CHARACTERIZATION OF *VIBRIO PARAHAEMOLYTICUS* ISOLATED FROM COASTAL SEAWATER IN PENINSULAR MALAYSIA

Gwendelynne Bulan Tanil[1], Son Radu[1], Mitsuaki Nishibuchi[4], Raha Abdul Rahim[2] Suhaimi Napis[3], Lesley Maurice[5] and Jurin Wolmon Gunsalam[1]

[1]Department of Food Science, Faculty of Food Science and Technology, University Putra Malaysia, Serdang, Selangor; [2]Department of Bioprocess Technology, [3]Department of Cell and Molecular Biology, Faculty of Biotechnology and Biomolecular Sciences, University Putra Malaysia, Malaysia; [4]Center for Southeast Asian Studies, Kyoto University, Japan; [5]Department of Biotechnology, Faculty of Engineering and Science, University Tunku Abdul Rahman, Genting Klang Road, Setapak, Selangor, Malaysia

Abstract. Twenty-one *Vibrio parahaemolyticus* isolates representing 21 samples of coastal seawater from three beaches in peninsular Malaysia were found to be sensitive to streptomycin, norfloxacin and chloramphenicol. Resistance was observed to penicillin (100%), ampicillin (95.2%), carbenicilin (95.2%), erythromycin (95.2%), bacitracin (71.4%), cephalothin (28.6%), moxalactam (28.6%), kanamycin (19.1%), tetracycline (14.3%), nalidixic acid (9.5%) and gentamicin (9.5%). Plasmids of 2.6 to 35.8 mDa were detected among plasmid-containing isolates. All isolates carried the *Vp-toxR* gene specific to *V. parahaemolyticus* and were negative for the *tdh* gene, but only one isolate was positive for the *trh* gene. DNA fingerprinting of the isolates using ERIC-PCR and PFGE showed that the isolates belong to two major clonal groups, with several isolates from different locations in the same group, indicating the presence of similar strains in the different locations.

INTRODUCTION

Vibrio parahaemolyticus is a well-known human pathogen and widely distributed in the marine and estuarine environment. *V. parahaemolyticus* is also associated with gastroenteritis from consumption of contaminated seafood (Nishibuchi and Kaper, 1995). A previous study showed that both *tdh* and/or *trh* genes are important virulence factors in *V. parahaemolyticus* (Shirai *et al*, 1990). Identification of *V. parahaemolyticus* strains isolated from the environment by standard biochemical tests is difficult, thus, the PCR method targeting the regulatory gene (*toxR*) was developed (Kim *et al*, 1999). The increasing prevalence of *V. parahemolyticus* demands an effective typing scheme to determine the origin and divergence of strains. In the present study, both the ERIC-PCR and PFGE methods were used to compare the relatedness of the strains isolated from different locations.

Correspondence: Son Radu, Department of Food Science, Faculty of Food Science and Technology, University Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.
Tel: 603-8946-8361; Fax: 603-8942-3552
E-mail: son@putra.upm.edu.my

MATERIALS AND METHODS

Sample collection, enrichment and isolation

Seawater samples were collected at three beaches (Morib, Kuala Lukut and Port Klang) on the west region of peninsular Malaysia from May to August, 2002. Samples were collected in presterilized bottles and examined 1 hour after sample collection. One milliliter of seawater was added to 9 ml of alkaline peptone water (APW). A series of four-fold dilution of each sample was made and the dilution of 10^{-4} bottle was incubated overnight at 37ºC. The next day, aliquots of 1 ml of the duplicate bottle (10^{-4}) was transferred into 5 ml of Salt Polymyxin Broth (SPB) and a series of four-fold dilution was prepared and further incubated overnight at 37ºC. Finally, 100 µl of the 10^{-4} dilution was plated onto the CHROMagar^{TM} *Vibrio* and incubated overnight

at 37ºC. Single mauve colored colonies were picked and enriched in SPB overnight before subjected to the *Vp-toxR* gene detection for confirmation as *V. parahaemolyticus* (Kim *et al*, 1999). *Vibrio parahaemolyticus* WP1 (positive control) and *Vibrio alginolyticus AQ*4045 (negative control) were obtained from Professor Mitsuaki Nishibuchi from University of Kyoto, Japan.

Antibiotics susceptibility testing

Antimicrobial susceptibility tests were performed by the disk diffusion method according to the National Committee for Clinical Laboratory Standard (1997). The *V. parahaemolyticus* were tested against the following antibiotics disks (BBL, Becton Dickinson, USA) on Mueller-Hinton agar (Oxoid, England): ampicillin, Am (10 µg); bacitracin, B (10 µg); carbenicillin, Car (100 µg); chloramphenicol, C (30 µg); erythromycin, E (15 µg); gentamicin, Gm (10 µg); kanamycin, K (30 µg); cephalothin, Kf (30 µg); moxalactam, Mox (30 µg); nalidixic acid, Na (30 µg); norfloxacin, Nor (30 µg); penicillin, P (10 µg); streptomycin, S (10 µg); and tetracycline, Te (30 µg). The control strain used was *Escherichia coli* (ATCC 25922). Multiple Antibiotic Resistance (MAR) index of the isolates was done as referred to Krumperman (1983). The MAR index is defined as a/b where 'a' represents the number of multiple antibiotics to which the particular isolates are resistant and 'b' the number of multiple antibiotics to which the particular isolates are exposed.

Plasmid DNA isolation

The isolates were screened for plasmid DNA according to the procedure as described by Sambrook *et al* (1989). Extracted plasmid DNA were electrophoresed in a 0.8% agarose gel and photographed under uv illumination. Reference plasmids from *Escherichia coli* V517 were included as molecular size markers.

Genomic DNA extraction

The isolates were grown in salt polymixin broth (Nissui, Japan) and incubated with shaking at 300 rpm for 24 hours at 37ºC. Cell pellet was collected by centrifugation and resuspended in 1 ml of sterile distilled water. Crude DNA extraction was carried out by the boiling method described by Hara-Kudo *et al* (2003).

PCR assays for the *vp-toxR, tdh* and *trh* genes detection

The boiled culture supernatant was used as the templates. Strain identification was done using species-specific primers targeting the presence of regulatory gene *vp-toxR* as described by Kim *et al* (1999). Confirmed *Vibrio parahaemolyticus* strains were then tested for the presence of *tdh* and *trh* genes using the primers described by Tada *et al* (1992). The PCR conditions were as follows; reaction mixture consisted of 3 µl of template DNA, 5 µl of 10x buffer, 4 µl of 25 mM $MgCl_2$, 0.25 µl of *Taq* polymerase, 4 µl of 2.5 mM dNTPs, 2 µl of each primer (10 pmol/µl) and 29.75 of sterile ultrapure water. The amplification conditions were 35 cycles of amplification consisting of denaturation at 94ºC for 1 minute, annealing at 63ºC (*Vp-toxR*) or 55ºC (*trh* and *tdh*) for 1 minute, and extension at 72ºC for 1 minute. Amplified products were resolved by electrophoresis in 1.2% agarose and photographed under uv illumination.

ERIC-PCR

The PCR amplifications were conducted by adding to the reaction mixture (25 µl per reaction) consisting of 7.5 µl of sterile distilled water, 2.5 µl of 10x PCR buffer, 1 µl of 25 mM $MgCl_2$, 0.5 µl of 1.25 mM dNTPs, 1 µl each of primers, ERIC1R 5´ ATGTAAGCTCCTGGGGATTCA3´ and ERIC2 5´ GTAAGTGACTGGGGTGAGCG 3´ (Hulton *et al*, 1991), 1 µl of *Taq* polymerase and 10 µl of the template DNA. An initial denaturation at 94ºC for 3 minutes was followed by 35 cycles of 94ºC for 1 minute, annealing at 36ºC for 1 minute, extension at 72ºC for 2 minutes, and a final extension at 72ºC for 5 minutes. Amplified products were separated by electrophoresis on 1.2% agarose gels and photographed under uv illumination. 1 kb DNA was used as the molecular size markers (Promega, Madison, WI USA).

Genomic DNA preparation for PFGE

The genomic DNA extraction protocol was done as described by the standardized PFGE protocol by Center for Disease Control and Prevention, Atlanta, Ga (CDC, 1998). DNA frag-

ments cleaved by restriction enzyme, *SfiI* were separated on 1.5% agarose gel by electrophoresis using the CHEF DR III apparatus (Bio-Rad). Electrophoresis was performed at 6V/cm at 14ºC for 25 hours. Bacteriophage lambda DNA ladder PFGE marker (New England Biolabs) was used as size marker. Following electrophoresis, the gel was stained with ethidium bromide and photographed under UV transillumination.

Analysis of ERIC and PFGE patterns

Computer-assisted fingerprint analysis, Gelcompar Version 5.0 (Applied Maths, Kortrijk, Belgium) was used for the comparative analysis of electrophoresis patterns.

RESULTS

A total of 21 strains representing 21 seawater samples from Morib (n=1), Kuala Lukut (n=3) and Port Klang (n=17) were confirmed by the *Vp-toxR* gene detection as *V. parahaemolyticus* were selected for further characterization. *In vitro* resistance profiles of the 21 environmental *V. parahaemolyticus* isolates against the antibiotics tested were presented in Table 1. They displayed resistance to nalidixic acid (9.5%), gentamicin (9.5%), tetracycline (14.3%), kanamycin (19.1%), cephalothin (28.6%), moxalactam (28.6%), bacitracin (71.4%), ampicillin (95.2%), erythromycin (95.2%), carbenicillin (95.2%) and penicillin (100%). All the isolates were susceptible to norfloxacin, streptomycin, and chloramphenicol. Table 1 also shows the MAR indices of the test strains that ranged from 0.29 to 0.57. Plasmids were detected in 13 isolates with sizes from 2.6 to 35.8 mDa. None of the isolates possessed the *tdh* gene and 0nly one isolate was positive for *trh* gene.

In ERIC-PCR, 2 to 15 bands with sizes ranging from < 1,000 bp to 4,200 bp were discernible in the strains except for strain PK 5 which was untypeable. In the PFGE analysis, 10 to 17 bands with sizes ranging from < 48.5 kbp to 339.4 kbp were distinct in the strains. Four strains were untypeable in this analysis due to persistent DNA degradation, despite repeating the DNA preparation and digestion three times. The clonal relationships among these *V. parahaemolyticus* strains were examined through the cluster analysis of the ERIC-PCR and PFGE generated patterns and were presented in dendrograms. In ERIC-PCR (Fig 1) the strains could be grouped into 2 clusters and 14 single isolates based on the 70% similarity level. In PFGE (Fig 2), the strains could be grouped into 4 clusters and 9 single isolates based on 70% similarity level. Interestingly, the third cluster grouped one strain each from Kuala Lukut and Morib together. One strain from Port Klang and one strain from Kuala Lukut were also located in the fourth cluster, respectively.

Table 1

Vibrio parahaemolyticus isolates used in this study.

Strain[a]	MAR index	Plasmid size (mDa)	Resistance pattern
PK4	0.29	3.5	AmCarEP
PK13	0.29	3.5,4.5	AmCarEP
W3	0.36	-[b]	AmBCarMoxP
PK11	0.36	3.1	AmBCarEP
PK7	0.36	-	AmBCarEP
PK3	0.36	4.8,35.8	AmBCarEP
PK5	0.36	3.4,4.5	AmCarEKP
PK12	0.36	2.6	AmCarEKP
PK8	0.36	2.8,3.4	AmBCarEP
PK9	0.36	3.5	AmBCarEP
PK14	0.36	3.4	AmBCarEP
PK15	0.36	-	AmBCarEP
PK17	0.36	-	AmBCarEP
W1	0.43	3.5	AmBCarEKfP
W2	0.43	4	AmCarEMoxNaP
PK10	0.43	3.5,4.5	AmBCarEMoxP
PK6	0.43	-	AmBCarEKP
PK2	0.43	3.4,4.5	BCarEKMoxP
A1	0.57	-	AmBCarEKfMoxPTe
PK16	0.57	-	AmBCarEKMoxPTe
PK1	0.57		AmCarEKfMoxNaTeP

[a] A, Morib; W, Kuala Lukut; PK, Port Klang. All isolates were *Vp-toxR* positive, *tdh* gene negative and only strain PK4 was *trh* gene positive.
[b] None detected

DISCUSSION

This study shows that potentially pathogenic *V. parahaemolyticus* are frequent contaminants in coastal seawater examined. The isolation of potentially pathogenic species among the

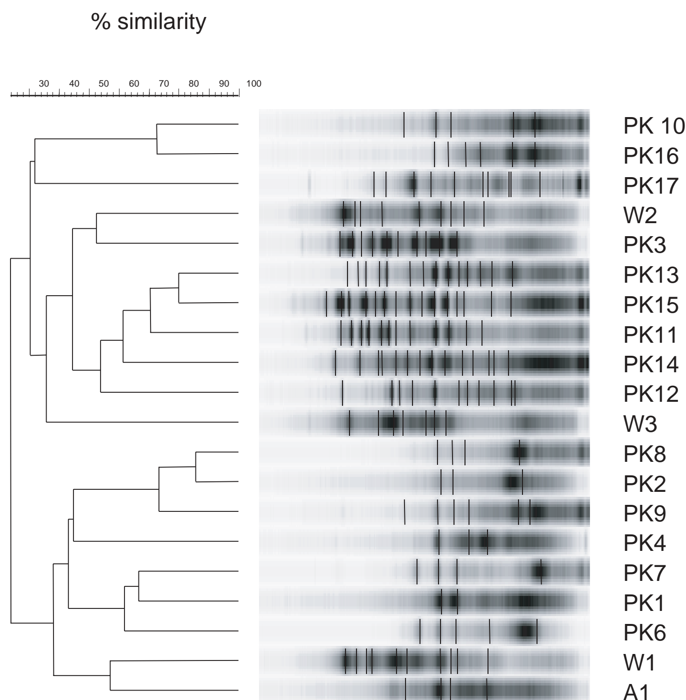

Fig 1–Dendrogram constructed from the ERIC-PCR profiles for the typable *V. parahaemolyticus* isolates.

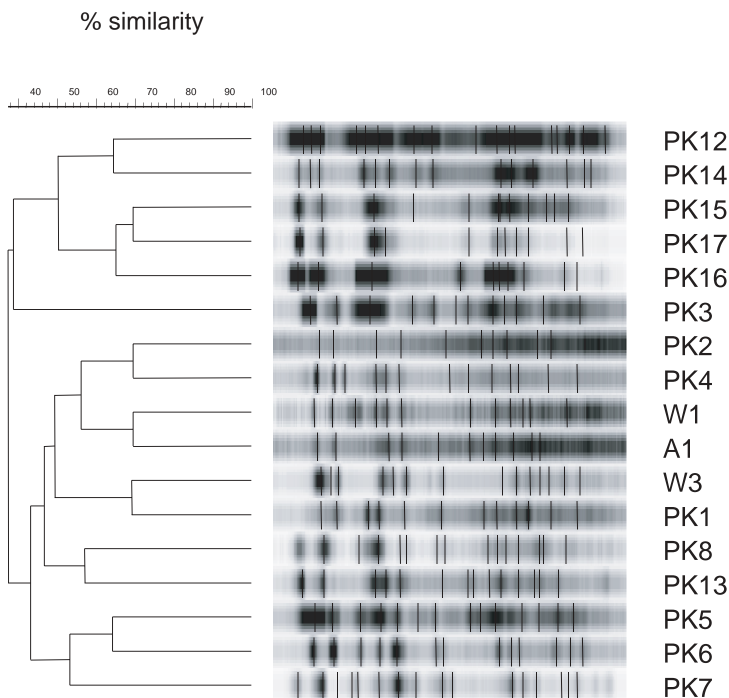

Fig 2–Dendrogram constructed from the PFGE profiles for the typable *V. parahaemolyticus* isolates.

strains represents a health hazard for individuals who consume raw seafood or direct contact with seawater from this area. However, during the sampling period there have been no reported cases or published data of any outbreaks associated with *V. parahaemolyticus* infection. PCR-based methods have been useful as genotypic tool in characterization and identification of the strains and have reduced the time spent on the research by eliminating the biochemical tests and toxin detection. The 21 isolates were confirmed as *V. parahaemolyticus* as shown by their positive results for the presence of the *Vp-toxR* gene. It is well known that the probability of isolation of *tdh* and or *trh* gene-positive strains from environmental or seafood samples are very low (Kishishita *et al*, 1992). In this study, only 4.8% (1 strain) of the environmental isolates was positive for *trh* gene. To the best of our knowledge, this is the first study to report on the presence of *trh* positive *V. parahaemolyticus* isolated from the environment source in Malaysia. According to Nishibuchi and Kaper (1995), only a small proportion of *V. parahaemolyticus* strains carry *tdh* or *trh* genes or both and these strains are considered virulent.

According to Rang and Dale (1987), bacteria may be resistant to many drugs simultaneously or to the drugs from the same antibiotics group. The results in this study also implicate some examples of resistance to the same group of drugs. Majority of the isolates were resistant to b-lactams used namely

penicillin, ampicilin and carbenicilin. This was strongly supported by other authors that also discovered multiple resistances of *Vibrio* spp to b-lactams (Joseph *et al,* 1978; French *et al*, 1989; Chai and Pace, 1994). None of the strains were resistant to chloramphenicol, streptomycin, and norfloxacin, which agreed with other authors that also reported the same antibiotics susceptibility of *V. parahaemolyticus* (French *et al*, 1989; Li *et al*, 1999). High MAR indices ranging from 0.29 to 0.57 were detected in this study (Table 1). MAR indices higher than 0.2 were rendered from high-risk sources (Krumperman, 1983) such as those from hospital sewage that somehow finds their way to the open sea via illegal dumping of waste or transferred by infected humans. In the plasmid profiling study, 61.9% of the isolates possessed plasmids ranging from 2.6 mDa to 35.8 mDa in size (Table 1). In this study, the plasmids profiling as an alternative for epidemiological analysis was not useful as there was high number of strains (38%), which were devoid of plasmids.

Based on the ERIC-PCR dendrogram (Fig 1), 2 clusters and 14 isolates were produced at the 70% similarity level indicating the presence of genetically heterogeneous strains. At this similarity level, none of the strains from different sampling sites were grouped together. ERIC-PCR analysis was able to resolve the *V. parahaemolyticus* strains untypeable by PFGE. Our results agreed with those of Marshall *et al* (1999) who reported that ERIC-PCR was useful for evaluating genetic and epidemiological relationships among *V. parahaemolyticus*. In this study, the PFGE method using *Sfi* I produced 10 to 17 bands and differentiated the isolates into 13 PFGE patterns was able to resolve the *V. parahaemolyticus* PK5 strain untypeable by ERIC. When Wong (2003) used *Sfi* I as restriction enzyme to type highly genetically diverse *V. parahaemolyticus* strains in seafood imported from Asian countries and strains from a nosocomial outbreak using PFGE, the 371 *V. parahaemolyticus* isolates were grouped into 57 patterns. The PFGE method was also able to clearly distinguish the O3:K6 strain from domestic strains (Wong, 2003). Elsewhere, PFGE was used effectively to discriminate 60 isolates of *V. parahaemolyticus* from a 1997 outbreak in British Columbia, Canada (Marshall *et al*, 1999).

For epidemiological purposes, PFGE and ERIC-PCR are suitable typing methods for *V. parahaemolyticus*. By combining both genotypic methods in characterizing bacteria, we can understand better about the ecology and diversity of environmental *Vibrio parahaemolyticus* as both typing approach were able to reveal the genetic proximity that existed among the strains from different origins. Generally, both dendrograms showed that majority of the isolates are grouped together despite coming from different places. Therefore, it is suggested that the strains could have originated from the same clonal linage of *V. parahaemolyticus.* There have been several possible means of transfer for *V. parahaemolyticus* including infected humans, tidal drift, ecological relationship with other marine organisms and via shipping vessels.

REFERENCES

Centers for Disease Control and Prevention. Standardized molecular subtyping of foodborne bacterial pathogens by pulsed field gel electrophoresis. CDC Training Manual. Atlanta, Ga: CDC, 1998.

Chai TJ, Pace J. *Vibrio parahaemolyticus*. In: Hui YH, Gorham JR, Murrel KD, Cliver DO, eds. Foodborne disease handbook. Disease caused by bacteria. Vol I. New York: Marcel Dekker, 1994.

French G, Woo ML, Hui YW, Chan KY. Antimicrobial susceptibilities of halophilic vibrios. *J Antimicrob Chemother* 1989; 24: 183-94.

Hara-KudoY, Sugiyama K, Nishibuchi M, *et al.* Prevalence of pandemic thermostable direct hemolysin-producing *Vibrio parahaemolyticus* O3:K6 in seafood and the coastal environment in Japan. *Appl Environ Microbiol* 2003; 69: 3883-91.

Hulton CSJ, Higgins CF, Sharp PM. ERIC sequences: a novel family of repetitive elements in the genomes of *Escherichia coli*, *Salmonella typhimurium* and other enterobacteria. *Mol Microbiol* 1991; 5: 825-34.

Joseph SW, Debel RM, Brown WP. *In vitro* response to chloramphenicol, tetracycline, ampicilin, gentamicin and β-lactamase production by halophiic vibrios from human and environmental sources. *Antimicrob Agents Chemother* 1978; 13:244-48.

Kim YB, Okuda J, Matsumoto C, Takahashi N, Hashimoto S, Nishibuchi M. Identification of *Vibrio parahaemolyticus* strains at species level by PCR targeted to the *toxR* gene. *J Clin Microbiol* 1999; 37: 1173-7.

Kishishita M, Matsuoka N, Kumagai K, Yamasaki S, Takeda Y, Nishibuchi M. Sequence variation in the thermostable direct hemolysin-related hemolysin (*trh*) gene in *Vibrio parahaemolyticus*. *Appl Environ Microbiol* 1992; 58: 2449-57.

Krumperman PH. Multiple antibiotic resistance indexing of *Escherischia coli* to identify high-risk sources of fecal contamination of food. *Appl Environ Microbiol* 1983; 46: 165-70.

Li J, Yie J, Rita FWT, Julia LML, Xu H, Norman WYS. Antibiotic resistance and plasmid profiles of *Vibrio* isolates from cultured silver sea bream, *Sparus sarba*. *Marine Pollut Bull* 1999; 39: 245-9.

Marshall S, Clark CG, Wang G, Mulvey M, Kelly MT, Johnson WM. Comparison of molecular methods for typing *Vibrio parahaemolyticus*. *J Clin Microbiol* 1999; 37: 2473-8.

National Committee for Clinical Laboratory Standards. Performance standards for antimicrobial disk susceptibility tests. Approved Standard M2-A6. Villanova, PA: National Committee for Clinical Laboratory, 1997.

Nishibuchi M, Kaper JB. Thermostable direct hemolysin gene of *Vibrio parahaemolyticus* : A virulence gene acquired by a marine bacterium. *J Infect Immun* 1995; 49: 181-6.

Rang HP, Dale MM. Pharmacology. London: English Language Book Society, 1987.

Sambrook J, Fritsch ES, Maniatis T. Molecular cloning: a laboratory manual. 2nd ed. Cold Spring Harbour, New York: Cold Spring Harbour, Laboratory Press, 1989.

Shirai H, Ito H, Hirayama T, *et al*. Molecular epidemiologic evidence for association of thermostable direct hemolysin (TDH) and TDH-related hemolysin of *Vibrio parahaemolyticus* with gastroenteritis. *J Infect Immun* 1990; 58: 3568-73.

Tada J, Ohashi T, Nishimura N, *et al.* Detection of the thermostable direct hemolysin gene (*tdh*) and the thermostable direct hemolysin-related gene (*trh*) of *Vibrio parahaemolyticus* by polymerase chain reaction. *Mol Cell Probes* 1992; 6: 477-87.

Wong HC. Detecting and molecular typing of *Vibrio parahaemolyticus*. *J Food Drug Anal* 2003; 11: 79-86.