Research Article

Multiple-Locus Variable-Number Tandem Repeat Analysis of *Vibrio cholerae* in Comparison with Pulsed Field Gel Electrophoresis and Virulotyping

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Molecular analysis of Malaysian *Vibrio cholerae* was carried out using a multiple-locus variable-number tandem repeat analysis (MLVA) assay based on 7 loci of *V. cholerae*. The discriminatory ability of the assay was compared with pulsed-field gel electrophoresis (PFGE) using 43 Malaysian *V. cholerae* isolated from various sources. In addition, the virulotypes of the strains were determined. Based on MLVA, 38 allelic profiles were obtained (F = 0.63) while PFGE generated 35 pulsotypes (F = 0.71). Simpson's index of diversity for different VNTR loci ranged from 0.59 to 0.92. The combined loci increased the discriminatory index to 0.99 which was comparable with PFGE (D = 0.99). Most of the environmental non-O1/non-O139 strains harbored *rtx*A, *rst*R, *tox*R, and *hly*A only, and the virulotype of this serogroup was significantly different ($P < .01$) from clinical/environmental O1 and environmental O139 strains. In conclusion, the MLVA assay developed in this study was a useful genotyping tool with comparable discriminatory power with PFGE. In addition, the combination of the two approaches can further distinguish the strains from different sources and geographical regions of isolation.

1. Introduction

Vibrio cholerae is a gram negative bacterium which lives freely in aquatic environment and causes cholera [1]. Cholera is endemic in many parts of the world, especially the countries which lack proper sanitation managements.

In Malaysia, cholera outbreaks due to the *V. cholerae* O1 serotype which occurs periodically [2]. The ratio of distribution of *V. cholerae* O139 to O1 serogroups isolated from seafood from 1998 to 1999 was 14 : 1. Non-O1/non-O139 *V. cholerae* is also frequently isolated from seafood and water sources but has not been implicated in any major outbreaks [3, 4]. Although non-O1/non-O139 *V. cholerae* is not associated with any major outbreak, it has been reported to be responsible for sporadic cases of diarrhea [5–7].

The well-known genes associated with colonization are *ctx*A and *tcp*A. These genes are commonly found in O1 and O139 serogroups. However, the mechanism of colonization for the non-O1/non-O139 strains remains relatively unknown as they do not harbor *ctx*A and *tcp*A [5]. Olivier et al. [8] had reported that accessory toxins such as hemolysin and multifunctional autoprocessing RTX toxin in El Tor *V. cholerae* are involved in prolonged colonization without cholera toxin (CT) or toxin-coregulated pili (TCP). As these accessory virulence genes are commonly found in all serogroups of *V. cholerae*, it is of interest to investigate the involvement of these accessory virulence genes for prolonged colonization in other serogroups of *V. cholerae*.

Molecular subtyping of pathogen is important for tracing a new or previously found virulent or multidrug-resistant clone. Genomic variation and epidemiological study for different serogroups of *V. cholerae* have been carried out using many DNA-fingerprinting tools. PFGE is the most common subtyping tool to define strains from outbreaks

and from sporadic cases of cholera as it has the highest discriminatory ability [4]. However, PFGE is time consuming and requires strict adherence to standardize protocols for interlaboratory comparison.

Multiple-locus variable-number of tandem repeat analysis (MLVA), a method based on the tandem repeats in multiple loci, was developed and has been popularly adopted for differentiation of bacterial pathogens since 2006 [9]. Recently, Olsen et al. [10] evaluated the usefulness of MLVA on *V. cholerae* isolated worldwide based on polymorphism in 6 VNTR loci and demonstrated a high level of polymorphism among the strains tested.

The objective of the study was to develop a novel MLVA assay for genotypic study and to determine the presence of virulence-associated genes among Malaysian *V. cholerae* strains of different serogroups.

2. Materials and Methods

2.1. Bacteria Strains and DNA Extraction for Polymerase Chain Reaction (PCR). Isolation of *V. cholerae* strains was carried out from June 2008 to April 2009. The identification and confirmation of the cultures were previously described in [11]. All together 43 *V. cholerae* strains were isolated from human stools ($n = 15$, from various public hospitals in Kuala Lumpur, Labuan, Kota Kinabalu, Kota Bharu, Tumpat, and Alor Setar), sea water ($n = 13$, from different locations in Klang, Banting, Morib, and Bachok), household wastewater ($n = 6$, from different locations in Petaling Jaya, Klang, Banting, Serdang, and Kajang), algae ($n = 2$, from costal water in Klang and Morib), and various raw seafood ($n = 7$) sold in wet markets in Petaling Jaya and used in this study. Crude DNA for each strain was prepared by suspending a loopful of bacterial colonies in $50\,\mu$l of deionized water and boiled at $99°$C for 5 minutes. After a quick centrifugation, $5\,\mu$l of the supernatant ($\sim$50 ng of DNA template) was used for PCR.

2.2. Variable Number Tandem Repeats (VNTRs) Search and Primers Design. A search for potential VNTR candidates in the genome of *V. cholerae* O1 biotype El Tor, N16961, was carried out (http://minisatellites.u-psud.fr/). Primers were designed for each VNTR based on a $\sim$500 bp sequence covering the flanking and tandem repeats region retrieved from the database. Primer 3 software [12] was used to design the primers. The specificity of the primers and VNTR polymorphisms were tested with *in silico* PCR (http://insilico.ehu.es/) and monoplex PCR using DNA templates from 5 *Vibrio* strains of different serogroups.

2.3. PCR and Capillary Electrophoresis. Primers labeled with fluorescent dyes in the $5'$-end for the VNTRs were synthesized, that is, VCTR1-FAM, VCTR2-VIC, VCTR3-PET, VCTR4-NED, VCTR5-FAM, VCTR6-VIC, and VCTR7-NED. Two multiplex PCRs were performed: VCTR1–VCTR4 (Multiplex 1) and VCTR5–VCTR7 (Multiplex 2). Each multiplex PCR mixture in a total volume of $25\,\mu$l contained 1X PCR buffer, 2 mM MgCl$_2$, $160\,\mu$M dNTPs, $0.4\,\mu$M of each

primer, $2\,\mu$l of *Taq* DNA Polymerase (Promega, USA), and 50 ng of DNA template. The multiplex PCR was run at $95°$C for 5 minutes (1 cycle), $95°$C for 30 s, $58°$C for 30 s, $72°$C for 50 s (30 cyclers), and $72°$C for 7 minutes (1 cycle).

The PCR product was then diluted in 1 : 100 ratio with ddH$_2$O. One microlitre of the dilution was mixed with $10\,\mu$l HiDi formamide and $0.2\,\mu$l GeneScan 500 LIZ size standard (Applied Biosystem). The mixture was boiled at $95°$C for 5 minutes and analyzed with ABI prism 3130xl Genetic Analyzer (Applied Biosystem). The amplicons were identified based on the peak height and colour while the size was determined with the GeneScan size standard using GeneMapper software (Applied Biosystem).

2.4. Data Analysis for MLVA. The peak tables obtained from Gene Mapper were imported into BioNumerics 6.0 (Applied Maths, Belgium). The character values for each VNTR were determined and mapped. A Minimum Spanning Tree (MST) was also constructed using the categorical coefficient and WARD algorithm. The discrimination power of each VNTR was calculated individually and combined using Simpson's index of diversity [13].

2.5. Pulsed-Field Gel Electrophoresis (PFGE). PFGE was performed according to established protocol from PulseNet USA [14]. Briefly, equal volumes of standardized cell suspension (OD$_{610}$ = 0.8) of 1% (w/v) Seakem Gold agarose (Cambrex Bio Science Rockland, Inc, USA) were mixed to form plugs. The plugs were lysed in cell lysis buffer (50 mM Tris; 50 mM EDTA [pH 8.0], 1% Sarcosyl, and 1 mg/ml proteinase K) and incubated at $54°$C for 3 hours. The plugs were then washed thoroughly with sterile deionised water (twice) and TE buffer (6 times). A slice of DNA plug was digested overnight with 10 U restriction enzyme *Not* I ($5'$-GCGGCCGC-$3'$) (Promega, Madison, Wis., USA) at $37°$C. The digested chromosomal DNA was subjected to PFGE on a 1.0 % (w/v) agarose gel (Sigma Type 1, St. Louis, Mo) with the CHEF DRIII (Bio-Rad, Hercules, CA) using the following conditions: 200 V for 13 hours with pulse times of 2–10 s and 6 hours with pulse time 20–25 s. Gel image was captured by using Gel Doc XR after staining with ethidium bromide ($0.5\,\mu$g/ml).

PFGE banding patterns were analyzed with BioNumerics 6.0 (Applied Maths, Kortrijk, Belgium). The quantitative differences among the banding patterns were defined by the Dice coefficient; F. Cluster analysis was carried out based on the unweighted pair group method with arithmetic averages (UPGMA) using the position tolerance of 0.15. Simpson's index of diversity was also calculated [13].

2.6. Virulence Genes Detection. Detections of *ctx*A, *zot*, *rtx*A, *rst*R, *tox*R, *tcp*A, *tcp*I, and *hly*A genes were carried out via PCR using published primers [6, 15–17]. The genes selected were either major virulence genes or virulence-associated genes. The PCR amplifications were carried out in a Master cycler (Eppendorf, USA). The PCR mixture in a total volume of $25\,\mu$l contained 50 ng of DNA template, 1X PCR buffer, 2 mM MgCl$_2$, $200\,\mu$M of each dNTP, $0.3\,\mu$M of each primer,

and 2.5 U of *Taq* DNA polymerase (Promega, USA). The cycling conditions were set at 94°C for 3 minutes (1 cycle), 94°C for 30 s, 60°C for 30 s, 72°C for 1 minute (30 cycles), and 72°C for 10 minutes (1 cycle). The products were then analyzed on 1.5% (w/v) agarose gel and run at 90 V for 1 hour. Gel images were captured and analyzed using Gel Doc XR (Bio Rad, USA). Representative amplicons of each gene were purified and sent to a commercial facility for sequencing to confirm the identity of amplicons.

3. Results

3.1. MLVA. Seven VNTR regions were chosen, and primers were designed based on the sequences retrieved from GenBank. Initially, the primers specificity was determined individually with monoplex PCR using 2 O1, 1 O139, and 2 non-O1/non-O139 strains. All 7 VNTR regions showed polymorphisms for those tested strains.

By using capillary electrophoresis, the sizes of DNA fragments could be determined more accurately based on the peaks. Overall, the size distributions of the 43 *V. cholerae* strains for each VNTR were 212–230 bp (VCTR1), 216–252 bp (VCTR2), 218–276 bp (VCTR3), 278–300 bp (VCTR4), 228–251 bp (VCTR5), 211–269 bp (VCTR6), and 328–369 bp (VCTR7).

Thirty-eight MLVA profiles (F = 0.63) were obtained based on the character types (allelic profiles) (Figure 3). The 43 strains were grouped into 3 major clusters in the MST (Figure 1). The first cluster (I) comprised of 14 O1 strains (8 clinical, 6 water) (90.6% similarity). In this cluster, 5 of the O1 strains shared the same profile (MLVA2: 09-08-09-12-05-02-23). These strains (VC4, VC52, VC63, VC65, and VC66) were isolated during a cholera outbreak in Kota Bahru, West Malaysia (*n* = 2) and Labuan, East Malaysia (*n* = 3) in 2008. Most of the environmental O1 strains were in this cluster. One environmental O1 strain, VC70, was highly similar (95 % similarity) with the O1 outbreak strains.

Cluster II consisted of 9 O1 strains, 1 non-O1/non-O139 strain, and 1 O139 strain. In this cluster, 4 strains (VC21, VC37, VC38, and VC40) isolated in 2004 from human stools were highly similar with only 1-2 alleles difference. VC40 (O1 clinical) has 2 and 3 alleles difference from VC11 (environmental O1) and VC4370 (environmental O139), respectively. The VCPSW (environmental non-O1/non-O139) also has 2 alleles difference from VC11 (environmental O1). The similarity shared among the O1 and O139 strains was 95%. Cluster III comprised of non-O1/non-O139 strains with 83.3% similarity or 1–5 alleles difference.

Simpson's index of diversity for each individual VCTR ranged from 0.59 to 0.92 (Table 1) while the index for the combined VCTR loci was 0.99.

3.2. PFGE Analysis. PFGE of *Not* I-digested chromosomal DNA yielded 15–22 fragments ranging from 40 to 400 kbp (Figure 2). Thirty-five pulsotypes (F = 0.71–1.0) were observed. At a similarity of 80%, there were 5 clusters (Figure 3). Cluster I comprised of all O1 strains, 1 O139 strain, and 7 non-O1/non-O139 strains. Clusters II and III comprised of 3 non-O1/non-O139 strains while Clusters IV and V were each composed of 2 non-O1/non-O139 strains each. Two non-O1/non-O139 strains were not clustered. The discriminatory index for PFGE was 0.99.

3.3. Virulotyping. Based on the different combination of virulence genes analysed, 5 virulotypes (V1–V5) were observed for the 43 *V. cholerae* strains (Figure 3). Virulotype 1 was the most common profile (V1: $ctxA^+$, zot^+, $rtxA^+$, $rstR^+$, $toxR^+$, $tcpA^{E+}$, $tcpI^+$, and $hlyA^{E+}$) and was exhibited by O139 and 22 O1 strains. Virulotype 2 (V2: $rtxA$, $rstR$, $toxR$, and $hlyA^{E+}$) comprised of 16 non-O1/non-O139 and 1 *V. cholerae* O1 strain. Among the non-O1/non-O139 serogroups, VC310 (V3: $rtxA$, $rstR$, $toxR$, $tcpI$, and $hlyA^{E+}$) and VC3477 (V4: $rtxA$, $rstR$, $toxR$, $tcpI$, and $hlyA^{C+}$) were negative for $tcpA$. Besides VC3477, classical $hlyA$ was also present in VC77 (V5: $rtxA$, $rstR$, $toxR$, and $hlyA^{C+}$). Analysis of the DNA sequences of the amplicons confirmed their identities as there was a 90–100% homology with the targeted genes sequence in the database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

When the strains were grouped according to serogroups and sources (clinical O1, environmental O1, environmental O139, and environmental non-O1/non-O139), significant difference in the aggregation was observed (one way-MANOVA F, Wilkes' λ = 0.0874, $F_{6,76}$ = 30.1700, and $P < .0001$). Further Post Hoc Tukey HSD test identified that there were significant differences between non-O1/non-O139 environmental *Vibrio cholerae* and the other 3 groups ($P < .01$). This was pertinent to the fact that all non-O1/non-139 strains lacked 4 virulent genes ($ctxA$, zot, $tcpA$, and $tcpI$). There was no significant difference in the virulotypes among the clinical O1, environmental O1, and environmental O139.

4. Discussion

The MLVA assay was developed based on the 7 loci in chromosomes I and II of *V. cholerae*. Among these loci, VCTR4 demonstrated the highest discriminatory ability (D = 0.92), followed by VCTR7 (D = 0.89). Both loci are situated in the small chromosome of *V. cholerae* genome (chromosome II) and suggest that there is a higher genetic variation in chromosome II of *V. cholerae* genome compared with chromosome I. Danin-Poleg et al. [18] and Olsen et al. [10] reported that the tandem repeat locus that showed the highest discriminating ability is physically located at 187 kb in chromosome II (such as VCTR7 in this study). However in our study, VCTR4 which is located at 303 kb in chromosome II showed even higher genetic variation (17 alleles, D = 0.92) among the 43 strains tested.

Both the PFGE and MLVA data showed that the *V. cholerae* O139 strain (VC4370) was closely related with an O1 strain with similarity of more than 90%. Strains VCGB and VCSW, isolated from river water and algae, respectively, were indistinguishable by both PFGE and MLVA. Both strains harbored the same virulence genes ($rtxA^+$, $rstR^+$, $toxR^+$, and $hlyA^{E+}$) although they were isolated from different locations. VCGB was isolated from river water in Banting, Selangor (August 2008) while VCSW was isolated from algae
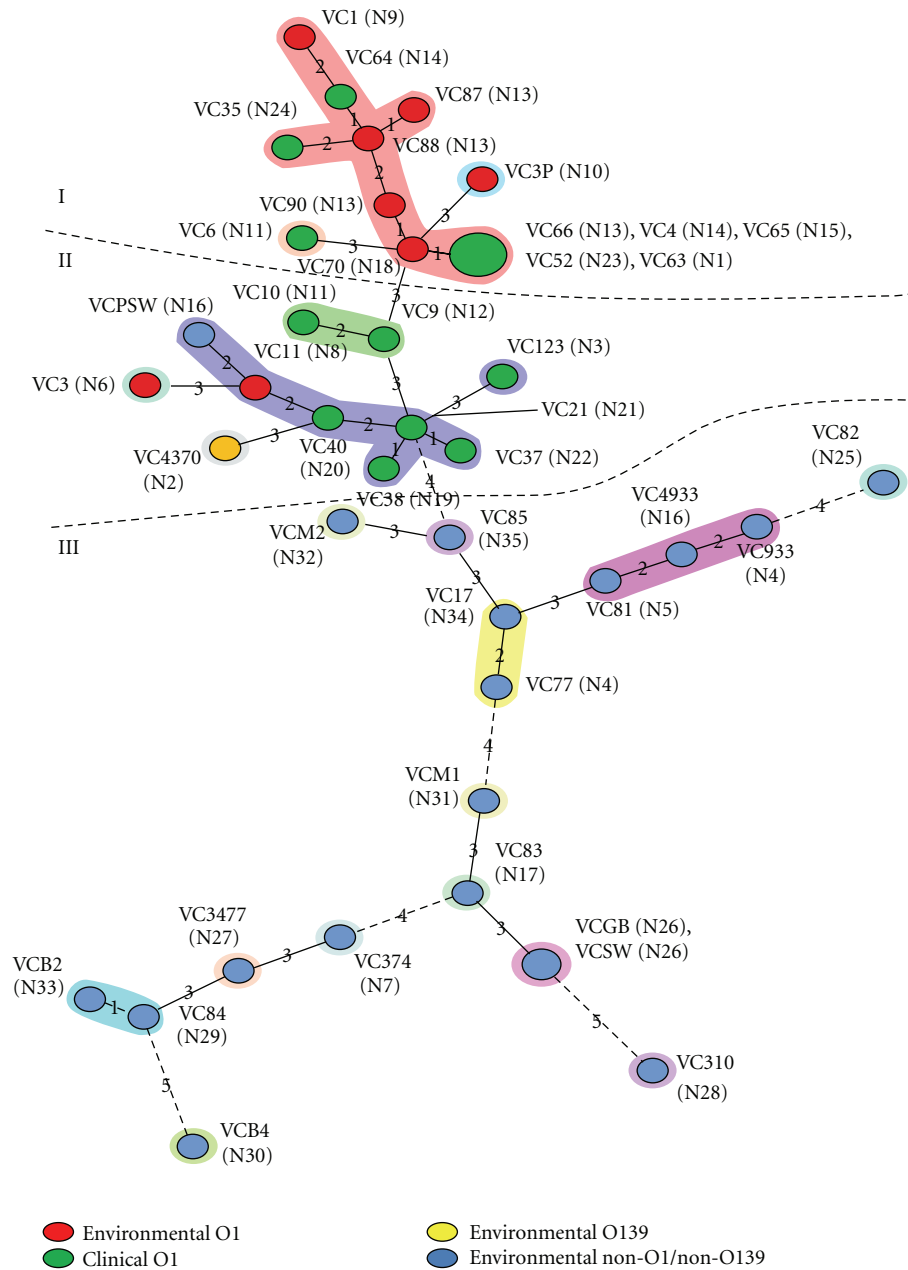
FIGURE 1: The relationship between 38 MLVA genotypes identified among 43 *V. cholerae* isolates using Minimum Spanning Tree analysis. The highlighted branches indicate the close relationship between strains (1-2 alleles difference). The dotted line shows the gap between O1/O139 strains and non-O1/non-O139 strains.

collected at Pantai Morib, Selangor in October 2008. The result suggests that both river water and algae were probably contaminated with the same clone of non-O1/non-O139 *V. cholerae*.

Among the environmental *V. cholerae* O1 strains isolated in 2008, 6 had close allelic similarity to the clinical O1 strains isolated in year 2008 (cluster I) while another 2 were indistinguishable to clinical strains isolated in year 2004 (cluster II). This phenomenon showed that microevolution might have taken place, resulting in new variance of strains since 2004. The *V. cholerae* O139 strain was also found to

be more clonally related to O1 strains isolated in 2004 than to the O1 strains which were isolated in 2008. This suggests that the O139 strains might have derived from the 2004 O1 strains due to horizontal gene transfer. With the exception of one strain (VC35), most of the 2004 clinical *V. cholerae* O1 strains were very similar with only 1-2 alleles difference and were different from the 2008 strains.

From the analysis of MST, those strains which belonged to the same PFGE profile (N11, N13, N14, and N16) were distinguishable by MLVA. However, some strains with same MLVA profiles could be further distinguished by PFGE.

TABLE 1: Characteristics of *V. cholerae* VNTR loci.

Character	Primers sequence	Size range of PCR products (bp)	VNTR position (kb)	Alleles	D index
VCTR 1	F: 5′-GAGAAAAGCCAAACCACTGC-3′ R: 5′-TTTAAGCGCGCAAAGAAACT-3′	212–230	137	9	0.82
VCTR 2	F: 5′-CGTTAGCATCGAAACTGCTG-3′ R: 5′-CCACTCAATCTCGTGGGAAA-3′	216–252	467	5	0.75
VCTR 3	F: 5′-AGTGGGCACAGAGTGTCAAA-3′ R: 5′-GCTGTACTCTGGCACATCCA-3′	218–276	1778	7	0.84
VCTR 4	F: 5′-TCGAATGTATGGGGAACATTT-3′ R: 5′-TTAATACCCCTTTCTTCCGATG-3′	278–300	303*	17	0.92
VCTR 5	F: 5′-GAAGAGACGGACCTTGATCG-3′ R: 5′-TCGCAGCAAGTTTGCTTAAC-3′	228–251	1685	6	0.65
VCTR 6	F: 5′-GTTTTATCGCTGATGCGTGA-3′ R: 5′-GCAAGCAAGTGCCGAATTAT-3′	211–269	540*	4	0.59
VCTR 7	F: 5′-GTTTGAGAGCTCGCCTCTTG-3′ R: 5′-CAATCTCGCCAATGCTTATG-3′	328–369	187*	11	0.89

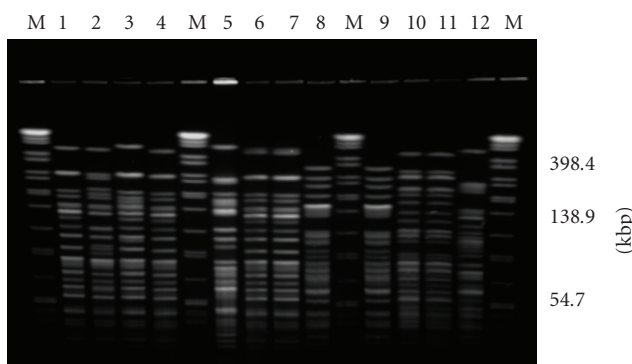*position in chromosome II of *V. cholerae* genom.

FIGURE 2: *Not*I-PFGE profiles of representative Malaysian *V. cholerae* of different serogroups. M: *Xba*I-digested *S. ser.* Braenderup H9812 standard; lanes 1–7: strains of O1 serogroup; lanes 8–12: strains of non-O1/non-O139 serogroup.

Different methods assessed the genetic variability in different parts of the chromosome. Mutations at the restriction sites may result in variation in PFGE profiles while gene mutations may affect the number and frequency of tandem repeats. Hence, a combination of PFGE and MLVA analysis may yield more information about the clonality of bacterial pathogens. For example, 3 environmental strains (VC87, VC88, and VC90) and 1 clinical strain (VC66) had identical pulsotype but were distinguishable by MLVA. Five strains (VC4, VC52, VC63, VC65, and VC66) were indistinguishable by MLVA as they shared identical allelic profile. However, PFGE was able to separate 2 of Kota Bahru outbreak strains (VC4 and VC65) (West Malaysia) from the Labuan outbreak strains in East Malaysia.

Cholera cases caused by O1 serogroup occur occasionally in Malaysia and the neighboring countries. PFGE is the most commonly used subtyping method to determine the epidemiological relatedness of the strains. In this study, the 23 O1 strains were subtyped into 18 pulsotypes. Based on visual comparison of the published pulsotypes [19], our pulsotypes N22 and N11 appeared to be similar to those pulsotypes of Hong Kong strains isolated during an outbreak in 2001. Pulsotype N13 which comprised of 3 environmental strains (VC87, VC88, and VC90) and 1 clinical strain (VC66) was similar to the pulsotype of a Philippine strain obtained during an outbreak in 2001. The Malaysian strains isolated from sea water had similar pulsotype with those Indonesian outbreak strains in 1995 [19]. Overall, comparison of the pulsotype distributions among *V. cholerae* in different neighboring countries would provide important information and further facilitate the epidemiological study of cholera in Southeast Asia. However, as different PFGE conditions were used by different researchers in the region, direct comparison was difficult. Adoption of a standardized PFGE protocol such as the PulseNet PFGE protocol proposed by CDC PulseNet, USA would greatly enhance interlaboratory comparison and improve tracking of *V. cholerae* strains among the endemic countries in the region.

In this study, 8 environmental strains harbored all virulence-associated genes, indicating the toxigenic potential and risk of infection from environmental sources [1]. The presence of accessory toxigenic genes in non-O1/non-O139 strains indicates the relative importance of these strains as they have the potential to cause infections [20].

Two non-O1/non-O139 strains, VC3477 and VC310 isolated from seawater and seafood, respectively, harbored *tcp*I but not *tcp*A. *tcp*I gene is the first gene in TCP cluster and is associated with the synthesis of *tcp*A. It may function as a regulator to determine the virulence of VPI [6, 21]. Therefore, the presence of this gene in the strains which do not harbor *tcp*A is not surprising. In addition, VC3477 harbored the classical *hly*A gene while VC310 harbored the El Tor *hly*A gene. However, these 2 strains could not be distinguished from other non-O1/non-O139 strains using MLVA, but PFGE distinguished VC3477 from other strains.

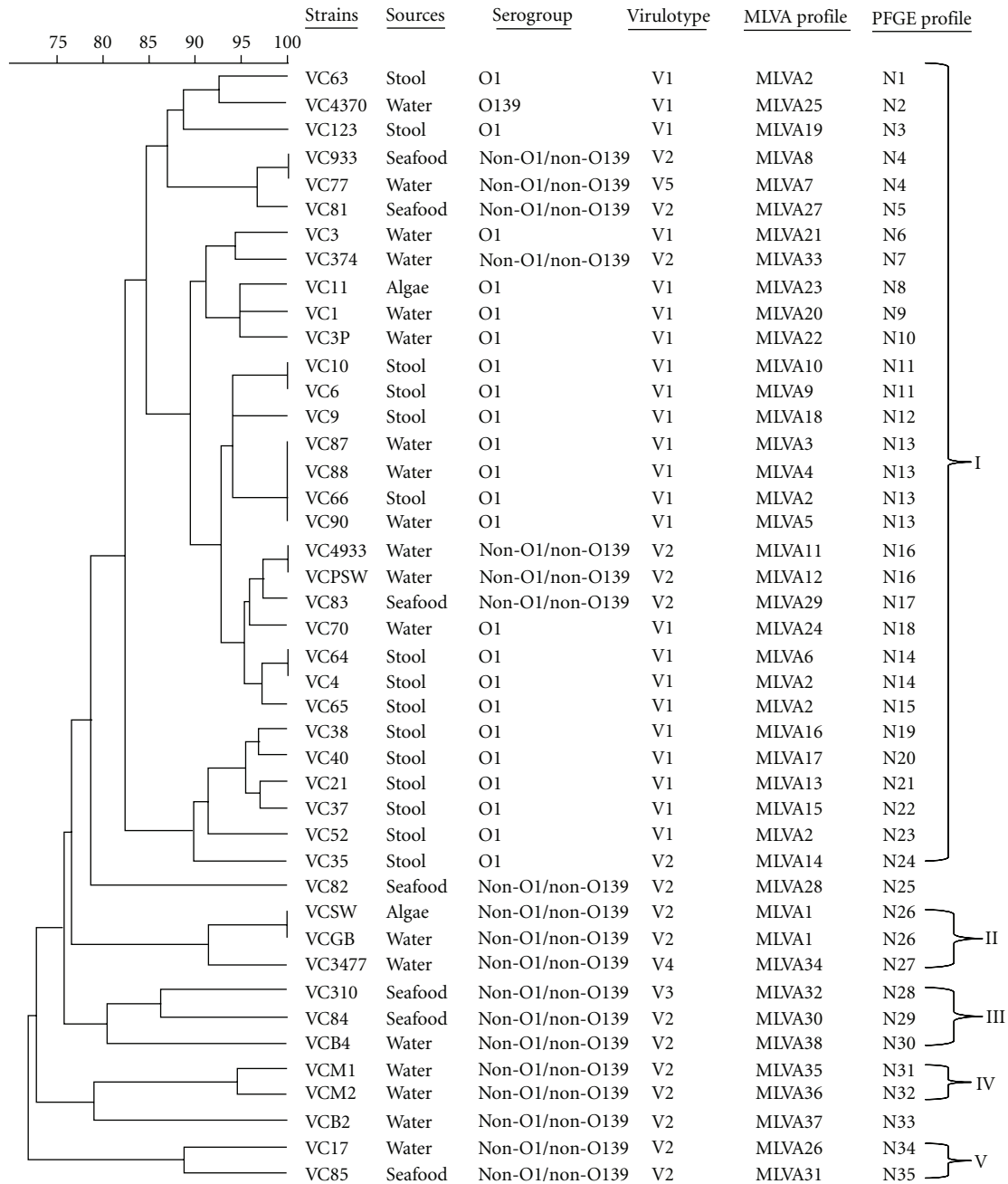|	Strains	Sources	Serogroup	Virulotype	MLVA profile	PFGE profile
	VC63	Stool	O1	V1	MLVA2	N1
	VC4370	Water	O139	V1	MLVA25	N2
	VC123	Stool	O1	V1	MLVA19	N3
	VC933	Seafood	Non-O1/non-O139	V2	MLVA8	N4
	VC77	Water	Non-O1/non-O139	V5	MLVA7	N4
	VC81	Seafood	Non-O1/non-O139	V2	MLVA27	N5
	VC3	Water	O1	V1	MLVA21	N6
	VC374	Water	Non-O1/non-O139	V2	MLVA33	N7
	VC11	Algae	O1	V1	MLVA23	N8
	VC1	Water	O1	V1	MLVA20	N9
	VC3P	Water	O1	V1	MLVA22	N10
	VC10	Stool	O1	V1	MLVA10	N11
	VC6	Stool	O1	V1	MLVA9	N11
	VC9	Stool	O1	V1	MLVA18	N12
	VC87	Water	O1	V1	MLVA3	N13
	VC88	Water	O1	V1	MLVA4	N13
	VC66	Stool	O1	V1	MLVA2	N13
	VC90	Water	O1	V1	MLVA5	N13
	VC4933	Water	Non-O1/non-O139	V2	MLVA11	N16
	VCPSW	Water	Non-O1/non-O139	V2	MLVA12	N16
	VC83	Seafood	Non-O1/non-O139	V2	MLVA29	N17
	VC70	Water	O1	V1	MLVA24	N18
	VC64	Stool	O1	V1	MLVA6	N14
	VC4	Stool	O1	V1	MLVA2	N14
	VC65	Stool	O1	V1	MLVA2	N15
	VC38	Stool	O1	V1	MLVA16	N19
	VC40	Stool	O1	V1	MLVA17	N20
	VC21	Stool	O1	V1	MLVA13	N21
	VC37	Stool	O1	V1	MLVA15	N22
	VC52	Stool	O1	V1	MLVA2	N23
	VC35	Stool	O1	V2	MLVA14	N24
	VC82	Seafood	Non-O1/non-O139	V2	MLVA28	N25
	VCSW	Algae	Non-O1/non-O139	V2	MLVA1	N26
	VCGB	Water	Non-O1/non-O139	V2	MLVA1	N26
	VC3477	Water	Non-O1/non-O139	V4	MLVA34	N27
	VC310	Seafood	Non-O1/non-O139	V3	MLVA32	N28
	VC84	Seafood	Non-O1/non-O139	V2	MLVA30	N29
	VCB4	Water	Non-O1/non-O139	V2	MLVA38	N30
	VCM1	Water	Non-O1/non-O139	V2	MLVA35	N31
	VCM2	Water	Non-O1/non-O139	V2	MLVA36	N32
	VCB2	Water	Non-O1/non-O139	V2	MLVA37	N33
	VC17	Water	Non-O1/non-O139	V2	MLVA26	N34
	VC85	Seafood	Non-O1/non-O139	V2	MLVA31	N35

Figure 3: Dendrogram generated using PFGE profiles showing the relatedness of 43 Malaysian *V. cholerae* with virulotypes and MLVA profiles. The dendrogram is based on the unweighted pair group method with arithmetic averages (UPGMA) using the position tolerance of 0.15. Five clusters were observed at the similarity of 80% with all O1 strains clustered in Cluster I. **V1:** ctxA$^+$, zot $^+$, rtxA$^+$, rstR$^+$, toxR$^+$, tcpA^{E+}, tcpI$^+$, and hlyA^{E+}; **V2:** rtxA$^+$, rstR$^+$, toxR$^+$, and hlyA^{E+}; **V3:** rtxA$^+$, rstR$^+$, toxR$^+$, tcpI$^+$, and hlyA^{E+}; **V4:** rtxA$^+$, rstR$^+$, toxR$^+$, tcpI$^+$, and hlyA^{C+}; **V5:** rtxA$^+$, rstR$^+$, toxR$^+$, and hlyA^{C+}.

VC 35 was isolated from a patient during a cholera outbreak in Kota Kinabalu, Sabah. This strain did not harbor the major virulence genes (*ctx*A, *zot*, *tcp*A, and *tcp*I) which are essential for colonization. Hence, the finding was in agreement with the finding of Olivier et al. [8] that secreted accessory toxins such as hemolysin and RTX toxin in El Tor *V. cholerae* would modify the host environment and prolong colonization without cholera toxin (CT) or toxin-coregulated pili (TCP).

Overall, the MLVA assay developed in this study was a promising method to study the relatedness of Malaysian *V. cholera,* and the discriminatory ability was comparable with PFGE. In addition, further combination of MLVA and PFGE

analysis may provide more information of the relatedness of strains and distinguish strains from different sources and geographical regions.

Acknowledgments

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