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Full Paper

Molecular characterization of serogrouping and virulence genes of Malaysian *Vibrio cholerae* isolated from different sources

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A pair of primers targeting the *hlyA* gene for *Vibrio cholerae* which could distinguish the classical from El Tor biotypes was designed and combined with other specific primers for *ompW*, *rfb* complex, and virulence genes such as *ctxA*, *toxR*, and *tcpI* in a multiplex PCR (m-PCR) assay. This m-PCR correctly identified 39 *V. cholerae* from clinical, water and seafood samples. The efficiency of this multiplex PCR (m-PCR) was compared with conventional biochemical and serogrouping methods. One O139 and 25 O1 *V. cholerae* strains including 10 environmental strains harbored all virulence-associated genes except 1 clinical strain which only had *toxR* and *hlyA* genes. Thirteen environmental strains were classified as non-O1/non-O139 and had the *toxR* and *hlyA* genes only. The detection limit of m-PCR was 7×10^4 cfu/ml. The m-PCR test was reliable and rapid and reduced the identification time to 4 h.

Key Words——multiplex PCR; serogroup; *Vibrio cholerae*; virulence gene

Introduction

Vibrio cholerae, the causative agent of cholera, is endemic in many parts of the world, especially in developing and less developed countries which lack clean water supplies and public health facilities (Alam et al., 2006). The recent cholera incidence in Zimbabwe with more than 60,000 cases and 3,100 deaths between August 2008 and February 2009 has reemphasized the need for the monitoring and management of cholera (World Health Organization, 2009).

Cholera is endemic in Malaysia and sporadic cases do occur with low mortality rates. In 2006, 237 cases of cholera and 2 deaths due to cholera were reported by the Department of Public Health (DPH), Ministry of Health Malaysia (http://www.dph.gov.my/survelans/Statistik). *V. cholerae* has occasionally been isolated from environmental sources such as water and seafood in the country (Chen et al., 2004).

Both classical and El Tor biotypes of *V. cholerae* O1 were responsible for diarrhea in humans and were associated with the 6th and 7th pandemics, respectively (Danin-Poleg et al., 2007; Singh et al., 2001). After the emergence of *V. cholerae* O139 which caused a major outbreak in India in 1992, this serogroup began to spread to other neighboring countries, especially in the Indian subcontinent (Danin-Poleg et al., 2007; Faruque et al., 1998). *V. cholerae* O139 was believed to be the result of horizontal gene transfer from serogroup O1 to non-O1, non-O139 (Faruque et al., 2004; Singh et al., 2001). This serogroup appeared to be as virulent as the El Tor O1 serogroup as they share the same

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virulence genes (Sack et al., 2001).

Strains of *V. cholerae* non-O1, non-O139 are frequently isolated from environmental sources which are generally non-toxigenic as virulence genes for colonization are absent in this group of organisms (Singh et al., 2001). However cases of diarrhea caused by *V. cholerae* non-O1, non-O139 have been reported and this suggests that the environmental strains which are presumed to be non-toxigenic may cause colonization as these strains are potential reservoirs for virulence genes (Uma et al., 2003).

There are two major virulence-associated elements in *V. cholerae*, namely the TCP gene cluster for colonization in the *V. cholerae* Pathogenicity Island (VPI) and the CTX genetic element for cholera toxin production (Rivera et al., 2001). These two elements are closely linked as TCP functions as the receptor for CTX phage to infect non-toxigenic *V. cholerae* strains (Chakraborty et al., 2000). The expression of both elements is coordinately regulated by the ToxR regulatory system (Faruque et al., 1998).

Identification of clinical and environmental strains of *V. cholerae* in Malaysia usually involves biochemical tests, followed by PCR detection of the *toxR* gene and agglutination test for serogrouping (Chen et al., 2004). A rapid method for specific identification of *V. cholerae* using the *ompW* gene was reported by Nandi et al. (2000) while Hoshino et al. (1998) introduced a single PCR for differentiation of *V. cholerae* O1and O139 serogroups. Many of the current m-PCR assays target the *ctxA* and *tcpA* genes for the rapid identification of toxigenic *V. cholerae* (Alam et al., 2006; Chen et al., 2004; Hoshino et al., 1998; Mantri et al., 2006; Nandi et al., 2000).

The objective of this work was to optimize a multiplex PCR by combining published primers for serogrouping together with the primers which target virulence-associated genes. This assay would improve the simultaneous identification of serogroups of O1, O139 and non-O1/non-O139 *V. cholerae* toxigenic associated strains. The efficiency of this PCR was also compared with conventional methods in identification of field isolates from water and retail seafood.

Materials and Methods

Strains collection. Clinical (n=15) and environmental (n=14) *V. cholerae* strains were retrieved from the strains collection of Biochemical Science Lab, University of Malaya and Institute of Medical Research (IMR), Kuala Lumpur. Identities of strains were confirmed using biochemical tests and O agglutination tests by the Bacteriology Unit, IMR. Six reference strains were provided by National Institute of Cholera and Enteric Diseases (NICED), Kolkata, India and Public Health Laboratory, Johor, Malaysia including 2 El Tor O1 *V. cholerae*, 2 Classical O1 *V. cholerae*, and 2 O139 *V. cholerae*.

Isolation of V. cholerae from water and seafood samples. *V. cholerae* were isolated from water and seafood samples using a modified method of Keymer et al. (2007) and Ottaviani et al. (2003). Briefly, 100 ml seawater was filtered through a 0.45 μm membrane (Millipore, USA) and enriched in 10 ml of APW (Alkaline Peptone Water, pH 8.0) for 16 h at 37°C with agitation. For seafood samples, the food homogenate was directly enriched in 10 ml of APW. A loopful of the overnight culture was streaked on TCBS (thiosulfate citrate-bile salts-sucrose) agar (Oxoid, UK) or CHROMagar Vibrio (Becton Dickinson, USA) and incubated overnight at 37°C. The presumptive 2−4 mm diameter yellow sucrose fermenting colonies on TCBS and blue colonies on CHROMagar were subcultured on non-selective agar for further conventional biochemical analysis such as the string test, Lysin Iron Agar test (LIA), Kliger Iron test (KIA), API 20E (Biomerieux, France) and PCR analysis. An agglutination test was also carried out to identify the serogroup of strains using commercial antisera (Becton, Dickinson).

Crude DNA template preparation. A single colony of *V. cholerae* from non-selective media was suspended in 50 μl of deionized water, boiled at 99°C for 5 min and snapped cold on ice for 10 min. The cell lysate was centrifuged at 10,000 ×g for 2 min and about 3 μl (∼10 ng) of supernatant was used as DNA template for PCR.

Primer design for hlyA gene. Based on the sequences retrieved from the Genbank (Accession no.: AE003853.1, AY427780.1, and CP000626.1), a pair of primers targeting the hemolysin gene was designed (using the Primer 3 program, http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) in this study. Initially, an in-silico PCR (http://insilico.ehu.es/PCR) was carried out to test the specificity of the designed primers (Hly 1, and Hly 2), followed by evaluation with a panel of 50 strains including 19 confirmed *V. cholerae*, 20 *Vibrio* spp., 6 *Aeromonas* spp., 3 *E. coli*, 1 *Salmonella* Enteritidis, and 1 *Klebsiella pneumoniae.* Select-

ed amplicons were purified and sequenced to verify the amplification products.

PCR assay. The Hly primers designed in this study were then combined with 5 other pairs of primers described in previous studies (Table 1) to form a m-PCR to target *omp*W, *toxR*, *tcpI*, *ctxA*, and *orf* complex. The amplification was carried out in a Corbett palm cycler (Australia) in a total volume of 25 μl per reaction. A single reaction contained 10 ng of DNA template, 1× PCR buffer (Promega, USA), 2 mM $MgCl_2$ (Promega), 200 μM each dNTPs (Promega), 0.3 μM of each primer and 2.5 U of *Taq* DNA polymerase (Promega). The cycling conditions included an initial denaturation at 95°C, 5 min, followed by 30 cycles of denaturation at 94°C (30 s), annealing at 59°C (30 s), extension at 72°C (1 min) and a final extension at 72°C (7 min).

The PCR products were analyzed by a 2.5% LE agarose gel and electrophoresed at 90 V in 0.5× TBE buffer. The gel was stained with ethidium bromide (0.5 μg/ml) and visualized with Gel Doc XR (Bio Rad, CA).

Sensitivity test. The procedure previously described by Teh et al. (2008) was adapted for the sensitivity test using bacterial cell dilutions of reference strains N16961, 569B and SG24. Briefly, a mid-log phase grown culture (OD_{600} of 0.5) was serially diluted 10-fold. One aliquot of 100 μl of each dilution was plated in duplicate onto LB plates to determine cell density. Another aliquot of 100 μl was boiled, snap-cooled, centrifuged and 3 μl (about 10 ng) was used as DNA template for m-PCR. The m-PCR was performed under optimized conditions.

Results

Specificity of the hlyA primer

An in-silico PCR using all the available *Vibrio* spp. in the public domain gave two amplicons, 255 bp and 244 bp for biotypes El Tor and classical, respectively. None of the non-*V. cholerae* gave any amplification. In in-vitro PCR, a slightly smaller band ~240 bp was observed for the Classical strain (569B), and strains other than *V. cholerae* had no amplification. Sequence analysis of the amplicons for Classical 569B and El Tor N16961 were aligned using the Bioedit sequence alignment editor (Tom Hall, CA) and the similarity was found to be 0.945. However, 11 bp deletions were observed in the Classical 569B strain in concordance with the report from Rader and Murphy (1988) (Fig. 1).

Multiplex PCR

Among the panel of 29 Malaysian strains tested, 1 was subtyped as O139 serogroup, 7 were non-O1/non-O139, and 21 were subtyped as O1 serogroup, with 20 strains harboring toxigenic genes *toxR* (779 bp), *ompW* (336 bp), *hlyA* (~250 bp), *ctxA* (564 bp) and *tcpI* (862 bp). One strain lacked the *ctxA* and *tcpI* gene. Representative results are shown in Fig. 2.

Only 6 out of 45 water samples and 5 out of 55 seafood samples showed the characteristic colonies on TCBS and CHROMagar. Thirty-eight presumptive *V.*

Table 1. Primers used in this study.

Primer	Sequence (5′–3′)	Amplicon size	Target	Reference
132F	TAGCCTTAGTTCTCAGCAGGCA	862 bp	*tcpI*	Rivera et al. (2001)
951R	GGCAATAGTGTCGAGCTCGTTA			
ompW2	GAACTTATAACCACCCGCG	336 bp	*ompW*	Nandi et al. (2000)
ompW3	CCACCTACCTTTATGGTCC			
101F	CCT TCGATCCCCTAAGCAATAC	779 bp	*toxR*	Rivera et al. (2001)
837R	AGGGTTAGCAACGATGCGTAAG			
94F	CGGGCAGATTCTAGACCTCCTG	564 bp	*ctxA*	Fields et al. (1992)
614R	CGATGATCTTGGAGCATTCCCAC			
hyA1	GTGCGTATCAGCCTAGATGA	255 (ET)/244 (C)	*hlyA*	This study. Modified from Rivera et al. (2001)
hlyA2	CCCAAGCTCAAAACCTGAAA			AE003853.1, AY427780.1, CP000626.1
O1*rfb*F	GTTTCACTGAACAGATGGG	192 bp	O1 serogroup	Hoshino et al. (1998)
O1*rfb*R	GGTCATCTGTAAGTACAAC			
O139*rfb*F	AGCCTCTTTATTACGGGTGG	449 bp	O139 serogroup	Hoshino et al. (1998)
O139*rfbR*	GTCAAACCCGATCGTAAAGG			

Table 2. Summary of characteristics of *V. cholerae* isolated from water and seafood samples.

Label	Morphology on TCBS agar/CHROMagar	Biochemical tests				Agglutination test (O1/O139)	Multiplex PCR
		String	LIA	KIA	API		
W01, W03, W11, W30	2–3 mm yellow colony	+	+	+	VC	O1 +ve	VC^+, $O1^+$, hly^+, tox^+, $ctxA^+$, $tcpI^+$
W17, W25, W27, W34	2–3 mm yellow colony	+	+	+	VC	−ve	VC^+, hly^+, tox^+
W02, W08, W09, W15, W20, W21, W22, W36, W29, W32, W37, W38	2–3 mm yellow colony	−	−	−	VP	−ve	−
W04, W05, W06, W07, W16, W26, W28	2–3 mm yellow colony	−	−	−	AH	−ve	−
W10, W12, W13, W14, W18, W19, W23, W24, W31, W33, W35	2–3 mm yellow colony	−	−	−	VA	−ve	−
S01, S5	Turquoise blue colony	−	−	−	VP	−ve	−
S02, S3	Turquoise blue colony	+	+	+	VC	−ve	VC^+, hly^+, tox^+
S04	Turquoise blue colony	−	−	−	VV	−ve	−

W1–38: presumptive *V. cholerae* isolated from water; S1–5: presumptive *V. cholerae* isolated from seafood; VC: *V. cholerae* ; VP: *V. parahaemolyticus*; VA: *V. alginolyticus*; VV: *V. vulnificus*; AH: *Aeromonas hydrophila.*

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          ....|....| ....|....| ....|....| ....|....| ....|....|
                10        20        30        40        50
El Tor    TGACAGCACG GGGGCCGGCA TTCATCTGAA TGATCAACTC GGTTATCGTC
Classical -----GTGCG GGGGC-GGCA TC--TCTGA- TG-TCA-CTC GGTTATCGTC

          ....|....| ....|....| ....|....| ....|....| ....|....|
                60        70        80        90        100
El Tor    AGTTTGGAGC CAGTTATACG ACGTTAGATG CCTATTTCCG TGAGTGGTCA
Classical AGTTTGGAGC CAGTTATACG ACGTTAGATG CCTATTTCCG TGAGTGGTCA

          ....|....| ....|....| ....|....| ....|....| ....|....|
                110       120       130       140       150
El Tor    ACCGATGCGA TTGCCCAAGA TTATCGCTTC GTGTTTAACG CATCGAACAA
Classical ACCGATGCGA TTGCCCAAGA TTATCGCTTC GTGTTTAACG CATCGAACAA

          ....|....| ....|....| ....|....| ....|....| ....|....|
                160       170       180       190       200
El Tor    TAAAGCGCAG ATCCTGAAAA CCTTTCCTGT CGATAACATT AACGAGAAAT
Classical TAAAGCGCAG ATCCTGAAAA CCTTTCCTGT CGATAACATT AACGAGAAAT

          ....|....| ....|....| ....|....| ....|.
                210       220       230
El Tor    TTGAGCGCAA AGAGGTTTCA GGTTTTGAGC TTGGG.
Classical TTGAGCGCAA AGAGGTTTCA GGTTTTGAGC TTGGG.
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Fig. 1. Sequence alignment for the *hlyA* amplicons of El Tor and Classical strains.

Eleven bp deletions were found in Classical strains.

cholerae were randomly selected from these selective media and subjected to biochemical tests such as the string test, LIA, KIA and API 20E tests. Only 10 isolates were confirmed as *V. cholerae.* Other vibrios identified based on biochemical tests were *V. parahaemolyticus* (n=14), *V. alginolyticus* (n=11) and *V. vulnificus* (n= 1). Seven isolates which appeared as yellow colonies on TCBS agar were identified as *Aeromonas hydrophila*. Based on agglutination tests on the 10 *Vibrio cholerae* isolated, 4 belonged to the O1 serogroup (from water samples) while 6 belonged to non-O1/non-O139. Simultaneously, a m-PCR assay was tested directly on the presumptive colonies from the selective media. Among the 38 isolates tested, 10 were identified as *V. cholerae*, with 4 belonging to serogroup O1 and 6 to non-O1, non-O139.

There was a concordance in the identification of the strains based on conventional and m-PCR methods as summarized in Table 2. The 4 O1 *V. cholerae* harbored all 4 virulence genes, while the other 6 non-O1, non-O 139 *V. cholerae* harbored only 2 virulence genes (*hlyA* and *toxR*) (Table 2).

Sensitivity test

The sensitivity of the m-PCR using 10 fold-dilutions of strains N16961, 569B, and SG24 was averaged at 7×10^4 cfu/ml (equivalent to approximately 210 cfu per PCR).

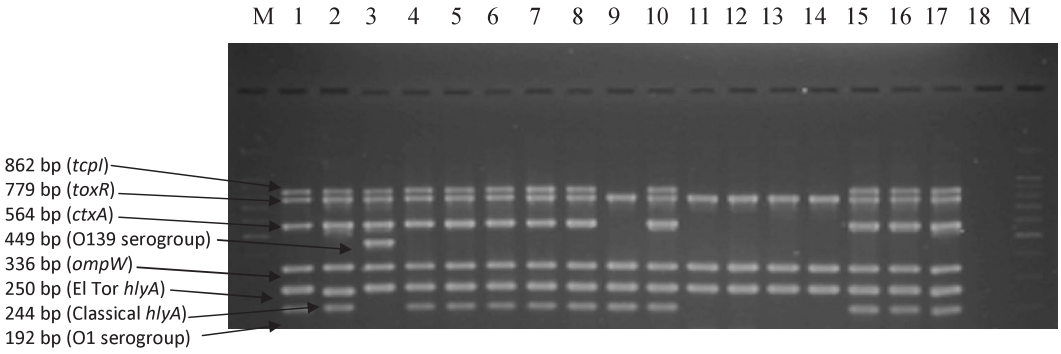

Fig. 2. Representative Gel of the Multiplex PCR amplification of toxigenic genes in *Vibrio cholerae*. Lane 1: O1 El Tor N16961 (Reference Strain, NICED: *tcpI*$^+$, *toxR*$^+$, *ctxA*$^+$, *ompW*$^+$, El Tor *hlyA*$^+$), Lane 2: O1 Classical 569B (Reference Strain, NICED: *tcpI*$^+$, *toxR*$^+$, *ctxA*$^+$, *ompW*$^+$, Classical *hlyA*$^+$), Lane 3: O139 SG24 (Reference Strain, NICED: *tcpI*$^+$, *toxR*$^+$, *ctxA*$^+$, *ompW*$^+$, El Tor *hlyA*$^+$), Lanes 4–8, 10: O1 Clinical isolates (*tcpI*$^+$, *toxR*$^+$, *ctxA*$^+$, *ompW*$^+$, El Tor *hlyA*$^+$), Lane 9: O1 Clinical isolates (*toxR*$^+$, *ompW*$^+$, El Tor *hlyA*$^+$), Lanes 11–14: non-O1/non-O139 environmental isolates (*toxR*$^+$, *ompW*$^+$, El Tor *hlyA*$^+$), Lanes 15–17: O1 environmental isolates (*toxR*$^+$, *ompW*$^+$, El Tor *hlyA*$^+$), Lane 18: negative control, Lane M: 100 bp DNA marker (Promega, USA).

Discussion

Isolation, detection and confirmation of *V. cholerae* by conventional methods have been complicated. *V. cholerae* is genetically closely related to other *Vibrio* spp. and *Aeromonas* spp. Therefore, ambiguous identification using biochemical tests occur frequently. Moreover, commercial antisera are only available for O1 and O139 *V. cholerae* (Nandi et al., 2000).

There is a lot of interest by scientists to improve on the detection system. Nandi et al. (2000) reported a PCR assay for detection of *ompW*, *rfb* complex, and *ctxA*, which was adopted by Alam et al. (2006). Chen et al. (2004) reported a PCR targeting the *rfb* complex and virulence genes of *V. cholerae*. A septaplex PCR was developed by Mantri et al. (2006) for the same purpose. These PCR assays differentiated *V. cholerae*, serogroup O1/O139, and virulence genes based on *ctxA* and *tcpA*. However, other important virulence-associated genes such as *toxR*, *hlyA*, and *tcpI* were not included.

In the present study, *tcpI* was used as the marker for detection of colonization factor. The *tcpI* gene is associated with the synthesis of *tcpA* and may function as a regulator to determine the virulence of VPI, and *tcpI* is included in the panel of virulence genes (Dalsgaard et al., 2001; Faruque et al., 1998). To detect the major colonization factor and cholera toxin production, both *ctxA* and *tcpI* should be combined. Therefore, this PCR would be useful for targeting potential toxigenic strains of *V. cholerae*.

Fifteen human stools and 10 environmental strains of serogroup O1 harbored all the virulence genes tested (*hlyA*, *toxR*, *ctxA*, and *tcpI*). *toxR* and *hlyA* genes were present in all strains. None of the non-O1, non-O139 strains harbored *ctxA* or *tcpI* genes. One clinical O1 strain was negative for both *ctxA* and *tcpI* genes. When re-tested by monoplex PCR reported in Rivera et al. (2001), this strain was negative for genes such as *tcpA*, *ctxA*, and *zot* (data not shown). The mechanism of colonization and pathogenicity of this strain needs further investigation in future work.

Although the classical biotype was responsible for the 6th pandemic and superseded by El Tor biotype O1 and O139, it is potentially concealed in the environment and the risk of being infected by this biotype remains due to horizontal gene transfer (Salim et al., 2005). Rivera et al. (2001) proposed a set of modified *hlyA* primers which enable biotype identification. These primers produced 481 bp/738 bp bands for El Tor and a 727 bp band for classical *V. cholerae*. However, the sizes of these amplicons were not suitable to be used in the multiplex assay in this study as it would be difficult to differentiate from the *toxR* band (779 bp) and O139 band (449 bp). Therefore, a novel pair of primers of *hlyA* for the detection of El Tor and classical biotypes was designed so that all the amplicons could be detected easily. However, no additional classical strain was isolated in the field to evaluate the efficiency of the m-PCR.

In this study, an *ompW* target was used to specifically distinguish *V. cholerae* from other closely related

species such as *V. vulnificus*. Both the selected media, TCBS and CHROMagar *Vibrio*, are useful for screening the target microorganisms but not for direct differentiation of the specific species of vibrios as the color and morphology of the colonies on these media were unspecific and variable (only 23% presumptive colonies were confirmed as *V. cholerae* using biochemical tests and PCR in this study). Moreover, biochemical tests used were based on the changes in color due to chemical reactions and sometimes these reactions were not reproducible. By targeting the specific gene of *V. cholerae*, identification of this pathogen is easier, faster and more definite.

By using this m-PCR, the time required to confirm *V. cholera*e and other *Vibrio* spp. from a presumptive culture was about 4 h as compared to the conventional methods (biochemical tests, API kit, and sera agglutination) which require about 4 days' time for completion. In addition, this m-PCR assay provided further information on the carriage of virulence genes. All strains were tested with monoplex PCR before the multiplex PCR was carried out to ensure the specificity of the multiplex PCR. The results for the multiplex PCR developed in this study were reproducible. However, a molecular approach cannot replace the conventional method as cultures are still needed for further testing such as determination of antibiograms. The m-PCR method may be used to complement the conventional culture method.

In conclusion, the m-PCR developed in this study provides an alternative and easier method to rapidly identify toxigenic and non-toxigenic *V. cholerae* with different serogroups and may complement the traditional biochemical tests.

Acknowledgments

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