more convenient study of bacterial attachment and colonization, various indirect methods of observation have been developed.

According to the isolates from the seafood samples in Figure 6, *V. parahaemolyticus* positive detection from bivalves (cockles and clams) possesses a potential stronger biofilm. This is due to the habitat for both bivalves normally found in sandy beaches throughout the earth which are usually restricted to estuarine and coastal marine waters and this ecosystem is suitable for *V. parahaemolyticus* to grow. As we know, cockle is a popular ingredient in local foods and as in many areas of Asian; they are frequently consumed in a partially-cooked condition. While most of the strains from cluster Type A were isolated from clam in wet market while the other derived from cockle in hypermarket. The higher percentage of strong biofilm producer shown *V. parahaemolyticus* was isolated from clam samples. As reported by Centre of Disease (CDC, 1996) stated there is outbreak of *V. parahaemolyticus* infection associated with eating clams harvested from Long Island Sound, USA. Hence, this could be other factor caused contamination of clam by *V. parahaemolyticus* such as strong biofilm producer which do not killed by any disinfectant as well.

In addition, if according to the sampling location, a total of 27.78% (10/36) isolates contained strong biofilm producer from wet market at 37°C and 16.67% (6/36) at room temperature. While other isolates were moderate and weak biofilm producers. Based on observation, the cleanliness of the display location at the wet market itself was unsatisfactory. In addition, from observations during sample collection, the level of hygiene practised by handlers at wet markets was lower compared to hypermarket. Another surveillance of the surroundings and places, the seafood displays at wet markets were not clean and tidy, besides the handlers were not wearing gloves while handling the seafood when compared to supermarkets in which most of their handlers wear gloves. This could contribute to cross-contamination and could be the possible cause of prevalence of *V. parahaemolyticus* in seafood. Cross contamination could occur at any stage during the long processing and distribution chain.

RAPD assay in this study was shown to be useful in subtyping of *V. parahaemolyticus* strains as it shows genetic diversity and similarities between isolates. Not only DNA-based typing such as RAPD is important for tracking epidemiological and causal relationship, this method is also helpful in distinguishing the biofilm formation capabilities in isolated from every sources. Our study strongly highlight that the application of RAPD fingerprinting as a useful tool in investigating sources of contamination even identified biofilm capabilities besides certainly useful in determining the critical points in HACCP.

Conclusions

The subtyping data obtained from this study useful as a comparison with the epidemiological data obtained from different location and sources through RAPD. Thus, provide a new information with regards to the presence of *V. parahaemolyticus* in seafood samples as well as it possesses a potential biofilm formation can cause further outbreak in future. Further investigation of *V. parahaemolyticus* forming biofilm is important to better ensure seafood safety level.

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