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Isolation, Detection and Genomic Differentiation of *Vibrio cholerae* and *Vibrio parahaemolyticus* in Bachok, Kelantan

Vimala B.[1,2], Thong Kwai Lin[1,2]* and Chong V. C.[1]

[1]Institute of Biological Sciences, Faculty of Science & [2]BioMedical Science and Molecular Typing Laboratory, Institute of Postgraduate Studies, University of Malaya, 50603 Kuala Lumpur, Malaysia.
* thongkl@um.edu.my (corresponding author)
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ABSTRACT The objective of this study was to determine the genetic diversity and virulence factors of *Vibrio cholerae* and *V. parahaemolyticus* from selected aquatic environments in Bachok, Kelantan, Malaysia. Out of 50 water samples, 33 were confirmed with the presence of presumptive *V. cholerae* and *V. parahaemolyticus* based on biochemical tests. Primers targeting *tox*R gene specific for *V. cholerae* (800bp) and *V. parahaemolyticus* (368bp), *tdh* (251bp) and *trh* (250bp) virulence genes were used in the PCR. Results of PCR showed that only 25 isolates of *V. parahaemolyticus* and 3 of *V. cholerae* had the *tox*R gene. None of the *V. parahaemolyticus* isolates had the *trh* and *tdh* genes. Repetitive extragenic palindromic PCR (REP-PCR) was used to determine the relatedness of these strains at the genomic level. Cluster analysis for REP-PCR showed 2 major clusters A-B, with several isolates from different locations grouped together indicating the geographical relatedness due to interconnected waterways. REP-PCR showed that the species of *Vibrios* are genetically diverse.

ABSTRAK Objektif kajian ini adalah untuk menentukan diversiti gen and faktor-faktor virulen *Vibrio cholerae* dan *Vibrio parahaemolyticus* yang diperolehi di sekitar perairan Bachok, Kelantan. Sejumlah 33 daripada 50 sampel air yang diuji melalui kaedah biokimia didapati positif untuk *V. cholerae* dan *V. parahaemolyticus*. Bagi amplifikasi PCR, primer *toxR* yang spesifik kepada *V. cholerae* (800bp) dan *V. parahaemolyticus* (368bp) serta primer-primer untuk mengesan gen-gen virulen seperti *tdh* (251bp) and *trh (*250bp) juga digunakan. Hasil PCR menunjukkan hanya 25 strain *V. parahaemolyticus* dan 3 strain *V. cholerae* mempunyai gen *toxR*. Tiada gen virulen dikesan pada mana-mana strain *V. parahaemolyticus.* REP-PCR diaplikasi untuk menentukan hubungan antara strain-strain yang telah diasingkan dalam kajian ini. Analisis dendrogram bagi REP-PCR menunjukkan 2 kumpulan utama, A-B. Strain-strain yang berasal dari lokasi yang berbeza dikumpulkan bersama. Ini mungkin disebabkan oleh faktor geografi di mana sistem perairan yang berkaitan. Keputusan REP-PCR menunjukkan variasi genetik yang nyata di dalam spesies *Vibrio.*

(**Keywords:** Vibrios, *toxR , tdh , trh ,* REP-PCR)

INTRODUCTION

Vibrio spp. are natural inhabitants of freshwater, estuarine and seawater environments. Therefore, the aquatic environment acts as a reservoir and source of their transmission [1]. In this study, we have focused on *V. cholerae* and *V. parahaemolyticus* since these two *Vibrio* spp. are potential, emerging water-borne pathogens responsible for negative impact to humans, marine animals and aquaculture [2, 3]. Few studies conducted in Malaysia, however, have indicated the prevalence of *V. parahaemolyticus* and *V. cholerae in* foods, environment and clinical samples [4, 5, 6, 7].

Vibrio cholerae lives naturally in riverine, brackish and estuarine ecosystems, and *V. cholerae* non-O1/non-O139 is frequently isolated from aquatic environments as well as in seafood. In Malaysia, cholera due to *V. cholerae* poses a public health problem as sporadic outbreaks occur periodically [8]. The recruitment of foreign labour force from other cholera-endemic countries may foster the migration of strains into Malaysia, thus causing further outbreaks [9].

On the other hand, *V. parahaemolyticus* has been recognized as the leading cause of human gastroenteritis associated with seafood consumption in Asia and other parts of the world [10, 11, 12, 13]. Many researchers reported the abundance of *V. parahaemolyticus* during summer in temperate zone when temperature was above 25°C [14, 15, 16], whereas the organism is expected to be prevalent throughout the year in the tropical zone like Malaysia [17]. It is known that most strains of pathogenic *V. parahaemolyticus* can produce either thermostable

direct hemolysin (TDH), TDH-related hemolysin (TRH) or both [18, 19, 20] recognized as two major virulence factors for the pathogenesis of *V. parahaemolyticus* [19, 21]. Application of PCR specific for the virulence genes (*tdh* & *trh*) will help in detection of pathogenic *V. parahaemolyticus*. Most of *V. parahaemolyticus* isolates from the environmental and seafood samples are known to be negative for *tdh* and *trh* and not pathogenic [21, 22].

*tox*R gene which is involved in the regulation of gene expression in *Vibrio* species has also been characterized. This gene is shown to be present in all of the *V. parahaemolyticus* and *V. cholerae* isolates and could be used as target gene to develop method for specific detection of *V. parahaemolyticus* [23, 24]. Molecular typing procedures are applied to show clonal and close relationship between isolates of one species. In this present study, REP-PCR genomic fingerprinting was used to determine relatedness among strains of *Vibrio* spp. from Bachok, Kelantan. Previous study [25] has shown that REP-PCR is the most discriminatory compared to RAPD, pulsed-field gel electrophoresis, and ribotyping.

The aims of this study were to detect the presence of *V. parahaemolyticus* and *V. cholerae* in various waterways of Bachok, Kelantan, to characterize the virulence properties of the recovered environmental *V. parahaemolyticus* isolates, and to analyse the genetic relatedness among the isolates by REP-PCR which targets conserved repetitive elements that are distributed throughout the genome.

MATERIALS AND METHODS

Sample collection

A total of 50 water samples were collected from various waterways including river water (n=39), sea water (n=9) and waterfall (n=2) in Bachok, Kelantan from June 16 to 18, 2008 [Table 1]. Samples were collected in pre-sterilized bottles and were kept on ice and transported to the laboratory to be processed within 3 hours.

Sample processing and isolation of *V. cholerae* and *V. parahaemolyticus*

100 ml of water samples (if turbid, prefiltered with sterile Whatman filter Type 1) were filtered through 0.45-µm-pore-size gridded membrane filters (Sartorius, Germany). The membrane filters were overlaid on CHROMagar^{TM} *Vibrio* agar and incubated for 16-18 h at 37°C. Visible colonies that were blue and purple were counted as presumptive *V. cholerae* and *V. parahaemolyticus,* respectively [26]. *Vibrio* concentrations were expressed as colony-forming units (cfu) per 100 ml of water.

Biochemical tests

Presumptive *V. cholerae* and *V. parahaemolyticus* colonies were picked and streaked on Nutrient agar (Oxoid, England) supplemented with 3% NaCl. These colonies were characterized using 3 types of biochemical tests consisting of Triple Sugar Iron Test, Oxidase Test and String Test before being subjected to PCR confirmation.

DNA extraction

A well isolated single colony grown on nutrient agar (3% w/v, NaCl) was picked and mixed thoroughly in 50 µl sterile distilled water. The cell suspension was then vortexed to disperse the cells in the water. The suspended cells were boiled at 99°C for 5 minutes to allow cell lysis. Immediately after that, the tubes were placed on ice for 10 minutes. Using a micro-centrifuge, the cell lysate were centrifuged and clear supernatant was transferred into new tubes for PCR analysis.

PCR confirmation

Primer sequences, expected amplicon sizes and annealing temperature are referred in Table 2. All PCRs were performed in 0.2 ml microfuge tubes using 25 ml of reaction volume in a Thermal cycler (Eppendorf Mastercycler, Germany). Polymerase chain reaction (PCR) targeting *tox*R genes unique to *V. cholerae* (*Vc-tox*R) and *V. parahaemolyticus* (*Vp-tox*R) were used to confirm biochemical results and for detection of virulence genes *tdh* and *trh* based on published protocols [24, 27, 28].

REP-PCR

The REP-PCR of *V. cholerae* and *V. parahaemolyticus* isolates were initially performed using four different REP primers to determine their usefulness. However, only primer REP I gave multiple and reproducible bands (data not shown) and hence it was used for subsequent analysis. Amplification was performed in 25 µl volume containing 5µl of 5X buffer (without $MgCl_2$), 2.5 mM dNTPs, 0.25µl primer (100 µM/µl), 5U/µl of *Taq* polymerase, 25 mM $MgCl_2$. The PCR conditions included an initial cycle of 94°C for 5 min, 33°C for 5 min, 68°C for 5 min followed by 35 cycles of 94°C, 1 min, 40°C, 1 min and 68°C, 2 min. Final extension step at 68°C for 1 min. The PCR amplification products were fractioned by electrophoresis through 1.5% agarose gel and detected by staining with ethidium bromide (0.5µg/ml).

Table 1. Sites and coordinates of sampling stations in the waterways of Bachok**.**

No.	Coordinate		Sites
	Latitude	Longitude	
1	N 05° 53.374'	E 102° 28.494'	Kg. Pak Makyong Jetty
2	N 05° 52.796'	E 102° 28.223'	Near Kg. Makyong Jetty
3	N 05° 52.467'	E 102° 27.707'	Kg.Siam River
4	N 05° 52.110'	E 102° 27.314'	Kg. Lembah River
5	N 05° 51.287'	E 102° 26.594'	Kg.Ajin river
6	N 05° 50.993'	E 102° 26.340'	Semerak River
7	N 05° 52.049'	E 102° 29.484'	Kg. Cerang Ruku
8	N 05° 52.281'	E 102° 29.310'	Semerak River
9	N 05° 52.328'	E 102° 29.325'	Semerak River
10	N 05° 52.543'	E 102° 29.343'	Semerak River
11	N 05° 52.650'	E 102° 28.900'	Semerak River
12	N 05° 53.099'	E 102° 28.871'	Semerak River
13	N 05° 53.477'	E 102° 28.739'	Semerak River
14	N 05° 53.724'	E 102° 29.060'	Semerak River
15	N 05° 53.442'	E 102° 28.636'	Opposite Kg.Makyong Jetty
16	N 06° 07.499'	E 102° 22.320'	Kuala Kemasin
17	N 06° 06.947'	E 102° 21.617'	Pantai Irama River Mouth
18	N 06° 04.163'	E 102° 23.889'	Kuala Melawi River mouth
19	N 05° 53.361'	E 102° 30.254'	Kg.Tok Bali
20	N 05° 52.991'	E 102° 52.170'	Kg. Semerak
21	N 05° 52.376'	E 102° 32.101'	Kg. Dalam
22	N 05° 55.113'	E 102° 29.179'	Tok Bali
23	N 05° 57.178'	E 102° 26.390'	Sg. Gali (Jetty)
24	N 05° 57.149'	E 102° 26.913'	Sg. Gali (Near River Mouth)
25	N 05° 51.901'	E 102° 29.637'	Semerak River
26	N 05° 51.094'	E 102° 25.231'	Semerak River
27	N 05° 54.807'	E 102° 22.533'	Temusu Telok Lubok
28	N 05° 54.524'	E 102° 22.623'	Sg. Tasek
29	N 05° 57.843'	E 102° 21.086'	Irrigation channel
30	N 05° 54.141'	E 102° 21.531'	Sg. Tasek (Mid River)
31	N 05° 54.391'	E 102° 20.953'	Upper Sg.Tasek
32	N 05° 49.872'	E 102° 25.017'	Near Bkt. Awang
33	N 05° 49.653'	E 102° 28.185'	Near Kg. Tok Luchar
34	N 06° 01.207'	E 102° 24.960'	Rekang River
35	N 06° 01.240'	E 102° 25.135'	Sg.Rekang
36	N 05° 59.755'	E 102° 25.814'	Sg.Dua (IOES)
37	N 05° 59.307'	E 102° 26.044'	Sg. Limau Nipis

38	N 04° 31.978'	E 101° 25.568'	Jeram Pasu
39	N 05° 44.450'	E 102° 22.615'	Jeram Linang
40	N 06° 01.903'	E 102° 25.780'	Kg. Rekang
41	N 06° 01.510'	E 102° 26.015'	Kuala Rekang
42	N 05° 56.811'	E 102° 27.488'	Kampung Sg. Dua
43	N 05° 55.667'	E 102° 28.540'	Tok Bali
44	N 05° 55.048'	E 102° 28.364'	Tok Bali
45	N 05° 43.190'	E 102° 15.628'	Hutan Lipur Bukit Bakar Semerak
46	N 06° 07.039'	E 102° 17.753'	Sg. Pengkalan Datu
47	N 06° 09.914'	E 102° 20.657'	Kuala Senok
48	N 06° 09.933'	E 102° 20.152'	Sg. Senok
49	N 06° 08.323'	E 102° 19.389'	Pantai Sabak
50	N 06° 07.678'	E 102° 22.127'	Kuala Kemasin

Table 2. List of primers, target genes and amplicon sizes.

	Primers and sequences (5' to 3')	Amplicon size (bp)	Annealing Temperature(°C)	Reference
Vp-*toxR*1	GTC TTC TGA CGC AAT CGT TG	368	63	[24]
Vp-*toxR*2	ATA CGA GTG GTT GCT GTC ATG			
Vc-*toxR*1	CCT TCG ATC CCC TAA GCA ATA	779	60	[28]
Vc-*toxR*2	AGG GTT AGC AAC GAT GCG TAA G			
tdh 1	CCA CTA CCA CTC TCA TAT GC	251	55	[27]
tdh 2	GGT ACT AAA TGG CTG ACA TC			
trh 1	GGC TCA AAA TGG TTA AGC G	250	55	[27]
trh 2	CAT TTC CGC TCT CAT ATG C			
REP I	GCG CCG ICA TGC GGC ATT	Variable	40	[38]

Gel documentation and image analysis

After de-staining, the gel was photographed using the GelDoc system (Bio-Rad). The fingerprints were analyzed using a computer software package, GelComparII Version 2.0 software (Applied Math, Belgium). After background subtraction and gel normalization, the fingerprints were subjected to cluster analysis using the unweighted pair group method with arithmetic mean (UPGMA). This program was used to construct a dendrogram and to calculate similarity values (Dice Coefficient, F).

RESULTS AND DISCUSSION

The aim of this study was to determine whether *Vibrio cholerae* and other *Vibrio* spp. such as *V.parahaemolyticus* were present in water bodies of Bachok, Kelantan. Such information may allow us to determine the possible influence of water sources on the infection and disease in the community.

Identification of *V. cholerae* and *V. parahaemolyticus*

A total of 50 water samples were screened for the presence of *V. cholerae* and *V. parahaemolyticus*. Based on the selective medium (CHROMagar™ *Vibrio*), distinct mauve coloured colonies (presumptive *V. parahaemolyticus*) and blue colonies (presumptive *V. cholerae/ V. vulnificus*) were present in 45 (average density of 2.4×10^4 cfu/100ml) and 49 (average density of 4.4×10^4 cfu/100ml) water samples, respectively. A total of 200 isolates were subjected to biochemical tests consisting of Triple Sugar Iron Test, Oxidase Test and String Test.

Table 3: Results of biochemical tests used to screen for *V. cholerae*[a] and *V.parahaemolyticus*[b]

Biochemical tests	*V. cholerae* reaction	No. of isolates with same reaction as *V.cholerae*	*V. parahaemolyticus* reaction	No. of isolates with same reaction as *V.parahaemolyticus*
Triple Sugar Iron Test	A/A	56	K/A	45
Oxidase Test	+	40	+	50
String Test	+	33	+	29

[a] A total of 86 bacterial isolates were subjected to each biochemical test
[b] A total of 114 bacterial isolates were subjected to each biochemical test
[c] A/A, acid slant (yellow) and acid butt (yellow)
[d] K/A, alkaline slant (purple) and acid butt (yellow)

Biochemical tests results showed that 33 isolates presented the characteristics of *V. cholerae* and 29 isolates for *V. parahaemolyticus* which were mostly found in river water samples (Table 3). All the presumptive *V. cholerae* and *V. parahaemolyticus* were subjected to PCR analysis for further confirmation.

PCR confirmation of *Vc-toxR*, *Vp-toxR*, *tdh* and *trh* genes

Based on PCR method, a 800bp fragment of *tox*R gene was amplified in 3 strains of *V. cholerae* (Figure 1) and a 368bp *tox*R gene in 25 strains of *V. parahaemolyticus* (Figure 2). Due to the limitation of the biochemical tests, there were a number of false positives. Since most of the *Vibrio* species showed the same appearance and characteristics, it was difficult to differentiate or confirm different species by just using biochemical tests. Moreover, presumptive blue colonies from the selective medium (CHROMagar^{TM} *Vibrio*) used in the study represent either *V. cholerae* or *V. vulnificus*. This further reduced the chances to pick the presumptive *V. cholerae* colonies. In future, more tests such as arginine dihydrolase and esculin hydrolysis tests [29] which are specific for *V. cholerae* should be included to reduce the number of false positives. The low isolation rate of *Vibrio cholerae* in the present study could, however, also have been affected by the isolation method used in this study which excluded the enrichment step. In this study, the direct plating technique was used because of time constraint in the field. Furthermore, *V. cholerae* strains under certain conditions assume spore-like or dormant forms (viable but non-culturable (VBNC)). These forms cannot readily be made culturable and so most conventional tests fail to detect them [30, 31].

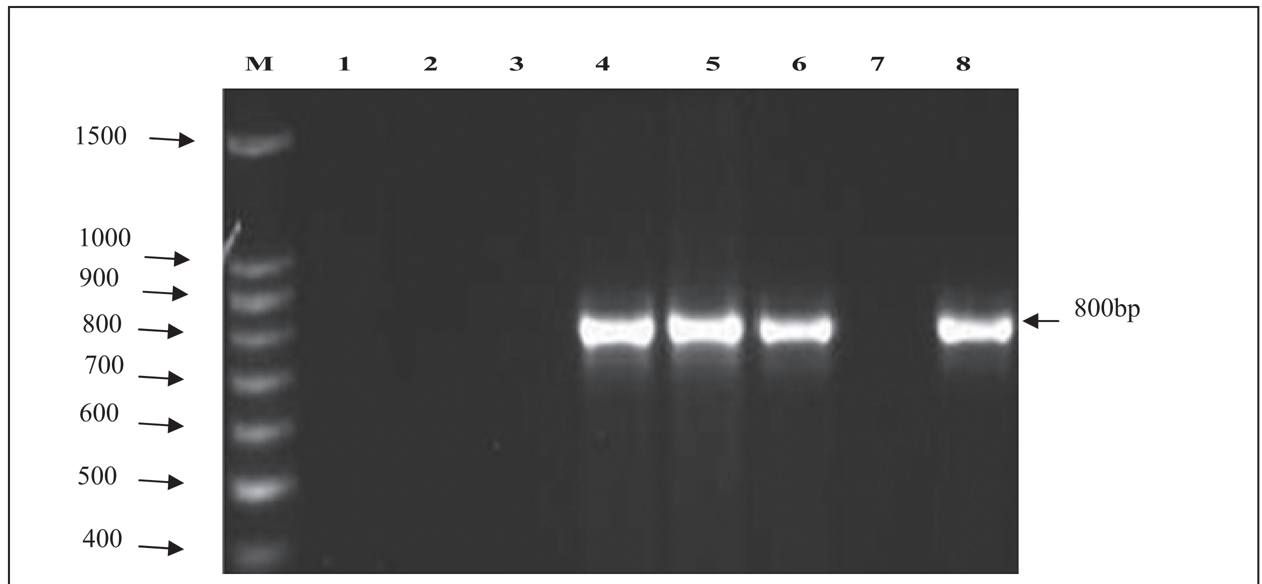

Figure 1. PCR for the detection of *toxR* gene, 800bp) unique to *V. cholerae* strain. Lane M: DNA ladder (100 bp); lane 7, negative control; lanes 4-6, *V.cholerae* isolates; lane 8, positive control.

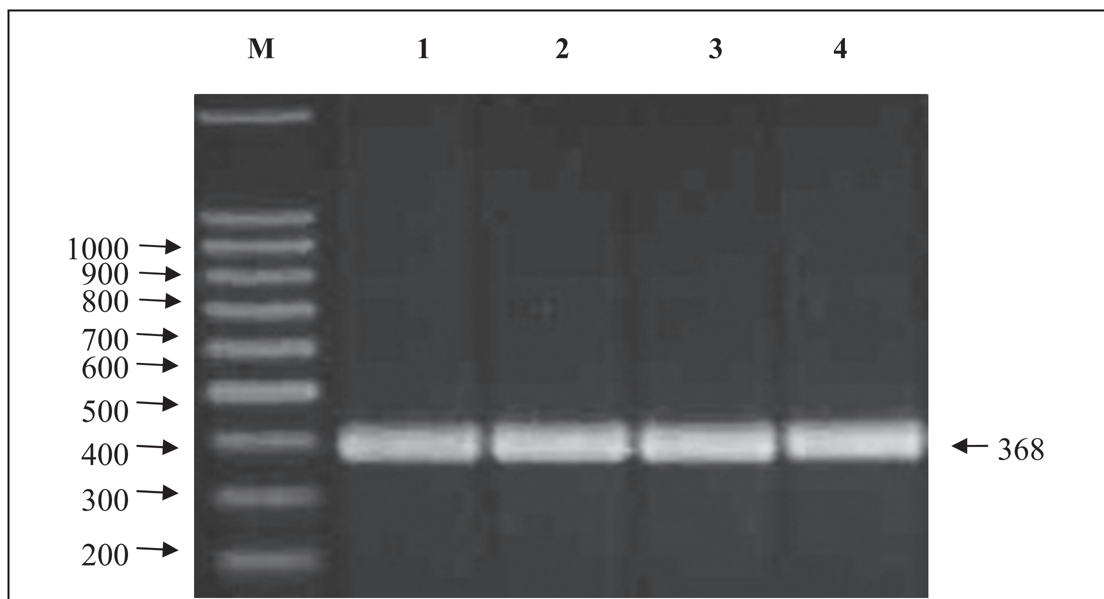

Figure 2. PCR for the detection of *toxR* gene (368bp). Lane M: DNA ladder (100bp), Lanes 1-3: *V. parahaemolyticus* isolates, Lane 4: PCR positive control

Although *V. parahaemolyticus* is widely distributed in the coastal environments all over the world, most of the environmental strains are not pathogenic to humans. In this study all the confirmed isolates of *V. parahaemolyticus* were tested for the presence of *tdh* and *trh* genes by PCR. None of the isolates were found to be positive for both virulence genes. This suggests that most of the environmental *V. parahaemolyticus* strains are non–toxin producers [32]. In addition, the prevalence of trh^+ strains in environmental samples and seafood products was reported to be very low in comparison to clinical strains [33, 34, and 35]. However, the presence of non-toxigenic *V. parahaemolyticus* in Bachok, Kelantan does not mean that the water is safe for human consumption as the guts serve as the site where these non-toxigenic strains can be converted into toxigenic ones [36].

REP-PCR

Molecular typing was used for epidemiological studies as it provides the information on genetic relatedness of different bacterial strains, the source of infection, molecular markers of virulent and host specific strains [37]. In this study, 28 strains of *Vibrio*s were subjected to REP-PCR analysis. Isolates from different water samples were found to be very diverse in their REP-PCR patterns, with 28 different patterns among 28 strains (Figure 3). Primer REP I successfully generated fingerprints consisted of 4 to 9 bands ranging from 250 to 4000 bp among the *V.parahemolticus* (Figure 4) and *V. cholerae* strains (Figure 5). It was evident that majority of the strains exhibited patterns with small number of REP-PCR products. However all the strains demonstrated a common band approximately 350 bp. The REP-PCR amplification was also repeated at least three times to evaluate its reproducibility. The results revealed consistent band amplification in all the REP-PCR assays. The feasibility of this molecular typing method depends on the use of various primers and experimental conditions.

Similarity analysis was performed by using the Dice coefficient and clustering was done by UPGMA. Based on 70 % similarity, the dendrogram constructed elucidated two main clusters, A and B (Figure 3). Cluster A consisted only *V. cholerae* strains and Cluster B with *V. parahaemolyticus* which was further subdivided into B1-B6. A distinct grouping between clusters was apparent pertaining to the different species, source of isolation and geographical location. Three pairs of the strains, VPH16 (Kuala Kemasin) and VPH25 (Semerak River), VPH41 (Kuala Rekang) and VPH44 (Tok Bali) and VPH40 (Kampung Rekang) with VPH42 (Kampung Sungai Dua) showed close relatedness by sharing more than 90% similarity, as shown in the dendrogram (Figure 3). Interestingly strain VPH 16 which was isolated from water sample obtained from mangrove site and strain VPH 25 from river samples were grouped together with similarity >90% in spite of different geographical areas. Therefore, it

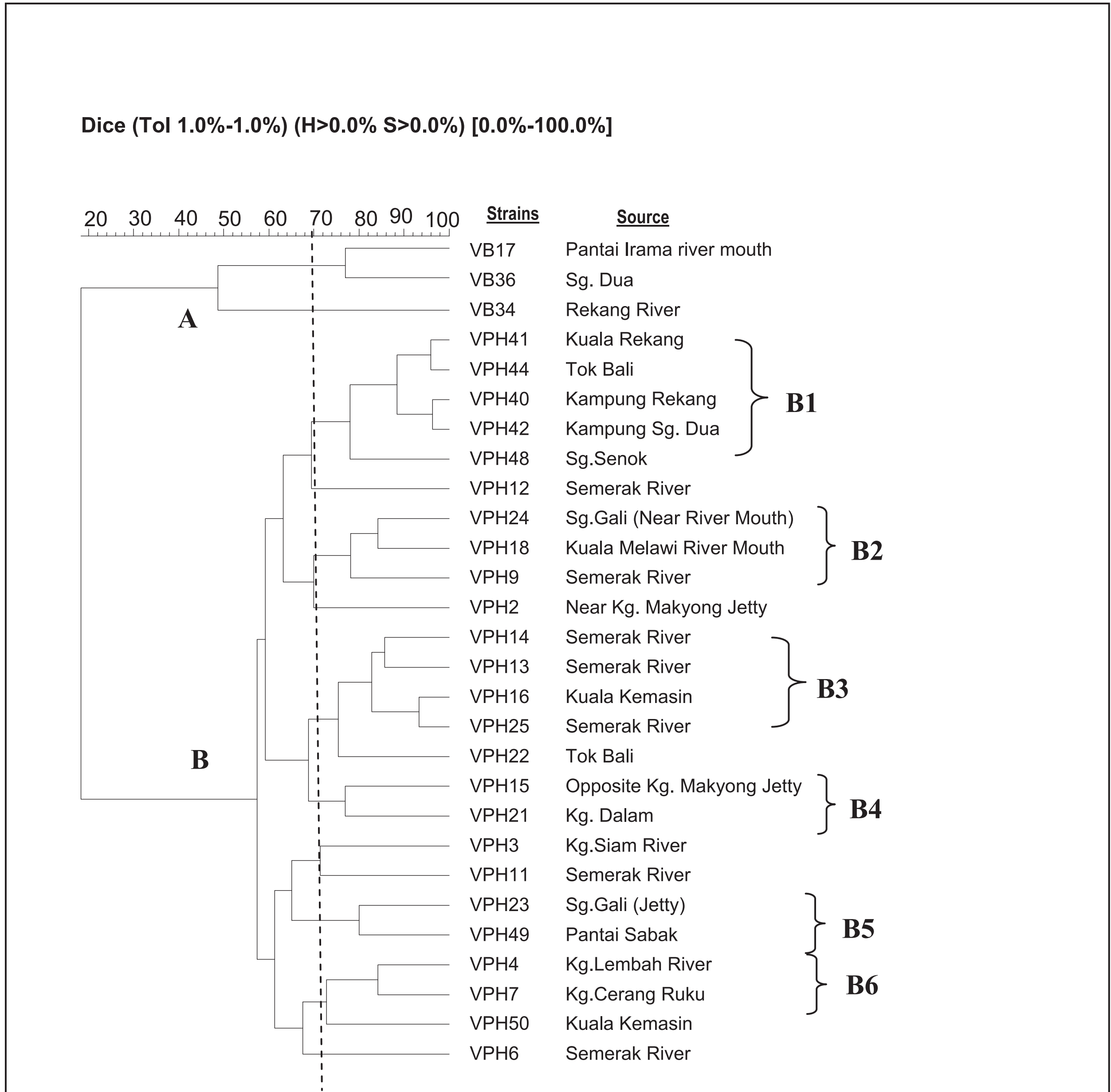

Figure 3. Dendrogram showing cluster analysis of REP-PCR profiles of selected environmental samples (Cluster A represents *V. cholerae* strains and cluster B represents *V. parahaemolyticus* strains). Within cluster B, there are 6 subclusters, B1-B6.

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, and ecological relationship with other marine organisms.

However, the REP-PCR patterns of the majority of the strains demonstrated similarities below 90%. In a previous study [39], strains with similarities below 90% are considered genetically unrelated. Thus, based on similarity using Dice coefficient, *Vibrio* spp. isolated from Bachok, Kelantan were genetically diverse.

In conclusion, this study provided new information with regards to the presence of *V. cholerae* and *V. parahaemolyticus* strains in various waterways of Bachok Kelantan. Although conventional culture methods can detect the bacteria, PCR technique was more reliable, since they are specific in the detection of a target bacterium and could differentiate

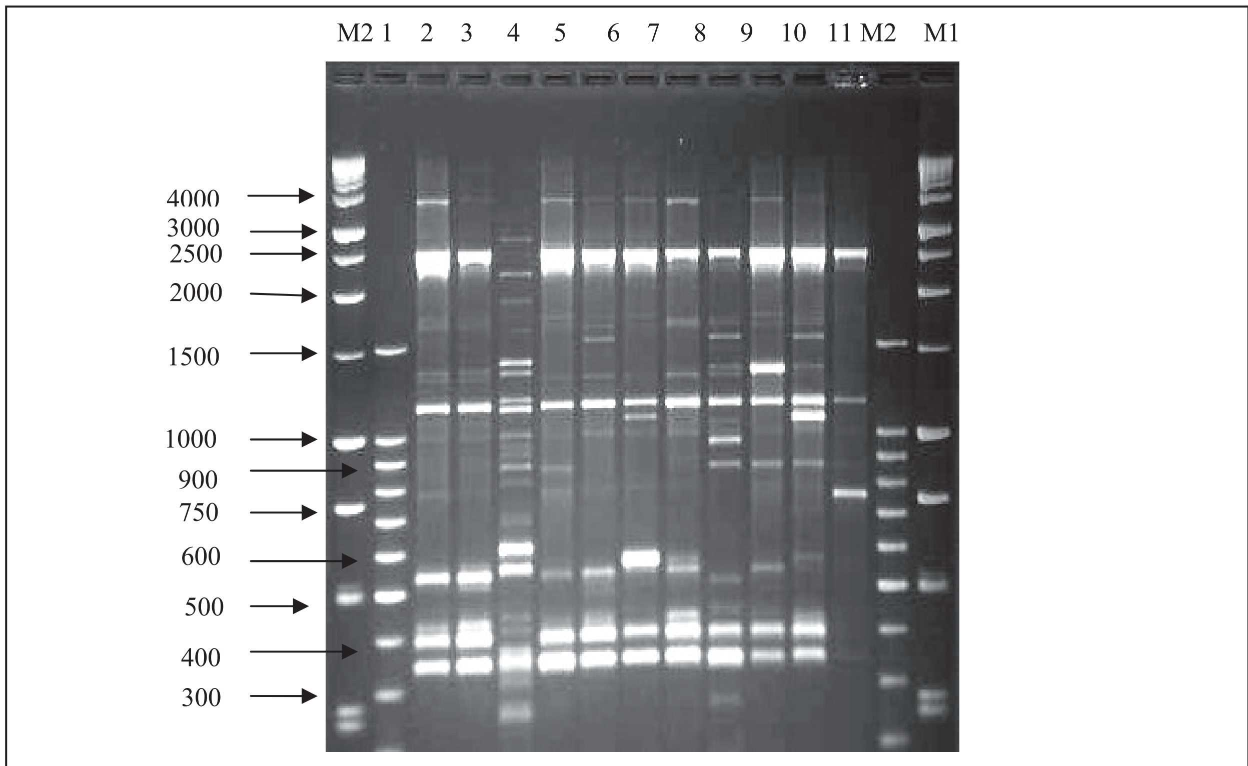

Figure 4. Representative REP-PCR Fingerprints of *V. parahaemolyticus* (VPH) M1=1kb DNA Standard, M2 =100bp DNA marker (Promega). Lanes 1-3,Positive control strains; Lane 4,VPH 12;Lane 5, VPH 2;Lane 6,VPH 3;Lane 7, VPH 4; Lane 8, VPH 6;Lane 9, VPH 7; Lane 10, VPH 9; Lane 11, VPH 11

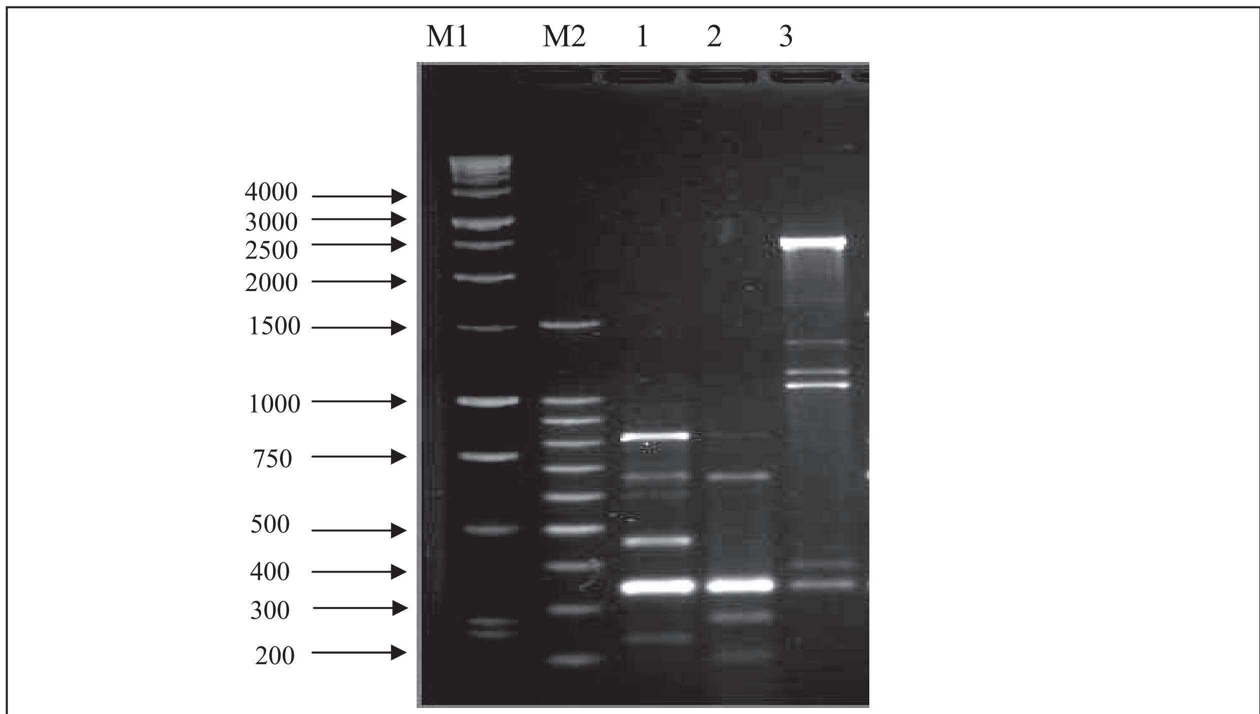

Figure 5. Representative REP-PCR fingerprints of *V.cholerae* strains M1 = 1kb DNA marker, M2 = 100bp DNA marker (Promega). Lane 1, VB 17; Lane 2, VB 34; Lane 3, VB 36.

pathogenic from non-pathogenic strains. REP-PCR generated fingerprints have been successfully used to discriminate the diverse bacterial population.

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