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ORIGINAL PAPER

Antibiotic resistance and molecular typing among cockle (*Anadara granosa*) strains of *Vibrio parahaemolyticus* by polymerase chain reaction (PCR)-based analysis

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Abstract Genomic DNA of *Vibrio parahaemolyticus* were characterized by antibiotic resistance, enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR) and random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) analysis. These isolates originated from 3 distantly locations of Selangor, Negeri Sembilan and Melaka (East coastal areas), Malaysia. A total of 44 (n = 44) of tentatively *V. parahaemolyticus* were also examined for the presence of *tox*R, *tdh* and *trh* gene. Of 44 isolates, 37 were positive towards *tox*R gene; while, none were positive to *tdh* and *trh* gene. Antibiotic resistance analysis showed the *V. parahaemolyticus* isolates were highly resistant to bacitracin (92 %, 34/37) and penicillin (89 %, 33/37) followed by resistance towards ampicillin (68 %, 25/37), cefuroxime (38 %, 14/37), amikacin (6 %, 2/37) and ceftazidime (14 %, 5/37). None of the *V. parahaemolyticus* isolates were resistant towards chloramphenicol, ciprofloxacin, ceftriaxone, enrofloxacin, norfloxacin, streptomycin and vancomycin. Antibiogram patterns exhibited, 9 patterns and phenotypically less heterogenous when compared to PCR-based techniques using ERIC- and RAPD-PCR. The results of the ERIC- and RAPD-PCR were analyzed using GelCompare software. ERIC-PCR with primers ERIC1R and ERIC2 discriminated the *V. parahaemolyticus* isolates into 6 clusters and 21 single isolates at a similarity level of 80 %. While, RAPD-PCR with primer Gen8 discriminated the *V. parahaemolyticus* isolates into 11 clusters and 10 single isolates and Gen9 into 8 clusters and 16 single isolates at the same similarity level examined. Results in the presence study demonstrated combination of phenotypically and genotypically methods show a wide heterogeneity among cockle isolates of *V. parahaemolyticus*.

Keywords Antibiotic resistance · Molecular typing · *Vibrio parahaemolyticus* · ERIC-PCR · RAPD-PCR

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Introduction

Vibrio parahaemolyticus is a marine microorganism which can be isolated from fish, shellfish and marine water (Li et al. 1999; Molina-Aja et al. 2002). The ingestion of this bacterium into human body can cause illness similar to a mild form of cholera with an average incubation period of 15 h with rapid onset of symptoms. *V. parahaemolyticus* shows profuse diarrhoea which is leading to dehydration, vomiting, fever and acute abdominal pain. The illness usually lasts for 2–5 days. Cockle associated with *V. parahaemolyticus* can cause gastroenteritis by the consumption of undercooked or raw cockle meat (Pruzzo et al. 2005).

Cockle (*Anadara granosa*) is among popular seafood and consider as one of important protein source in Malaysia (Ang et al. 2005). Cockle has been produced commercially along the west coast of Pulau Pinang, Selangor and Perak in which it contributes RM54 millions to Malaysia's economic value from aquaculture production in year 2004 (Awang-Hazmi et al. 2007; Fariduddin 2008). The value is believed to increase in every year due to its

demand in local food industry such as tourism, raw and processed food.

The consumption of cockles is increasing due to its delicious to eat, in Malaysia it being served as *sambal kerang* (cockle with chilli paste), *kerang rebus* (boiled cockle) and *kerang bakar* (burned cockle). Therefore, the exposure of *V. parahemolyticus* to consumer becomes greater. No data has been reported on the outbreaks of this bacterium in Malaysia. However, the incidence of *V. parahaemolyticus* in cockle has been reported in year 1997 (Son et al. 1998) and these bacteria have also been reported present in black tiger shrimp (*Panaeus monodon*) (Ariyawansa 2008). The prevalence of *V. parahaemolyticus* in cockle is rather alarming and recently has also been reported by Ahmad Azuhairi et al. (2011). The continuous assessment should being carried out due to health public concern especially cockles are potentially serve as a vehicle for the transmission of disease to man.

In this study, we examined the presence of *tox*R, *tdh* and *trh* gene among *V. parahaemolyticus* isolates in cockles from 3 distantly locations of west coastal areas in Malaysia namely, Selangor, Negeri Sembilan and Melaka. The detection of *V. parahaemolyticus* in any samples can be analyzed through biochemical tests and PCR approaches by targeting the *tox*R gene on the DNA chromosomal of bacteria. *Tox*R gene is useful in specific identification of *V. parahaemolyticus* in foods and clinical samples (Kim et al. 1999). The *tox*R gene is a regulatory gene of cholera toxin (CT) operon and appears to be well conserved among *Vibrio* species. *Tox*R gene is also necessary for transcription of gene encoding a second regulatory protein, *tox*R which is the direct transcriptional activator of CT and toxin coregulated pilus (TPC) in *Vibrio cholerae*. The relation between virulence regulatory protein *tox*R in *V. cholarae* and other pathogenic *Vibrio* species including *V. parahaemolyticus* is well described by Provenzano et al. (2000) and Kim et al. (1999). Besides *tox*R gene, the ability of *V. parahaemolyticus* which can cause a disease is also related with the presence of a thermostable direct hemolysin (*tdh*) and the thermostable direct hemolysin-related gene (*trh*), both are associated with gastrointestinal illnesses especially in clinical strains (Shirai et al. 1990; Kishishita et al. 1992). While, typing among *V. parahaemolyticus* isolates are carried out using antibiotic resistance, enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR) and random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) fingerprinting. Strains differentiation of *V. parahaemolyticus* is important in surveillance of possible public health risk for predictive value in epidemiological control. Besides that, it also allows the identification of virulent strains and changing pattern of this bacterium to be monitored using PCR-based technology (Sahilah et al. 2010). These approaches are helpful to examine the genetic diversity and epidemiological relationships of the bacteria.

Materials and methods

Vibrio parahaemolyticus

A total of 44 (n = 44) tentatively *V. parahaemolyticus* isolates were obtained from the Laboratory of Food Sciences, Universiti Kebangsaan Malaysia, Selangor. *V. parahaemolyticus* isolates were isolated from cockle samples in 3 states of Peninsular Malaysia (East coastal areas: Selangor, Negeri Sembilan and Melaka). *V. parahaemolyticus* isolates VPS1–VPS19 were from Selangor; isolates VPN1–VPN18 were from Negeri Sembilan and isolates VPM1–VPM7 were from Melaka.

Detection of *tox*R gene

The detected of *tox*R gene and PCR condition was conducted as described by Kim et al. (1999). A pair of primer was used as sequences described toxR4 for forward, 5′-GTC TTC TGA CGC AAT-3′ and toxR7 for reverse 5′-ATA CGA GCG GTT GCT GTC ATG-3′. The assay was performed in a 20 µl volume containing 4.0 µl of 10× PCR buffer (100 mM of Tris-HCl, 20 mM of $MgCl_2$, 750 mM of KCl, pH 8.8), 1.6 µl of 2 mM of dNTPs (Vivantis, MY), 0.8 µl of 100 pmol of primer toxR4 and toxR7 each, 0.1 µl of 0.5 units of *Taq* DNA polymerase (Fermentas, MY), 10.7 µl of sterile ultrapure deionized water and 2.0 µl of 50 ng DNA template. A negative-DNA control was performed by adding 1 µl of sterile ultrapure deionized water, no positive control was performed. Amplification was performed in Eppendorf thermal-cycler (Eppendorf, Germany) with a temperature program consisting of the initial denaturation at 96 °C for 5 min followed by 30 cycles of denaturation at 94 °C for 1 min, annealing for 1.5 min at 63 °C and polymerization at 72 °C for 1.5 min. Final elongation was at 72 °C for 7 min. The amplification products were analyzed by electrophoresis in a 1.5 % (w/v) agarose 1X TAE (40 mM Tris-OH, 20 mM of acetic acid and 1 mM EDTA; pH 7.6) at 90 V for 40 min. Gels were stained with ethidium bromide. The amplified fragments were visualized with UV transilluminator (AlphaImager™ Gel Documentation). The 100 bp DNA ladder (Vivantis, MY) was used as a DNA size marker.

Detection of *tdh* and *trh* gene

The detection of *tdh* and *trh* gene and PCR condition was examined as described by Marlina et al. (2007). The assay was performed in a 20 µl volume containing, 4.0 µl

of 10× PCR buffer (100 mM of Tris-HCl, 20 mM of $MgCl_2$, 750 mM of KCl, pH 8.8), 1.6 µl of 2 mM of dNTPs (Vivantis, MY), 0.8 µl of 100 pmol of primer toxR4 and toxR7 each, 0.1 µl of 0.5 units of *Taq* DNA polymerase (Fermentas, MY), 10.7 µl of sterile ultrapure deionized water and 2.0 µl of 50–100 ng DNA template. A negative-DNA control was performed by adding 1 µl of sterile ultrapure deionized water, no positive control was performed. Amplification was performed in Eppendorf thermal-cycler (Eppendorf, Germany) with a temperature program consisting of the initial denaturation at 96 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing for 1 min at 55 °C and polymerization at 72 °C for 1 min. Final elongation was at 72 °C for 7 min (Marlina et al. 2007; Ariyawansa 2008). The amplification products were analyzed by electrophoresis in a 1.5 % (w/v) agarose in 1X TAE buffer (40 mM Tris-OH, 20 mM acetic acid and 1 mM of EDTA; pH 7.6) at 90 V for 40 min and stained by ethidium bromide. The 100 bp DNA ladder (Vivantis, MY) was used as size reference. The gels were visualized using UV transilluminator (Alpha Innotech, USA).

Antibiotic susceptibility

Antibiotic susceptibility test was carried out using the standard disc diffusion method by NCCLS (1997). Disc containing the following antibiotics was used: Ampicilin at 10 µg, Amikacin at 30 µg, Bacitracin at 10 µg, Chloramphenicol at 30 µg, Ceftazidime at 30 µg, Ciprofloxacin at 5 µg, Ceftriaxone at 30 µg, Cefuroxime at 30 µg, Enrofloxacin at 5 µg, Norfloxacin at 10 µg, Penicillin at 10 µg, Streptomycin at 10 µg and Vancomycin at 30 µg (OXOID). The plates were incubated at 37 °C for 24 h. The sensitivity or resistance of each isolate towards these antibiotics was determined by measuring the diameter of the inhibition zone around the antibiotic disc.

DNA extraction for PCR analysis

DNA extraction was done involving boiling, chilling and centrifugation (Jothikumar and Griffiths 2002; Sahilah et al. 2010). The cells were grown in 1.5 ml of Lauria–Bertani (LB) (tryptone, 4.0 g l^{-1}, yeast extract, 5.0 g l^{-1}, sodium chloride, 10.0 g l^{-1}) at 35 °C for 20 h were harvested and centrifuged at 12,000 rpm for 1 min. The supernatant was discarded. The pellet was then washed with 1.0 ml sterile distilled water and vortex. Then, it was boiled at 97 °C for 10 min and immediately was kept frozen at −20 °C for 10 min. The tube was centrifuged at 10,000 rpm for 3 min. The supernatant was used as a template.

ERIC-PCR analysis

The primer used were ERIC1R (5′-CACTTAGGGGTCCT CGAATGTA-3′) and ERIC2 (5′-AAGTAAGTGACTGGG GTGAGCG-3′) as described by Versalovic et al. (1991). PCR amplification reactions consisted of 25 µl volume containing 2 µl of 50 ng of genomic DNA, 2.5 µl 10× PCR buffer (500 mM KCl, 100 mM Tris-HCl, pH 9.1) and 0.1 % Triton™ X-100 (Vivantis, MY), 0.5 µl of 5 unit *Taq* polymerase (Vivantis, MY), 2.5 µl of 5 µM each of the forward and reverse primers and 0.5 µl of dNTPs and 14.5 sterile ultrapure deionized water. Amplification was performed in Eppendorf thermal-cycler (Eppendorf, Germany) with a temperature program consisting of the initial denaturation at 95 °C for 5 min followed by 35 cycles of denaturation at 92 °C for 45 s, annealing for 1 min at 52 °C and polymerization at 70 °C for 20 min. Final elongation was at 70 °C for 10 min. The amplification products were analyzed by electrophoresis in a 1.5 % (w/v) agarose in 1× TAE (40 mM Tris-OH, 20 mM acetic acid and 1 mM of EDTA; pH 7.6) at 90 V for 40 min. Gels were stained with ethidium bromide. The amplified fragments were visualized with UV transilluminator (Alpha Innotech, USA). The 1 kb DNA ladder (Vivantis, MY) was used as a DNA size marker.

RAPD-PCR analysis

The discriminatory ability and stability of RAPD-PCR fingerprinting were conducted with primer Gen8 (5′-GGAAGACAAC-3′) and Gen9 (5′-AGAAGCGATG-3′) which showed the greatest stability and discriminatory ability among the *V. parahaemolyticus* isolates and was therefore used in this study. The RAPD-PCR fingerprinting assay was performed in a 25 µl volume containing 2.5 µl of 10X PCR buffer (500 mM KCl, 100 mM Tris-HCl, pH 9.1) and 0.1 % Triton™ X-100 (Vivantis, MY), 0.5 µl of 2.5 mM dNTPs (Vivantis, MY), 2.5 µl of 100 mM primer, 0.5 µl of 2.5 units of *Taq* DNA polymerase (Vivantis, MY), 14.0 µl deionized water and 5.0 µl of 10 ng DNA template. A negative-DNA control was performed by adding 1 µl of sterile ultrapure deionized water. Amplification was performed in Eppendorf thermal-cycler (Eppendorf, Germany) with a temperature program consisting of the initial denaturation at 94 °C for 5 min followed by 45 cycles of denaturation at 94 °C for 1 min, annealing for 1 min at 35 °C and polymerization at 72 °C for 2 min. Final elongation was at 72 °C for 7 min (Sahilah et al. 2010). The amplification products were analyzed by electrophoresis in a 1.5 % (w/v) agarose in 1.0× TAE (0.1 M Tris, 0.1 M Boric acid, 0.1 mM EDTA) at 100 V for 40 min. Gels were stained with ethidium bromide. The amplified fragments were visualized with UV

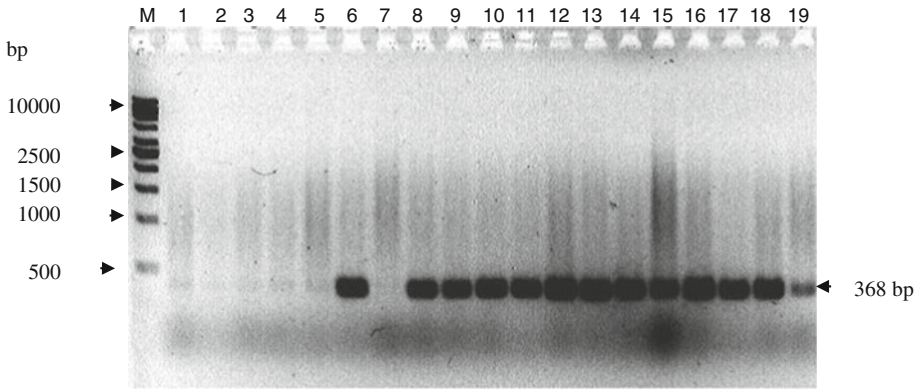

Fig. 1 Detection of *tox*R gene of *V. parahaemolyticus* isolates from Selangor by polymerase chain reaction (PCR) electrophoresed on 1.5 % (w/v) agarose gel. *M* molecular weight sizes (base pairs, bp) are indicated by *numbers* on the *left*, *lanes 1–19* VPS1–VPS19

transilluminator (Alpha Innotech, USA). The 1 kb DNA ladder (Vivantis, MY) was used as a DNA size marker.

Data analysis

Clonal relatedness of the *V. parahaemolyticus* was estimated by ERIC-PCR and RAPD-PCR from image of gel photographs using Gel ComparII image analysis software (Applied Math, Kortjik, Belgium) with the band matching coefficient of Dice (Sahilah et al. 2000) The position of the markers run in both ERIC and RAPD were used to normalize the sample data by correcting lane-to-lane and gel-to-gel variation. Isolates were clustered using average linkage (UPGMA, unweighted group pair method with arithmetic averages) and displayed in dendrogram form.

Results

Detection of *tox*R, *tdh* and *trh* gene

A total of 44 (n = 44) of *V. parahaemolyticus* isolates were examined for the presence of *tox*R gene on the chromosomal DNA of the bacteria, only 37 isolated showed positive towards *tox*R gene which produced 368 bp amplicons (Figs. 1, 2, 3). As indicated in Figs. 1, 2 and 3, of 19 *V. parahaemolyticus* strains isolated from Selangor, 6 were negative towards *tox*R gene (strains VPS1–VP5 and VPS7). A single strain of *V. parahaemolyticus* isolated from Melaka was negative towards *tox*R gene (strain VP1). While, all strains from Negeri Sembilan were positive towards *tox*R gene (strains VP10–VP29). Thus, only 37 isolates of *V. parahaemolyticus* were examined for antibiotic resistance, ERIC- and RAPD-PCR analysis. All strains which were positive toward *tox*R gene were analyzed for the presence of *tdh* and *trh* gene on the chromosomal DNA of bacteria. However, none of the *V. parahaemolyticus* strains were positive towards both gene.

Antibiotic susceptibility test

All *V. parahaemolyticus* strains (n = 37) were tested for their susceptibility towards 13 antibiotics as tabulated in Table 1. Antibiotic susceptibility analysis showed that all 37 *V. parahaemolyticus* isolates were resistant to one or more antibiotics which produced 9 antibiogram patterns, namely R1 to R9. They were highly resistant to bacitracin (92 %, 34/37) and penicillin (89 %, 33/37). Resistance towards ampicillin and cefuroxime were of 68 % (25/37) and 38 % (14/37), respectively. While, 6 % (2/37) and 14 % (5/37) were resistant towards amikacin and ceftazidime, respectively. None of the *V. parahaemolyticus* isolates were resistant towards chloramphenicol, ciprofloxacin, ceftriaxone, enrofloxacin, norfloxacin, streptomycin and vancomycin.

ERIC-PCR analysis

ERIC-PCR analysis indicated that the 37 isolates of *V. parahaemolyticus* were differentiated into 36 strains, namely E1–E36. Strain VPS6 and VPS8 were showed similar patterns. Data analyzed using Gel ComparII has formed dendrogram as shown in Fig. 4. As indicated in Fig. 4, at 80 % similarity level the 37 *V. parahaemolyticus* isolates produced 6 clusters and 21 single isolates.

RAPD-PCR analysis

In RAPD-PCR analysis, using primer Gen8 and Gen9, the 37 *V. parahaemolyticus* isolates were differentiated into 28 and 35 strains, namely P1–P28 and Q1–Q35, respectively. Analysis using Gel ComparII, dendrogram formed by primer Gen8 has shown 11 clusters and 10 single isolates at 80 % similarity level as shown in Fig. 5. While, primer Gen9 produced 8 clusters and 16 singles isolates at the same level examined as shown in Fig. 6.

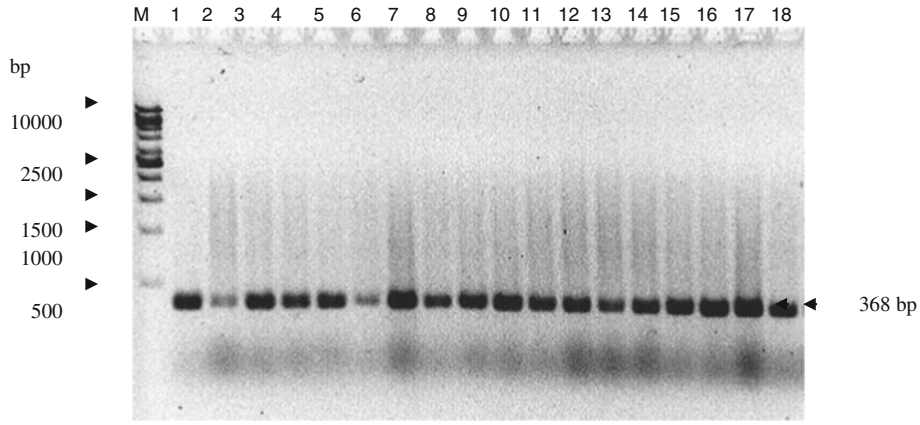

Fig. 2 Detection of *tox*R gene of *V. parahaemolyticus* isolates from Negeri Sembilan by polymerase chain reaction (PCR) electrophoresed on 1.5 % (w/v) agarose gel. *M* molecular weight sizes (base pairs, bp) are indicated by *numbers* on the *left*, *lanes 1–18* VPN1–VPN18

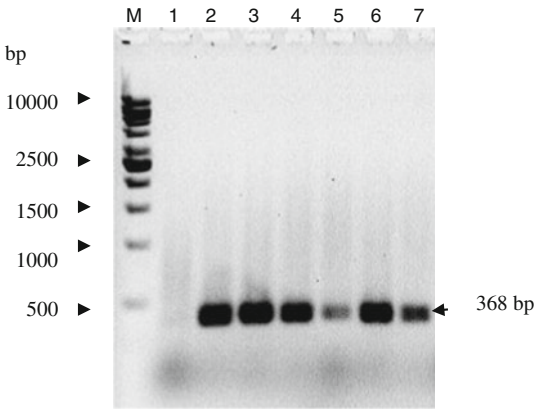

Fig. 3 Detection of *tox*R gene of *V. parahaemolyticus* isolates from Melaka by polymerase chain reaction (PCR) electrophoresed on 1.5 % (w/v) agarose gel. *M* molecular weight sizes (base pairs, bp) are indicated by *numbers* on the *left*, *lanes 1–7* VP1–VP7

Discussion

Large outbreaks of *V. parahaemolyticus* infection in human have not been reported in Malaysia. However, the incidence of this bacterium associated with seafood especially cockles were first reported in year 1998 (Son et al. 1998) and 2011 (Ahmad Azuhairi et al. 2011). The presence of these bacteria has also been reported in black tiger shrimp (Ariyawansa 2008). Study on the genetic diversity among cockle isolates of *V. parahaemolytica* isolated from Malaysia using random amplified polymorphic DNA-PCR was reported by Son et al. (1998), since then, no there are no extensively studies on the genetic diversity of *V. parahemolyticus* isolates especially from east coastal areas of 3 different states in Malaysia (Selangor, Negeri Sembilan and Melaka). There was study conducted on the antibiotic resistance and plasmid profiling *of V. parahaemolyticus* by Lesley et al. (2011), but they were from single location of Tanjung Karang, Selangor. Precise determination of *V. parahaemolyticus* clones is now well accepted as an important part in the trace of epidemiological investigation and DNA-based approaches is one way of examining *V. parahaemolyticus* that may assist to elucidate the epidemiology of *V. parahaemolyticus* strains in Malaysia.

In the presence study, a total of 44 (n = 44) tentatively *V. parahaemolyticus* isolates isolated from cockle were examined for the presence of *tox*R gene as specific identification for these bacteria. All isolates were previously isolated onto thiosulfate citrate bile salts sucrose (TCBS) medium and showed morphological colonies characterization as mucoid and were blue-green in colour. The identification using biochemical test has been conducted previously as described by Ahmad Azuhairi et al. (2011). Of 44 *V. parahaemolyticus* isolates only 37 colonies showed positive toward *tox*R gene which confirmed as *V. parahaemolyticus* colonies. Our result was consistent in repeated experiments and in agreement with Kim et al. (1999) whose explained the specific identification of *V. parahaemolyticus* isolates produced 368 bp amplicons in size (Figs. 1, 2, 3). Thus, all colonies which were positive towards *tox*R were further characterized using molecular typing of ERIC- and RAPD-PCR fingerprinting.

The bacteria isolate which were confirmed as *V. parahaemolyticus* isolates were then examined for the presence of a thermostable direct hemolysin (*tdh*) and the thermostable direct hemolysin-related gene (*trh*). However, none of the isolates were positive towards both genes. This result was expected since it was reported only clinical isolates of *V. parahaemolyticus* co-existed carried both gene was high which showed 91 % isolates as reported by Annick et al. (2004). However, in contrast as reported by Shirai et al. (1990) and Kishishita et al. (1992) reported the co-existed of both genes was low in clinical samples. The chances of *V. parahaemolyticus* isolates from seafood which carried both gene was also low as demonstrated by Honda and Iida (1993), Annick et al. (2004), Marlina et al. (2007) and Roque et al. (2009). Similar finding was also shown by Nair et al. (2007) whose reported the pathogenic isolates of *V. parahaemolyticus* which were capable inducing

Table 1 Typing among cockle (*A. granosa*) isolates of *V. prahaemolyticus* using antibiotic resistance profiles and PCR-based techniques

Strains no.[a]	*tox*R	*tdh*	*trh*	Antibiotic resistance (patterns)[b] and their groups	ERIC profiles	RAPD profiles using different primer		Genome types[c]
						Gen8	Gen9	
VPS1	−	−	−	N	N	N	N	N
VPS2	−	−	−	N	N	N	N	N
VPS3	−	−	−	N	N	N	N	N
VPS4	−	−	−	N	N	N	N	N
VPS5	−	−	−	N	N	N	N	N
VPS6	+	−	−	AmpBP (R1)	E1	P1	Q1	1
VPS7	−	−	−	N	N	N	N	N
VPS8	+	−	−	CazBP (R2)	E1	P2	Q2	2
VPS9	+	−	−	AmpBP (R1)	E2	P3	Q3	3
VPS10	+	−	−	AmpCxmCazBP (R3)	E3	P4	Q4	4
VPS11	+	−	−	CazBP (R2)	E4	P4	Q5	5
VPS12	+	−	−	CazBP (R2)	E5	P5	Q6	6
VPS13	+	−	−	CazBP (R2)	E6	P6	Q7	7
VPS14	+	−	−	B (R4)	E7	P7	Q8	8
VPS15	+	−	−	BP (R5)	E8	P8	Q7	9
VPS16	+	−	−	AmpBP (R1)	E9	P9	Q9	10
VPS17	+	−	−	BP (R5)	E10	P10	Q9	11
VPS18	+	−	−	BP (R5)	E11	P11	Q10	12
VPS19	+	−	−	N N	E12	P12	Q11	13
VPN1	+	−	−	AmpCxmBP (R6)	E13	P13	Q12	14
VPN2	+	−	−	AmpCxmBP (R6)	E14	P14	Q13	15
VPN3	+	−	−	AmpCxmP (R7)	E15	P14	Q14	16
VPN4	+	−	−	AmpCxmBP (R6)	E16	P14	Q15	17
VPN5	+	−	−	AmpCxmBP (R6)	E17	P15	Q16	18
VPN6	+	−	−	AmpCxmBP (R6)	E18	P16	Q17	19
VPN7	+	−	−	AmpBP (R1)	E19	P14	Q18	20
VPN8	+	−	−	AmpBP (R1)	E20	P17	Q19	21
VPN9	+	−	−	AmpBP (R1)	E21	P18	Q20	22
VPN10	+	−	−	AmpBP (R1)	E22	P19	Q21	23
VPN11	+	−	−	BP (R5)	E23	P20	Q22	24
VPN12	+	−	−	AmpBP (R1)	E24	P21	Q23	25
VPN13	+	−	−	AmpBP (R1)	E25	P14	Q24	26
VPN14	+	−	−	AmpB (R8)	E26	P14	Q25	27
VPN15	+	−	−	AmpBP (R1)	E27	P22	Q26	28
VPN16	+	−	−	AmpCxmBP (R6)	E28	P17	Q27	29
VPN17	+	−	−	AmpBP (R1)	E29	P11	Q287	30
VPN18	+	−	−	B (R4)	E30	P23	Q29	31
VPM1	−	−	−	N	N	N	N	N
VPM2	+	−	−	AmpCxmBP (R6)	E31	P24	Q30	32
VPM3	+	−	−	AmpCxmBP (R6)	E32	P25	Q31	33
VPM4	+	−	−	AmpCxmBP (R6)	E33	P26	Q32	34
VPM5	+	−	−	AmpCxmBP (R6)	E34	P27	Q33	35
VPM6	+	−	−	AmpCxmAkBP (R9)	E35	P26	Q34	36
VPM7	+	−	−	AmpCxmAkBP (R9)	E36	P28	Q35	37
Total	37	0	0	9	36	28	35	37

N not determined, *ND* not detected, + positive result, − negative result

[a] *V. parahaemolyticus* isolates VPS1–VPS19—isolates from Selangor, VPN1–VPN18—isolates from Negeri Sembilan, VPM1–VPM7—isolates from Melaka

[b] Tested for Ampicillin (Amp), Amikacin (Ak), Bacitracin (B), Chloramphenicol (C), Ceftazidime (Caz), Ciprofloxacin (Cip), Ceftriaxone (Cro), cefuroxime (Cxm), Enrofloxacin (Enr), Norfloxacin (Nor), Penicillin (P), Streptomycin (S), and Vancomycin (Va)

[c] Combination within antibiotic susceptibility, ERIC- and RAPD-PCR profiles

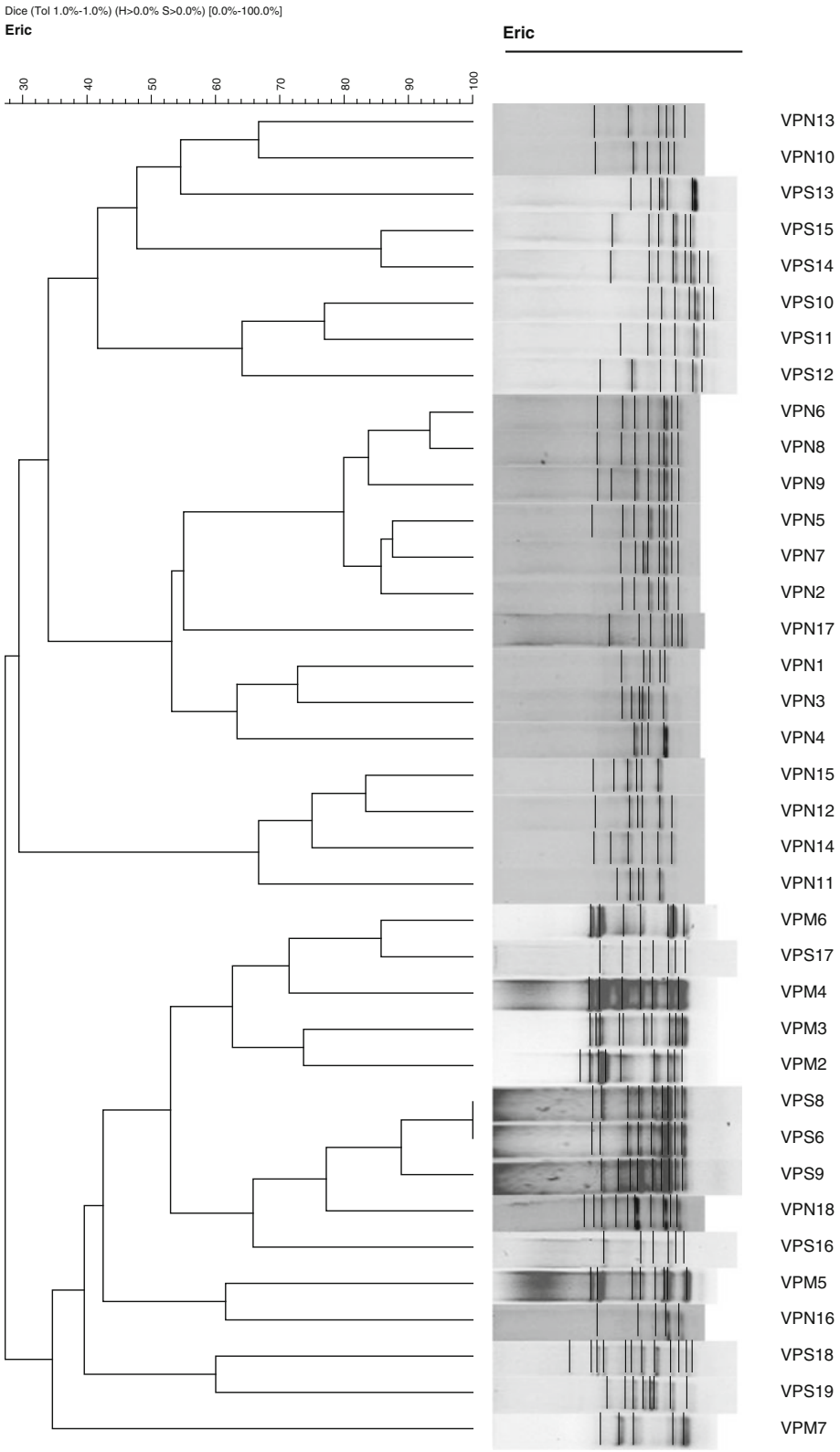

Fig. 4 *Dendrogram* of typable *V. parahaemolyticus* isolates produced from ERIC analysis using average linkage unweighted group pair method with arithmetic averages (UPGMA)

gastroenteritis in humans are rare in environmental samples (2–3 %) or are often not detected (Honda and Iida 1993). Thus, none occurrence of both pathogenic genes in *V. parahaemolyticus* may due to the less isolate examined. More isolates are tested more *tdh* and *trh* genes could be detected.

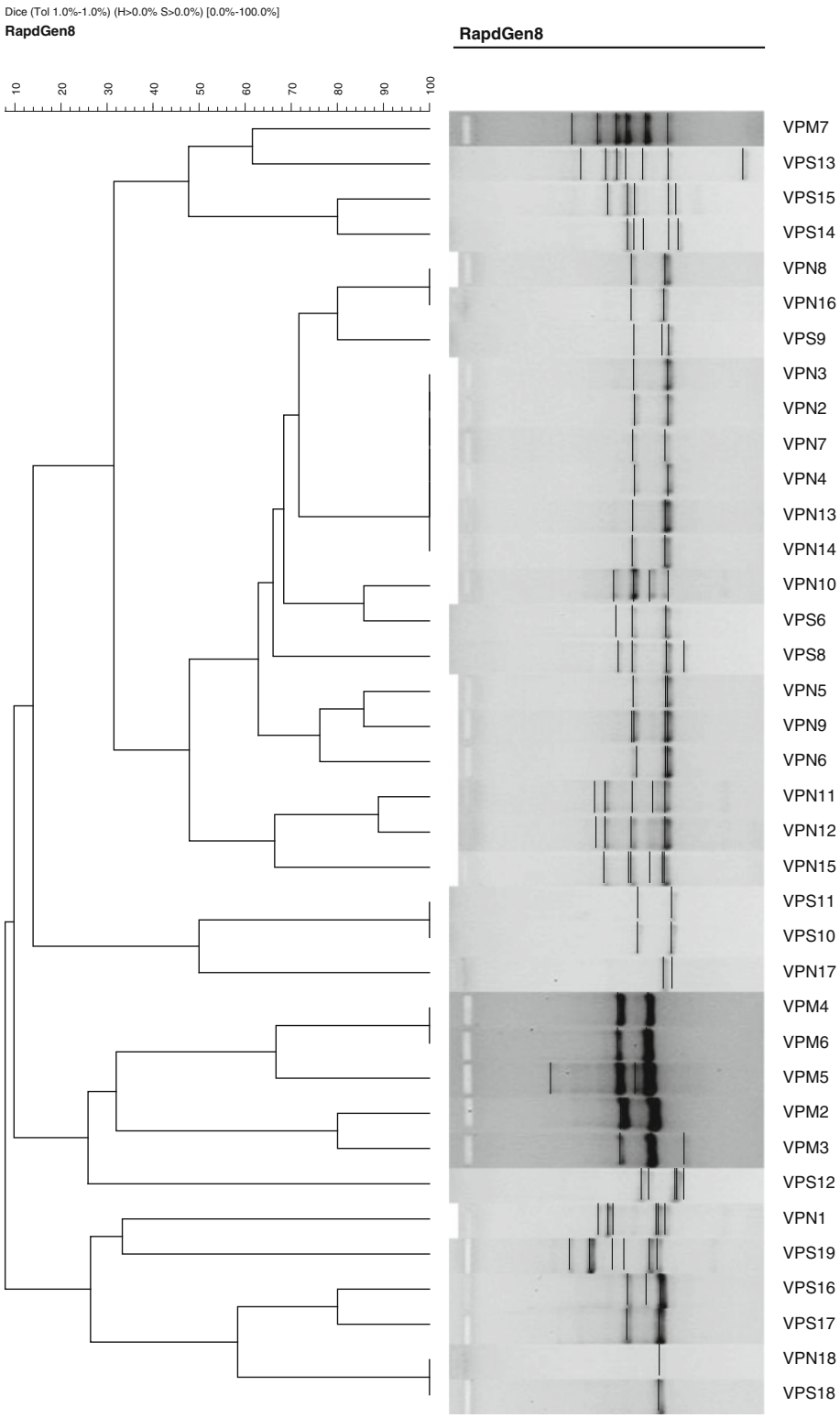

Fig. 5 *Dendrogram* of typable *V. parahaemolyticus* isolates produced from RAPD analysis (Gen8) using average linkage unweighted group pair method with arithmetic averages (UPGMA)

The 37 *V. parahaemolyticus* isolates which were confirmed and identified as *V. parahaemolyticus* were then examined for antibiogram. As demonstrated in Table 1, the frequent antibiotic resistance profiles of *V. parahaemolyticus* strains isolated from Selangor showed by pattern R2 (Ceftazidime, Bacitracin and Penicillin), isolates from Negeri Sembilan dominated by patterns R1 (Amplicillin, Bacitracin and Penicillin) and isolates from Melaka by patterns R6 (Ampicillin, Cefuroxime, Bacitracin and Penicillin). Those antibiotic resistance profiles were therefore found to be and acceptable

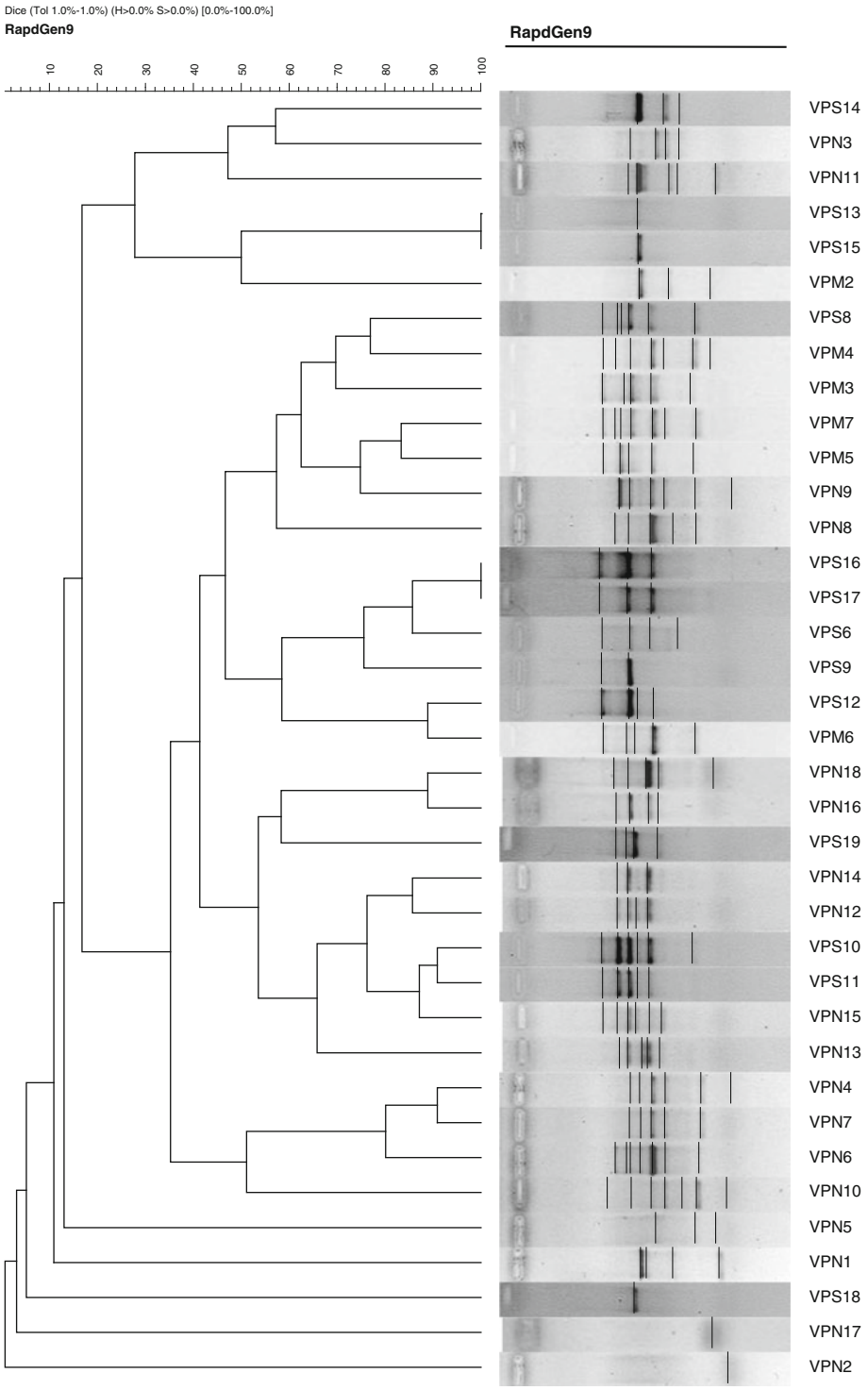

Fig. 6 *Dendrogram* of typable *V. parahaemolyticus* isolates produced from RAPD analysis (Gen9) using average linkage unweighted group pair method with arithmetic averages (UPGMA)

markers in 3 different distantly locations set of isolates. The antibiotic resistance profiles, however phenotypically exhibited less heterogenous. Antibiotic resistance test revealed, 9 antibiograms namely R1 to R9 (Table 1). Thus, for epidemiological purposes, antibiotic resistance profiles only showed limited degree of strain differentiation. Besides, using antibiogram as one approaches of strain differentiation, study on the antibiotic resistance was also important for more effective therapy antibiotic management of infections of *V. parahaemolyticus* in human. Majority of the isolates displayed resistant towards bacitracin (92 %, 34/37), penicillin (89 %, 33/37), ampicillin (68 %, 25/37) and cefuroxime (38 %, 14/37). While, only 6 % (2/37) and 14 % (5/37) were resistant towards amikacin and ceftazidime, respectively. None of the *V. parahaemolyticus* isolates were resistant towards chloramphenicol, ciprofloxacin,

ceftriaxone, enrofloxacin, norfloxacin, streptomycin and vancomycin. Our results confirm data reported by other authors demonstrating that the frequency of antibiotic resistance in *V. parahaemolyticus* has shown to be high (Zulkifli et al. 2009a, b; Lesley et al. 2011). Multiresistance of *V. parahaemolyticus* isolates (Table 1) was also observed in the presence study. The prevalence of such antibiogram in cockle isolates in 3 different distantly locations were inconclusive.

Comparing with PCR-based analysis (ERIC- and RAPD-PCR) and antibiotic resistance profiles, demonstrated that these approaches was shown genotypically very heterogenous. ERIC-PCR analysis for instance, the 37 *V. parahaemolyticus* isolates showed 36 ERIC profiles. While, in RAPD-PCR analysis, using two different primers of Gen8 and Gen9 produced, 28 and 35 RAPD profiles, respectively. Analysis derived from these data in combination (antibiotic resistance, ERIC- and RAPD-PCR), all 37 isolates were distinct into 37 individual isolates. Both DNA-based techniques in this study showed differences in the capacity to differentiate between 37 *V. parahaemolyticus* isolates. ERIC-PCR was shown to be a typing method with slightly higher degree of discriminative power that was superior to RAPD-PCR. However, it is evident combination of both results increase level of strain differentiation. These results were consistent as reported by Sahilah et al. (2010) where ERIC- and RAPD-PCR analysis increased the discrimination level when both techniques were used in combination for strains differentiation among egg and raw meat isolates of *Escherichia coli*.

Comparison of both PCR-based techniques (ERIC- and RAPD-PCR) and antibiotic resistance profiles, PCR-based techniques showed high discriminating power and undoubtedly to be of great help in strain differentiation study. This was support by the finding reported by Zulkifli et al. (2009a, b) whose demonstrated that ERIC- and RAPD-PCR analysis is useful and showed high degree of strain discrimination in *V. parahaemolyticus* isolates.

Data analyzed using average linkage (UPGMA, unweighted group pair method with arithemetic averages) and displayed in dendrogram form, demonstrated that ERIC-PCR analysis of 37 *V. parahaemolyticus* isolates produced 6 clusters and 21 single isolates at 80 % similarity level (Fig. 4). While, in RAPD-PCR analysis the Gen8 and Gen9, have produced 11 clusters and 10 single isolates; and 8 clusters and 16 single isolates at the same level examined (Figs. 5, 6). We could not see a specific pattern or clusters to be associated within 3 distantly origin of *V. parahaemolyticus* isolates using both techniques (ERIC- and RAPD-PCR). Neither strains from Selangor, Negeri Sembilan nor Melaka, the results exhibited a wide heterogeneity within *V. parahaemolyticus* isolates.

Conclusion

In conclusion, our data exhibit high level of local geographical genetic variation of *V. parahaemolyticus* isolates. It is an evident ERIC-PCR and RAPD-PCR typing methods have shown to be useful for intraspecies discrimination.

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