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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 *rtxA*, *rstR*, *toxR*, and *hlyA* 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 *ctxA* and *tcpA*. 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 *ctxA* and *tcpA* [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.
2. Materials and Methods

2.1. Bacteria Strains and DNA Extraction for Polymerase Chain Reaction (PCR). Isolation of \textit{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 \textit{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 coastal 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 μl of the supernatant (∼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 \textit{V. cholerae} O1 biotype El Tor, N16961, was carried out (http://minisatellites.u-psud.fr/). Primers were designed for each VNTR based on a ∼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 \textit{in silico} PCR (http://insilico.ehu.es/) and monoplex PCR using DNA templates from 5 \textit{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 μl contained 1X PCR buffer, 2 mM MgCl₂, 160 μM dNTPs, 0.4 μM of each primer, 2 μl of \textit{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 ddH₂O. One microlitre of the dilution was mixed with 10 μl HiDi formamide and 0.2 μl 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 ampiclons 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 (OD₆₀₀ = 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'-GGGCCC-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 DR III (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 μg/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 ctxA, zot, rtxA, rstR, toxR, tcpA, tcpI, and hlyA 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 μl contained 50 ng of DNA template, 1X PCR buffer, 2 mM MgCl₂, 200 μM of each dNTP, 0.3 μM of each primer,
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 pulotypes (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 I was the most common profile (V1: ctxA+, zot+, rtxA+, rstR+, toxR+, tcpA⁰⁷⁻, tcpI⁺) and was exhibited by O139 and 22 O1 strains. Virulotype 2 (V2: rtxA, rstR, toxR, and hlyAE⁰) 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 hlyAE⁰) and VC3477 (V4: rtxA, rstR, toxR, tcpI, and hlyAE⁰) were negative for tcpA. Besides VC3477, classical hlyA was also present in VC77 (V5: rtxA, rstR, toxR, and hlyAC⁰). 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-ANOVA F, Wilkes’ λ = 0.0874, F₆,₇₆ = 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-O139 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 hlyAE⁺) although they were isolated from different locations. VCGB was isolated from river water in Banting, Selangor (August 2008) while VCSW was isolated from algae.
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.

**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.
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 tcpI but not tcpA. tcpI gene is the first gene in TCP cluster and is associated with the synthesis of tcpA. 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 tcpA is not surprising. In addition, VC3477 harbored the classical hlyA gene while VC310 harbored the El Tor hlyA 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.

### Table 1: Characteristics of V. cholerae VNTR loci.

<table>
<thead>
<tr>
<th>Character</th>
<th>Primers sequence</th>
<th>Size range of PCR products (bp)</th>
<th>VNTR position (kb)</th>
<th>Alleles</th>
<th>D index</th>
</tr>
</thead>
<tbody>
<tr>
<td>VCTR 1</td>
<td>F: 5’-GAGAAAAAGCCAAACCACGTGC-3’&lt;br&gt; R: 5’-TTAAGGGGCGAGAACT-3’</td>
<td>212–230</td>
<td>137</td>
<td>9</td>
<td>0.82</td>
</tr>
<tr>
<td>VCTR 2</td>
<td>F: 5’-CGTTAGATCGAAACTGCTG-3’&lt;br&gt; R: 5’-CCACCACTCCTGTCAGGAA-3’</td>
<td>216–252</td>
<td>467</td>
<td>5</td>
<td>0.75</td>
</tr>
<tr>
<td>VCTR 3</td>
<td>F: 5’-AGTGGGCACAGAGTGCAAA-3’&lt;br&gt; R: 5’-GCTGATCTGCCACATCCA-3’</td>
<td>218–276</td>
<td>1778</td>
<td>7</td>
<td>0.84</td>
</tr>
<tr>
<td>VCTR 4</td>
<td>F: 5’-TCGAATGTATGGGAAACATT-3’&lt;br&gt; R: 5’-TTAATCCGCTTTCTTCGATG-3’</td>
<td>278–300</td>
<td>303*</td>
<td>17</td>
<td>0.92</td>
</tr>
<tr>
<td>VCTR 5</td>
<td>F: 5’-GAAGAGACGGACCTTGATCG-3’&lt;br&gt; R: 5’-TGCACGACAGTTTGGCTTAC-3’</td>
<td>228–251</td>
<td>1685</td>
<td>6</td>
<td>0.65</td>
</tr>
<tr>
<td>VCTR 6</td>
<td>F: 5’-GTTTTATCGCTGATGCGTGA-3’&lt;br&gt; R: 5’-CCAAGGAAGTGCGTAATTAT-3’</td>
<td>211–269</td>
<td>540*</td>
<td>4</td>
<td>0.59</td>
</tr>
<tr>
<td>VCTR 7</td>
<td>F: 5’-GAGAAAAGCCAAACCACGTGC-3’&lt;br&gt; R: 5’-CAATCTCGCCAATGCTTATG-3’</td>
<td>328–369</td>
<td>187*</td>
<td>11</td>
<td>0.89</td>
</tr>
</tbody>
</table>

*position in chromosome II of V. cholerae genom.

**Figure 2**: NotI-PFGE profiles of representative Malaysian V. cholerae of different serogroups. M: XbaI-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 tcpI but not tcpA. tcpI gene is the first gene in TCP cluster and is associated with the synthesis of tcpA. 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 tcpA is not surprising. In addition, VC3477 harbored the classical hlyA gene while VC310 harbored the El Tor hlyA 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.
VC 35 was isolated from a patient during a cholera outbreak in Kota Kinabalu, Sabah. This strain did not harbor the major virulence genes (*ctxA*, *zot*, *tcpA*, and *tcpI*) 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. cholerae*, 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.

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