Chapter One

Introduction

1.1 Research Background

Vibrio cholerae, the causative agent of cholera, is a gram negative bacterium which lives freely in aquatic environments. Cholera is endemic in developing and less developed countries which lack clean water supplies and public health facilities (Alam *et al.*, 2006). Cholera remains a public health concern in the perspective of morbidity and mortality as well as economic concerns for the presence of *V. cholerae* in seafood (Radu *et al.*, 2002). Nevertheless, isolation of *V. cholerae* is problematic. Conventional isolation of *V. cholerae* normally depends on culture-based biochemical tests (Harwood *et al.*, 2004). As *Vibrio* species are genetically similar, *V. cholerae* is frequently misidentified as *V. mimicus* (Kaysner and DePaola, 2004). Biotyping and serogrouping of *V. cholerae* are carried out to determine the biotypes and serogroups, respectively. The overall cultural, biochemicals and serotyping process is time consuming (3-5 days) and laborious. Therefore, the development of a rapid and specific approach for identification, biotyping, and serogrouping of *V. cholerae* is necessary.

In addition to rapid identification, molecular subtyping of *V. cholerae* is also important for tracing a new or multidrug-resistant clone (Thong *et al.*, 2002). Traditional PCR-based fingerprinting approaches such as arbitrarily primed PCR (AP-PCR), Box-PCR, random amplified polymorphic DNA (RAPD)-PCR, enterobacterial repetitive intergenic consensus sequence (ERIC)-PCR, repetitive extragenic palindromic (REP)-PCR are not reliable and reproducible (van Belkum *et al.*, 2001). Tokunaga *et al.* (2010) reported a

relatively new *V. cholerae r*epeats PCR (VCR-PCR). However, this newly developed method has never been tried on the *V. cholerae* of non-O1/non-O139 serogoups.

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 (Chen *et al.*, 2004). Moreover, the methodology is standardized for interlaboratory comparison. However, PFGE is time consuming and requires strict adherence to standardized protocols for inter-laboratory comparison. Hence, newer subtyping approaches such as the multiple-locus variable-number of tandem repeat analysis (MLVA) and Multilocus sequencing typing (MLST) were developed. MLVA is based on the variable-number tandem repeats (VNTR) in multiple loci, each VNTR consists of DNA elements that are repeated in tandem and polymorphism is dependent on the number and size of the repeats (van Belkum *et al.*, 2007).

MLST usually examines the nucleotide changes in the housekeeping genes. However, compared to housekeeping genes which diversify slowly, virulence genes are more polymorphic (Danin-Poleg *et al.*, 2007; Kotetishvili *et al.*, 2003). Virulence markers could also be used to compare groups of strains that are either toxigenic or non-toxigenic and ultimately translated into clinically relevant diagnostic targets (van Belkum *et al.*, 2007).

The virulence-genes associated with colonization for O1 serogroups are well-defined. Two major virulence-associated elements such as toxin-coregulated pili (TCP) cluster in *Vibrio cholerae* pathogenicity island (VPI) which coordinately regulated by ToxR regulatory system (Faruque *et al.*, 1998) are the important factors for colonization. However, little is known about colonization for non-O1/non-O139 serogroups because

non-toxigenic O1 and non-O1/non-O139 *V. cholerae* which lack TCP and CT are also capable of colonization (Faruque *et al.*, 2004; Dalsgaard *et al.*, 2001). Therefore, it is important to investigate the influence of host environment and the difference of virulence and virulence-associated genes expressions in toxigenic and non-toxigenic *V. cholerae* during colonization.

1.2 Objectives:

The objectives of this study were:

1. To develop a multiplex PCR to differentiate *V. cholerae* from other *Vibrio* species
2. To compare the efficiency of multiplex PCR, biochemical tests and API 20E for differential detection of *V. cholerae* from water sources.
3. To develop a multiplex PCR for biotyping, serogrouping and virulotyping of *V. cholerae*.
4. To determine the genetic diversity of *V. cholerae* using ERIC, RAPD, REP and VCR-PCR and to compare the discriminatory ability of these methods.
5. To study the relatedness of *V. cholerae* by using a novel MLVA and PFGE and to compare the discriminatory ability of both methods.
6. To study the genetic variations of *V. cholerae* by using MLST and MVLST.

7. To study the *in-vivo* expression of colonization-associated virulence genes for different variants of *V. cholerae* and the influence of host environment for colonization.

Chapter Two

Literature Review

2.1 *Vibrio cholerae*

Vibrio cholerae is a gram negative bacterium which is also a member of the *Vibrionaceae* family. It is anaerobic, curved rod with a single polar flagellum (Salyers and Whitt, 2002). O1 and O139 *V. cholerae* are the causative agent of cholera. *V. cholerae* does not require mammalian host for its only survival but also inhabits in estuarine environments and associated with phytoplankton blooms (Colwell, 1994). The survival and persistence of *V. cholerae* to various stresses in different environment can be due to the formation of biofilm (Reidl and Klose, 2002).

The basis of *V. cholerae* classification was first described by Gardner and Venkatraman in 1935 based on polysacharide of the heat-stable surface somatic 'O' antigen. *V. cholerae* is now classified into 206 'O' serogroups (WHO, 2002).

The first cholera pandemic started in Indian subcontinent in 1817 and spread to Asia. In 1830s, the second pandemic occurred and spread to Asia, United Kingdom and Canada from the Indian subcontinent (Salyers and Whitt, 2002). During the second pandemic, Lord John Snow discovered that this disease is water-borned with his historic Broad Street pump study but the causative agent remained unknown (Tison, 1999; Thompson *et al.*, 2004). The third and fourth pandemic started in 1852 and 1879, respectively; both originated from Indian subcontinent and reached United States (Salyers and Whitt, 2002).

In 1880s, the fifth cholera pandemic happened in Indian subcontinent and spread to Asia and Europe. In 1883, the bacterial etiology of cholera was confirmed by Robert Koch when he isolated the cholera bacillus from pond water during an outbreak in Egypt (Tison, 1999; Thompson *et al.*, 2004). The strain thatcaused the fifth pandemic was later named as O1 Classical *V. cholerae*. Similar to previous cholera pandemics, the sixth pandemic started in Indian subcontinent in 1889 and later spread to Asia. This pandemic was also caused by O1 Classical *V. cholerae* (Salyers and Whitt, 2002).

The seventh pandemic started in Indonesia and reached South America in 1960 and continues to the present. This cholera pandemic introduced a new strain, O1 El Tor which harbours different biochemical properties and phage susceptibility patterns compared to Classical strain (Salyers and Whitt, 2002). In addition, altered El Tor strains of *V. cholerae* O1 which produce cholera toxin of the Classical biotype have been reported (Olsvik *et al.*, 1993). This variant strain is also known as hybrid strains as it carried the Classical cholera toxin sequence at the 39 and 68 position of the gene (*ctxB*) (Nair *et al.*, 2006).

In 1992, there was a rise of a new variant strain, O139 resulting from the horizontal gene transfer event between O1 and non-O1 serogroups (Faruque *et al.*, 2004; Singh *et al.*, 2001). This serogroup began to spread to other neighbouring countries, especially in Indian subcontinental after the major outbreak in India (Danin-Poleg *et al.*, 2007; Faruque *et al.*, 1998). The recorded pandemic of cholera is summarized in **Table 2.1**.

Since the recognition of O139 serogroup, the designation of non-O1/non-O139 for those strains which do not belong to O1 and O139 *V. cholerae* has been used (Nair *et al.*, 1994). Non-O1/non-O139 *V. cholerae* is frequently isolated from environmental sources and is generally non-toxigenic as virulence genes for colonization are absent in this group

of organisms (Singh *et al.*, 2001). However cases of diarrheal caused by non-O1/non-O139 *V. cholerae* 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).

Table 2.1: Recorded pandemics for cholera, sources and extent of pandemics, and the associated *V. cholerae* strains (Salyers and Whitt, 2002).

Pandemic	**Year**	**Origin and extent**	**Strains**
First	1817-1823	Indian subcontinent, spread to Asia	Unknown
Second	1830s	Indian subcontinent, spread to Asia, United Kingdom, and Canada	Unknown
Third	1852-1859	Indian subcontinent, reached United State	Unknown
Fourth	1870s	Indian subcontinent, reached United State	Unknown
Fifth	1880s	Indian subcontinent, spread to Asia and Europe	O1 Classical
Sixth	1889-1923	Indian subcontinent, spread to Asia	O1 Classical
Seventh	1961-to present	Indonesia, reached South America	O1 El Tor
Eighth	1992-1993	Indian subcontinent, spread to Asia	O139

2.2 Cholera

V. cholerae, particularly O1 and O139 serogroups are the main causative agents of cholera, a gastrointestinal disease. Nevertheless, reports of sporadic cases of cholera-like disease caused by non-O1/non-O139 *V. cholerae* have also been published (Faruque *et al.*, 2004). In Malaysia, cholera outbreaks due to the *V. cholerae* O1 serogroup occur periodically while non-O1/non-O139 *V. cholerae* is frequently isolated from seafood and aquatic sources but has not been implicated in any major outbreak (Vadivelu *et al.*, 2000; Nandi *et al.*, 2000; Rivera *et al.*, 2001; Faruque *et al.*, 1998). In Malaysia, a cholera outbreak reported in November 2009 involved 187 cases with one death (http://www.myhealth.gov.my/myhealth). *V. cholerae* has occasionally been isolated from environmental sources such as water and seafood in the country (Chen *et al.*, 2004).

Contamination of drinking water and improper food preparation are the cause of *V. cholerae* infection (Reidl and Klose, 2002). The infectious dose of *V. cholerae* is stated to be 10^8 cfu (Steffen *et al.*, 2003). *V. cholerae* must be able to go through and survive when exposed to low pH in the gastric compartment (Reidl and Klose, 2002). The viable *V. cholerae* will attach to the mucosal cells in the small intestine and produce an exotoxin (cholera toxin) to alter the balance of net flow of ions from the lumen to tissue. This directly causes massive diarrhea accompanied by vomiting and acidosis, electrolyte imbalance lost of 20 litres of water in a day is possible (Steffen *et al.*, 2003). The stools produced are colorless, oudourless, and flecked with mucous and known as 'rice watery stool'. As the water is rapidly lost, patients may die within 2 hours from the circulatory collapse if no treatment provided. *Vibrio cholerae* infection can also leads to shock

accompanied by drowsiness or unconscious in 4–12 hours and death in 18 hours to several days (Steffen *et al.*, 2003). Therefore, fluid replacement therapy for cholera patients is to prevent lethal dehydration (Salyers and Whitt, 2002).

Patients who recover from cholera are immuned to reinfection. However, when reinfected with *V. cholerae* again, these people will be at risk of being asymptomic carriers of *V. cholerae* for transmission of the disease to others and being the contributor of *V. cholerae* contamination (Salyers and Whitt, 2002).

Cholera remains the major problem to travellers who frequently travel to developing countries where cholera is endemic. It is difficult to estimate the true incidence of cholera in travellers from the official statistics. As this disease severity is dependent on several factors including size of the inocula and immune system of the travellers, those who are healthy are probably less severe compared to others (Steffen *et al.*, 2003).

2.3 *V. cholerae* and other *Vibrio* species

V. cholerae and other *Vibrio* species live naturally in aquatic environments. They are commonly isolated from marine organisms and seafood. Besides *V. cholerae,* pathogenic *Vibrio* species such as *V. parahaemolyticus* and *V. vulnificus* are of major concern as they are responsible for infectious diseases in humans (Tracz *et al.*, 2007). Although other *Vibrio* species, such as *V. harveyi*, *V. anguillarum,* and *V. alginolyticus* are less pathogenic to humans, they can cause massive mortality or infection in aquatic organisms (Cam *et al.,* 2009; Caipang *et al.,* 2009). Vibrios are naturally occurring in

aquatic environments which are often associated with infectious disease in humans and shellfish (Tracz *et al.*, 2007). In general, contamination of *Vibrio* species in fish, shrimp, squid and shellfish has direct impact on the public health policy and the economy of aquaculture (Noriega-Orozco *et al.*, 2007; Panicker *et al.*, 2004).

Most of the *Vibrio* species are genetically related and can not be easily differentiated based on the phenotypical features (Choopun *et al.*, 2002; Di Pinto *et al.*, 2005). For example, *V. mimicus*, a member of the *Vibrio* species is highly similar to *V. cholerae*. It was classified as sucrose-negative *V. cholerae* before being designated as a new species. The characteristics of gastrointestinal infection caused by *V. mimicus* are very similar to non-O1 *V. cholerae* (Tison, 1999).

2.4 Current methods for identification and confirmation of *V. cholerae*

Traditional isolation of *Vibrio* species normally depends on culture-based biochemical tests (Harwood *et al.*, 2004). However, *V. cholerae* could switch into viable but non-culturable (VNC) state in response to nutrient deprivation in the environment (Reidl and Klose, 2002). This leads to the difficulty of isolation of *V. cholerae* from the environmental sources.

Isolations of *Vibrio* species are often carried out using media formulated with alkaline pH as this facilitates the recovery of 'injured cell' from the natural environments (Kaysner and Depaola, 2004; Panicker *et al.*, 2004). Next, the enriched culture will be subjected for further confirmation. Generally, there are three major approaches for *V.*

cholerae confirmation such as culture-based identification, biochemical tests, and molecular approaches.

2.4.1 Cultured-based identification

CHROMagar *Vibrio* and thiosulfate-citrate-bile salt-sucrose (TCBS) agar are two commonly used selective media for *Vibrio* species selection and differentiation. Both media enable visually differentiation of various *Vibrio* species based on the colour of the colonies. On TCBS agar, sucrose-fermanting species such as *V. cholerae* and *V. alginolyticus* will produce yellow colonies. Species which do not ferment sucrose such as *V. parahaemolyticus, V. vulnificus*, and *V. mimicus* will produce blue-green colonies (Tison, 1999). Similarly, based on the color of colonies, CHROMagar *Vibrio* could be used to differentiatie *V. parahaemolyticus* (mauve), *V. vulnificus* (green blue to turquoise blue), *V. cholerae* (green blue to turquoise blue) and *V. alginolyticus* (colorless). A comparison between these two selective media was carried out by Di Pinto *et al.* (2010). The authors showed that CHROMagar *Vibrio* has higher accuracy and specificity than TCBS. However, such colour-based identification of the *Vibrio* species on the selective agar is sometimes inconsistent, and hence various biochemical tests such as oxidase test, triple sugar iron (TSI) test, Voges-Proskauer (VP) test, string test, salt tolerance and other tests are required for further confirmation (Elliot *et al.*, 2001; Kaysner and Depaola, 2004). For example, it is difficult to differentiate *Aeromona*s from Vibrios based on conventional culture method using selective media. This is because *Aeromonas* species can grow on the same selective

media and share high similarity in genetics, colony morphology and chemical reaction with *Vibrios* (Di Pinto *et al.*, 2005).

2.4.2 Biochemical tests

Presumptive *V. cholerae* colonies on the selective media are selected and subjected for conventional biochemical tests such as gram-stain, oxidase reaction, salt tolerance test, string test, triple sugar iron (TSI) test, kligler iron (KIA) test, lysine iron agar (LIA), Voges-Proskauer (VP) test, arginine dihydrolase test, and agglutination test. Gram-staining is commonly used for differentiation of gram-negative and gram positive bacteria while oxidase test is used to distinguish oxidase positive *Vibrio* species from other oxidase negative *Enterobacteriaceae*. String test is also useful to differentiate *Aeromonas* from *Vibrio* species based on the ability to lyse and produce a viscous suspension (Tison, 1999).

KIA and TSI are used to rule out *Pseudomonas* species and certain members of *Enterobacteriaceae*. The bacterial colony was picked by using an inoculating needle. Inoculation was carried out by thrusting the inoculating needle straight down into the butt and streaking on the surface of the medium. The slants are then incubated at 35ºC-37ºC and examined after 18-24 h. The caps on the tubes should be loosened to prevent anaerobic condition. On KIA, *V. cholerae* which is lactose non-fermenting produce alkaline (red) slant/acid (yellow) butt without gas and H_2S production while on TSI, *V. cholerae* produce acid (yellow) slant/acid (yellow) butt without gas and H_2S production (CDC, 2006).

LIA is used for detection of *V. cholerae* for determination of decarboxylate lysine reaction. LIA is inoculated by stabbing the butt and streaking surface of the slant. The slant is examined after incubation at 35ºC to 37ºC. After 18-24 h of incubation, *V. cholerae* will produce an alkaline reaction (purple color) in the butt of the tube but neither gas nor H_2S will be produced (CDC, 2006).

Sometimes, VP test may also be carried out to biotype the O1 *V. cholerae*. However, the results obtained from VP test are varied and not applicable to all serogroups of strains (Kay *et al.*, 1994). According to Choopun *et al.* (2002), arginine dihydrolase test is useful to detect *V. cholerae*. To perform the test, LB broth containing 1% (wt/vol) $_L$-arginine (pH6.8) with phenol red as indicator was used. After inoculation, the medium was covered with sterile mineral oil and incubated for 24 h. The medium can also be incubated for up to seven days. The presence of red color in the medium indicates positive reaction.

Alternatively, Analytical Profile Index (API) 20E (Biomerieux, SA, France) can be used as a more rapid and easy biochemical-based test for *V. cholerae* confirmation. API 20E (Biomerieux, SA, France) is widely used for Enterobactereacea and is efficient to identify commonly isolated species of *Vibrionaceae* (Overman *et al.*, 1985; O'hara *et al.*, 2003).

Once the identity of *V. cholerae* isolate is confirmed, agglutination tests may be carried out for serogrouping. Polyvalent O1 or O139 antisera are used for the slide agglutination test. The colonies which used for the agglutination tests must be grown on non-selective media to prevent false-negative result (CDC, 2006). A specific strain will be identified as non-O1/non-O139 if it does not agglutinate in O1 and O139 antiserum.

2.4.3 Polymerase chain reaction (PCR)

For specific detection of *V. cholerae*, PCR-based methods are widely used. Various PCRs have been reported for *V. cholerae* or multiple species detection. Markers such as 16S rRNA, *rpoB, dnaJ* and other housekeeping genes have been used for the detection and phylogenetic analysis of *Vibrios*. 16S rRNA target is not able to distinguish *Aeromonas* from *Vibrio* species (Tarr *et al.*, 2007). The *rpoB* gene is able to differentiate *Vibrio* strains up to species level with further DNA sequencing (Tarr *et al.*, 2007). Nhung *et al.* (2007) had proposed the use of *dnaJ* gene-based PCR approach to differentiate *Vibrio* species and further developed a PCR method to identify five human pathogenic *Vibrio* species based on the *dnaJ* gene (Nhung *et al.*, 2007).

For multiple spesies detection, Panicker *et al.* (2004) had developed a multiplex PCR approach which targets the *vvh* and *viu*B for *V. vulnificus*, *ompU*, *toxR*, *tcpI*, and *hlyA* for *V. cholerae*; and *tlh*, *tdh*, *trh*, for *V. parahaemolyticus*. A multiplex PCR approach for detection of *Vibrio* species, *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, and *V. mimicus* was also developed by Tarr *et al.* (2007). In addition, based on the collagenase gene, a PCR approach for *V. alginolyticus*, *V. cholerae* and *V. parahemolyticus* was developed by Di Pinto *et al.* (2005).

To differentiate O1 and O139 serogroups, Hoshino *et al.* (1998) proposed the use of *rfb* complex. Later in 2000, Nandi *et al.* (2000) had developed a PCR for *V. cholerae* specific detection based on *ompW* and incorporated the *rfb* complex to differentiate the serogroups of *V. cholerae*.

2.5 Molecular studies and characterization of *V. cholerae*

The relatedness characterization of *V. cholerae* outbreak strains remains important in order to trace the origin and geographical distribution of *V. cholerae* (Thong *et al.*, 2002). It is also very important to monitor the environmental *V. cholerae* strains with epidemic potential (Faruque *et al.*, 2006) and to study the genetic variation in a population (van Belkum *et al.*, 2007).

Classification of *V. cholerae* based on serological and antimicrobial susceptibility patterns may be relevant to disease treatment but is not suitable for epidemiology study as these methods are based on phenotypes which are not necessarily related to genetic descent. Therefore, molecular-based genotyping methods would be more useful. A good genotyping method should be able to differentiate unrelated strains and allow the recognition of the clonal grouping (Achtman, 2001). Basically, current genotyping methods are based on the repeat variation, point mutation, mobile DNA, multiple mutations (van Belkum *et al.*, 2007). Examples of the methods are PCR-based fingerprinting, pulsed-field gel electrophoresis (PFGE), multilocus variable number of repeats analysis (MLVA), and multilocus sequencing typing (MLST).

2.5.1 PCR-based fingerprinting of *V. cholerae*

Various PCR-based fingerprinting approaches have been described for *V. cholerae*. Among the methods, random amplified polymorphic DNA (RAPD) is based on the homologies of the chromosomal DNA to the short oligonucleotide primers. The DNA

elements which complement to the sequence of primers will be amplified. Bands of different intensity will be produced and the reliability is dependent on standardized conditions and is useful for rapid disrimination of unrelated strains. Nevertheless, it is less reliable for the grouping of close related isolates (Achtman, 2001).

ERIC-PCR employs the enterobacterial repetitive intergenic consensus (ERIC) sequence as the target and has been used to resolve *V. cholerae* clonal lineages. The ERIC sequences, also known as intergenic repeat units (IRUs) are present in the genomes of *Enterobacteriaceae* (Zulkifli *et al.*, 2009). The position of ERIC elements in enterobacterial genomes varies between different species and has been used as a genetic marker to characterize isolates within a bacterial species (Radu *et al.*, 2002). ERIC-PCR constitutes a powerful tool for determination of the virulence potential of *V. cholerae* O1 strains isolated in surveillance programs and for molecular epidemiological investigations (Rivera *et al.*, 1995). Rivera *et al.* (1995) had employed ERIC-PCR for 83 *V. cholerae* strains and found significant difference in the banding patterns for toxigenic and nontoxigenic strains. Similar to ERIC-PCR, repetitive extragenic palindromic (REP)-PCR is also a method based on the repetitive sequence in the genome. Both methods are widely used for epidemiological studies.

A relatively new approach, *V. cholerae* Repeats PCR (VCR-PCR) was developed by Tokuaga *et al.* (2010). This method targets the DNA sequences 'sandwiched' between the repetitive sequences of '*V. cholerae* repeats (VCR)' in the integron Island. This method was reported to be useful for genotyping of toxigenic *V. cholerae* O1 El Tor and O139 strains from different geographic regions and recognize strains with epidemic potential.

2.5.2 Pulsed-field gel electrophoresis (PFGE)

Pulsed-field gel electrophoresis (PFGE) has been considered as a gold standard for subtyping bacterial pathogens since it is highly discriminative and reproducible. It has been used to differentiate strains from outbreaks and from sporadic cases of cholera (Chen *et al.*, 2004). The principle of this approach is to use an infrequent cutting restriction enzyme that cut the genomic DNA into a few restriction fragments which later resolved by PFGE. PFGE facilitates the differential migration of large DNA fragments through agarose gels by constantly changing the direction of the electrical field during electrophoresis (CDC, 2009).

PFGE provides a robust genomic interpretation and the protocol is standardized by CDC, PulseNet. Validation of PFGE protocol for subtyping of *V. cholerae* was carried out in PulseNet International activities. The protocol was derived from the existing PulseNet protocol for *Escherichia coli* O157 and optimized for *V. cholerae* (Cooper *et al.*, 2006). However, strict adherence to the protocol is necessary. Once the methodologies and data anlaysis are standardized, PFGE would be very useful for effective epidemiological surveillance, prevention and control of important bacterial infection (Thong *et al.*, 2002).

2.5.3 Multilocus variable number of repeats analysis (MLVA)

Multilocus variable number of repeats analysis (MLVA) is a typing method which is based on the tandem repeats in multiple loci. It is widely used for genomic inference of different bacterial pathogens since 2006 (van Belkum, 2007). In this analysis, number of

repeats in a set of variable number of tandem repeats (VNTR) loci is assessed by performing PCR of the VNTR loci followed by accurate sizing of the PCR products using capillary electrophoresis on an automated DNA sequencer. The number of repeat units in each locus is then being calculated based on the size without flanking region. The calculated numbers of repeats of the VNTR loci (alleles) are combined into a string and given a MLVA type designation. This method can be enhanced by locus-specific DNA sequencing to recognise the mutations in the individual repeat units (van Belkum, 2007). A public database created by Institute Pasteur is used for exchange of knowledge on the geographic and temporal distribution of strain types for surveillance and epidemiology as well as evolution study (http://www.pasteur.fr/recherche/genopole/PF8/mlva/).

Recently, Olsen *et al.* (2009) had evaluated the usefulness of MLVA on *V. cholerae* isolated worldwide based on polymorphism in six VNTR loci and demonstrated a high level of polymorphism among the strains tested. This assay is potential to differentiate between various strains and identify the isolated from common outbreak. In addition, Choi *et al.* (2010) also reported the usefulness of MLVA to categorize hybrid strains from El Tor strains of seventh cholera pandemic.

2.5.4 Multilocus sequencing typing (MLST)

Multilocus sequencing typing (MLST) was developed in 1998 for bacteria characterization and allows rapid and global comparisons (Cooper and Feil, 2004). Multilocus sequence typing (MLST) exploits the unambiguous nature of nucleotide

sequence in the housekeeping genes for the characterization of microorganisms (Maiden *et al.*, 1998).

A few MLST analyses have been carried out for *V. cholerae*. For example, Salim *et al.* (2005) had reported the ability of MLST to distinguish the *V. cholerae* strains in sixth pandemic clones from the seventh pandemic *V. cholerae.* By using MLST analysis, Garg *et al.* (2003) had found 64 novel alleles distributed among O139 *V. cholerae* isolated from patients at the Infectious Diseases Hospital, Calcutta, from 1992 to 2000. Nevertheless, compared to the housekeeping genes which are more conserved, Kotetishvili *et al.* (2003) had reported the incorporation of virulence genes in the sequence analysis. MLST is an excellent tool with respect to fine-tune the genetic comparison among the *V. cholerae* of different genetic background (Chokesajjawatee *et al.*, 2008).

2.6 Colonization factors and gene expression studies

There are three major virulence-associated elements in *V. cholerae* such as toxin corregulated pili (TCP) gene cluster for colonization in *Vibrio cholerae* pathogenicity island (VPI), cholera toxin (CTX) element for cholera toxin production (Rivera *et al.*, 2001), and repeat in toxin (RTX) element which associated with cytotoxicity in Hep-2 cells (Chow *et al.*, 2001; Rivera *et al.*, 2001). The two former elements are closely linked as the transciption of genes in both elements is coordinately activated by ToxT and closely linked to the mechianism of colonization by toxigenic *V. chole*rae (Matson *et al.*, 2007).

ToxRS and TcpPH are 2 regulatory complexes which response to host environment to maximize the expression of *tox*T. When ToxT is expressed in large scale, the production of CT and TCP will also be up-regulated (Liu *et al.*, 2008). ToxR is a bitopic membrane protein that contains a cytoplasmic localized DNA binding while ToxS is also localized to the inner membrane and serve as the effector of ToxR function (Hase and Mekalanos, 1998; Matson *et al.*, 2007). TcpP acts in conjunction with ToxR in the presence of its membrane-bound effector protein TcpH. However, the transcription of *tcpP* and *tcpH* is also depends on AphA and AphB activators. Similar mechanism is employed by AphAB in activating *tcpPH* to the activation of *toxT* by ToxRS and TcpPH (Matson *et al.*, 2007). When ToxT is expressed, all virulence-associated elements are activated, including CTX and TCP gene clusters, and other accessory toxins. Hence, the strains without *tox*T gene do not produce CT and TCP and are avirulent (Reidl and Klose, 2002).

In addition to the well-defined colonization factors, the role of accessory toxins or virulence genes in colonization was documented. Olivier *et al.* (2007) reported that accessory toxins are associated with prolonged colonization in mice. Fullner *et al.* (2002) also suggested that the contribution of accessory toxin to the acute inflammatory response in a murine pulmonary model and further proved that severity of acute inflammatory responses is modulated by RTX toxin.

The biofilm formed in *V. cholerae* enable its survival in different environments. Besides biofilm formation, the pathogens must be able to regulate certain genes in coordination as moving from the environment to the host. *In-vitro* study may provide the knowledge of gene expression in the pathogens, but it does not reflect the gene expression

in a host during infection (Boyce *et al.*, 2004). Moreover, both Xu *et al.* (2003) and Faruque *et al.* (2004) had reported the significant enhancement of certain virulence genes expression in the intestinal environment.

Microarray technology has been applied to the analysis of *in-vivo* gene expression of *V. cholerae* (Merrell *et al.*, 2002; Xu *et al.*, 2003; Bina *et al.*, 2003). The validation of microarray depends on data centering and statistical procedures (Hinton *et al.*, 2004). In addition, Real-time Reverse Transcription (RT)-PCR appears to be an alternative method to estimate the expression level of genes of interest (Shelburne and Musser, 2004; Bustin, 2000). However, the utility of Real-time RT-PCR gene expression of *V. cholerae* is relatively new. Quantifying the relative changes in gene expression requires certain equations, assumptions and the testing of these assumptions prior to analysis of data. The $2^{-\Delta\Delta CT}$ method is used for quantifying the relative changes in gene expression (Livak and Schmittgen, 2001). The reliability of this method depends on the choice of housekeeping gene for normalization. The selected gene must always yield a same level in expression in all samples (Klein, 2002).

Chapter Three

Development of a Multiplex PCR assay for *Vibrio cholerae, Vibrio parahaemolyticus, Vibrio vulnificus* and *Vibrio* species differentiation

3.1 Background

Current culture and biochemical based methods for *Vibrio cholerae* (*V. cholerae*) confirmation are problematic. Thiosulfate-citrate-bile salt-sucrose (TCBS) agar and CHROMagar *Vibrio* are the main media used for confirmation of *Vibrio* species, followed by various biochemical tests to identify the presumptive colonies based on their phenotypic profiles. However, these phenotypic-based assays are laborious, and require several days to perform. Moreover, the sensitivity of the conventional standard biochemical tests is still questionable as *V. cholerae* is not only genetically close to other *Vibrio* species but also *Aeromonas* (Di Pinto *et al.*, 2005).

In parallel with the recent trend in bacterial identification through molecular approaches, various PCR-based approaches have been reported for *Vibrio* species identification. For *Vibrio* species differentiation, Nhung *et al.* (2007) had proposed the use of *dna*J gene-based PCR approach to differentiate *Vibrio* species and developed a PCR method to identify five human pathogenic *Vibrio* species based on the *dnaJ* gene. However, this particular PCR approach was only specific for pathogenic Vibrios but failed to identify non-pathogenic Vibrios. Tarr *et al.* (2007) developed a multiplex PCR approach for detection of *Vibrio* species, and differentiate *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, and *V. mimicus* based on their sequence. The authors showed that 16S rRNA

was not an ideal marker for *Vibrio* detection as it could not distinguish *Aeromonas* from other *Vibrio* species. Besides, there are some other PCR approaches for single *Vibrio* species identification or multiple detections. Due to such limitations with the current PCR detection techniques for *Vibrios*, development of an alternative PCR approach which provides more specificity for *Vibrio* species differentiation was needed.

Transhydrogenase alpha subunit (*pntA*) gene is one of the common target used in sequence-based analysis and could potentially be used for differential detection purpose. This gene encode transhydrogenase alpha subunit which couple the proton transport across the membrane to the reversible transfer of hydride ion equivalents between NAD and NADP (Bisharat *et al.*, 2005; Ansaruzzaman *et al.*, 2008; Gonzalez-Escalona *et al.*, 2008; Hazen *et al.*, 2009). On another hand, DNA gyrase subunit B (*gyrB*) is the first polypeptide of Type IIA topoisomerases which associated with ATP-binding activities (http://www.ncbi.nlm.nih.gov/sites/entrez). As there is limited horizontal transmission in *gyrB* gene (Le Roux *et al.*, 2004), hence it is a potential to be a genus-specific marker for *Vibrio* species detection.

The aim of the study was to develop an alternative PCR method for differentiation of *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* and genus-specific detection of Vibrios.

3.2 Materials and Methods

3.2.1 Bacterial strains

Nineteen *V. cholerae* were collected from Institute for Medical Research and 111 strains of *Vibrio* species including *V. parahaemolyticus* (n=64), *V. vulnificus* (n= 2), *V. alginolyticus* (n=10), *V. mimicus* (n=1) and other *Vibrio* species (n=34) were retrieved from the culture collection of Biomedical Science and Molecular Typing Laboratory, Institute of Graduates Studies, University of Malaya. Besides that, 50 non-*Vibrio* strains were also used in the study, including 30 *Aeromonas* species, three *Salmonella* species, two *Acinetobacter baumanii*, five *Escherichia coli*, two *Pseudomonas aeruginosa*, two *Klebsiella pneumoniae*, two *Staphylococcus aureus*, two *Shigella flexneri*, one *Pasteurella multocida*, and one *Listeria monocytogenes*. TCBS agar (Becton Dickson, NJ, USA) was used to check for the purity of all the *Vibrio* strains before commencement of bench work.

3.2.2 Oligonucleotide primer design for *V. cholerae, V. parahaemolyticus, V. vulnificus,* and other *Vibrio* species detection

To design primers for *pntA* gene, sequences of *pntA* gene from *V. parahaemolyticus* RIMD 2210633 (BA00032), *V. cholerae* (AE003853, CP001234, CP001236, CP001486), *V. vulnificus* (AE016796, BA000038) were retrieved from National Center for Biotechnology Information (NCBI) Genbank (http://www.ncbi.nlm.nih.gov).

To design primers for *gyrB* gene, sequences of *gyrB* were retrieved for *V. cholerae* N16961 (AE003852), *V. mimicus* ATCC 33653 (EF380259), *V. vulnificus* CMCP6 (AE076795), *V. splendidus* TNNIII7 (AJ515265), *V. splendidus* (AJ515264), *V. changasii* (AJ577820), *V. kanaloe* (AJ577821), *V. pomeroyi* (AJ577822), *V. tasmaniensis* (AJ577823), *V. gigantis* LGP37 (AJ582793), *V. crassostreae* LGP7 (AJ582799), *V. tapetis* (AM118101), *V. parahaemolyticus* ATCC 17802 (AY527390), *V. vulnificus* YJ016 (BA000037), *V. fischeri* ES III4 (CP000020), *V. harveyi* ATCC BAA 1116 (CP000789), *V. fischeri* MJ11 (CP001139), *V. cholerae* M66-2 (CP001233), *V. cholerae* O395 (CP001235), *V. cholerae* MJ1236 (CP001485), *V. fischeri* (EF380252), *V. logei* ATCC 29985 (EF380255), *V. salmonicida* ATCC 43839 (EF380256), *V. wodanis* ATCC BAA-104 (EF380257), *V. orientalis* ATCC 33934 (EF380258), *V. splendidus* ATCC 33125 (EF380261), *V. alginolyticus* YJ0666 (EF542801), *V. rotiferianus* LMG21460 (EU112810), *V. splendidus* LGP32 (FM954972) and *V. moditerranei* ATCC 43341 (EF380258).

The two sets of sequences for *pntA* and *gyrB* were then aligned using CHROMAS PRO (Technelysium Pty Ltd, Australia). The regions which potentially differentiate individual species were selected for *pntA* gene while the conserved region for *gyrB* was selected from the alignments. Primers were designed using the Primer 3 software (Rozen and Skaletsky, 2000) and *in-silico* PCR (http://insilico.ehu.es/PCR/Amplify) was carried out prior to PCR evaluation in the laboratory.

3.2.3 DNA template preparation

A loopful of bacterial colonies was picked and resuspended in 100 µl of ddH_2O. The DNA suspension was boiled at 99°C for 5 min and snapped cold on ice for 10 min. The cell lysate was then briefly centrifuged at high speed and the concentration was quantified (A_{260}) by using a Biophotometer (Eppendorf, Milan, Italy). Approximately 3 µl (≈50 ng) of DNA were used for PCR reaction.

3.2.4 Monoplex PCR for detection of *V. cholerae, V. parahaemolyticus, V. vulnificus,* and other *Vibrio* species

All the strains described in **Section 3.2.1** were used for evaluation of the specificity of the designed primers in monoplex and multiplex PCR. All the reagents for PCR master mixture preparation were removed from -20°C and thawed on ice. The reagents were vortexed and centrifuged briefly before used. Monoplex PCR was carried out for each pair of primers in a single tube containing 1x PCR buffer, 1 mM $MgCl_2$, 40 µM each dNTPs, 0.3 µM each primers, ~50 ng DNA template and 0.5 U of *Taq* DNA polymerase (Promega, Madison, USA). The PCR mixture was vortexed and centrifuged briefly. A DNA blank without DNA template was included in each PCR reaction. The PCR cycling condition consisted of 95°C for 1 min (pre-denaturation), followed by 30 cycles of 94°C for 30 sec (denaturation), 55°C for 30 sec (annealing) 72°C for 45 sec (extension) and a final extension at 72°C for 8 min.

3.2.5 Agarose gel electrophoresis

LE analytical agarose was added in 0.5 X TBE buffer to a final concentration of 2.0% (w/v). The mixture was heated in a microwave for 1-2 min until the agarose was fully dissolved in 0.5 X TBE buffer. The mixture was cooled to around 50°C before pouring into a gel casting tray. The solidified gel was placed in a horizontal gel tank and 0.5 X TBE was added until the gel was completely covered. Five µl of PCR product (~500 ng) was mixed with 2 µl of 6 X loading dye (Promega, Madison, USA) and loaded into the wells. Five µl of 100 bp DNA marker (1:10 dilution) was used as DNA molecular weight standards in each electrophoresis. The electrophoresis was carried out at 100 V for 45 min.

The gel was stained with ethidium bromide (0.5 µg/ml) and destained in distilled water for 10 min. The gel image was captured and analyzed with Gel documentation system (Bio-rad, Hercules, CA).

3.2.6 DNA extraction from agarose gel

QIA quick Gel Extraction Kit (Qiagen, Venlo, Netherlands) was used to extract DNA from agarose gel according to the manufacturer's instructions. Firstly, DNA fragment was excised from the agarose gel and weighed. Three volumes of buffer QG was added into one volume of gel (100 mg: 100 µl). The sample was incubated at 50°C in a water bath for 10 min or until the gel slice was completely dissolved. The sample was vortexed every 2-3 min during the incubation to dissolve the gel. When the gel was

completely dissolved, the yellow colour of the mixture was checked in order to determine the optimal pH for DNA binding in gel extraction.

Then, one gel volume of isopropanol was added into the sample. The sample was applied to the QIAquick spin column which was placed in a 2 ml collection tube. The sample was centrifuged for 1 min at 13,400 rpm (14110 x g) and the flow-through was discarded. The QIAquick spin column was placed back in the same collection tube and 500 µl of QG buffer was added into the column. The sample was centrifuged for another minute at 13.400 rpm. The flow-through was discarded and 750 µl PE buffer was added into the spin column. The sample was centrifuged for 1 min at 13,400 rpm and the flow-through was discarded after the centrifugation. The sample was centrifuged for an additional 1 min to completely remove the residual ethanol in PE buffer.

Finally, the QIAquick spin column was placed into a 1.5 ml microcentrifuge tube and 30 µl of ddH_2O was added to the centre of the QIAquick membrane for elution of DNA. The column was allowed to stand for 1 min and the DNA was collected by centrifugation for 1 min. The extracted DNA was stored at -20°C.

3.2.7 Confirmation of sequences amplified

The extracted DNA was sent to a laboratory (1^{st} base, Seri kembangan, Malaysia) for sequencing together with the forward and reverse PCR primers. All sets of forward and reverse sequencing results were aligned and analyzed using Bioedit Sequence Alignment software. Standard nucleotide-nucleotide BLAST search program

(http://www.ncbi.nih.gov/BLAST) was used to evaluate the sequence with published sequence in GenBank.

3.2.8 Optimization of multiplex PCR for differentiation of *V. cholerae, V. parahaemolyticus, V. vulnificus,* and other *Vibrio* species

All the four pairs of primers were combined and re-tested in a multiplex PCR format. Various conditions such as annealing temperatures and concentration of primers were modified to obtain specific, unique amplicons. Once the multiplex PCR conditions were optimized, the assay was evaluated with all the strains.

3.2.9 Sensitivity test of the multiplex PCR for differentiation of *Vibrio cholerae, Vibrio parahaemolyticus, Vibrio vulnificus* and *Vibrio* species

The procedure previously described by Teh *et al.* (2008) was adapted for the sensitivity test with minor modification. The sensitivity of the *pntA* primers was evaluated with *V. cholerae*, *V. parahemolytic*us, and *V. vulnificus* while the sensitivity of *gyrB* primer was evaluated with *V. mimicus.* Briefly, cell culture of each *Vibrio* species (OD_{600}= 0.5) was serially diluted 10-fold. Crude DNA was prepared by direct boiling of 100 µl of each dilution. An aliquot of 100 µl from each dilution was plated on an LB agar plate and incubated overnight at 37°C in order to determine the cell density. The multiplex PCR was carried out under optimized condition described in **Section 3.2.8**.

3.2.10 Specifity of *gyrB* compared with *dnaJ* for *Vibrio* species detection

PCR using *dnaJ* primers as described by Nhung *et al.* (2007) was performed using the same sets of 130 *Vibrio* species and 50 non-*Vibrio* strains to compare the specificity of *gyrB* primers that developed in this study. The conditions for PCR targeting *dnaJ* was based on Nhung *et al.* (2007) with minor modification. In brief, the PCR reaction was carried out in a total volume of 25 µl containing 1x PCR buffer, 1.5 mM $MgCl_2$, 50 µM each dNTPs, 0.4 µM each primers, ~50 ng DNA template and 0.5 U of *Taq* DNA polymerase (Promega, Madison, USA). The PCR cycling condition consisted of 95°C for 1 min (pre-denaturation), followed by 30 cycles of 94°C for 1 min (denaturation), 56°C for 30s (annealing) 72°C for 1 min (extension) and a final extension at 72°C for 8 min. The PCR products were then electrophoresed on 2% LE agarose gel for 2 hours at 100V. The gel was finally stained with ethidium bromide (0.5 µg/ml) and visualized under UV.

3.3 Results

3.3.1 Oligonucleotide Design

Primer choice was constrained by the intention to use all four primer pairs in a multiplex PCR assay that generated amplicons of differing sizes based on the sequence alignment of *gyrB* and *pntA* targeting different species of Vibrios. Candidate primers were then checked by BLASTN against the available *Vibrio* genome sequences to minimize the likelihood of non-specific amplification from non-target loci. The oligonucleotides designed and the expected sizes of amplicons are summarized in **Table 3.1**. Overall, *in-*

silico PCR showed 100% specificity for all the self-designed oligonucleotides. The primers were further investigated for its specificity in actual laboratory experiments.

Table 3.1: Oligonucleotide primers designed and used for differentiation of *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, and other *Vibrio* species.

Oligonucleotide primer	Sequence	Target	Amplicon size
pntA 1C	5’-CAGTAAAGAAACGACCAAACTC-3’	*Vibrio cholerae*	338 bp
*pntA*2 C	5’-TGCCAGTTTCGATGATGCCG-3’		
pntA 1P	5’-AGCAAGTTTCGATGATGCTG-3’	*Vibrio parahaemolyticus*	409 bp
pntA 2P	5’-ACCAGCAACCAAAACTTTCGCT-3’		
pntA 1V	5’-CTGTAACAAGGCACCGACAA-3’	*Vibrio vulnificus*	656 bp
pntA 2V	5’-TCACAACCGCACTGATTCCAG-3’		
gyrB 1	5'-AGCCAAACNAAAGAYAARYT-3'	*Vibrio* species	493 bp
gyrB 2	5'-CGYARYTTRTCYGGRTTRTRYTC-3'		

3.3.2 Monoplex PCR for detection of *V. cholerae, V. parahaemolyticus, V. vulnificus,* and other *Vibrio* species

Monoplex PCR was first carried out for all 4 pairs of primers in a single tube as described as **Section 3.2.4.** PCR targeting *gyrB* for *Vibrio* species and *pntA* for *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus* worked well as shown in **Figure 3.1**.

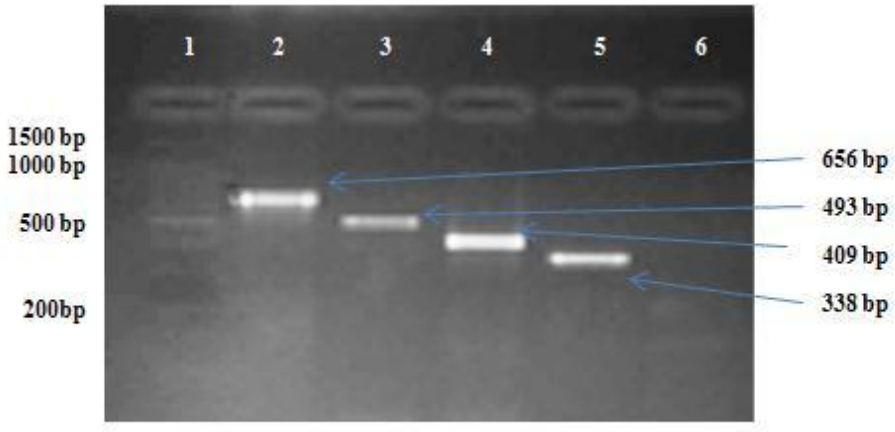


Figure 3.1 Monoplex PCR targeting *gyrB* for *Vibrio* species and *pntA* for *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus*, respectively. Lane 1: 100 bp marker; Lane 2: *V. vulnificus* (656 bp band specific for *V. vulnificus*); Lane 3: *V. parahaemolyticus* (493 bp band specific for *Vibrio* species); Lane 4: *V. parahaemolyticus* (409 bp band specific for *V. parahaemolyticus*); Lane 5: *V. cholerae* (338 bp band specific for *V. cholerae*); Lane 6: DNA blank (ddH_2O).

3.3.3 Sequence Analysis

PCR products of 493 bp (*Vibrio* species), 409 bp (*V. parahaemolyticus*), 338 bp (*V. cholerae*) and 656 bp (*V. vulnificus*) were purified and sequenced to verify the content of the fragments. The sequences obtained were evaluated with the published sequences in GenBank by using standard BLAST search option. As expected, the sequences for all amplicons were confirmed to be the desired fragments belonging to the *gyrB* and *pntA* genes of *Vibrio* species

3.3.4 Optimization of a Multiplex PCR for differentiation of *V. cholerae, V. parahaemolyticus, V. vulnificus*, and other *Vibrio* species

The problem of non-specific bindings was encountered in the first multiplex optimization. The non-specific amplicon was approximately 350 bp and the intensity of the band for *V. vulnificus* strains tested was high (**Figure 3.2**).

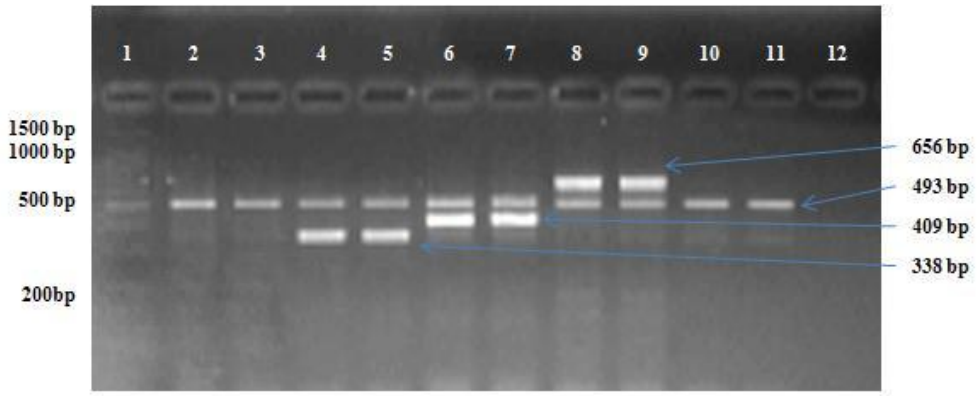


Figure 3.2 First optimization of multiplex PCR for differential detection of *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus* and *Vibrio* species. Each reaction containing 1 X buffer, 2.5 mM $MgCl_2$, 200 µM of each dNTP, 2.5 U *Taq* DNA

Polymerase (Promega, Madison, USA), 0.4 µM of each primer, 3 µl (~50 ng) of DNA template and appropriate ddH_2O with 5 min of initial denaturation at 95 °C, followed by 30 cycles of denaturation, annealing, and extenion for 94°C (30 sec), 57°C (30 sec) and 72.0°C (1 min), respectively and a final extension at 72.0°C for 5 min. All targeted bands were present but non-specific bands were detected. Lane 1: 100 bp marker ; Lane 2: DNA blank (ddH_2O) ; Lane 3-4: *V. parahaemolyticus*; Lane 5-6: *V. vulnificus*; Lane 7-8: *V. cholerae*; Lane 9: *Vibrio* species

To overcome this problem, various approaches were taken. One of them was to increase the annealing temperature to 58℃. The concentration of $MgCl_2$, dNTP, and each primer was also decreased to 2 mM, 160 µM and 0.3 µM, respectively. The result for the second optimization of multiplex PCR is shown in **Figure 3.3**.

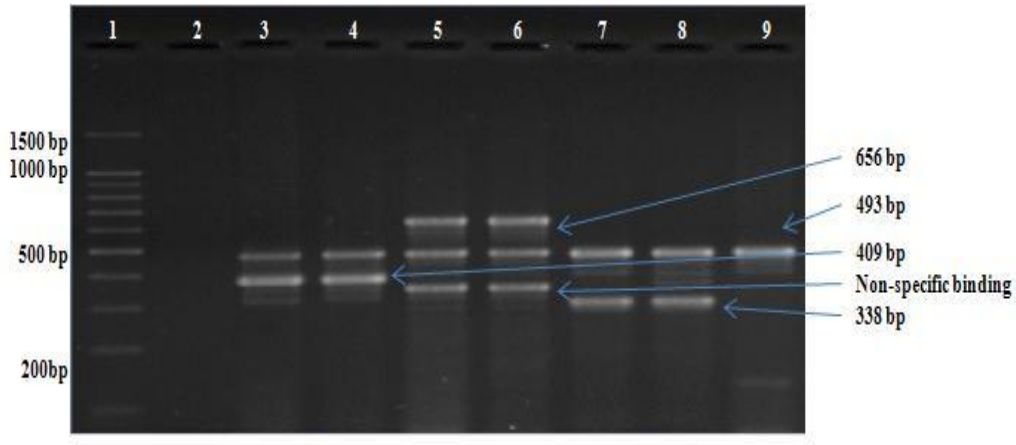


Figure 3.3 Second optimization of multiplex PCR for differential detection of *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus* and *Vibrio* species. Each reaction contained 1 X buffer, 2.0 mM $MgCl_2$, 160 µM of each dNTP, 2.5 U *Taq* DNA Polymerase (Promega, Madison, USA), 0.3 µM of each primer, 3 µl (~50 ng) of DNA template and appropriate ddH_2O with 5 min of initial denaturation at 95°C, followed by 30 cycles of denaturation, annealing, and extension for 94°C (30 sec), 58°C (30 sec) and 72.0°C (1 min), respectively and a final extension at 72.0°C for 5 min. All targeted bands were present. Non-specific bands were still detected but the intensity of non-specific bands for *V. vulnificus* (lanes 5-6) at ~350 bp was decreased. Lanes 1 and 10: 100 bp marker; Lane 2: *Vibrio* species; Lane 3-4: *V. cholerae*; Lane 5-6: *V. vulnificus*; Lane 7-8: *V. parahaemolyticus*; Lane 9: DNA blank (ddH_2O)

To remove the non-specific bindings, the concentration of *Taq* DNA Polymerase was decreased to 2.0 U/ml while the annealing temperature was increased to 59℃. The result for the third optimization of multiplex PCR is shown in **Figure 3.4**.

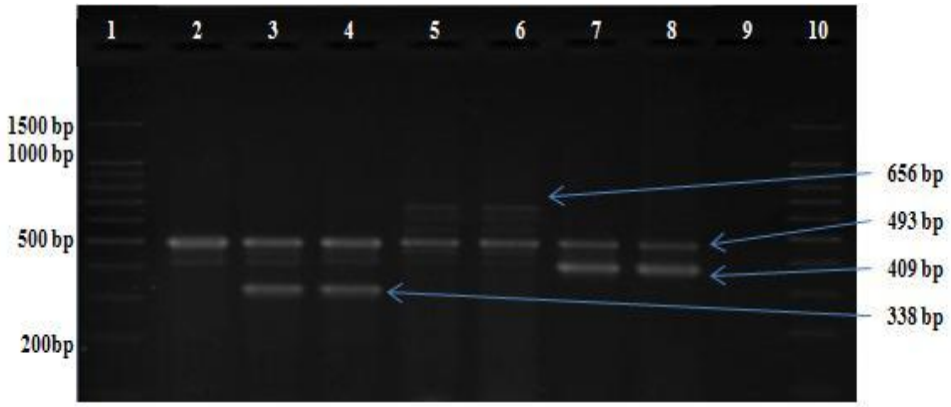


Figure 3.4 Third optimization of multiplex PCR for differential detection of *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus* and *Vibrio* species. Each reaction contained 1 X buffer, 2.0 mM $MgCl_2$, 160 µM of each dNTP, 2.0 U *Taq* DNA Polymerase (Promega, Madison, USA), 0.3 µM of each primer, 3 µl (~50 ng) of DNA template and appropriate ddH_2O with 5 min of initial denaturation at 95°C, followed by 30 cycles of denaturation, annealing, and extension for 94°C (30 sec), 59°C (30 sec) and 72.0 °C (1 min), respectively and a final extension at 72.0°C for 5 min. All targeted bands were present. The 656 bp band for *V. vulnificus* was faint. Most of the non-specific bands were eliminated. Lanes 1 and 10: 100 bp marker; Lane 2-3: *V. parahaemolyticus*; Lane 4-5: *V. cholerae*; Lane 6-7: *V. vulnificus*; Lane 8: *Vibrio* species; Lane 9: DNA blank (ddH_2O).

To increase the intensity of 656 bp band, the concentration of dNTP was increase to 200 µM. The result for the forth optimization of multiplex PCR is shown in **Figure 3.5**.

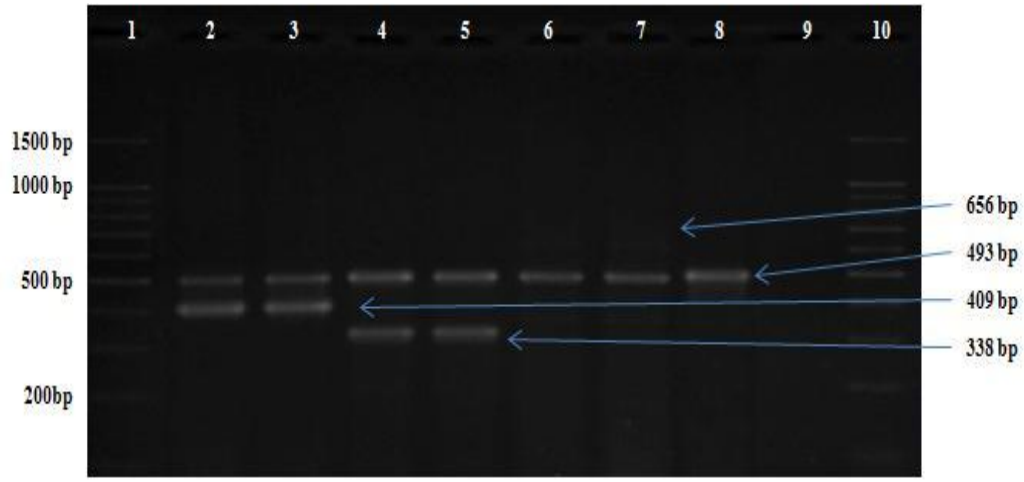


Figure 3.5 Fourth optimization of multiplex PCR for differential detection of *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus* and *Vibrio* species. Each reaction contained 1 X buffer, 2.0 mM $MgCl_2$, 200 µM of each dNTP, 2.0 U *Taq* DNA Polymerase (Promega, Madison, USA), 0.3 µM of each primer, 3 µl (~50 ng) of DNA template and appropriate ddH_2O with 5 min of initial denaturation at 95°C, followed by 30 cycles of denaturation, annealing, and extension for 94°C (30 sec), 59°C (30 sec) and 72.0°C (1 min), respectively and a final extension at 72.0°C for 5 min. All targeted bands were present. Lane 1: 100 bp marker; Lane 2-3: *Vibrio* species; Lane 4-5: *V. cholerae*; Lane 6-7: *V. parahaemolyticus*; Lane 8-9: *V. vulnificus*; Lane 10-11: *Vibrio* species; Lane 12: DNA blank (ddH_2O).

Finally, the multiplex PCR was optimized. The optimized multiplex PCR conditions for the simultaneous detection of *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus* and *Vibrio* species comprised of an initial denaturation of 95°C for 3 min, followed by 30 cycles of 94°C for 30 sec (denaturation), 59°C for 30 sec (annealing) and 72.0°C for 1 min (extension), and a final extension at 72.0°C for 5 min. One

reaction of this multiplex PCR contained 1X buffer, 2.0 mM $MgCl_2$, 200 μM of each dNTP, 2.0 U *Taq* DNA polymerase (Promega, Madison, USA), 0.3 μM of each primer and 3μl of DNA (~ 50 ng) .

The optimized multiplex PCR was applied on 180 strains. All *Vibrio* species gave an amplicon size of 493 bp with the *gyrB* primers. *pntA* primers amplified *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* at 338 bp, 409 bp and 656 bp, respectively. The multiplex-PCR targeting both *gyrB* and *pntA* genes successfully identified the 130 *Vibrio* strains (100% detection) and differentiated the strains into *V. cholerae* (493/338 bp), *V. parahaemolyticus* (493/409 bp), *V. vulnificus* (493/656 bp), and *Vibrio* species (493 bp). All 30 *Aeromonas* species were not amplified. However, one *Salmonella* strain and one *E. coli* strain was also amplified with the *gyrB* primer.

3.3.5 Sensitivity of the multiplex PCR

The sensitivity of the *pntA* primers evaluated with *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* was found to be 2 x 10^3 cfu/mL (10 cfu per PCR), 2 x 10^3 cfu/mL (10 cfu per PCR), and 1 x 10^4 cfu/mL (50 cfu per PCR), respectively. The sensitivity of *gyrB* primers evaluated with *V. mimicus* was 4 x 10^4 cfu/mL (200 cfu per PCR).

3.3.4 Comparison of the specificity of *gyrB* and *dnaJ* primers

PCR using primers targeting *dnaJ* gene failed to identify five *Vibrio* strains among the 180 strains in this study and showed positive amplification for one

Aeromonas species, one *E. coli*, two *Salmonella* species and one *Klebsiella pneumoniae*. The *gyrB* primers were 100% specific as it amplified all *Vibrio* strains. However, it also amplified one *Salmonella* species and one *E. coli* strain. The amplicons for these two strains were sequenced and confirmed as part of *gyrB* gene. This may be due to the gene transfer or mutation within the family of *Enterobacteriacea*. The overall results showed that the *gyrB* primers were more specific than *dnaJ* in *Vibrio* species detection. The comparison results of both PCR methods are summarized in **Table 3.2**.

Table 3.2: Comparison results between multiplex PCR based on the *gyrB* and *pntA* genes with the *dnaJ*-based PCR on *Vibrio* species and other bacteria strains

Strains	No. of strains (n)	PCR developed in this study (n)				*dnaJ*-based PCR*
		338 bp (*pntA*)	409 bp (*pntA*)	656 bp (*pntA*)	493 bp (*gyrB*)	559 bp
V. cholerae	19	+ (19)	-	-	+ (19)	+ (19)
V. parahaemolyticus	2	-	+ (64)	-	+ (64)	+ (64)
V. vulnificus	2	-	-	+ (2)	+ (2)	+ (2)
V. alginolyticus	10	-	-	-	+ (10)	+ (9)
V. mimicus	1	-	-	-	+ (1)	+ (1)
Other *Vibrio* species	34	-	-	-	+ (34)	+ (30)
Aeromonas species	30	-	-	-	-	+ (1)
Escherichia coli	5	-	-	-	+ (1)	+ (1)
Salmonella species	3	-	-	-	+ (1)	+ (2)

Acinetobacter baumanii	2	-	-	-	-	-
Pseudomonas aerugenosa	2	-	-	-	-	-
Klebsiella pneumoniae	2	-	-	-	-	+(1)
Staphylococcus aureus	2	-	-	-	-	-
Shigella flexneri	2	-	-	-	-	-
Pasteurella multocida	1	-	-	-	-	-
Listeria monocytogenes	1	-	-	-	-	-

* Nhung *et al.* (2007); '+' positive; '-' negative

3.4 Discussion

The multiplex PCR approach developed in this study provides an alternative attempt for wider *Vibrio* species detection including human pathogenic Vibrios such as *V. cholerae*, *V. parahaemolyticus, V. vulnificus*, other predominantly non-pathogenic *Vibrio* species. Collagenase-targeted PCR for *V. alginolyticus*, *V. cholerae* and *V. parahemolyticus* developed by Di Pinto *et al.* (2005) yielded high specificity. However, that PCR assay did not include *V. vulnificus*, an organism of public health concern, and many other *Vibrio* species. The PCR developed by Nhung *et al.* (2007) was specific for pathogenic Vibrios only and could not differentiate non-pathogenic Vibrios including some non-O1/non-O139 serogroup *V. cholerae.* In this study, *pntA* gene was used as a target for *V. parahaemolyticus, V. cholerae* and *V.vulnificus* differentiation and the usefulness was demonstrated.

Many genes such as 16S rRNA, *rpoB, dnaJ* and other housekeeping genes have been used for the detection and phylogenetic analysis of Vibrios. Tarr *et al.* (2007) reported that 16S rRNA is not able to distinguish *Aeromonas* from *Vibrio* species. The *rpoB* gene could differentiate *Vibrio* strains up to species level but require further DNA sequencing of the amplicon. This additional step nullifies the objective of shortening the detection period. In this study, the usefulness of *gyrB* and *dnaJ* gene for *Vibrio* species detection was focused and compared. Overall, the primers targeting *gyrB* gene as evaluated in this study were more specific as they correctly identified all 250 *Vibrio* strains as compared to *dnaJ* primers which fail to detect five *Vibrio* strains.

It is difficult to differentiate *Aeromonas* from Vibrios based on conventional culture method using selective media such as TCBS or CHROMagar *Vibrio*. This is

because *Aeromonas* species can grow on the same selective media and share high similarity in genetics, colony morphology and chemical reaction with Vibrios (Di Pinto *et al.*, 2005). Nonetheless, molecular detection prior to isolation of Vibrios using selective media would help to reduce the misidentification of non-*Vibrio* species such as *E. coli* and *Salmonella* species (except for *Aeromonas* species).

The multiplex PCR developed in this study could distinguish *V. cholerae* from *V. mimicus* and *V. parahaemolyticus* from *V. alginolyticus. V. mimicus* is always misidentified as *V. cholerae* as both are genetically similar. *V. mimicus* is also phenotipically similar to *V. cholerae* especially the atypical non-O1 *V. cholerae* isolates which unable to utilize sucrose (Thompson *et al.*, 2005). Moreover, *V. parahaemolyticus* is also frequently misinterpreted as *V. alginolyticus* due to the high similar genetic content (Tracz *et al.*, 2007). In this study, the multiplex PCR assay successfully identified 19 *V. cholerae* 46 and *V. parahaemolyticus* while *V. mimicus* and *V. alginolyticus* were identified as *Vibrio* species. This shows the versatility of PCR test to distinguish as both species are genetically similar.

In conclusion, the multiplex PCR developed in this study was able to differentiate *Aeromonas* species from *Vibrio* species and provides a simultaneous differential detection of different *Vibrio* species in a single reaction.

Chapter Four

Comparison of PCR, Biochemical Tests and API 20E for Detection of Aquatic *Vibrio cholerae* in Malaysia

4.1 Background

Colour-based identification of the *Vibrio* species on the selective agar is sometimes inconsistent and dependent on further confirmation using various biochemical tests (Elliot *et al.*, 2001; Kaysner and Depaola, 2004). However, these biochemical tests are tedious and sometimes unreliable. As an alternative method, API 20E was used to identify *Enterobactereaceae* and was reported as the most accurate and efficient system to identify commonly isolated species of *Vibrionaceae* (Overman *et al.* 1985).

In order to hasten and improve the identification procedures, several molecular approaches for identification of *Vibrio* species have been proposed recently. A multiplex PCR approach for differentiation of *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus* and *Vibrio* species based on *pntA* and *gyrB* genes was developed as described in **Chapter 3**. This multiplex PCR could specifically differentiate 19 *V. cholerae* from other strains tested, hence could be useful for *V. cholerae* detection.

The objective of this chapter was to further evaluate the usefulness of the *pntA*/*gyrB*-based multiplex PCR approach for the detection of *V. cholerae* compared to API 20E and conventional biochemical tests.

4.2 Materials and Methods

4.2.1 Samples collection and *V. cholerae* strains isolation

A total of 100 water samples were collected from rivers, coasts, drains, and ponds in the state of Selangor, Malaysia for a period of three months from September to December 2008. During the same period of time, 15 algae samples were also collected.

Isolation of *V. cholerae* was carried out by using modified method of Keymer *et al.* (2007) and Ottaviani *et al.* (2009). 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 algae samples, the homogenate was directly enriched in 10ml of APW. A loopful of the overnight APW grown culture was streaked on thiosulfate citrate-bile salts-sucrose (TCBS) agar (Becton Dickinson, NJ, USA) and incubated overnight at 37°C. The presumptive 2-4 mm diameter yellow sucrose fermenting colonies on TCBS (Becton Dickinson, NJ, USA) were subcultured on non-selective agar for further conventional biochemical analysis, API 20E (Biomerieux, SA, France) and PCR analysis.

4.2.2 Biochemical tests

Based on the study carried out by Choopun *et al.* (2002) which described 13 difererent biochemical tests for identification of *V. cholerae* and the guideline of isolation from CDC (2006), five tests were selected and used for identification of *V.*

cholerae. These tests included salt tolerance, oxidase test, string test, triple sugar iron (TSI) test and arginine dihydrolase test.

Firstly, the presumptive *V. cholerae* colonies were subjected to the tolerance of 0%, 3%, 6%, and 10% NaCl in triptone broth. The inoculated broth was incubated at 37°C for up to 7 days. Interpretation was based on the turbidity of the broth. Next, oxidase test was carried out. For this purpose, two drops of oxidase reagent were allowed to absorb on a moistened filter paper. Single colony of culture was picked and rubbed onto the moistened filter paper. The change of colour due to the reaction was recorded. Then, string test was performed. Overnight growth of bacteria was suspended in a drop of 0.5% aqueous solution of sodium deoxycholate. Any formation of mucoid 'string' was recorded.

Triple Sugar Iron (TSI) tests were carried out to detect the acid and gas production of the isolates. The inoculation was done by using stab-and-streak technique. The slant agar (with supplement of 3 % NaCl) was incubated at 37°C for 24 hours. The changes of colours of the slant and butt were recorded. Finally, arginine dihydrolase test was performed. LB broth containing 1% (wt/vol) L-arginine (pH6.8) with phenol red as indicator was used. After inoculation, the medium was covered with sterile mineral oil and incubated for up to seven days. The presence of red color in the medium indicates positive reaction.

4.2.3 API 20E identification

API 20E (Biomerieux, SA, France) identification test was carried out according to the manufacturer's instruction. Briefly, a fresh single colony of bacterial culture on

non-selective agar plate was inoculated in 5 ml of 0.85% NaCl. The suspension was distributed into the tube of the strip and fill both strip and cupule of CIT (citrate utilization), VP (acetoin production), and GEL (GELatinase). The tube for ADH (argine DiHydrolase), LDC (Lysine DeCarboxylase), ODC (Ornithine DeCarboxylase), H_2S (H_2S Production) and URE (UREase) were created for anaerobiosis by overlaying with mineral oil after filling of the bacterial suspension. The strip was placed in the incubation box which filled with ddH_2O and incubated at 37°C for 24 h. The next day, one drop of TDA reagent, JAMES reagent, and VP1/VP2 was added in TDA, IND and VP, respectively. The results of all reactions in a strip was recorded and analyzed with apiweb^{TM} identification software (Biomerieux, SA, France). Based on the manufacturer's instruction, identity of less than 80% homology is not acceptable.

4.2.4 DNA extraction and PCR differentiation of *V. cholerae* from other *Vibrio* species

A loopful of bacterial culture obtained from a LB agar plate with 3% NaCl was suspended in 100 µl ddH_2O. The suspension was boiled at 99°C for 5 min, snapped cool on ice, and centrifuged at high speed for 2 min as described in **Section 3.2.3**. PCR targeting *pntA* and *gyrB* genes for *V. paraharmolyticus*, *V. cholerae*, *V. vulnificus* and *Vibrio* species detection was carried out as described in **Chapter 3**.

4.2.5 The evaluation of effectiveness of biochemical tests, API 20E and *pntA*/*gyrB*-based multiplex PCR

ompW-based PCR was developed in 2000 and showed high specificity in *V. cholerae* identification (Nandi *et al.*, 2000). Hence this PCR assay was performed on the 37 isolates and used as the standard for sensitivity and specificity calculations of biochemical test, API 20E (Biomerieux, SA, France) and *pntA/gyrB*-based multiplex PCR for *V. cholerae* detection. The multiplex PCR was carried out in a total volume of 25 µl containing 1x PCR buffer, 1 mM $MgCl_2$, 40 µM each dNTPs, 0.3 µM each primers, ~50 ng DNA template and 0.5 U of *Taq* DNA polymerase (Promega, Madison, USA). The PCR cycling condition consisted of 95 °C for 1 min (pre-denaturation), followed by 30 cycles of 94 °C for 30 sec (denaturation), 55 °C for 30 sec (annealing) 72°C for 45 sec (extension) and a final extension at 72°C for 8 min. The PCR products were then electrophoresed on 2% LE agarose gel for two hours at 100V. The gel was finally stained with ethidium bromide (0.5 µg/ml) and visualized under UV.

Sensitivity and specificity for each method was calculated based on Altman and Bland (1994) with the formula below:

$$\text{specificity} = \frac{\text{number of true negative}}{\text{number of true negative + number of false positive}}$$

$$\text{sensitivity} = \frac{\text{number of true positive}}{\text{number of true positive + number of false positive}}$$

4.3 Results

Out of 100 analyzed water samples, only 27% showed presumptive *V. cholerae* on the selective TCBS agar (Becton Dickinson, NJ, USA) as indicated by the morphology of the colony which was yellow, flat and approximately 2-3 mm diameter. Twenty-nine yellow colonies and four green colonies (flat and 2-3 mm diameter) were picked and subjected to conventional biochemical tests, API 20E (Biomerieux, SA, France), and PCR confirmations (*ompW* and *pntA/gyrB*-based).

Out of 15 algae sample, only three were positive for presumptive *V. cholerae* based on the morphology on the selective TCBS agar (Becton Dickinson, NJ, USA). Four yellow colonies which were flat and have ~2 mm diameter were picked for further screening.

4.3.1 Biochemical Tests

The interpretation of biochemical tests results was based on Elliot *et al.* (2001) and Choopun *et al.* (2002). Seventeen isolates were able to grow without NaCl while all the isolates were able to tolerate 3%-6% NaCl. Only seven isolates could tolerate 10% NaCl.

For the string test, one isolate was negative and two showed fairly weak reaction to the string test. All isolates were positive for the oxidase test. For TSI test, one isolate produced alkaline slant and alkaline butt (K/K), nine produced alkaline slant and acid butt (K/A) and 27 produced acid slant and acid butt (A/A).

Among the 37 isolates which subjected to the arginine dehydrolase test, six were observed with colour change in the medium after one day of incubation. Five isolates showed colour change in the medium after two days of incubation. There were 26 isolates which did not show colour change in the medium after seven days of incubation.

The biochemical tests results for the 37 isolates are summarized in **Table 4.1**. Based on combined biochemical tests, only 17 isolates were identified as *V. cholerae.*

Table 4.1: Biochemical characteristic of the 37 presumptive *V. cholerae* isolates from water and algae samples.

No.	Code for Isolates	Morphology on TCBS agar	Biochemical tests														Interpretation
			Tolerance to NaCl				Oxidase test	String test	TSI test	Arginine dihyrolase test							
			0 %	3 %	6 %	10 %				Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	
1	Vib 1/08w	Yellow, flat, 2-3 mm diameter	+	+	-	-	+	+	A/A	-	-	-	-	-	-	-	*V. cholerae*
2	Vib 3/08w	Yellow, flat, 2-3 mm diameter	+	+	-		+	+	A/A	-	-	-	-	-	-	-	*V. cholerae*
3	Vib 9/08w	Yellow, flat, 2-3 mm diameter	-	+	+	-	+	+	A/A	+							*Vibrio* species
4	Vib 10/08w	Yellow, flat, 2-3 mm diameter	-	+	+	+	+	+	A/A	-	-	-	-	-	-	-	*V. alginolyticus*
5	Vib 17/08w	Yellow, flat, 2-3 mm diameter	+	+	-	-	+	+	A/A	-	-	-	-	-	-	-	*V. cholerae*
6	Vib 77/08w	Yellow, flat, 2-3 mm diameter	+	+	-	-	+	+	A/A	-	-	-	-	-	-	-	*V. cholerae*
7	Vib 199/08w	Yellow, flat, 2-3 mm diameter	-	+	+	+	+	+	A/A	-	-	-	-	-	-	-	*V. alginolyticus*

8	Vib365 /08w	Yellow, 2-3 diameter	flat, mm	-	+	+	-	+	+	K/A	+								*Vibrio* species
9	Vib 370/08w	Yellow, 2-3 diameter	flat, mm	-	+	+	+	+	+	A/A	-	-	-	-	-	-	-	-	*V. alginolyticus*
10	Vib 374/08w	Yellow, 2-3 diameter	flat, mm	+	+	-	-	+	+	A/A	-	-	-	-	-	-	-	-	*V. cholerae*
11	Vib1114/08 w	Yellow, 2-3 diameter	flat, mm	-	+	-	-	+	+	K/A	-	+							*Vibrio* species
12	Vib 3034/08w	Yellow, 2-3 diameter	flat, mm	-	+	-	-	+	+ (weak)	K/K	+								*Vibrio* species or *Aeromonas hydrophila*
13	Vib 3477/08w	Yellow, 2-3 diameter	flat, mm	+	+	-	-	+	+	A/A	-	-	-	-	-	-	-	-	*V. cholerae*
14	Vib 3577/08w	Yellow, 2-3 diameter	flat, mm	-	+	+	-	+	+	K/A	-	-	-	-	-	-	-	-	*V. parahaemolyticus*
15	Vib 4130/08w	Yellow, 2-3 diameter	flat, mm	-	+	+	-	+	+ (weak)	K/A	-	+							*Vibrio* species
16	Vib 4365/08w	Yellow, 2-3 diameter	flat, mm	-	+	-	-	+	+	K/A	-	+							*Vibrio* species
17	Vib 4370/08w	Yellow, 2-3 diameter	flat, mm	+	+	-	-	+	+	A/A	-	-	-	-	-	-	-	-	*V. cholerae*

18	Vib 4372/08w	Yellow, 2-3 diameter	flat, mm	-	+	+	+	+	+	A/A	-	-	-	-	-	-	-	*V. alginolyticus*
19	Vib 4784/08w	Yellow, 2-3 diameter	flat, mm	-	+	+	-	+	+	A/A	+							*Vibrio* species
20	Vib 4888/08w	Yellow, 2-3 diameter	flat, mm	-	+	+	+	+	+	A/A	-	-	-	-	-	-	-	*V. alginolyticus*
21	Vib 4900/08w	Yellow, 2-3 diameter	flat, mm	-	+	+	-	+	+	K/A	+							*Vibrio* species
22	Vib 4933/08w	Yellow, 2-3 diameter	flat, mm	+	+	-	-	+	+	A/A	-	-	-	-	-	-	-	*V. cholerae*
23	Vib 2P/08w	Yellow, 2-3 diameter	flat, mm	-	+	+	+	+	+	A/A	-	-	-	-	-	-	-	*V. alginolyticus*
24	Vib 3P/08w	Yellow, 2-3 diameter	flat, mm	+	+	-	-	+	+	A/A	-	-	-	-	-	-	-	*V. cholerae*
25	Vib B2/08w	Yellow, 2-3 diameter	flat, mm	+	+	-	-	+	+	A/A	-	-	-	-	-	-	-	*V. cholerae*
26	Vib B4/08w	Yellow, 2-3 diameter	flat, mm	+	+	-	-	+	+	A/A	-	-	-	-	-	-	-	*V. cholerae*
27	Vib H1/08w	Yellow, 2-3 diameter	flat, mm	-	+	+	-	+	+	A/A	-	+						*Vibrio* species

28	Vib M1/08w	Yellow, 2-3 diameter	flat, mm	+	+	-	-	+	+	A/A	-	-	-	-	-	-	-	*V. cholerae*
29	Vib M2/08w	Yellow, 2-3 diameter	flat, mm	+	+	-	-	+	+	A/A	-	-	-	-	-	-	-	*V. cholerae*
30	Vib GB/08w	Green, 2-3 diameter	flat, mm	+	+	-	-	+	+	A/A	-	-	-	-	-	-	-	*V. cholerae*
31	Vib GH/08w	Green, 2-3 diameter	flat, mm	-	+	+	-	+	+	K/A	-	-	-	-	-	-	-	*V. parahaemolyticus*
32	Vib PA/08w	Green, 2-3 diameter	flat, mm	-	+	+	-	+	+	K/A	-	+						*Vibrio species*
33	Vib PSW/08w	Green, 2-3 diameter	flat, mm	+	+	-	-	+	+	A/A	-	-	-	-	-	-	-	*V. cholerae*
34	Vib 11/08al	Yellow, 2-3 diameter	flat, mm	+	+	-	-	+	+	A/A	-	-	-	-	-	-	-	*V. cholerae*
35	Vib 12/08al	Yellow, 2-3 diameter	flat, mm	-	+	+	+	+	+	A/A	-	-	-	-	-	-	-	*Vibrio* species
36	Vib SS/08al	Yellow, 2-3 diameter	flat, mm	-	+	+	-	+	-	K/A	+							Non-*Vibrio*
37	Vib SW/08al	Yellow, 2-3 diameter	flat, mm	+	+	-	-	+	+	A/A	-	-	-	-	-	-	-	*V. cholerae*

4.3.2 API 20E

There were 16 *V. cholerae* identified by API 20E (Biomerieux, SA, France) test with the likelihood values from 82%-99.9%. The API 20E (Biomerieux, SA, France) also identified five *V. alginolyticus* (97.8%-99.4 %), two *V. parahaemolyticus* (97.9%), one *V. mimicus* (84.9%), one *A. hydrophila* (89.8%), and one *Pasteurella pneumotropica* (87.3%). Three isolates gave likelihood value less than 80%: *V. cholerae* (70.3%), *V. vulnificus* (54.0%) and *V. alginolyticus* (75.8%). Eight isolates were not identified but suggested as *V. fluvialis* by the software. The API 20E (Biomerieux, SA, France) results are summarized in **Table 4.2**.

Table 4.2: API 20E ID and percentage of identity of the 37 presumptive *V. cholerae* isolates from water and algae samples.

No	Code for Isolates	Morphology on TCBS agar	API 20E ID	% of identity
1	Vib 1/08w	Yellow, flat, 2-3 mm diameter	5347124	99.9 % *V. cholerae*
2	Vib 3/08w	Yellow, flat, 2-3 mm diameter	5045124	88.8 % *V. cholerae*
3	Vib 9/08w	Yellow, flat, 2-3 mm diameter	5347105	54.0 % *V. vulnificus*
4	Vib 10/08w	Yellow, flat, 2-3 mm diameter	4047124	97.8 % *V. alginolyticus*
5	Vib 17/08w	Yellow, flat, 2-3 mm diameter	5155124	99.9 % *V. cholerae*
6	Vib 77/08w	Yellow, flat, 2-3 mm diameter	5145126	91.3 % *V. cholerae*
7	Vib 199/08w	Yellow, flat, 2-3 mm diameter	4045124	99.3 % *V. alginolyticus*
8	Vib365 /08w	Yellow, flat, 2-3 mm diameter	5244144	Possibility of *V. fluvialis*
9	Vib 370/08w	Yellow, flat, 2-3 mm diameter	4347124	75.8 % *V. alginolyticus*
10	Vib 374/08w	Yellow, flat, 2-3 mm diameter	5346124	99.6 % *V. cholerae*

11	Vib1114/08w	Yellow, flat, 2-3 mm diameter	6315124	Possibility of *V. fluvialis*
12	Vib 3034/08w	Yellow, flat, 2-3 mm diameter	3047125	89.8% *Aeromonas hydrophyla*
13	Vib 3477/08w	Yellow, flat, 2-3 mm diameter	5257124	82.0 % *V. cholerae*
14	Vib 3577/08w	Yellow, flat, 2-3 mm diameter	4344144	*97.9% V. parahemolyticus*
15	Vib 4130/08w	Yellow, flat, 2-3 mm diameter	3315124	Possibility of *V. fluvialis*
16	Vib 4365/08w	Yellow, flat, 2-3 mm diameter	5515124	Possibility of *V. fluvialis*
17	Vib 4370/08w	Yellow, flat, 2-3 mm diameter	5347124	99.9 % *V. cholerae*
18	Vib 4372/08w	Yellow, flat, 2-3 mm diameter	4045124	99.3 % *V. alginolyticus*
19	Vib 4784/08w	Yellow, flat, 2-3 mm diameter	6315124	Possibility of *V. fluvialis*
20	Vib 4888/08w	Yellow, flat, 2-3 mm diameter	4155124	99.4% *V. alginolyticus*
21	Vib 4900/08w	Yellow, flat, 2-3 mm diameter	5244144	Possibility of *V. fluvialis*
22	Vib 4933/08w	Yellow, flat, 2-3 mm diameter	5346124	99.6 % *V. cholerae*
23	Vib 2P/08w	Yellow, flat, 2-3 mm diameter	4355124	99.1 % *V. alginolyticus*
24	Vib 3P/08w	Yellow, flat, 2-3 mm diameter	5147124	99.9 % *V. cholerae*
25	Vib B2/08w	Yellow, flat, 2-3 mm diameter	5045124	88.0 % *V. cholerae*
26	Vib B4/08w	Yellow, flat, 2-3 mm diameter	5255124	88.8 % *V. cholerae*
27	Vib H1/08w	Yellow, flat, 2-3 mm diameter	5315124	Possibility of *V. flubvialis*
28	Vib M1/08w	Yellow, flat, 2-3 mm diameter	5245124	91.1 % *V. cholerae*
29	Vib M2/08w	Yellow, flat, 2-3 mm diameter	5045124	88.0 % *V. cholerae*
30	Vib GB/08w	Green, flat, 2-3 mm diameter	6347124	70.3% *V. cholerae*
31	Vib GH/08w	Green, flat, 2-3 mm diameter	4344144	97.9% *V. parahaemolyticus*
32	Vib PA/08w	Green, flat, 2-3 mm diameter	5315124	Possibility of *V. fluvialis*
33	Vib PSW/08w	Green, flat, 2-3 mm diameter	5347124	99.9 % *V. cholerae*
34	Vib 11/08al	Yellow, flat, 2-3 mm diameter	5347124	99.9 % *V. cholerae*
35	Vib 12/08al	Yellow, flat, 2-3 mm diameter	5144144	84.9 % *V. mimicus*

36	Vib SS/08al	Yellow, flat, 2-3 mm diameter	5001124	87.3% Pasteurella pneumotropica
37	Vib SW/08al	Yellow, flat, 2-3 mm diameter	5346124	99.6 % *V. cholerae*

4.3.3 *gyrB*/*pntA*-based multiplex PCR for detection of *V. cholerae*

Based on the multiplex PCR targeting the *pntA* and *gyrB* genes, only 17 isolates showed amplification at 493 bp and 338 bp and identified as *V. cholerae*. Besides, two *V. parahaemolyticus* and 16 *Vibrio* spp were also detected based on the amplification at 493 bp and 409 bp or 493 bp, respectively (**Figure 4.1**). Two non-*Vibrio* species were also detected The PCR results are summarized in **Table 4.3**.

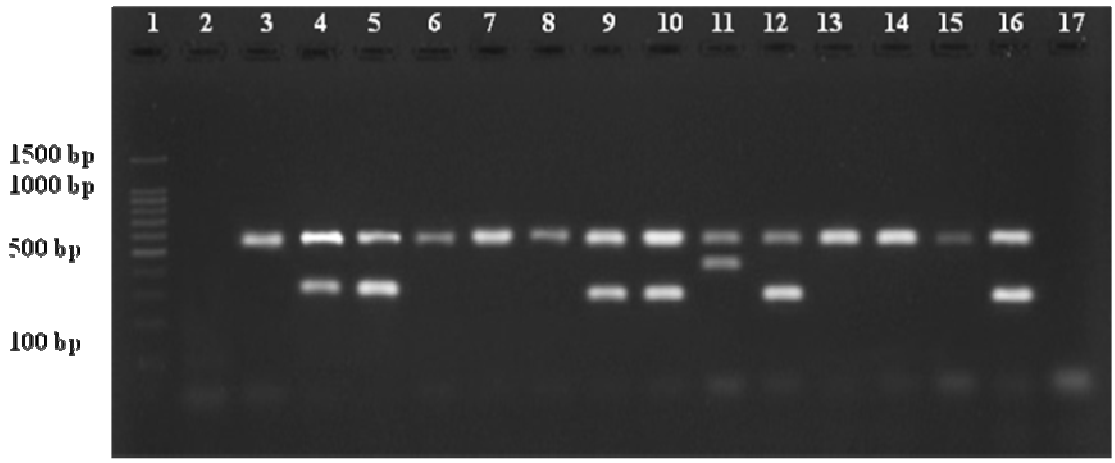


Figure 4.1: Representative agarose gel showing the PCR results based on *pntA* and *gyrB* gene. Lane 1: 100 bp DNA marker; Lane 2: non-*Vibrio* species (Negative Control) (no amplification); Lanes 3, 6-8, 13-15: *Vibrio* species (493 bp); Lanes 4-5, 9-10, 12 and 16: *V. cholerae* (493 bp/338 bp); Lane 11: *V. parahaemolyticus* (493 bp/ 409 bp); Lane 17: DNA blank (ddH_2O).

Table 4.3: *gyrB* and *pntA*-based multiplex PCR results for the 37 presumptive *V. cholerae* isolates from water and algae samples.

No	Code for Isolates	Morphology on TCBS agar	PCR				Interpretation
			338 bp (*pntA*)	409 bp (*pntA*)	656 bp (*pntA*)	493 bp (*gyrB*)	
1	Vib 1/08w	Yellow, flat, 2-3 mm diameter	+	-	-	+	*V. cholerae*
2	Vib 3/08w	Yellow, flat, 2-3 mm diameter	+	-	-	+	*V. cholerae*
3	Vib 9/08w	Yellow, flat, 2-3 mm diameter	-	-	-	+	*Vibrio* species
4	Vib 10/08w	Yellow, flat, 2-3 mm diameter	-	-	-	+	*Vibrio* species
5	Vib 17/08w	Yellow, flat, 2-3 mm diameter	+	-	-	+	*V. cholerae*
6	Vib 77/08w	Yellow, flat, 2-3 mm diameter	+	-	-	+	*V. cholerae*
7	Vib 199/08w	Yellow, flat, 2-3 mm diameter	-	-	-	+	*Vibrio* species
8	Vib365 /08w	Yellow, flat, 2-3 mm diameter	-	-	-	+	*Vibrio* species
9	Vib 370/08w	Yellow, flat, 2-3 mm diameter	-	-	-	+	*Vibrio* species
10	Vib 374/08w	Yellow, flat, 2-3 mm diameter	+	-	-	+	*V. cholerae*
11	Vib1114/08w	Yellow, flat, 2-3 mm diameter	-	-	-	+	*Vibrio* species

12	Vib 3034/08w	Yellow, diameter	flat,	2-3	mm	-	-	-	-	*Non-Vibrio* species
13	Vib 3477/08w	Yellow, diameter	flat,	2-3	mm	+	-	-	+	*V. cholerae*
14	Vib 3577/08w	Yellow, diameter	flat,	2-3	mm	-	+	-	+	*V. parahaemolyticus*
15	Vib 4130/08w	Yellow, diameter	flat,	2-3	mm	-	-	-	+	*Vibrio* species
16	Vib 4365/08w	Yellow, diameter	flat,	2-3	mm	-	-	-	+	*Vibrio* species
17	Vib 4370/08w	Yellow, diameter	flat,	2-3	mm	+	-	-	+	*V. cholerae*
18	Vib 4372/08w	Yellow, diameter	flat,	2-3	mm	-	-	-	+	*Vibrio* species
19	Vib 4784/08w	Yellow, diameter	flat,	2-3	mm	-	-	-	+	*Vibrio* species
20	Vib 4888/08w	Yellow, diameter	flat,	2-3	mm	-	-	-	+	*Vibrio* species
21	Vib 4900/08w	Yellow, diameter	flat,	2-3	mm	-	-	-	+	*Vibrio* species
22	Vib 4933/08w	Yellow, diameter	flat,	2-3	mm	+	-	-	+	*V. cholerae*
23	Vib 2P/08w	Yellow, diameter	flat,	2-3	mm	-	-	-	+	*Vibrio* species
24	Vib 3P/08w	Yellow, diameter	flat,	2-3	mm	+	-	-	+	*V. cholerae*
25	Vib B2/08w	Yellow, diameter	flat,	2-3	mm	+	-	-	+	*V. cholerae*
26	Vib B4/08w	Yellow, diameter	flat,	2-3	mm	+	-	-	+	*V. cholerae*

27	Vib H1/08w	Yellow, diameter	flat,	2-3	mm	-	-	-	+	*Vibrio* species
28	Vib M1/08w	Yellow, diameter	flat,	2-3	mm	+	-	-	+	*V. cholerae*
29	Vib M2/08w	Yellow, diameter	flat,	2-3	mm	+	-	-	+	*V. cholerae*
30	Vib GB/08w	Green, diameter	flat,	2-3	mm	+	-	-	+	*V. cholerae*
31	Vib GH/08w	Green, diameter	flat,	2-3	mm	-	+	-	+	*V. parahaemolyticus*
32	Vib PA/08w	Green, diameter	flat,	2-3	mm	-	-	-	+	*Vibrio* species
33	Vib PSW/08w	Green, diameter	flat,	2-3	mm	+	-	-	+	*V. cholerae*
34	Vib 11/08al	Yellow, diameter	flat,	2-3	mm	+	-	-	+	*V. cholerae*
35	Vib 12/08al	Yellow, diameter	flat,	2-3	mm	-	-	-	+	*Vibrio* species
36	Vib SS/08al	Yellow, diameter	flat,	2-3	mm	-	-	-	-	Non-*Vibrio species*
37	Vib SW/08al	Yellow, diameter	flat,	2-3	mm	+	-	-	+	*V. cholerae*

4.3.4 Evaluation of specificity and sensitivity for biochemical tests, API 20E and *gyrB*/*pntA*-based multiplex PCR

Among the 37 isolates, only 17 showed amplification at 336 bp in the PCR based on *ompW*. This showed that only 17 isolates were *V. cholerae* (**Figure 4.2**).

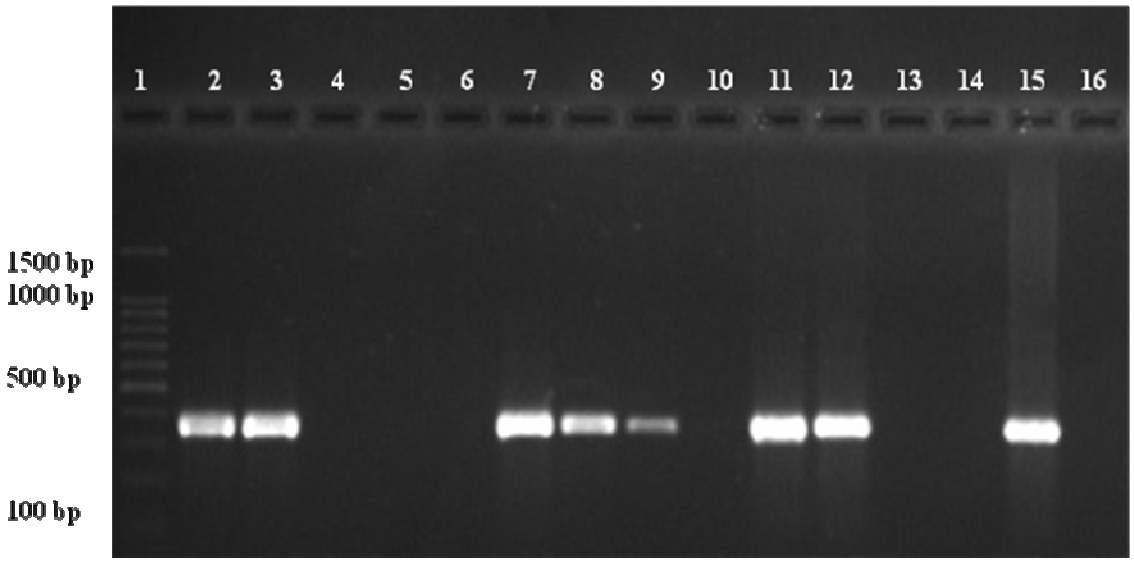


Figure 4.2: Representative agarose gel showing the PCR results based on *ompW* gene. Lane 1: 100 bp DNA marker; Lanes 2-3, 7-9, 11-12 and 15: *V. cholerae* (338 bp) Lanes 4-6, 10, 13-14: non-*Vibrio* species (Negative Control) (no amplification); Lane 16: DNA blank (ddH_2O).

Sensitivity and specificity for both conventional biochemical test and *gyrB*/*pntA*-based PCR were 1.0 (**Table 4.4** and **Table 4.5**). API 20E (Biomerieux, SA, France) was specific (1.0) but less sensitive (0.94) (**Table 4.5)**.

Table 4.4: Binary classification table showing the calculation of specificity, sensitivity for biochemical test.

		V. cholerae detected (as determined by biochemical test)	
		True	False
PCR Result (*ompW*)	Positive	TP =**17**	FP =**0**
	Negative	FN =**0**	TN =**20**
		Sensitivity =TP / (TP+FN) =**1**	Specificity =TN / (FP+TN) =**1**

Table 4.5: Binary classification table showing the calculation of specificity, sensitivity for *gyrB*/*pntA*-based multiplex PCR.

		V. cholerae detected (as determined by *gyrB*/*pntA*-based multiplexPCR)	
		True	False
PCR Result (*ompW*)	Positive	TP =**17**	FP =**0**
	Negative	FN =**0**	TN =**20**
		Sensitivity =TP / (TP+FN) =**1**	Specificity =TN / (FP+TN) =**1**

Table 4.6: Binary classification table showing the calculation of specificity, sensitivity for API 20E test.

		V. cholerae detected (as determined by API 20E)	
		True	False
PCR Result (*ompW*)	Positive	TP =**16**	FP =**0**
	Negative	FN =**1**	TN =**20**
		Sensitivity =TP / (TP+FN) =**0.94**	Specificity =TN / (FP+TN) =**1**

4.4 Discussion

V. cholerae is genetically close to other *Vibrio* species and live freely in the aquatic environment and established ecological relationship with the other organisms in the same habitat. Vibrios have been isolated in raw seafood, phytoplankton and zooplankton (Armstrong *et al.*, 1998, Dumontet *et al.*, 2000). The halotolerance of *Vibrio* species in the environment indirectly increase the public health concern as virulence gene circulation may occurr among the different species of halophilic Vibrios (Canigral *et al.*, 2011). Some *Vibrio* species are non-halophilic but most species require salt for growth (Kaysner and DePaola, 2004). Different species of *Vibrio* require different salt level for growth but *Vibrio cholerae* is the only *Vibrio* spp. which can tolerate with 0% NaCl. This is an important parameter for differentiation of *V. cholerae* from other *Vibrio* species.

According to the morphology of *V. cholerae* on the TCBS agar, presumptive colonies are yellow, flat, and approximately 2-3 mm diameter. In this study, only

colonies which showed the expected morphology were selected for further tests. Among the 17 confirmed *V. cholerae* based on *ompW*-based PCR, *pntA/gyrB*-based PCR and conventional biochemical tests, two showed characteristic of green, flat and 2-3 mm diameter on TCBS agar (Becton Dickinson, N, USA). Twenty presumptive *V. cholerae* as confirmed on TCBS agar (Becton Dickinson, N, USA) was not *V. cholerae*, but identified as other *Vibrio* species and even non-*Vibrio* species. This is in agreement with Choopun *et al.* (2002) that the appearance of colony on TCBS agar produced by other environmental *Vibrio* species was similar to *V. cholerae*. According to Di Pinto *et al.* (2005), the morphology of *Aeromonas hydrophilla* colonies on TCBS was also very similar with other *Vibrio* species because high similarity in genetics and chemical reaction was shared. This suggests that the morphology of colonies on the selective agar is sometimes not reliable and further confirmations are needed for final identification.

Based on Choopun *et al.* (2002), arginine dihydrolase test was useful to screen *V. cholerae*. However, 26 isolates showed negative result in the test in this study and most of the *Vibrio* species such as *V. cholerae*, *V. alginolyticus*, *V. parahaemolyticus*, *V. vulnificus*, and *V. mimicus* were negative in the arginine dihydrolase test (Elliot *et al.*, 2001; Kaysner and Depaola, 2004). Moreover, *V. alginolyticus* and *V. cholerae* shared the same characteristic in most of the tests. The only test which could differentiate these two bacteria was the tolerance to NaCl. In brief, *V. cholerae* could grow in the media with 0% NaCl while *V. alginolyticus* could not tolerate 0% NaCl but able to grow in media with 10% NaCl. This suggests that the confirmation of bacteria should not depend on a single biochemical test.

API 20E may be easy to perform, however the result may not be satisfactory and the evaluation of biochemical reactions were very subjective (Nucera *et al.*, 2006; Chakraborty *et al.*, 2006). Overman *et al.* (1985) had reported that API 20E was the most accurate and efficient system to identify commonly isolated species of *Vibrionaceae* compared to API RapidE and API RapidNFT although previous study had shown that the accuracy of identification using this test strip was only 88% (Holmes *et al.*, 1978). Misidentification by API 20E is also not new. Several misidentification cases using API 20E had been reported by O'hara *et al.* (2003) and Chakraborty *et al.* (2006). However, there was no misindentification of *V. cholerae* in our study. One isolate was identified as *V. cholerae* at a low likelihood value (70.3%), but was confirmed as *V. cholerae* by biochemical tests, *gyrB*/*pntA*-based PCR and *ompW*-based PCR.

The *gyrB*/*pntA*-based multiplex PCR was repeated twice and similar results were obtained. The results obtained by *gyrB*/*pntA*-based multiplex PCR was in concordance with the results showed by *ompW*-based PCR. This indicates the high specificity of the *gyrB*/*pntA*-based multiplex PCR in *V. cholerae* detection. Furthermore, this multiplex PCR could also differentiate *V. cholerae* from other isolates such as *V. parahaemolyticus, Vibrio* species and non-*Vibrio* species *gyrB*/*pntA*-based PCR was also more rapid as it took only three hours to confirm the identity of a presumptive colony on non-selective agar compared to the conventional biochemical tests (7 days) and API 20E (2 days).

Based on the sensitivity and specificity results, biochemical tests are useful for confirmation of the bacterial isolates although they require longer experimental time and is tedious when compared to multiplex PCR. Although biochemical tests were

laborious, the result was more reliable than API 20E. API 20E was specific, but not sensitivite enough to identify *V. cholerae* due to the subjective interpretation of color change in the reaction.

In conclusion, PCR remains the best detection or identification method in terms of specificity, sensitivity, ease of operation and duration. The results obtained also showed that the optimized conditions for *gyrB*/*pntA*-based multiplex PCR in **Chapter 3** were robust and stable.

Chapter Five

Development of a Multiplex PCR for Biotyping, Serogrouping and Virulotyping of *Vibrio cholerae*

5.1 Background

Both Classical and El Tor biotypes of *V. cholerae* O1 were responsible for diarrhea in humans and were associated with the sixth and seventh pandemics, respectively (Singh *et al.*, 2001; Danin-Poleg *et al.*, 2007). The two biotypes can be differentiated conventionally based on the phenotypic traits. More recently, comparative genomic analysis had revealed the variations of different genes in these two biotypes such as *hlyA, ctxB, tcpA*, and *rtxC* (Alam *et al.*, 1988; Safa *et al.*, 2006; Raychoudhuri *et al.*, 2009). After the emergence of *V. cholerae* O139 which caused a major outbreak in India in 1992, O139 serogroup of *V. cholerae* 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 El Tor O1 serogroup as they share the same 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).

Identification of clinical and environmental strains of *V. cholerae* in Malaysia usually involves biochemical tests, followed by PCR detection of *toxR* gene and agglutination test for serogrouping (Chen *et al.*, 2004). A rapid PCR approach for specific identification of *V. cholerae* using *ompW* gene was reported by Nandi *et al.* (2000) while Hoshino *et al.* (1998) introduced a PCR approach for differentiation of *V. cholerae* O1 and O139 serogroups based on *orf* complex. Many of the current PCR approaches 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), but non-toxigenic strains which lack of virulence genes might not be detected (Dalmasso *et al.*, 2009).

The environmental sources remain a reservoir for a variety of *V. cholerae* strains harbour different virulence profiles (Colwell *et al.*, 1994). The virulence genes carried will determine the ability of colonization when the cells enter the host. Since pathogenic strains of *V. cholerae* evolved from nonpathogenic environmental strains, it is important to identify intermediate strains that are likely to carry some of the virulence-related genes (Rahman *et al.*, 2008).

The main objective of this Chapter was to develope a PCR for simultaneous identification of biotypes, serogroups and virulence genes harboured in *V. cholerae* strains.

5.2 Materials and methods

5.2.1 Bacterial strains and template preparation

Fourty-three *V. cholerae* strains were used in this study: 19 were collected from Institute for Medical Research (IMR), Malaysia, seven were retrieved from the culture collections of The laboratory of Biomedical Science and Molecular Microbiology (LBSMM), University of Malaya, and seventeen were isolated as previously described in **Chapter 4**. The strains are listed in **Appendix A**. Two O1 *V. cholerae* (El Tor and Classical biotypes) and one O139 serogroup reference strains from the National Institute of Cholera and Enteric Diseases (NICED), Kolkata, India were used as control strains in this study.

DNA templates for PCR were prepared as previously described in **Chapter 3, Section 3.2.3.**

5.2.2 Primer design for *hly*A gene

Based on the sequences retrieved from the Genbank (Accession no: AE003853.1, AY427780.1, and CP000626.1), a pair of primers targeting the *hly*A gene was designed using Primer 3 program (Rozen and Skaletsky 2000) in this study. Initially, an in-silico-PCR (http://insilico.ehu.es/PCR) was carried out to test the specificity of the designed primers (*hlyA* 1, and *hlyA* 2), followed by evaluation with a panel of strains including *V. cholerae*, *Vibrio species*, and non-*Vibrio* species

Monoplex PCR was also carried out for *hlyA* primers using N16961 and 569B as template in a single tube containing 1x PCR buffer, 1 mM $MgCl_2$, 40 µM each

dNTPs, 0.3 µM each primers, ~50 ng DNA template and 0.5 U of *Taq* DNA polymerase (Promega, Madison, USA). The PCR cycling condition consisted of 95°C for 1 min (pre-denaturation), followed by 30 cycles of 94°C for 30 sec (denaturation), 57°C for 30 sec (annealing) 72°C for 1 min (extension) and a final extension at 72°C for 8 min.

The PCR products were analyzed by a 1% (w/v) LE agarose gel and electrophoresed at 90V in 0.5 X TBE buffer. The gel was then stained with ethidium bromide (0.5 µg/ml) and visualized with Gel Doc XR (Bio Rad, Hercules, CA) as described in **Chapter 3, Section 3.2.5**.

DNA was extracted from gel as described in **Chapter 3**, **Section 3.2.6**. The extracted DNA was sent to a laboratory (1st Base, Seri Kembangan, Malaysia) for sequencing together with the PCR primers.

5.2.3 Conventional Voges-Proskauer (VP) test and Agglutination test

Voges-Proskauer (VP) test was performed as described in Kay *et al.* (1994) with minor modification. Briefly, a bacterial colony was inoculated into MRVP broth with 1% NaCl and incubated 24 hours. The next day, 0.6 ml of α-naphthol and 0.2 ml of 40% potassium hydrochloride KOH (with 0.3% creatine) were added into 1 ml of culture. The production of red color within 5–30 min indicates positive reaction while yellow to orange or no color development indicates negative reaction.

Difco *Vibrio cholerae* Antiserum Poly (Becton Dickinson, NJ, USA) was used to screen possible O1 *V. cholerae* isolates. The antiserum was reconstituted by adding 3 ml of sterile 0.85% saline. Firstly, a drop of the antiserum was dispensed on an agglutination slide. Then, a loopful of *V. cholerae* colony was transfered to the drop of antiserum and mixed thoroughly. Next, the slide was rotated for 1 min and read for agglutination. Strains which do give negative result in agglutination test were sent to Institute for Medical Research, Kuala Lumpur for serogroups determination.

5.2.4 Optimization of multiplex PCR based on *hlyA, ompW, toxR, tcpI, ctxA, and orf complex* markers

The *hly*A primers designed in this study were combined with five other pairs of primers described in previous studies (**Table 5.1**) to form a multiplex PCR targeting *ompW*, *toxR*, *tcpI*, *ctxA*, and *orf* complex. Various conditions such as annealing temperatures and concentration of primers were modified to obtain specific, unique amplicons. Once the multiplex PCR conditions were optimized, the assay was evaluated with all the strains.

The PCR products were analyzed by a 2.5% LE agarose gel and electrophoresed at 90V in 0.5 X TBE buffer. The gel was then stained with ethidium bromide (0.5 µg/ml) and visualized with Gel Doc XR (Bio Rad, Hercules, CA) as described in **Chapter 3, Section 3.2.5**.

Table 5.1: Primers used for biotyping, serogrouping and virulotyping study.

Primer	Sequence (5'-3')	Amplicon size	Target gene	Reference
132F	TAGCCTTAGTTCTCAGCAGGCA	862 bp	*tcpI*	Rivera *et al.*, 2001
951R	GGCAATAGTGTCGAGCTCGTTA			
*ompW*2	GAACTTATAACCACCCGCG	336 bp	*ompW*	Nandi *et al.*, 2000
*ompW*3	CCACCTACCTTTATGGTCC			
101F	CCT TCGATCCCCTAAGCAATAC	779 bp	*toxR*	Rivera *et al.*, 2001
837R	AGGGTTAGCAACGATGCGTAAG			
94F	CGGGCAGATTCTAGACCTCCTG	564 bp	*ctxA*	Fields *et al.*, 1992
614R	CGATGATCTTGGAGCATTCCCAC			
*hlyA*1	GTGCGTATCAGCCTAGATGA	255 (ET) / 244 (C)	*hlyA*	This study. AE003853.1,
hlyA 2	CCCAAGCTCAAAACCTGAAA			AY427780.1, CP000626.1
O1*rfb*F	GTTTCACTGAACAGATGGG	192 bp	*orf* complex	Hoshino *et al.*, 1998
O1*rfb*R	GGTCATCTGTAAGTACAAC			
O139*rfb*F	AGCCTCTTTATTACGGGTGG	449 bp	*orf* complex	Hoshino *et al.*, 1998
O139*rfbR*	GTCAAACCCGATCGTAAAGG			

5.2.5 Sensitivity of the multiplex PCR

Sensitivity test was performed using bacterial cell dilutions of reference strains N16961, 569B and SG24 as described in **Chapter 3, Section 3.2.9**. The multiplex PCR was carried out under optimized condition in **Section 5.2.3**.

5.3 Results

5.3.1 Specificity of the *hlyA* primer

An *in-silico* PCR performed using all the public domain available *Vibrio species* resulted in two amplicons, 255 bp and 244 bp for biotypes El Tor and Classical, respectively. No amplification was found for non-*V. cholerae* strains.

In *in-vitro* PCR, a slightly smaller band ~240 bp was observed for Classical 569B strain (**Figure 5.1**). Strains other than *V. cholerae* had no amplification. Sequence analysis of the amplicons for Classical 569B and El Tor N16961 were aligned using 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. This observation is in concordance with the report from Rader and Murphy *et al.* (1988) (**Figure 5.2**).

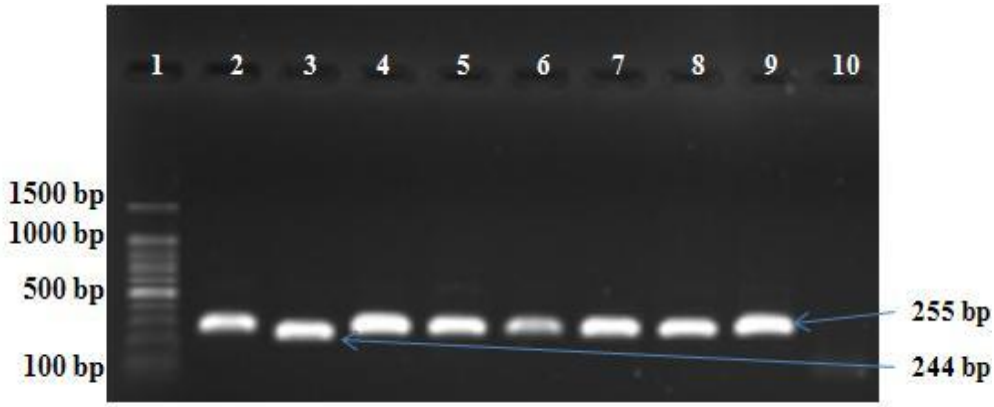


Figure 5.1 Monoplex PCR targeting El Tor and Classical *hlyA* gene. Lane 1: 100 bp marker; Lane 2: El Tor O1 *V. cholerae* (N16961); Lane 3: Classical *V. cholerae* (569B); Lane 4: O139 *V. cholerae* (SG24); Lane 5-9: *V. cholerae* strains; Lane 10: DNA blank (ddH_2O).

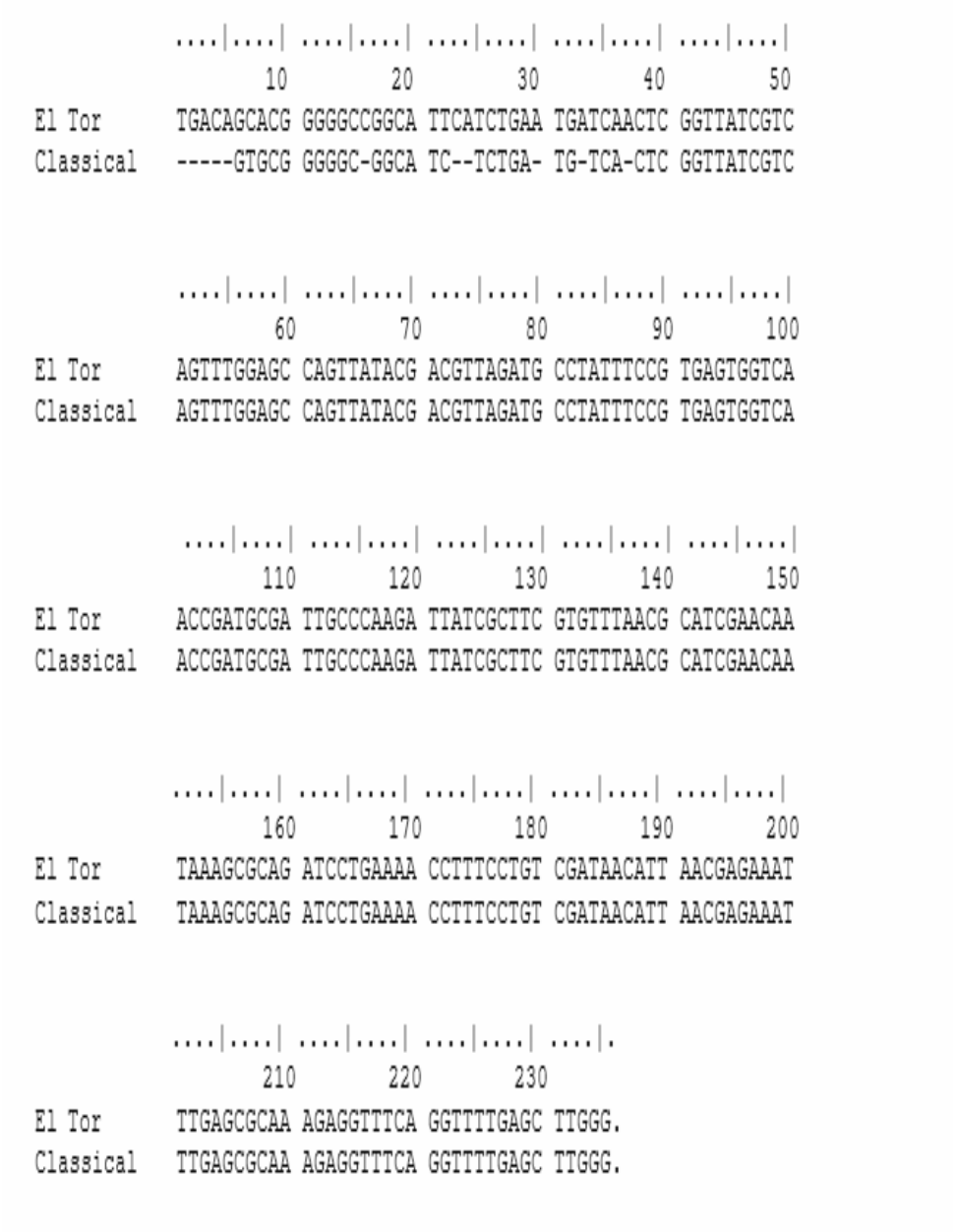


Figure 5.2. Sequence alignment for the *hlyA* amplicons of El Tor and Classical strains. 11 bp deletions were found in Classical strains.

5.3.2 Biotypes and serogroups determination

All strains showed positive reaction for VP test, except for the control Classical strain and two strains tested (17/08 and B2/08). Twenty-three *V. cholerae* strains showed positive reaction in the agglutination test while 20 strains were sent to Institute for Medical Research for serogroups determination. As a result, one of the strains was defined as O139 *V. cholerae* and the rest were non-O1/non-O139 *V. cholerae*. The results are summarized in **Table 5.2**.

5.3.3 Multiplex PCR for biotyping, serogrouping and virulotyping

Two problems were encountered in the first optimization of multiplex PCR. Firstly, the targeted 862 bp band for *tcpI* was absent. Secondly, non-specific band was detected at approximately 500 bp. The gel for first optimization is shown in **Figure 5.3**.

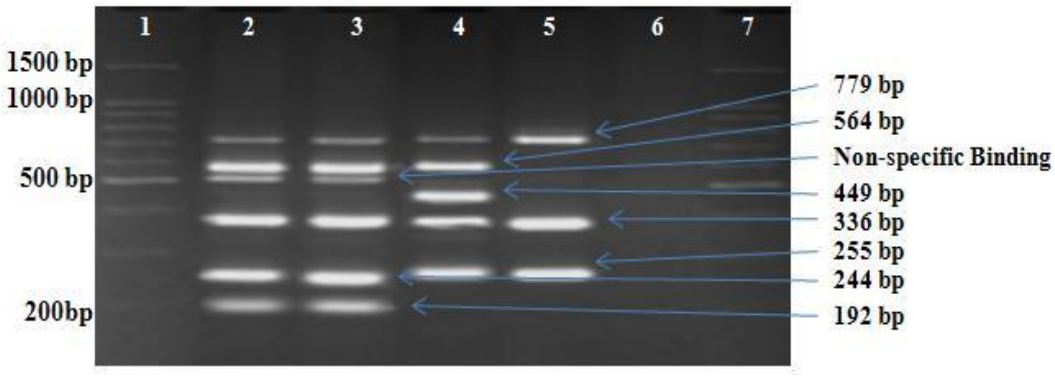


Figure 5.3 First optimization of multiplex PCR for biotyping, serogrouping and virulotyping. Each reaction contained 1 X buffer, 2.5 mM $MgCl_2$, 200 µM of each dNTP, 2.0 U *Taq* DNA Polymerase (Promega, Madison, USA), 0.3 µM of each primer, 3 µl (~60 ng) of DNA template and appropriate ddH_2O with 5 min of initial denaturation at 95°C, followed by 30 cycles of denaturation, annealing, and extension for 94°C (30 sec), 59°C (30 sec) and 72.0°C (1 min), respectively and a final extension at 72.0°C for 5 min. The 862 bp band for *tcp*I gene was absent and non-specific bands were detected. Lanes 1 and 7: 100 bp marker; Lane 2: El Tor O1 *V. cholerae* (N16961); Lane 3: Classical *V. cholerae* (569B); Lane 4: O139 *V. cholerae* (SG24); Lane 5: non-O1/non-O139 *V. cholerae*; Lane 6: DNA blank (ddH_2O).

To overcome these problems, various approaches were taken. The concentration of *Taq* DNA Polymerase and template were increased to 2.5 U/ml and 100ng, respectively. The result for the second optimization of multiplex PCR was shown in **Figure 5.4**.

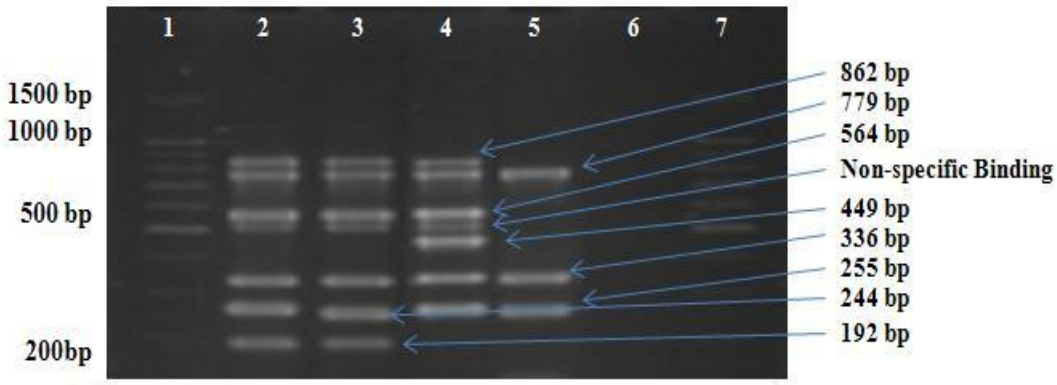


Figure 5.4 Second optimization of multiplex PCR for biotyping, serogrouping and virulotyping. Each reaction contained 1 X buffer, 2.5 mM $MgCl_2$, 200 µM of each dNTP, 2.5 U *Taq* DNA Polymerase (Promega, Madison, USA), 0.3 µM of each primer, 5 µl (~100 ng) of DNA template and appropriate ddH_2O with 5 min of initial denaturation at 95°C, followed by 30 cycles of denaturation, annealing, and extension for 94°C (30 sec), 58°C (30 sec) and 72.0°C (1 min), respectively and a final extension at 72.0°C for 5 min. All targeted bands were present and non-specific bands were detected. Lanes 1 and 7: 100 bp marker; Lane 2: El Tor O1 *V. cholerae* (N16961); Lane 3: Classical *V. cholerae* (569B); Lane 4: O139 *V. cholerae* (SG24); Lane 5: non-O1/non-O139 *V. cholerae*; Lane 6: DNA blank (ddH_2O).

To eliminate the non-specific binding, the concentration of $MgCl_2$ was decreased to 2.0 mM while annealing temperature was increased to 59°C. The result for the second optimization of multiplex PCR is shown in **Figure 5.5**.

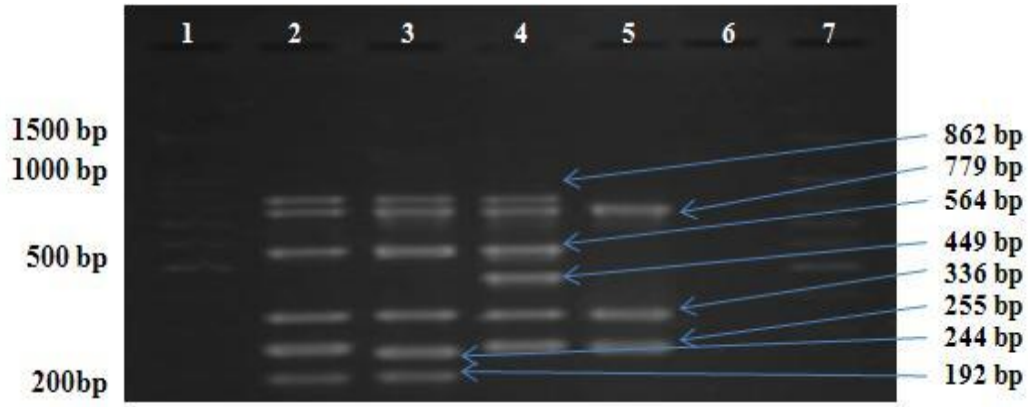


Figure 5.5 Third optimization of multiplex PCR for biotyping, serogrouping and virulotyping. Each reaction contained 1 X buffer, 2.0 mM $MgCl_2$, 200 µM of each dNTP, 2.5 U *Taq* DNA Polymerase (Promega, Madison, USA), 0.3 µM of each primer, 5 µl (~100 ng) of DNA template and appropriate ddH_2O with 5 min of initial denaturation at 95°C, followed by 30 cycles of denaturation, annealing, and extension for 94°C (30 sec), 59°C (30 sec) and 72.0°C (1 min), respectively and a final extension at 72.0°C for 5 min. All targeted bands were present and most of the non-specific bindings were eliminated. Lanes 1 and 7: 100 bp marker; Lane 2: El Tor O1 *V. cholerae* (N16961); Lane 3: Classical *V. cholerae* (569B); Lane 4: O139 *V. cholerae* (SG24); Lane 5: non-O1/non-O139 *V. cholerae*; Lane 6: DNA blank (ddH_2O).

Finally, the multiplex PCR for biotyping, serogrouping and virulotyping was optimized. The optimized conditions comprised of an initial denaturation of 95°C for 5 min, followed by 30 cycles of 94°C for 30 sec (denaturation), 59°C for 30 sec (annealing) and 72.0°C for 1 min (extension), and a final extension at 72.0°C for 7 min. One reaction of this multiplex PCR contained 1X buffer, 2.0 mM $MgCl_2$, 200

μM of each dNTP, 2.5 U DNA polymerase (Promega, Madison, USA), 0.3 μM of each primer and 5μl of DNA (~ 100 ng) .

The optimized multiplex PCRwas applied on 43 strains collected and isolated in Malaysia. Among the panel of 43 strains tested, one strain was subtyped as O139 serogroup, 19 strains were non-O1/non-O139, the rest were subtyped as O1 serogroup, with 18 strains harboring toxigenic genes *toxR* (779 bp), *ompW* (336 bp), *hlyA* (~250 bp), *ctxA* (564bp) and *tcpI* (862bp). One strain lacked *ctxA* and *tcpI* gene. Results are summarized in **Table 5.2**.

Table 5.2 Comparison of conventional method and multiplex PCR for biotyping, serogrouping of *V. cholerae*.

Strains code	Conventional Methods			Multiplex PCR Method			
	V. cholerae determination (As described in Chapter 4)	VP test (Biotypes determination)	Agglutination test (Serogroups determination)	*ompW* (*V. cholerae*)	*hly*A (biotypes determination)	*orf* complex (serogroups determination)	Virulence genes
N16961	+	+ (El Tor)	O1	+	255 bp (El Tor)	192 bp (O1)	*tox*R^{+}, *tcp*I^{+}, *ctx*A^{+}
569B	+	- (Classical)	O1	+	244 bp (Classical)	192 bp (O1)	*tox*R^{+}, *tcp*I^{+}, *ctx*A^{+}
SG24	+	+ (El Tor)	O139	+	255 bp (El Tor)	449 bp (O139)	*tox*R^{+}, *tcp*I^{+}, *ctx*A^{+}
4/08	+	+ (El Tor)	O1	+	255 bp (El Tor)	192 bp (O1)	*tox*R^{+}, *tcp*I^{+}, *ctx*A^{+}
6/08	+	+ (El Tor)	O1	+	255 bp (El Tor)	192 bp (O1)	*tox*R^{+}, *tcp*I^{+}, *ctx*A^{+}
9/08	+	+ (El Tor)	O1	+	255 bp (El Tor)	192 bp (O1)	*tox*R^{+}, *tcp*I^{+}, *ctx*A^{+}
10/08	+	+ (El Tor)	O1	+	255 bp (El Tor)	192 bp (O1)	*tox*R^{+}, *tcp*I^{+}, *ctx*A^{+}
21/04	+	+ (El Tor)	O1	+	255 bp (El Tor)	192 bp (O1)	*tox*R^{+}, *tcp*I^{+}, *ctx*A^{+}

35/04	+	+ (El Tor)	O1	+	255 bp (El Tor)	192 bp (O1)	*tox*R$^+$
37/04	+	+ (El Tor)	O1	+	255 bp (El Tor)	192 bp (O1)	*tox*R$^+$, *tcp*I$^+$, *ctx*A$^+$
38/04	+	+ (El Tor)	O1	+	255 bp (El Tor)	192 bp (O1)	*tox*R$^+$, *tcp*I$^+$, *ctx*A$^+$
40/04	+	+ (El Tor)	O1	+	255 bp (El Tor)	192 bp (O1)	*tox*R$^+$, *tcp*I$^+$, *ctx*A$^+$
52/08	+	+ (El Tor)	O1	+	255 bp (El Tor)	192 bp (O1)	*tox*R$^+$, *tcp*I$^+$, *ctx*A$^+$
63/08	+	+ (El Tor)	O1	+	255 bp (El Tor)	192 bp (O1)	*tox*R$^+$, *tcp*I$^+$, *ctx*A$^+$
64/08	+	+ (El Tor)	O1	+	255 bp (El Tor)	192 bp (O1)	*tox*R$^+$, *tcp*I$^+$, *ctx*A$^+$
65/08	+	+ (El Tor)	O1	+	255 bp (El Tor)	192 bp (O1)	*tox*R$^+$, *tcp*I$^+$, *ctx*A$^+$
66/08	+	+ (El Tor)	O1	+	255 bp (El Tor)	192 bp (O1)	*tox*R$^+$, *tcp*I$^+$, *ctx*A$^+$
70/08	+	+ (El Tor)	O1	+	255 bp (El Tor)	192 bp (O1)	*tox*R$^+$, *tcp*I$^+$, *ctx*A$^+$
87/08	+	+ (El Tor)	O1	+	255 bp (El Tor)	192 bp (O1)	*tox*R$^+$, *tcp*I$^+$, *ctx*A$^+$
88/08	+	+ (El Tor)	O1	+	255 bp (El Tor)	192 bp (O1)	*tox*R$^+$, *tcp*I$^+$, *ctx*A$^+$
90/08	+	+ (El Tor)	O1	+	255 bp (El Tor)	192 bp (O1)	*tox*R$^+$, *tcp*I$^+$, *ctx*A$^+$
123/08	+	+ (El Tor)	O1	+	255 bp (El Tor)	192 bp (O1)	*tox*R$^+$, *tcp*I$^+$, *ctx*A$^+$
310/08	+	+ (El Tor)	Non-O1/non-	+	255 bp (El Tor)	NA	*tox*R$^+$, *tcp*I$^+$

			O139				
933/08	+	+ (El Tor)	Non-O1/non-O139	+	255 bp (El Tor)	NA	*tox*R^{+}
81/08	+	+ (El Tor)	Non-O1/non-O139	+	255 bp (El Tor)	NA	*tox*R^{+}
82/08	+	+ (El Tor)	Non-O1/non-O139	+	255 bp (El Tor)	NA	*tox*R^{+}
83/08	+	+ (El Tor)	Non-O1/non-O139	+	255 bp (El Tor)	NA	*tox*R^{+}
84/08	+	+ (El Tor)	Non-O1/non-O139	+	255 bp (El Tor)	NA	*tox*R^{+}
85/08	+	+ (El Tor)	Non-O1/non-O139	+	255 bp (El Tor)	NA	*tox*R^{+}
1/08	+	+ (El Tor)	O1	+	255 bp (El Tor)	192 bp (O1)	*tox*R^{+}, *tcp*I^{+}, *ctx*A^{+}
3/08	+	+ (El Tor)	O1	+	255 bp (El Tor)	192 bp (O1)	*tox*R^{+}, *tcp*I^{+}, *ctx*A^{+}

17/08	+	- (Classical)	Non-O1/non-O139	+	255 bp (El Tor)	NA	*tox*R$^+$
77/08	+	+ (El Tor)	Non-O1/non-O139	+	244 bp (Classical)	NA	*tox*R$^+$
374/08	+	+ (El Tor)	Non-O1/non-O139	+	255 bp (El Tor)	NA	*tox*R$^+$
3477/08	+	+ (El Tor)	Non-O1/non-O139	+	244 bp (Classical)	NA	*tox*R$^+$, *tcp*I$^+$
4370/08	+	+ (El Tor)	O139	+	255 bp (El Tor)	449 bp (O139)	*tox*R$^+$, *tcp*I$^+$, *ctx*A$^+$
4933/08	+	+ (El Tor)	Non-O1/non-O139	+	255 bp (El Tor)	NA	*tox*R$^+$
3P/08	+	+ (El Tor)	O1	+	255 bp (El Tor)	192 bp (O1)	*tox*R$^+$, *tcp*I$^+$, *ctx*A$^+$
B2/08	+	- (Classical)	Non-O1/non-O139	+	255 bp (El Tor)	NA	*tox*R$^+$
B4/08	+	+ (El Tor)	Non-O1/non-	+	255 bp (El Tor)	NA	*tox*R$^+$

			O139				
M1/08	+	+ (El Tor)	Non-O1/non-O139	+	255 bp (El Tor)	NA	*tox*R^{+}
M2/08	+	+ (El Tor)	Non-O1/non-O139	+	255 bp (El Tor)	NA	*tox*R^{+}
GB/08	+	+ (El Tor)	Non-O1/non-O139	+	255 bp (El Tor)	NA	*tox*R^{+}
PSW/08	+	+ (El Tor)	Non-O1/non-O139	+	255 bp (El Tor)	NA	*tox*R^{+}
11/08	+	+ (El Tor)	O1	+	255 bp (El Tor)	192 bp (O1)	*tox*R^{+}, *tcp*I^{+}, *ctx*A^{+}
SW/08	+	+ (El Tor)	Non-O1/non-O139	+	255 bp (El Tor)	NA	*tox*R^{+}

NA = no Amplification = non-O1/non-O139 serogroup

5.3.4 Sensitivity of the multiplex PCR

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

5.4 Discussion

Isolation, detection and confirmation of *V. cholerae* by conventional methods are complicated. *V. cholerae* is genetically closely related to other *Vibrio* species and *Aeromonas* species. Therefore, ambiguous identification using biochemical tests occur frequently. Moreover, the commercial antisera are only available for O1 and O139 *V. cholerae* (Nandi *et al.*, 2000). In the present study, VP test was used for biotyping. Three strains were classified as Classical strains based on VP test. However, the results did not 100% concurred with PCR as one of the strains which gave negative result in VP test amplified El Tor *hlyA*. Moreover, a strain which amplified Classical *hlyA* was detected as El Tor variant by VP test. Notwithstanding, biotyping is not appropriate for non-O1 strains including O139 based on Kay *et al.* (1994) as variable results will be obtained.

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*, *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 *V. cholerae* pathogenecity island (VPI) (Faruque *et al.,* 1998) and *tcpI* is included in the panel of virulence genes. 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*.

Although the Classical biotype was responsible for the sixth 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 481bp/738bp bands for El Tor and a 727 bp band for Classical *V. cholerae*. 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), hence, 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. The *ctxB* and *tcpA* could be the marker for biotype differentiation, but there are toxigenic and non-toxigenic strains which do not harbour these two genes (Faruque *et al.,* 2004; Dalmasso *et al.,* 2009). In this study, one of the *V. cholerae* was confirmed as El Tor O1 by PCR but did not harbour virulence genes. This indicates the inappropriateness of virulence genes as specific marker for biotyping in PCR. However, no additional Classical strain was

isolated and the efficiency of the multiplex PCR for biotyping was not able to be further validated.

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

In conclusion, the multiplex PCR developed in the study provided an alternative and easier method for rapid identification of toxigenic and non-toxigenic *V. cholerae* with different serogroups and might complement the traditional biochemical tests.

Chapter Six

Comparison of PCR-based Fingerprinting for Relatedness Study of *Vibrio cholerae*

6.1 Background

Studies on the genomic variation and molecular epidemiology of O1 and O139 *V. cholerae* are usually carried out to track sources and spread of the pathogen (Ravi Kumar *et al.*, 2007). PCR-based DNA fingerprinting approaches such as arbitrarily primed PCR, Box-PCR, random amplified polymorphic DNA (RAPD)-PCR, enterobacterial repetitive intergenic consensus sequence (ERIC)-PCR, repetitive extragenic palindromic (REP)-PCR have been used to study epidemiological relationship among *V. cholerae* isolates (Rivera *et al.*, 2001; Singh *et al.*, 2001; Thong *et al.*, 2002). Although these methods are occasionally associated with poor reproducibility and low discriminatory ability, there are reports of the usefulness of RAPD-PCR, ERIC-PCR, and REP-PCR to differentiate toxigenic and non-toxigenic (Castaneda *et al.*, 2005) or serogroups of *V. cholerae* isolates (Chakraborty *et al.*, 2000).

A relatively new approach, *V. ch*olerae Repeats PCR (VCR-PCR) developed by Tokunaga *et al.* (2010) was reported to be useful for differentiating *V. cholerae* strains from different geographic regions and serogroups. This method targets the interspatial region of the repetitive sequences of the integron island in *V. cholerae*. However, this was only tested on a panel of toxigenic O1 El Tor and O139 *V. cholerae* (Tokunaga *et al.*, 2010).

Hence in this study, the relatedness of 43 *V. cholerae* strains of different serogroups was investigated by using RAPD-PCR, ERIC-PCR, REP-PCR and VCR-PCR. The discriminatory ability, typeability, stability and reproducibility of RAPD-PCR, ERIC-PCR, REP-PCR, and VCR-PCR were compared.

6.2 Materials and Methods

6.2.1 Bacterial Strains

Strains used in this Chapter were previously described in **Chapter 5** and listed in **Appendix A**.

6.2.2 DNA preparation for PCR

DNA preparation was carried out as described in **Chapter 3**, **Section 3.2.3**.

6.2.3 Random Amplified Polymorphic DNA (RAPD)-PCR

RAPD-PCR was performed using a single primer 1281 (5'-AACGCGCAAC) as described in Chakraborty *et al.* (2000) with minor modification. A PCR mixture contained 1× PCR buffer, 3 mM $MgCl_2$, 200 µM each dNTP, 1.0 µM primer, 2 U *Taq* DNA polymerase (Promega, Madison, USA) in a total volume of 25 µl. The PCR program was set at 94 °C, 5 min for initial denaturation, followed by 45 cycles of 94 °C

for 1 min; 36 °C for 1 min; 72 °C for 2 min, and one cycle of final extension step at 70 °C for 10 min.

6.2.4 Enterobacterial Repetitive Intergenic Consensus sequence (ERIC)-PCR

The ERIC-PCR was performed in a volume of 25 µl using 1× PCR buffer, 1.5 mM $MgCl_2$, 125 µM each dNTP, 1.0 µM each primer, 2.5 U *Taq* DNA polymerase (Promega, Madison, USA). The primers used were ERIC1R (5'-ATGTAAGCTCCTGGGGATTCAC3') and ERIC2 (5'-AAGTAAGTGACTGGGGTGAGCG3') (Versalovic et al. 1991). The PCR program consisted of an initial denaturation of 95°C for 5 min, followed by another 35 amplification cycles of 92°C for 45 sec, 52°C for 1 min, 70°C for 10 min and a final extension at 70 °C for 20 min.

6.2.5 Repetitive Extragenic Palindromic (REP)-PCR

REP-PCR was performed using REP primer (5'-GCG CCG ICA TGC GGC ATT-3') as previously described in Navia *et al.* (1999). PCR was carried out in a total of volume of 25 µl containing 1X buffer, 2.5 mM $MgCl_2$, 50 µM of each dNTPs, 0.6 µM of primer, 1.0 U *Taq* DNA polymerase (Promega, Madison, USA). The PCR program consisted of an initial denaturation of 4 min at 94ºC, followed by 35 cycles of 94ºC for 1 min, 42ºC for 1 min, 68ºC for 8 min and a final extension of 8 min at 72ºC .

6.2.6 *Vibrio cholerae* Repeats (VCR)-PCR

VCR-PCR was carried out as described by Tokunaga *et al.* (2010) with a minor modification in PCR condition. Briefly, the PCR mixture (25 µl) contained 1 X buffer, 2.5 mM $MgCl_2$, 50 µM of each dNTPs, 0.5 µM of primer (VCR-5' TCCCTCTTGAGGCGTTTGTTAC; VCR-3' AGCCCCTTAGGCGGGCGTTAA), 1.5 U *Taq* DNA polymerase (Promega, Madison, USA). The PCR program consisted of 25 cycles of 94ºC for 30 sec, 65ºC for 30 sec, and 72ºC for 3 min.

6.2.7 Analysis of PCR amplicons

The amplicons generated by RAPD-PCR, ERIC-PCR, REP-PCR and VCR-PCR were electrophoresed on a 1.2% (w/v) agarose gel at 90 V for 6 hours. A 1 kb DNA marker (Promega, Madison, USA) was used as the molecular size standard. The gels were stained with ethidium bromide (0.5 µg/ml) and then visualized by a Gel documentation (Bio-rad, Hercules, CA) system as described in **Chapter 3**, **Section 3.2.5**.

6.2.8 Analysis of Fingerprinting Patterns

Banding patterns generated by RAPD-PCR, ERIC-PCR, REP-PCR and VCR-PCR were analyzed with BioNumerics 6.0 (Applied Maths, Kortrijk, Belgium). All the PCR fingerprints profiles were assigned arbitrary designation. The quantitative differences among the profiles were defined by the Dice coefficient, F. Cluster analysis

was carried out based on the unweighted pair group with arithmetic averages (UPGMA) using the position tolerance of 0.15.

6.2.9 Comparison of PCR-based fingerprinting methods

The typeability, reproducibility, stability, and discriminatory power of RAPD-PCR, ERIC-PCR, REP-PCR and VCR-PCR were compared based on the fomulars described in Castaneda *et al.* (2005) and Hunter and Gaston (1988). The analysis was repeated three times on different days using freshly prepared DNA templates.

6.3 Results

6.3.1 RAPD-PCR

RAPD-PCR subtyped the 43 *V. cholerae* strains into 38 profiles (F=0.4–1.0) consisting of 14 to 24 bands ranging from 250 bp to 2000 bp (**Figure 6.1**). The banding patterns were reproducible for all except four strains which showed the same banding pattern in two out of three repeated tests. Two major clusters (RAPD1 and RAPD2) were observed at the similarity of 80% (**Figure 6.2**). RAPD1 comprised of 14 O1 *V. cholerae* strains isolated from human stool (2004 and 2008) and water (2008). One *V. cholerae* strain isolated from human stool (66/08) showed same banding pattern with two strains isolated from water (88/08 and 90/08). Four *V. cholerae* strains isolated from human stool (three isolated in 2008 and one in 2004) also shared the same profile in this cluster.

RAPD2 was composed by six non-O1/non-O139, one O1 and one O139 *V. cholerae* strains. All the strains were isolated from water, algae and seafood. Strain 6/08 (O1 serogroup) was distinctly different from all the strains with similarity less than 50%.

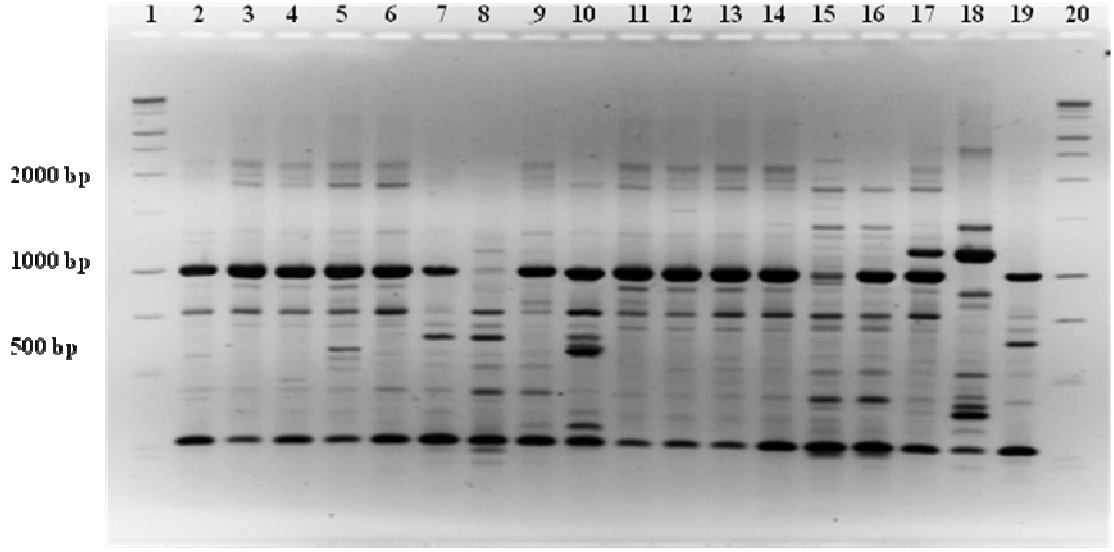


Figure 6.1 Representative agarose gels of RAPD profiles of *V. cholerae* strains using primer 1281. Lanes 1 & 20: 1kb DNA marker; Lane 2: 10/08 (O1); Lane 3: 64/08 (O1); Lane 4: 87/08 (O1); Lane 5: 1/08 (O1); Lane 6:123/08 (O1); Lane 7: 6/08 (O1); Lane 8: 85/08 (non-O1/non-O139); Lane 9: 70/08 (O1); Lane 10: 11/08 (O1); Lane 11: 37/04 (O1); Lane 12: 66/08 (O1); Lane 13: 63/08 (O1); Lane 14: 21/04 (O1); Lane 15: SW/08 (O1); Lane 16: 4370/08 (O139); Lane 17: 35/04 (O1); Lane 18: 77/08(non-O1/non-O139); Lane 19: 933/08 (non-O1/non-O139).

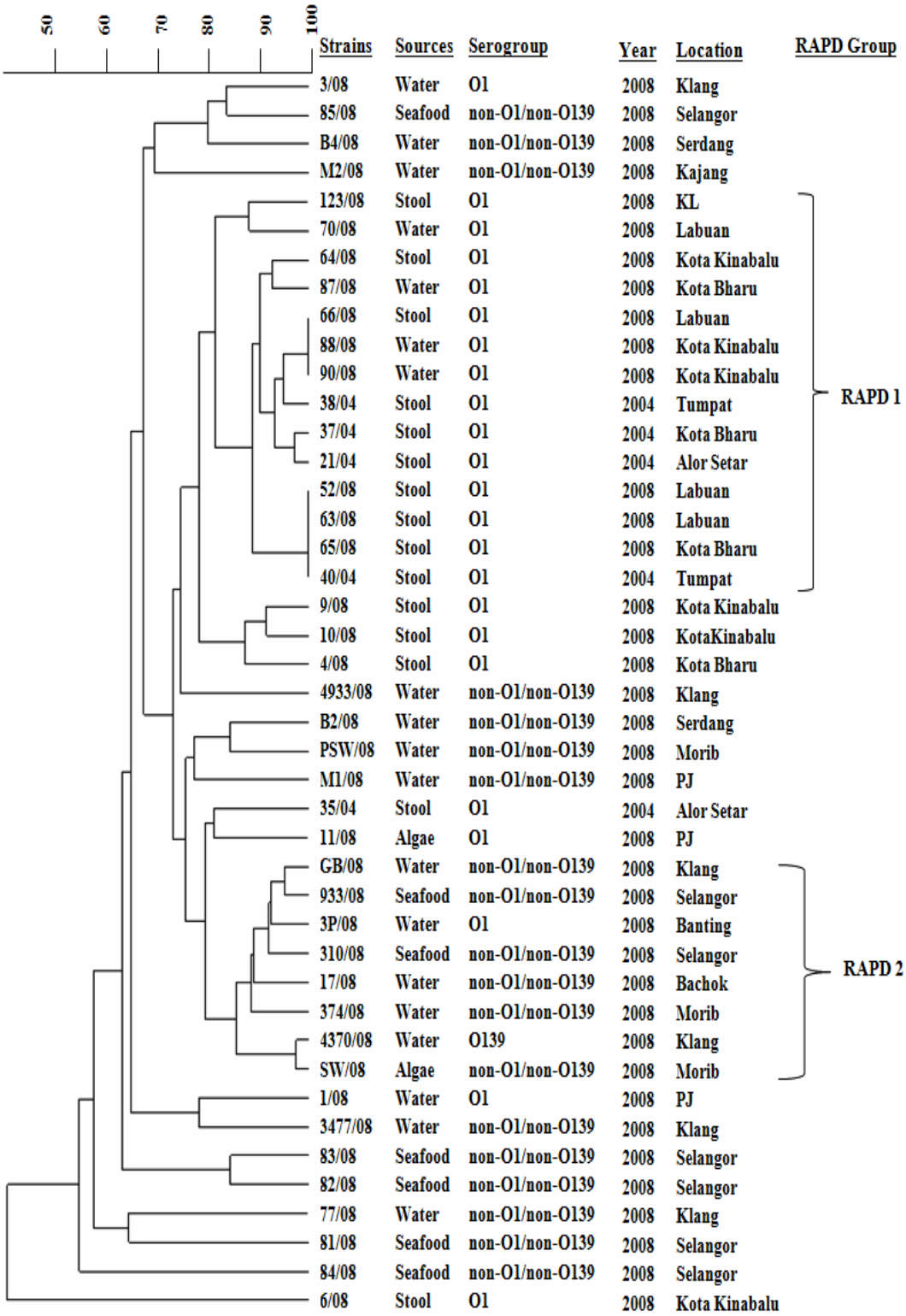


Figure 6.2 Dendrograms derived from banding patterns of RAPD-PCR for 43 *V. cholerae* strains.

6.3.2 ERIC-PCR

ERIC-PCR subtyped 43 *V. cholerae* strains into 40 profiles (F=0.67-1.0) comprising of 12-14 bands (**Figure 6.3**). The banding patterns were reproducible in three repeated tests. At the similarity of 80%, three clusters were observed (ERIC1-ERIC3) (**Figure 6.4**).

ERIC1 comprised of 11 O1 and one non-O1/non-O139 strains. The O1 *V. cholerae* strains in this cluster were isolated from algae, water and human stool samples in 2004 and 2008. The non-O1/non-O139 *V. cholerae* strain (SW/08) was isolated from algae sample and closely related (similarity of 90%) to strain 11/08 (O1 serogroup), which was also isolated from algae.

ERIC2 was the largest cluster which comprised of 12 O1 and three non-O1/non-O139 *V. cholerae* strains. All the strains in this cluster were isolated in 2008 from water, human stool, and seafood samples. In this cluster, two O1 *V. cholerae* strains (6/08 and 10/08) isolated from human stool in 2008 shared the same profile while another O1 strain isolated from water also showed same banding pattern with two *V. cholerae* isolated from human stool (9/08 and 4/08).

ERIC3 comprised of six non-O1/non-O139 strains and one O139 *V. cholerae* strain. All members of this cluster were isolated from aquatic sources in 2008 except for strain 84/08 which isolated from seafood. All the strains were from Selangor.

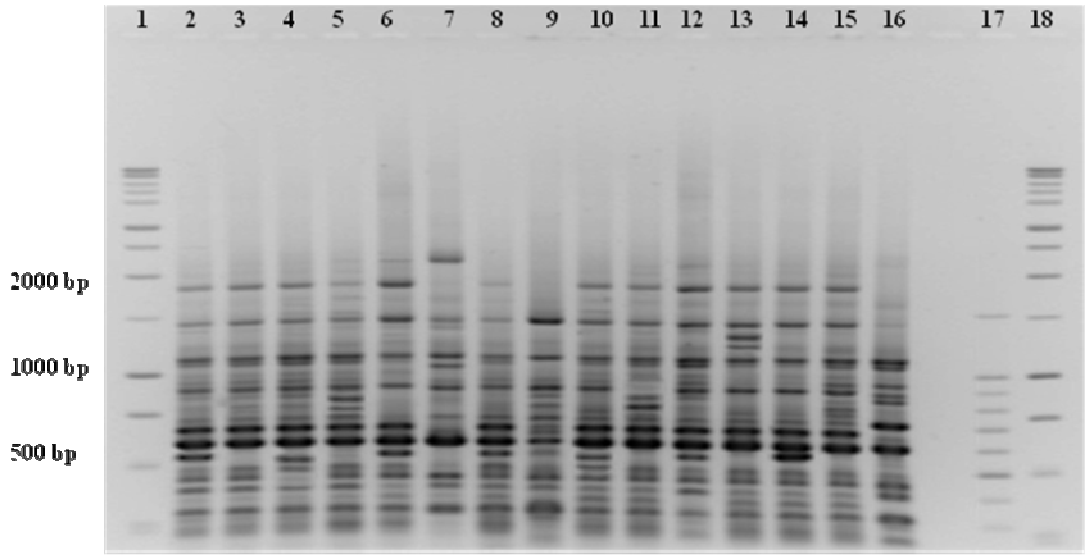


Figure 6.3 Representative agarose gels of ERIC profiles of *V. cholerae* strains using ERIC 1R and ERIC 2. Lanes 1 & 18: 1kb DNA marker; Lane 2: 52/08 (O1); Lane 3: 35/04 (O1); Lane 4: 38/04 (O1); Lane 5: 21/04 (O1); Lane 6:GB/08 (non-O1/non-O139); Lane 7: 3477/08 (non-O1/non-O139); Lane 8: 17/08 (non-O1/non-O139); Lane 9: M1/08 (non-O1/non-O139); Lane 10: 88/08 (O1); Lane 11: 4370/08 (O139); Lane 12: 63/08 (O1); Lane 13: 11/08 (O1); Lane 14: 64/08 (O1); Lane 15: 123/08 (O1); Lane 16: 83/08 (non-O1/non-O139); Lane 17: 100 bp DNA marker

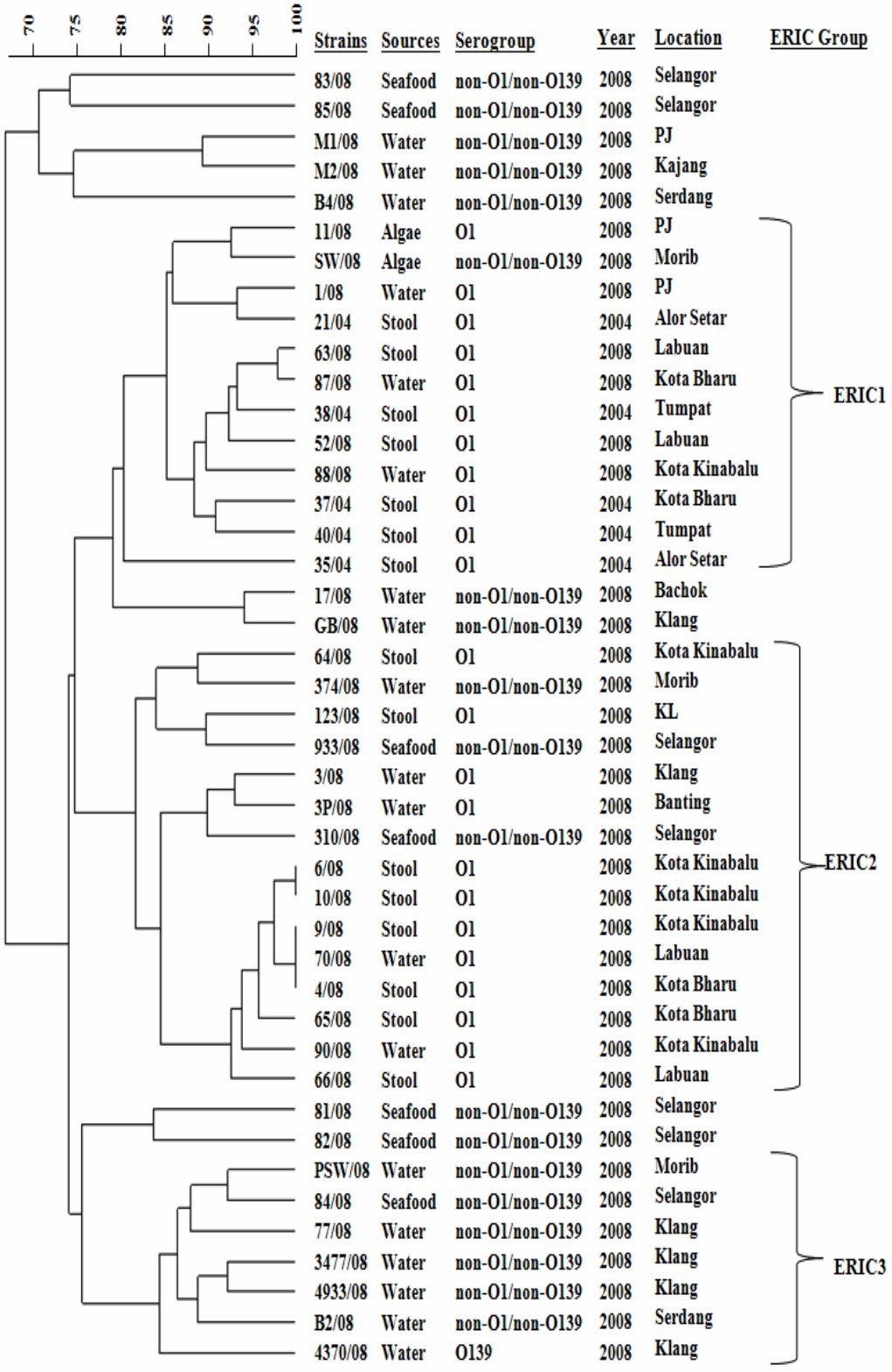


Figure 6.4 Dendrograms derived from banding patterns of ERIC-PCR for 43 *V. cholerae* strains.

6.3.3 REP-PCR

REP-PCR subtyped 43 *V. cholerae* into 35 profiles (F=0.44-1.0) comprising of 12-20 bands (**Figure 6.5**). The banding patterns were reproducible for all except two strains which showed the same banding pattern in two out of three repeated tests. The strains were grouped into four clusters (REP1-REP4) at the similarity of 80% (**Figure 6.6**).

Four non-O1/non-O139 *V. cholerae* strains which isolated from water samples were clustered with one non-O1/non-O139 *V. cholerae* isolated from seafood in REP1. All the strains were from Selangor. REP2 was composed by four non-O1/non-O139 *V. cholerae* strains. Among the four strains, two showed same banding pattern but isolated from different sources (algae and water).

REP3 was formed by nine O1 *V. cholerae* strains isolated in year 2008. There were seven profiles in this cluster. Strain 11/08 (algae) shared 100% similarity with strain 90/08 (water) while strain 9/08 (human stool) showed same banding pattern with strain 6/08 (human stool).

REP4 also comprised of nine O1 *V. cholerae*. The strains in this cluster were either isolated in 2004 or 2008 from human stool, except for strain 87/08 which isolated from water. Although not isolated in the same year, both strains 40/04 (human stool) and 65/08 (human stool) were identical (100% similarity). Strain 87/08 which isolated from water was also 100% similar to strains 63/08 and 64/08 which isolated form human stool.

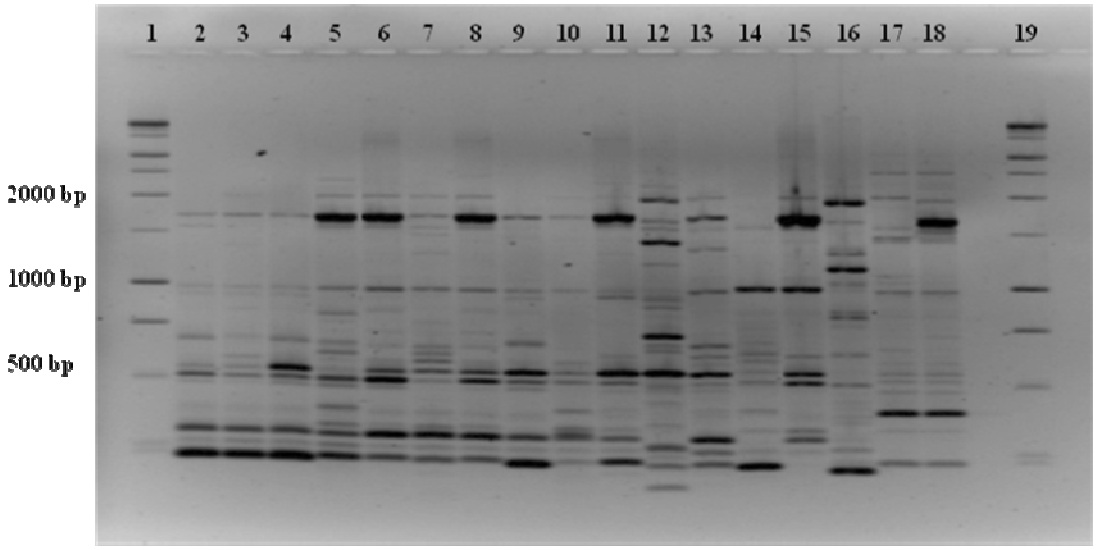


Figure 6.5 Representative agarose gels of REP profiles of *V. cholerae* strains using primer REP. Lanes 1 & 20: 1kb DNA marker; Lane 2: 52/08 (O1); Lane 3: 1/08 (O1); Lane 4: 90/08 (O1); Lane 5: 35/04 (O1); Lane 6:10/08 (O1); Lane 7: 40/04 (O1); Lane 8: 123/08 (O1); Lane 9: 38/04 (O1); Lane 10: 3P/08 (O1); Lane 11: 63/08 (O1); Lane 12: 4933/08 (non-O1/non-O139); Lane 13: 4370/08 (O139); Lane 14: 3477/08 (non-O1/non-O139); Lane 15: 81/08 (non-O1/non-O139); Lane 16: B2/08 (non-O1/non-O139); Lane 17: 374/08 (non-O1/non-O139); Lane 18: 933/08 (non-O1/non-O139).

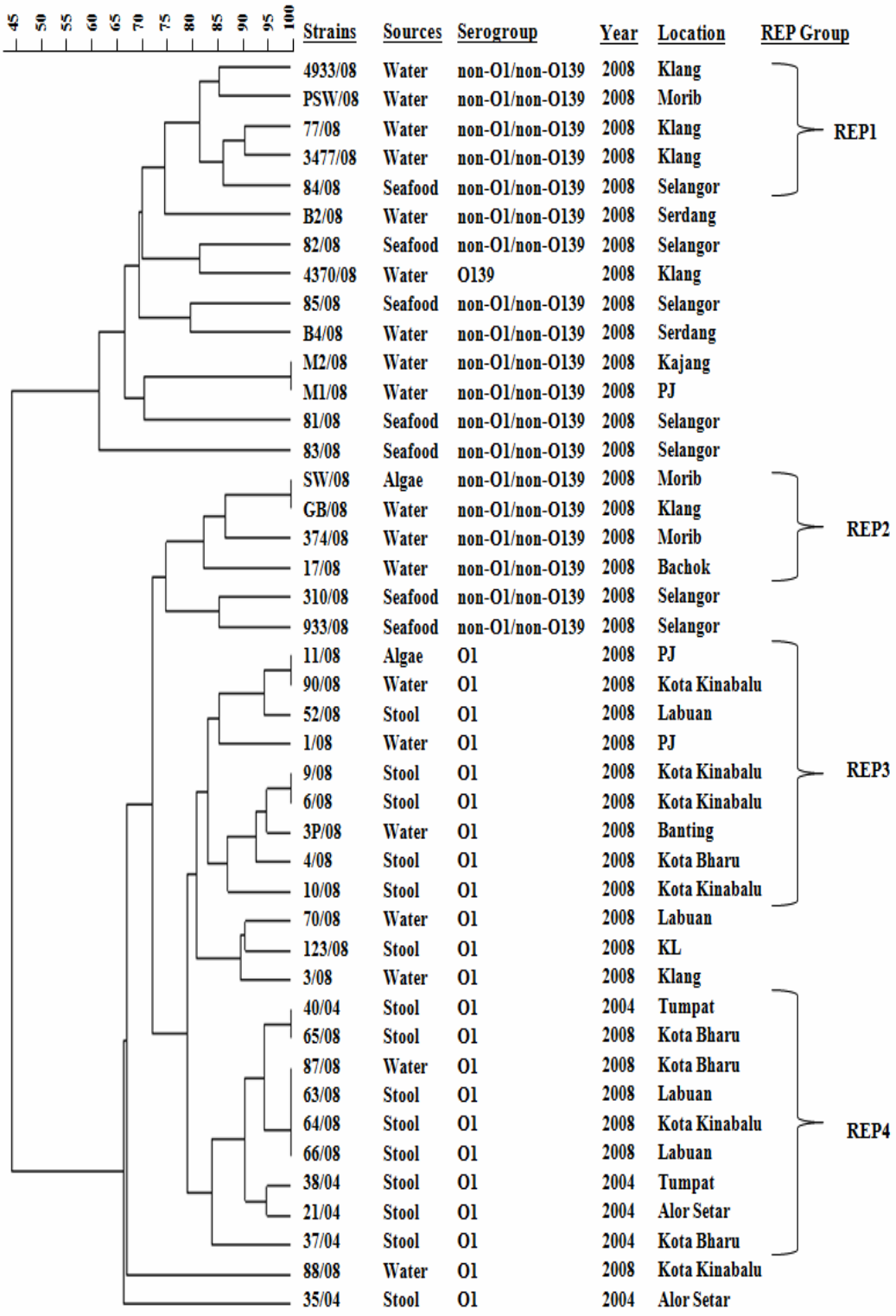


Figure 6.6 Dendrograms derived from banding patterns of REP-PCR for 43 *V. cholerae* strains.

6.3.4 VCR-PCR

VCR-PCR gave reproducible results in three repeated tests and subtyped 43 strains into 31 profiles (F=0.56-1.0) (**Figure 6.7**). Based on the similarity of 80%, all the 43 *V. cholerae* strains were grouped according to their serogroups (O1 and non-O1/non-O139) and three clusters were obtained (VCR1-VCR3) (**Figure 6.8**). VCR1 and VCR3 were composed by non-O1/non-O139 strains while all O1 strains were clustered in VCR 2.

VCR1 was the largest cluster for non-O1/non-O139 *V. cholerae* (n=10) with eight profiles obtained. In this cluster, strain 81/08 and 82/08 (both form seafood) shared 100% similarity while strain M1/08 and strain M2/08 (both from water) were also 100% related. All the strains were isolated around Selangor.

VCR2 comprised of 23 O1 *V. cholerae* isolated from various sources, such as human stool, water and algae samples. Thirteen profiles were obtained in this cluster with four profiles shared by at least two strains:

1. Six strains isolated from human stool in 2004 (21/04, 40/04 and 37/04) and 2008 (52/08, 63/08 and 65/08) shared the same profile.
2. Two clinical strains isolated in 2004 (38/04) and 2008 (64/08) were 100% similar.
3. Strain 66/08 isolated from human stool was 100% similar to strains 90/08 and 88/08 from water sample. All strains were from East Malaysia.
4. Strains 70/08 (water), 11/08 (algae) and 123/08 (human stool) from different sources shared same profile.

Five non-O1/non-O139 *V. cholerae* strains were clustered in VCR3. All the strains were isolated from water samples.

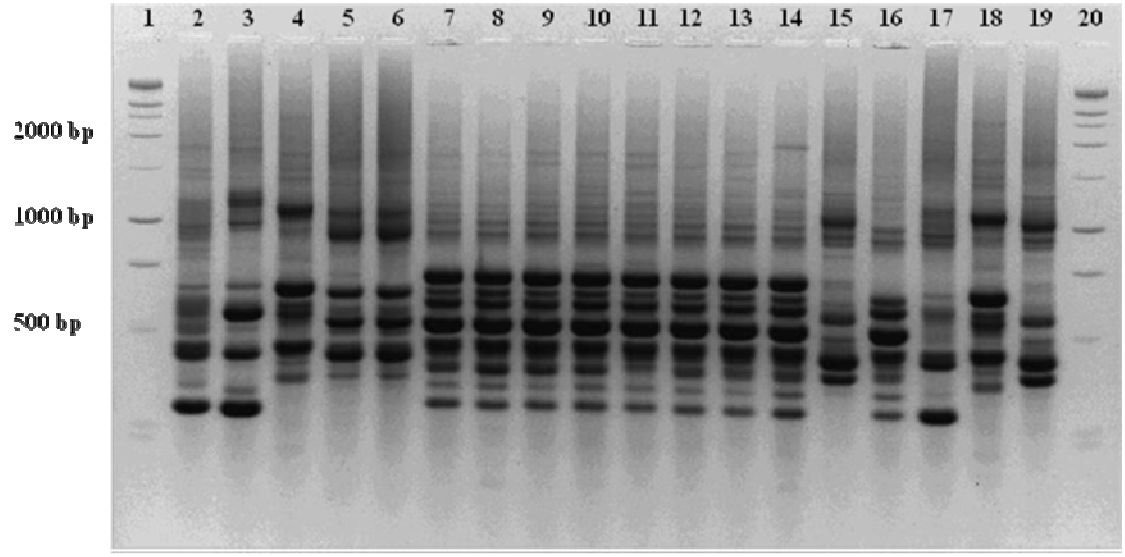


Figure 6.7 Representative agarose gels of VCR profiles of *V. cholerae* strains using primer VCR. Lanes 1 & 20: 1kb DNA marker; Lane 2: PSW/08 (non-O1/non-O139); Lane 3: B2/08 (non-O1/non-O139); Lane 4:83/08 (non-O1/non-O139); Lane 5: 10/08 (O1); Lane 6: 1/08 (O1); Lane 7: 123/08 (O1); Lane 8: 3P/08(O1); Lane 9: 11/08 (O1); Lane 10: 70/08 (O1); Lane 11: 4/08 (O1); Lane 12: 38/04 (O1); Lane 13: 37/04 (O1); Lane 14: 35/04 (O1); Lane 15: 4370/08 (O139); Lane 16: 933/08 (non-O1/non-O139); Lane 17:SW/08 (non-O1/non-O139); Lane 18: 374/08 (non-O1/non-O139); Lane 19: B2/08 (non-O1/non-O139).

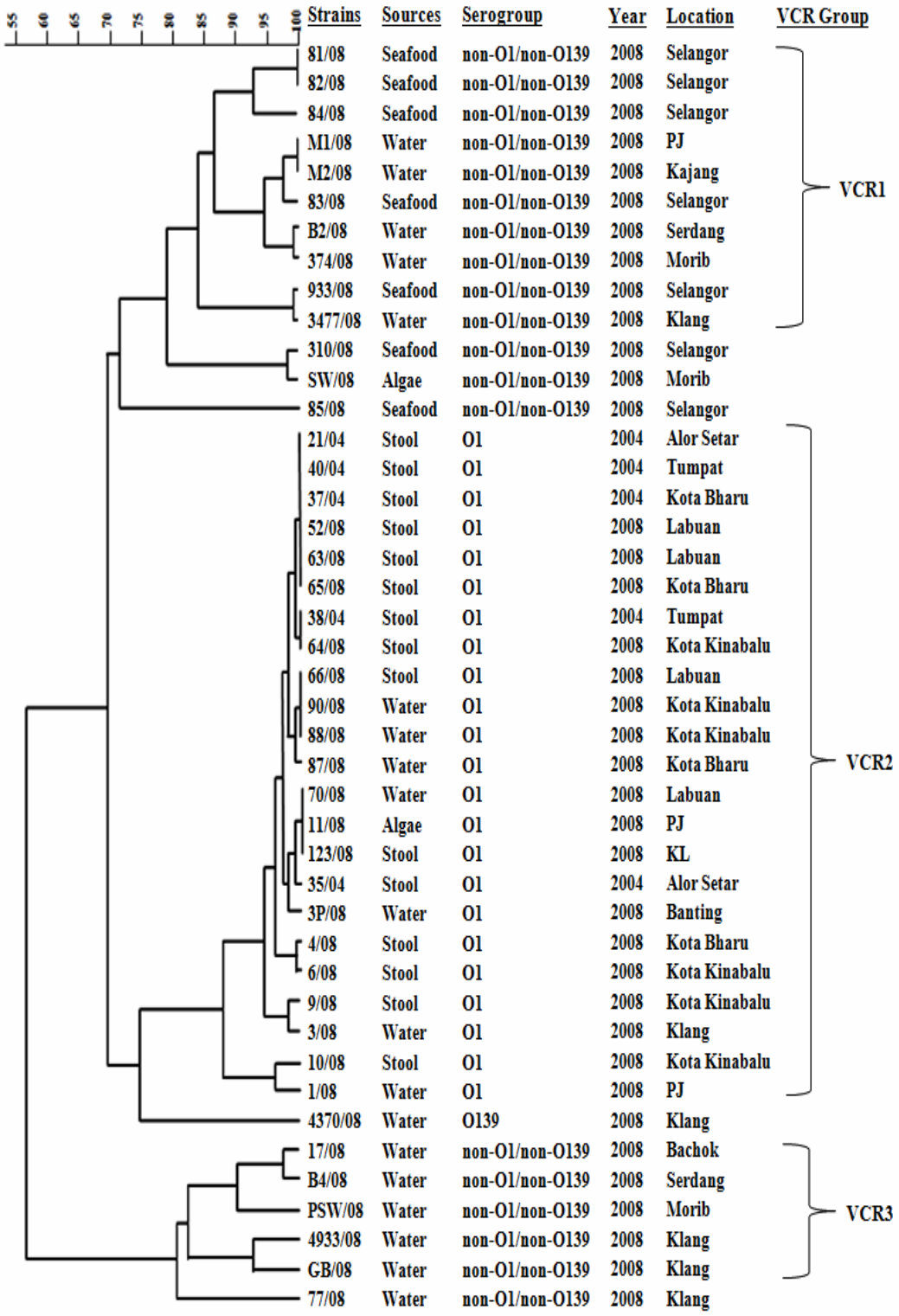


Figure 6.8 Dendrograms derived from banding patterns of VCR-PCR for 43 Malaysian *V. cholerae* strains.

6.3.5 Comparison of the methods based on typeability, reproducibility, stability and discriminatory power

ERIC-PCR scored the highest (0.93) in typeability, followed by RAPD-PCR (0.88), REP-PCR (0.81) and VCR-PCR (0.72). For reproducibility test, ERIC-PCR and VCR-PCR correctly assigned the 43 strains into same profiles on three repeated tests (1.0) while RAPD-PCR (0.9) and REP-PCR (0.95) were less reproducibile.

Due to the consistency in banding pattern, ERIC-PCR and VCR-PCR also scored 1.0 in stability while both RAPD-PCR and REP-PCR scored 0.6 only. Among the four approaches, ERIC-PCR was the most discriminative (0.996), followed by RAPD-PCR (0.990), REP-PCR (0.988) and VCR-PCR (0.973).

The typeability, reproducibility, stability and discriminatory power of the four approaches are summarized in **Table 6.1**.

Table 6.1: Comparison of RAPD-PCR, ERIC-PCR, REP-PCR, and VCR-PCR based on typeability, reproducibility, stability and discriminatory power.

	Typeability* $T= N_t /N$	Reproducibility* $R= N_r /N$	Stability* $S= N_s /N$	Discriminatory power** $D= 1 - \frac{1}{N(N-1)} \sum_{j=1}^{S} n_j (n_j-1)$
RAPD-PCR	0.88	0.90 (n=39)	0.6 (2/3)	0.990
ERIC-PCR	0.93	1.00 (n=43)	1.00 (3/3)	0.996
REP-PCR	0.81	0.95 (n=41)	0.6 (2/3)	0.988
VCR-PCR	0.72	1.00 (n=43)	1.00 (3/3)	0.973

N_t , number of strains assigned to a profile; N, number of strains; N_r , number of strains assigned as the same profile on repeated testing; N_s , number of tests in which the same strains were correctly assigned to the same profile on repeated testing; S , number of different profile; n_j , number of strains belong to the $_j$th type

* Formula obtained from Castaneda *et al.* (2005). ** Formula obtained from Hunter and Gaston (1988).

6.4 Discussion

Rapid identification of geographical or historical origin of the strains is very important to avoid prolonged public health problem (van Belkum *et al.*, 2001). Four PCR-based methods were evaluated and applied to determine the relatedness of the *V. cholerae* strains in this study. The total time required for all the PCR analysis including agarose gel electrophoresis were less than 14 hours.

Overall, VCR-PCR was useful to differentiate strains of non-O1/non-O139 from the O1 and O139 strains. Therefore, this method is useful to categorize O1, O139 and non-O1/non-O139 *V. cholerae* serogroups. In the study of Tokunaga *et al.* (2010), all O139 strains formed a homogenous genotype regardless of the geographic origin or time of isolation. However, only one strain isolated in this study, therefore the comparison of types for O139 could not be performed.

Besides VCR-PCR, REP-PCR also showed distinct clustering between O1 and non-O1/non-O139 *V. cholerae*. Among the four clusters obtained in REP-PCR analysis, REP1 and REP2 were comprised of non-O1/non-O139 *V. cholerae* isolated from aquatic environment while REP4 consisted of strains isolated from human stool (except for 87/08). This suggests that the genetic elements analyzed in REP-PCR were possibly associated with the key process which affects the population dynamics and the ecosystem in its living environment.

RAPD-PCR and ERIC-PCR concordantly showed clustering of three non-O1/non-O139 *V. cholerae* (374/08, 310/08, and 933/08) with O1 strains. Although REP-PCR and VCR-PCR had distinguished the 43 strains into clusters according to the serogroups, both analyses showed that strains 374/08, 310/08 and 933/08 were more

related to O1 *V. cholerae* compared to other non-O1/non-O139 strains. This observation is also in concordance with Li et al (2002) that there were some genetic elements shared between O1 and non-O1/non-O139 *V. cholerae*. All the methods concordantly clustered six O1 strains (40/04, 37/04, 38/04, 21/04, 63/08 and 87/08) together. Besides strains 63/08 and 87/08, all were isolated in 2004. This suggests that there is conserved genetic elements shared by 2004 strains and the conserved region in the genome might be descended in strains 63/08 and 87/08.

The generated dendrograms based on RAPD-PCR, ERIC-PCR and REP-PCR showed that the O139 strain was closely related with strains of non-O1/non-O139 serogroup. However, the O139 strain was distinguished from other O1 and non-O1/non-O139 strains by VCR-PCR analysis and was more related to O1 strains. Hence, this particular O139 strain which was isolated from aquatic environment could be the result of horizontal gene transfer of O1 antigen biosynthesis and housekeeping genes of O1 El Tor strains into non-O1/non-O139 environmental strain. This observation is in agreement with Faruque *et al.* (2004) and Singh *et al.*, (2001) who described the derivation of O139 strain from O1 and environmental non-O1/non-O139 strains.

There were 19 non-O1/non-O139 *V. cholerae* strains in this study. All the strains were isolated from environmental samples collected around Selangor, except for 17/08 which was from Bachok, Kelantan. Hence, it is not significant to show the relatedness of non-O1/non-O139 *V. cholerae* based on the geographical variation. Moreover, all the four approaches failed to show the geographical relatedness of O1 *V. cholerae* strains. A genetic relatedness study of non-O1/non-O139 *V. cholerae* was carried out by Chakraborty *et al.*, (2001). A wide genetic variation with no correlation

within the strains of non-O1/non-O139 *V. cholerae* was observed. This is in concurred with our findings on the relatedness of non-O1/non-O139 *V. cholerae* in this study.

Overall, all the four methods gave comparable discriminatory power to distinguish *V. cholerae* strains (>0.90). Although RAPD-PCR and REP-PCR showed higher discriminatory power than VCR-PCR, both were not reproducible in certain cases as shown in this study. Based on the criteria evaluated (typeability, reproducibility, stability and discriminatory power), ERIC-PCR was the best PCR-based fingerprinting method for discriminating purpose. VCR-PCR scored the least in typeability, and discriminatory power but it might be more useful for differentiating serogroups of *V. cholerae*.

In conclusion, there were six O1 *V. cholerae* strains which might be closely related while only four non-O1/non-O139 strains were more related to the O1 strains. The O139 strain was genetically related to both O1 and non-O1/non-O139 *V. cholerae.*

Chapter Seven

Development of Multiple-locus variable-number of tandem repeat analysis (MLVA) for relatedness study of *Vibrio cholerae* in comparison with Pulsed-field Gel Electrophoresis (PFGE)

7.1 Background

Molecular subtyping of pathogen is important for tracing a new or previously found virulent or multidrug-resistant clone (van Belkum *et al.*, 2007). Genomic variation and epidemiological study for different serogroups of *V. cholerae* have been carried out using many DNA-fingerprinting tools. Pulsed-filed gel electrophoresis (PFGE) is the most common subtyping method to define strains from outbreaks and from sporadic cases of cholera as it has the highest discriminatory ability (Chen *et al.*, 2004). However, PFGE is time consuming and requires strict adherence to standardize protocols for inter-laboratory 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 (van Belkum, 2007). Six polymorphic variable number of tandem repeats (VNTR) loci were used to evaluate the polymorphism of *V. cholerae* isolated worldwide by Olsen *et al.* (2009) and a high discriminatory power of this method in defining the strains was desmonstrated. Besides, Choi *et al.* (2010) also used MLVA to characterize 74 hybrid strains into three groups which were distant from the seventh pandemic El Tor strains. The MLVA studies carried out was either more discriminative or comparable to PFGE. However, little is known about the ability of MLVA to subtype non-O1/non-O139 *V. cholerae*. Moreover,

based on the *V. cholerae* tandem repeat database (http://minisatellites.u-psud.fr/), there are numbers of loci which remain uninvestigated.

The objective of the study was to develop a MLVA assay for relatedness study of *V. cholerae*. A comparison of discriminatory ability between MLVA and PFGE assays for genotyping of *V. cholerae* strains from different serogroups was performed.

7.2 Materials and Methods

7.2.1 Bacterial Strains

Strains used in this Chapter were previously described in **Chapter 5** and listed in **Appendix A**.

7.2.2 DNA preparation for PCR

DNA preparation was carried out as described in **Chapter 3**, **Section 3.2.3**.

7.2.3 VNTR 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 ~500 bp sequence covering the flanking and tandem repeat regions retrieved from the database. Seven loci were selected based on

the unit length, copy number and percentage matches stated in the database. Primer 3 software (Rozen and Skaletsky 2000) was used to design the primers.

7.2.4 PCR and capillary electrophoresis

Primers labeled with fluorescent dyes in the 5'-end for the VNTRs were synthesized and used for PCR. The multiplex PCR mixture was prepared in a total volume of 25 µl contained 1X PCR buffer, 2 mM $MgCl_2$, 160 µM dNTPs, 0.4 µM each primers, 2 µl of *Taq* DNA Polymerase (Promega, Madison, USA) and ~50 ng of DNA template. The multiplex PCR was run at 95°C for 5 min (1 cycle); 95°C for 30 sec, 58°C for 30 sec, 72°C for 50 sec (30 cycles); 72°C for 7 min (1 cycle).

Preparation of sample for loading into the ABI prism® 3130xl Genetic Analyzer (Applied Biosystems, Carlsbad, CA) was based on the protocol obtained from MLVA.net (http://www.mlva.net/bpertussis/documents/Protocol_MLVA) with minor modification. In brief, the PCR product was then diluted in 1:100 ratios with ddH_2O. One microlitre of the dilution was mixed with 10 µl of HiDi formamide and 0.2 µl of GeneScan™ 500 LIZ size standard (Applied Biosystems, Carlsbad, CA). The mixture was boiled at 95°C for 5 min and analyzed with Genetic Analyzer (Applied Biosystems, Carlsbad, CA). The amplicons were identified based on the peak height and colour while the size was determined with the GenScan™ size standard using GeneMapper software (Applied Biosystems, Carlsbad, CA). Further optimization was carried out based on the peak generated in the electropherogram. The optimized condition were used and applied on all *V. cholerae* strains.

7.2.5 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. Cluster analysis was carried out based on the unweighted pair group method with arithmetic averages (UPGMA). The discrimination power of each VNTR was calculated individually and combined using fomular described by Hunter and Gaston (1988) as previously mentioned in **Chapter 6**.

7.2.6 Pulsed-Field Gel Electrophoresis (PFGE)

PFGE was performed according to established protocol from CDC PulseNet, (2009). Briefly, the growth on agar plate was transferred to 2 ml of cell suspension buffer (CSB) and the cell density was adjusted to OD_{610} = 0.9. An aliquout of 100 µl of standardized cell suspension buffer was then transferred to a 1.5 ml microfuge tube. Two µl of Proteinase K (20 mg/ml stock solution) was added to the cell suspension. Then, 100 µl of 1% Seakem Gold agarose (Cambrex Bio Science Rockland, Inc, USA) were mixed with 100 µl of the cell suspension. Immediately, the mixture of agarose and cell suspension was dispensed into the well of plug mold to form plugs. The plugs were allowed to solidify in refrigerator (4°C) for 5 min. The bacterial cells were lysed within the plugs with cell lysis buffer (50 mM Tris: 50 mM EDTA [pH 8.0] + 1% Sacrosine + 1 mg/ml proteinase K) and incubated at 54ºC for 3 h. 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, USA) at 37ºC. 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 h with pulse times of 2-10 s and 6 h 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. Discriminatory index (DI) was calculated as described by Hunter and Gaston (1988) as previously mentioned in **Chapter 6**.

7.3 Result

7.3.1 MLVA

Two multiplex PCR were performed: VCTR1-FAM, VCTR2-VIC, VCTR3-PET, VCTR4-NED (Multiplex 1); VCTR5-FAM, VCTR6–VIC, and VCTR7-NED. (Multiplex 2). The initial PCR performed showed satisfactory peaks of amplicons in the electropherogram and no further optimization was needed.

By using capillary electrophoresis, the sizes of DNA fragments could be determined more accurately based on the peaks (**Figure 7.1** and **Figure 7.2**) compared to the gel-based determination of band size. Overall, MLVA showed high polymorphism among the 43 *V. cholerae* strains based on the size distributions of the for each VNTR: 212-230 bp (VCTR1), 216-252 bp (VCTR2), 218-276 bp (VCTR3), 278-300 bp (VCTR4), 228-251bp (VCTR5), 211-269 bp (VCTR6) and 328-369 bp (VCTR7) (**Table 7.2**).

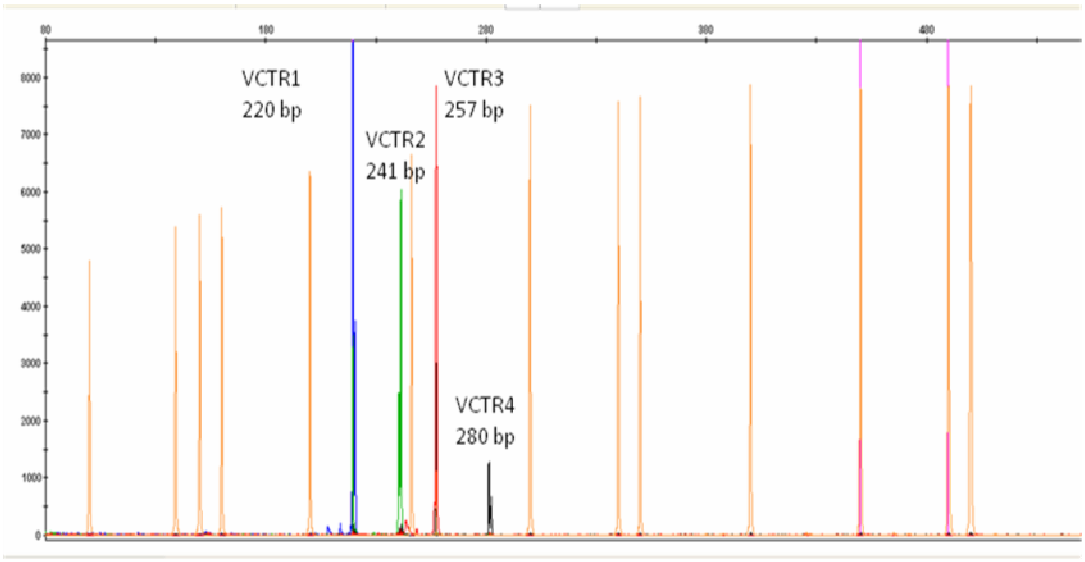


Figure 7.1 Electropherogram showing separation of four fragments for *V. cholerae* O1 isolates (52/08) from multiplex 1 defined by the height and colours of the peaks.

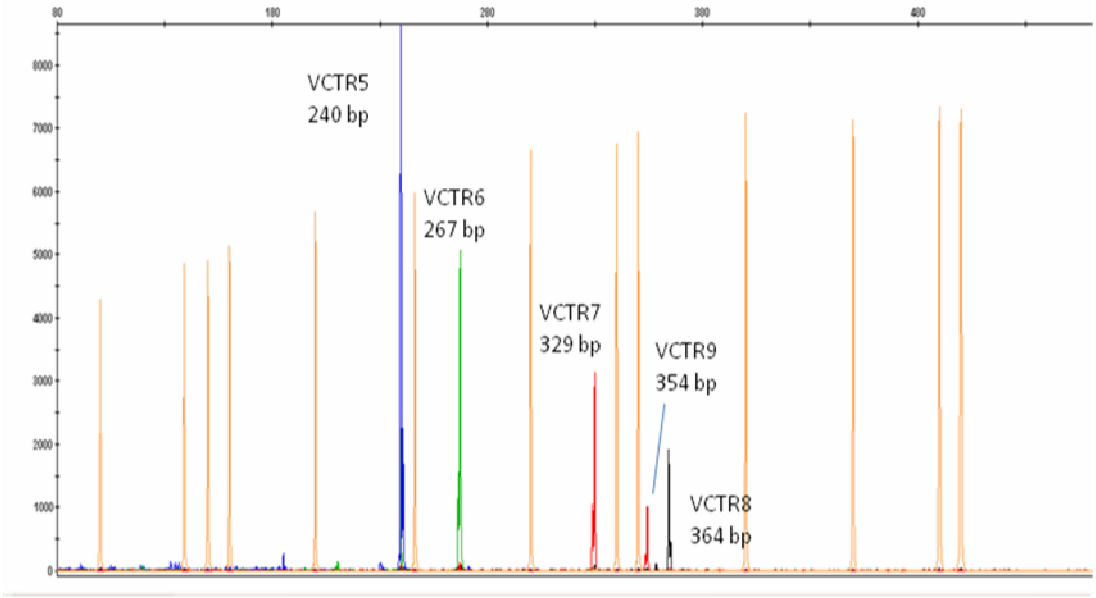


Figure 7.2 Electropherogram showing separation of four fragments for *V. cholerae* O1 isolates (52/08) from multiplex 2 defined by the height and colours of the peaks.

Table 7.1: Characteristics of *Vibrio cholerae* VNTR loci in the MLVA study.

Character	Primers sequence	Size of fragment (bp)	VNTR position (kb)	Alleles	Discriminatory ability
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* genome

7.3.2 Application of MLVA on *Vibrio cholerae* strains

Thirty-eight MLVA profiles (F=0.63) were obtained based on the character types (allelic profiles). The 43 strains were grouped into 3 major clusters (MLVA1-MLVA3) in the MST (**Figure 7.3**).

The first cluster (MLVA1) comprised of 13 O1 strains (eight clinical, five environmental) (90.6% similarity). In this cluster, five of the O1 strains shared the same profile (M1: 09-08-09-12-05-02-23). These strains (4/08, 52/08, 63/08, 65/08, and 66/08) were isolated in Kota Bahru, West Malaysia (n=2) and Labuan, East Malaysia (n=3) in 2008.

MLVA2 consisted of 10 O1 strains, one non-O1/non-O139 strain and one O139 strain. In this cluster, four strains (21/04, 37/04, 38/04, 40/04) isolated in 2004 from human stools were highly similar except for one to two alleles difference. Strain 40/04 (O1 clinical) have two and three alleles difference from strains 11/08 (environmental O1) and 4370/08 (environmental O139), respectively. Strain PSW/08 (environmental non-O1/non-O139) also has two different alleles from strain 11/08 (environmental O1). There was 95% similarity among the O1 and O139 strains.

MLVA3 comprised of non-O1/non-O139 strains with 83.3% similarity. All the non-O1/non-O139 *V. cholerae* strains were from Selangor, except for strain 17/08 which was isolated from water sample collected in Bachok, Kelantan.

Discrimnatory ability of each individual VCTR ranged from 0.59-0.92 (**Table 7.2**) while the discriminatory index for the combined VCTR loci was 0.988.

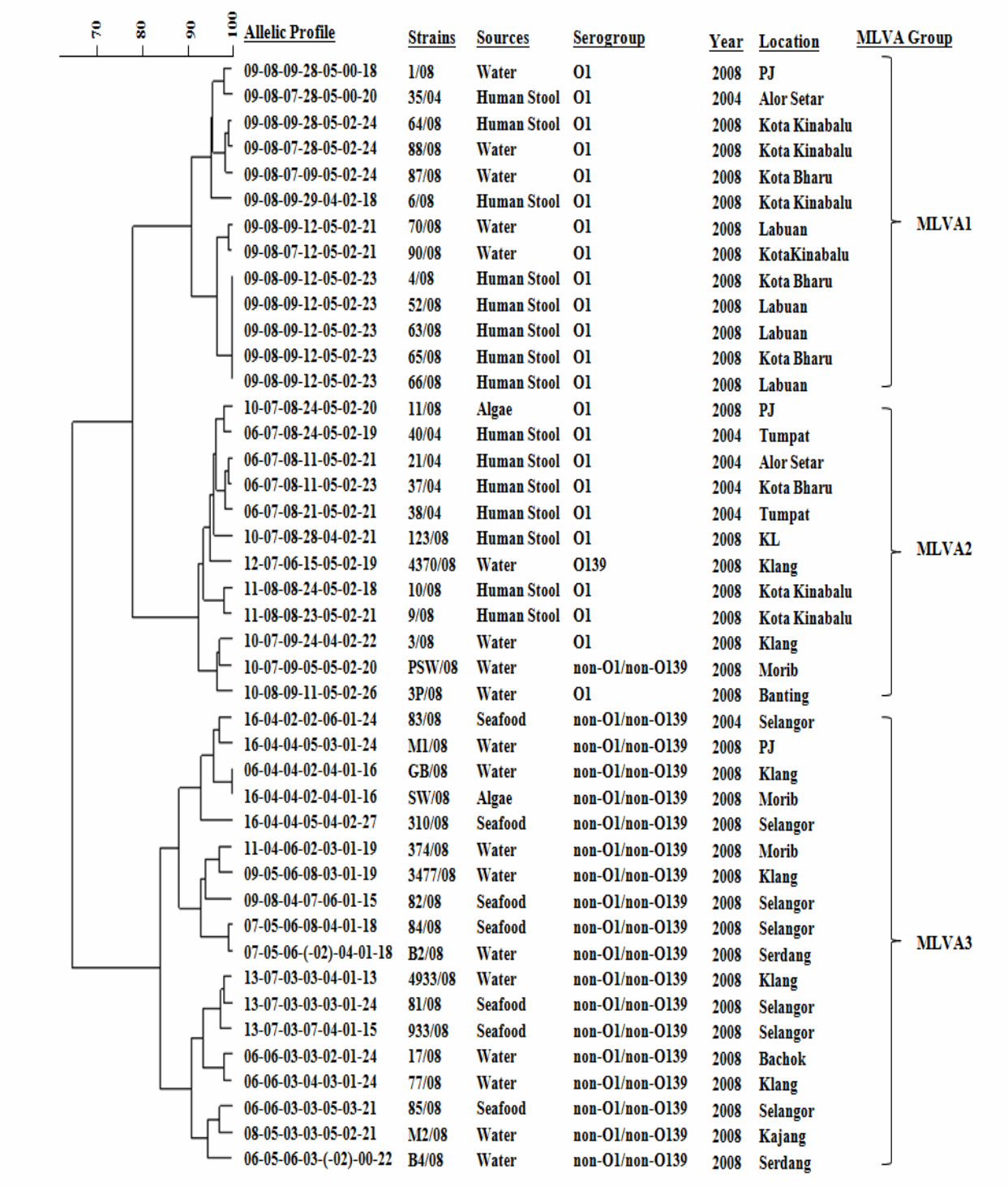


Figure 7.3 Dendrogram derived from the 38 MLVA profiles identified among 43 *V. cholerae*. The strains were well differentiated based on serogroups.

7.3.3 PFGE Analysis

PFGE of *Not* I-digested chromosomal DNA yielded 15-22 fragments ranging from 40 to 400 kbp (**Figure 7.4**). Thirty-five pulsotypes (F= 0.71) were observed. At a similarity of 80%, one major cluster was observed, comprised of all O1, one O139 and seven non-O1/non-O139 strains. Twenty-four profiles were obtained in this cluster with five profiles shared by at least two strains:

1. Both 933/08 (seafood) and 77/08 (water) of non-O1/non-O139 serogroup were 100 % similar.

2 Strains isolated from human stool in 2008 (10/08 and 6/08) showed same banding pattern. Both strains were from Kota Kinabalu.

3. Strain 66/08 isolated from human stool was 100% to strains 87/08, 88/08 and 90/08 from water sample.
4. Strain 64/08 (human stool) showed same banding pattern with strain 4/08 (human stool).

The non-O1/non-O139 *V. cholerae* strains were heterogenous based on PFGE analysis (**Figure 7.5**). The discriminatory index for PFGE was 0.989.

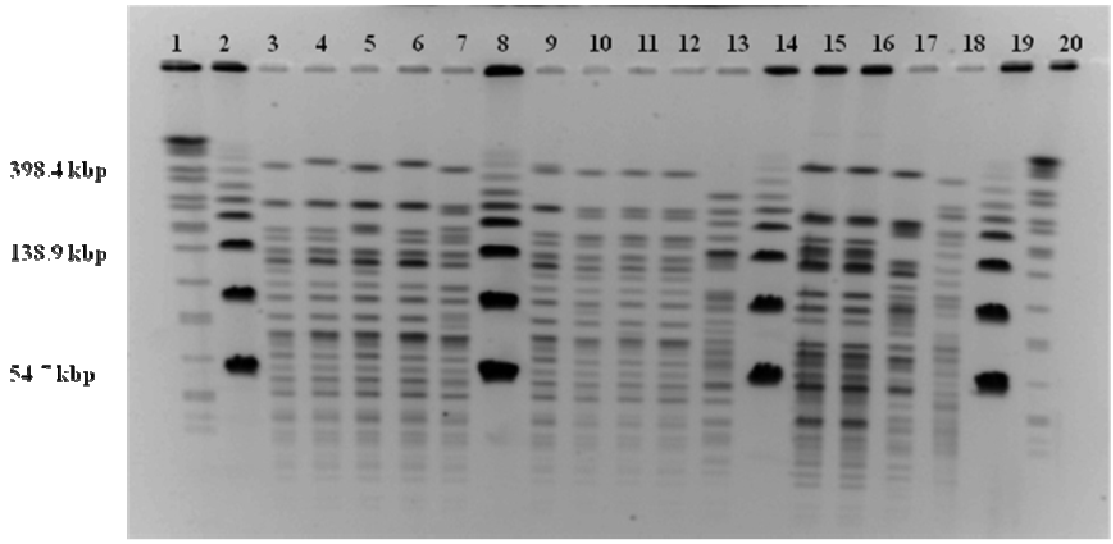


Figure 7.4 *Not*I-PFGE profiles of representative *V. cholerae* strains. Lanes 1 & 20: *Xba*lI-digested *S.* Braenderup H9812standard; Lanes 2, 8, 14 &19: Lamda marker; Lane 3: 4/08 (O1); Lane 4: 6/08 (O1); Lane 5: 9/08 (O1); Lane 6: 10/08 (O1); Lane 7: 123/08 (O1); Lane 9: 70/08(O1); Lane 10: 1/08 (O1); Lane 11: 11/08 (O1); Lane 12: 3P/08 (O1); Lane 13: 17/08 (non-O1/non-O139); Lane 15: GB/08 (non-O1/non-O139); Lane 16: SW/08 (non-O1/non-O139); Lane 17: 310/08 (non-O1/non-O139); Lane 18: 933/08 (non-O1/non-O139).

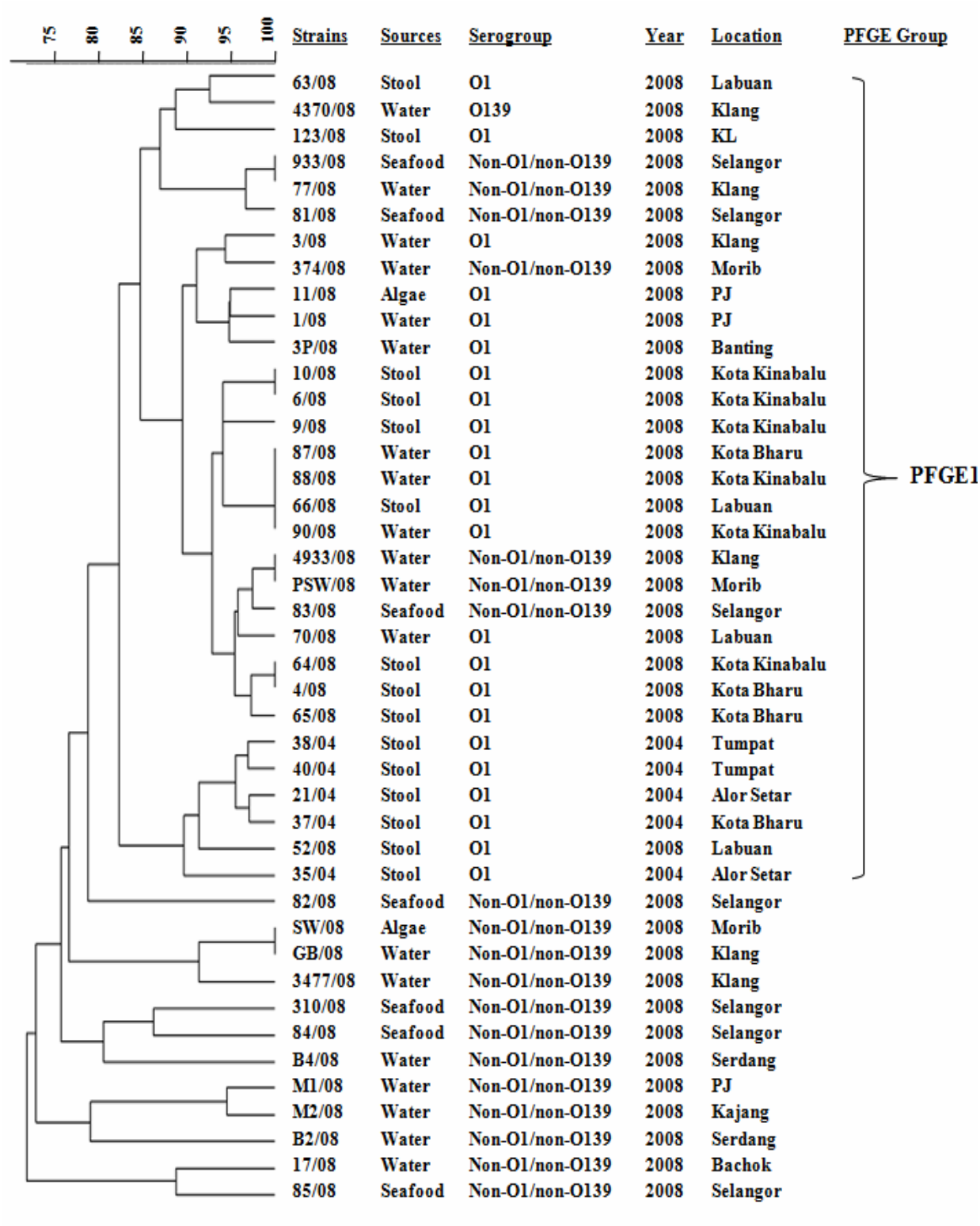


Figure 7.5 Dendrogram generated using PFGE profiles showing the relatedness of 43 *V. cholerae* strains.

7.4 Discussion

The MLVA assay was developed based on the seven 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 suggests that there is a higher genetic variation in chromosome II of *V. cholerae* genome compared with chromosome I. Danin-Poleg *et al.* (2007) and Olsen *et al.* (2009) 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 this 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.

In addition, VCTR4 was also able to differentiate the O1 and non-O1/non-O139 *V. cholerae* strains where O1 *V. cholerae* strains exhibited more copy numbers in VCTR4 than non-O1/non-O139 *V. cholerae* strains. Nine to 28 copy numbers of tandem repeats were observed for O1 *V. cholerae* strains while two to eight copy numbers of tandem repeats observed for non-O1/non-O139 *V. cholerae* strains. Therefore, it is indispensable to emphasize the important role of this locus for its high discriminative value and also its ability for serogroups differentiation.

Both the PFGE and MLVA data showed that the *V. cholerae* O139 strain (4370/08) was closely related with an O1 strain with similarity of more than 90%. Strains GB/08 and SW/08, isolated from river water and algae, respectively, were indistinguishable by both PFGE and MLVA. The result suggests that both river water and algae were probably contaminated with the same clone of non-O1/non-O139 *V. cholerae* based on the definition of relatedness among the strains by van Belkum *et al.* (2007).

With the exception on one strain (35/04), most of the 2004 *V. cholerae* O1 strains were very similar with only 1-2 alleles' difference and were different from the 2008 strains in MLVA analysis. Similarly, PFGE also showed a closer relationship (94%) between the 4 2004 *V. cholerae* O1 strains compared to 35/04.

The strains which belonged to the same PFGE profile were distinguishable by MLVA. However, some strains with same MLVA profiles could be further distinguished by PFGE. 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, five strains (4/08, 52/08, 63/08, 65/08 and 66/08) were indistinguishable by MLVA as they shared identical allelic profile. However, PFGE was able to separate two of Kota Bahru outbreak strains (4/08 and 65/08) (West Malaysia) from the Labuan outbreak strains in East Malaysia. In addition, three environmental strains (87/08, 88/08, and 90/08) and one clinical strain (66/08) had identical pulsotype. This is in agreement with Chakraborty *et al.* (2001) that it is very difficult to differentiate whether an isolate was deposited in the aquatic environment by infected individual or whether from aquatic environment to man. However, these strains (66/08, 87/08, 88/08, and 90/08) were distinguishable by MLVA in this study.

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. 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 conclusion, a MLVA assay was developed in this study and was useful to study the relatedness of *V. cholerae*. The discriminatory ability of MLVA was comparable with PFGE. Overall, MLVA and PFGE concurred that all strains isolated in 2004 were closely related, except for strain 35/04. The O139 strain was closely related to O1 *V. cholerae* and two non-O1/non-O139 *V. cholerae* (GB/08 and SW/08) were clonally related. In addition, combination of MLVA and PFGE analyses may provide more information of the relatedness of strains and to distinguish strains from different sources and geographical regions.

Chapter Eight

Multilocus Sequencing Typing (MLST) and Multi-virulence-locus Sequence Typing (MVLST) Assays for Gene Variation Study of *Vibrio cholerae*

8.1 Background

Strain typing and phylogeny study of *V. cholerae* using sequencing analysis such as Multilocus Sequencing Typing (MLST) assay has been widely used in addition to other gel-based fingerprint methods such as random amplified polymorphic DNA (RAPD)-PCR, enterobacterial repetitive intergenic consensus sequence (ERIC)-PCR, repetitive extragenic palindromic (REP)-PCR, Pulsed-field Gel Electrophoresis (PFGE) and BOX-PCR (van Belkum *et al.,* 2007). MLST was originally described to determine the nucleotide changes in the housekeeping genes (Aanensen and Spratt, 2005). MLST was easy to perform and more discriminative (Kotetishvili *et al.*, 2003).

MLST was able to distinguish the *V. cholerae* of the 6th pandemic from the 7th pandemic clones and the evolutionary relationship of the strains from different pandemic clones from Asia and other countries (Salim *et al.,* 2005). Kotetishvili *et al.* (2003) had included *tcp*A and *ctx*AB as two loci in their study as *ctxAB* and *tcpA* were found to be present in non-epidemic *V. cholerae* serogroups. In addition, compared to housekeeping genes which diversify slowly, virulence genes are more polymorphic and have been suggested to be used in the sequence-analysis (Danin-Poleg *et al.,* 2007; Kotetishvili *et al.*, 2003). Furthermore, it is important to include multiple loci scattered around the chromosome to ensure that strains are reliably grouped based on genetic relatedness (Achtman, 2001). For example, Zhang *et al.,* (2004) had developed a Multi-virulence-locus Sequence Typing

(MVLST) for *Listeria monocytogenes* and reported a higher discriminatory power of MVLST compared to MLST and PFGE.

In this study, we investigated the genetic variation of 43 Malaysian *V. cholerae* based on the six housekeeping genes and six virulence and virulence-associated genes. Cluster analysis was also carried out for MLST, MVLST and combined MLST/MVLST to study the relatedness of the strains isolated in Malaysia.

8.2 Materials and Methods

8.2.1 Bacterial Strains

Strains used in this Chapter were previously described in **Chapter 5** and listed in **Appendix A**.

8.2.2 DNA preparation for PCR

DNA preparation was carried out as described in **Chapter 3**, **Section 3.2.3**.

8.2.3 MLST Analysis

Six housekeeping genes (*dnaE, lap*, *recA*, *gyrB*, *cat* and *gmd*) were selected for the MLST analysis (**Table 8.1**). Monoplex PCRs were carried out for each gene with an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation (94°C for 30 sec), annealing (59°C for 30 sec), extension (72°C for 1 min) and a final extension at 72°C for 5 min. A single reaction of PCR consisted of 1x PCR buffer, 2 mM $MgCl_2$, 200 µM each dNTPs, 0.3 µM each primers, 50 ng DNA template and 2 U of *Taq* DNA polymerase (Promega, Madison, USA).

Table 8.1: Primers used for MLST of *V. cholerae* strains, numbers of alleles and discriminatory indices for different genes.

Gene	Primers Sequence (5'-3')	Size of Fragment analyzed (bp)	Coverage of complete CDS	Position (kb)	No of Alleles	Discriminatory index (D.I)
dnaE [a]	F: CGRATMACCGCTTTCGCCG	~533	~15 %	2397	5	0.46
	R: GAKATGTGTGAGCTGTTTGC					
lap [a]	F: GAAGAGGTCGGTTTGCGAGG	~467	31%	758*	10	0.45
	R:GTTTGAATGGTGAGCGGTTTGCT					
recA [b]	F: GAAACCATTTCGACCGGTTC	~699	56%	574	11	0.57
	R: CCGTTATAGCTGTACCAAGCGCCC					
gyrB [b]	F: GAAGGBGGTATTCAAGC	~401	~17 %	11	11	0.74
	R: GAGTCACCCTCCACWATGTA					
cat [a]	F: ATGGCTTATGAATCGATGGG	~482	~22 %	1671	10	0.69
	R: TCCCATTGCCATGCACC					
gmd [a]	F: CCTTATGCKGTGGCRAA	~452	~40 %	249	4	0.14
	R: CTWGGATCACCTAACA					

[a] Garg *et al.* 2003 [b] Kotetishvili *et al.*, 2003

8.2.4 MVLST Analysis

Three virulence genes (*ctxA*, *tcpA* and *tcpI*) and three virulence-associated genes (*hlyA*, *toxR* and *rtxA*) were selected for MVLST analysis (**Table 8.2**). Monoplex PCRs were carried out for each gene in a total volume of 25 µl containing 1x PCR buffer, 2 mM $MgCl_2$, 200 µM each dNTPs, 0.3 µM each primers, 50 ng DNA template and 2 U of *Taq* DNA polymerase (Promega, Madison, USA). The PCR cycling condition consisted of 95°C for 3 min (pre-denaturation), followed by 35 cycles of 94°C for 30 sec (denaturation), 59°C for 30 sec (annealing) 72°C for 1 min (extension) and a final extension at 72°C for 5 min.

8.2.5 DNA extraction from agarose gel and DNA sequencing

DNA was extracted from gel as described in **Chapter 3**, **Section 3.2.6**. The extracted DNA was sent to a laboratory (1^{st} Base, Seri Kembangan, Malaysia) for sequencing together with the PCR primers.

Table 8.2: Primers used for MVLST of *V. cholerae* strains, numbers of alleles and discriminatory indices for different genes.

Gene	Primers Sequence (5'-3')	Size of Fragment analyzed (bp)	Coverage of complete CDS	Position (kb)	No of Alleles	Discriminatory index (D.I)
ctxAB [a]	F: GGCTGTGGGTAGAAGTGAAACGG	~1080	94%	1566 (*ctxB*)	3	0.44
	R: CTAAGGATGTGGAATAAAAACATC			1567 (*ctxA*)		
hlyA [b]	F: GTGCGTATCAGCCTAGATGA	~216	~10%	237*	5	0.64
	R: CCCAAGCTCAAAACCTGAAA					
rtxA [c]	F: CTGAATATGAGTGGG TGACTTACG	~332	2%	1550	2	0.09
	R: TATTGTTCGATATCCGCTACG					
tcpA[d]	F: CACGATAAGAAAACCGGTCAAGAG	~390	58 %	890	2	0.47
	R1: CGAAAGCACCTTCTTTCACGTTG					
	R2: TTACCAAATGCAACGCCGAATG					
toxR [d]	F: CCTTCGATCCCCTAAGCAATAC	713	81 %	1047	8	0.51
	R: AGGGTTAGCAACGATGCGTAAG					

tcpI [d]	F: TAGCCTTAGTTCTCAGCAGGCA R: GGCAATAGTGTCGAGCTCGTTA	799	43%	886	4	0.30

[a] Li *et al.* 2002

[b] Teh *et al.* 2009

[c] Chow *et al.* 2001

[d] Rivera *et al.* 2001

8.2.6 Data Analysis

The DNA sequences obtained were analyzed and Blast against the published sequence in the GenBank (http://www.ncbi.nih.gov/BLAST). Multiple sequence alignments, transition/transversion ratio, and Tajima's neutrality test were performed using Mega 4 (Tamura *et al.*, 2007). The transition/transversion ratio test is important for determining the bias of substitution types and mutational patterns in a genome (Wakeley, 1996), while Tajima's neutrality test was applied to determine the neutrality of evolution (Tamura *et al.*, 2007). Strains with no amplification (designated as '0' allele) were not included in Tajima's test.

Arbitrary numbers (to denote allelic type, AT) were assigned for different allelic sequences (at least one nucleotide difference), with '1' for the most common allele for a gene and '0' for no amplification. The sequence type was assigned for MLST (*dnaE*, *lap*, *recA*, *gyrB*, *cat*, and *gmd*), MVLST (*ctxAB*, *hlyA*, *rtxA*, *tcpA*, *toxR*, and *tcpI*) and the combined MLST/MVLST (*dnaE*, *lap*, *recA*, *gyrB*, *cat*, *gmd*, *ctxAB*, *hlyA*, *rtxA*, *tcpA*, *toxR*, and *tcpI*) analyses for all tested strains. Dendrograms for MLST and MVLST were computed using the categorical unweighted pair group method with arithmetic mean (UPGMA) of sequence types using BioNumerics 6.0 (Applied Maths NV, Belgium). A minimum spanning tree (MST) was also constructed using the categorical data for combined MLST/MVLST data using BioNumeric 6.0 (Applied Maths NV, Belgium). The discriminatory index (DI) was calculated as described by Hunter and Gaston (1988) for individual genes and combined analyses.

8.2.7 Nucleotide Sequence Accession Numbers

The DNA sequences obtained in this study were deposited in GenBank under accession numbers HM042636-HM042677 and HQ452867-HQ452899.

8.3 Results

8.3.1 Allelic polymorphism of housekeeping genes

For MLST analysis, 3,034 bp (30% coverage of the six complete CDS) were analyzed for each strain. For each of the loci examined, the numbers of alleles obtained were the following: 1-5 for *dnaE*, 1-10 for *lap*, 1-11 for *recA*, 1-11 for *gyrB*, 1-10 for *cat* and 1-4 for *gmd*. The most common allele for *dnaE* was present in 72% of strains, 74% for *lap*, 65% for *recA*, 49% for *gyrB*, 53% for *cat* and 93% for *gmd*.

All of the allelic types (ATs) were blasted against the NCBI GenBank database. AT6 for the *recA* gene in the non-toxigenic non-O1/non-O139 *V. cholerae* strain was 100% similar to the allelic type of a Brazilian non-toxigenic O1 *V. cholerae* strain (accession no. EU085353) (Mohapatra *et al.*, 2009). Of the allelic types of the *gyrB* gene, AT6 and AT8 were also 100% similar to the allelic types of two non-O1/non-O139 *V. cholerae* strains isolated in Argentina (accession no. EU101418 and EU101417, respectively) (González-Fraga *et al.*, 2008).

A total of 42 novel ATs with single -nucleotide polymorphism (SNP) mutations were found for the six analyzed genes. One novel sequence each for the *lap* (HM042651), *recA* (HM042658), and *gmd* (HQ452884) genes was found in the O1 strains, while four novel O1 sequences were obtained for *gyrB* (HM042661, HM042662, HM042663, HM042667) and *cat*

(HM042668, HM042669, HM042670, HM042672) genes. Only one allele (AT1) was observed for the *dnaE* gene in 23 O1 *V. cholerae* strains.

Based on the **Figure 8.1** which showed the combined mutation points for the six housekeeping genes, *recA* gene was the most polymorphic with 54 polymorphic sites observed, followed by *cat*, *gyrB*, *dnaE*, *lap* and *gmd* genes.

There were two and 11 common polymorphic sites for *dnaE* (AT2-AT5) and *recA* genes (AT 2-11), respectively (**Figure 8. 1**). The two polymorphic sites for *dnaE* are located at the 978' (TA<u>T</u> → TA<u>C</u>) and 1251' (GA<u>T</u> → GA<u>C</u>) nucleotide of 12 non-O1/non-O139 *V. cholerae*. For *recA* gene, the mutations were located at 420' (GC<u>A</u> → GC<u>G</u>), 490' (TTG → <u>C</u>TG), 585' (GT<u>G</u> → GT<u>A</u>), 591' (GT<u>C</u> → GT<u>G</u>), 594' (AT<u>C</u> → AT<u>T</u>), 609' (GT<u>A</u> → GT<u>C</u>), 972' (GC<u>T</u> → GC<u>G</u>), 930' (GC<u>G</u> → GC<u>A</u>), 978' (AA<u>T</u> → AA<u>C</u>), 1017' (AA<u>A</u> → AA<u>G</u>) and 1026' (GA<u>G</u> → GA<u>A</u>) nucleotide in the sequence. Except for AT 2 (n = 1), AT3 – AT11 were comprised of 14 *V. cholerae* of non-O1/non-O139 serogroup. The allelic profiles and MLST types of the strains are summarized in the **Table 8.3**.

Transitional biases (A ↔ T or C ↔ G) were predominantly observed for all genes (transition/transversion ratio, R>0.5), except for *gmd*. Overall, silent mutations were commonly observed in the *dnaE*, *lap*, *recA*, *gyrB*, and *cat* genes. Three polymorphic sites with primarily transversional substitution in the 452-bp fragment of the *gmd* gene were non-synonymous, while no deletion was observed. Tajima's neutrality test showed negative D values in all analyzed genes except for the *dnaE* gene (D=0.06233), indicating a potential balancing selection for the 43 strains.

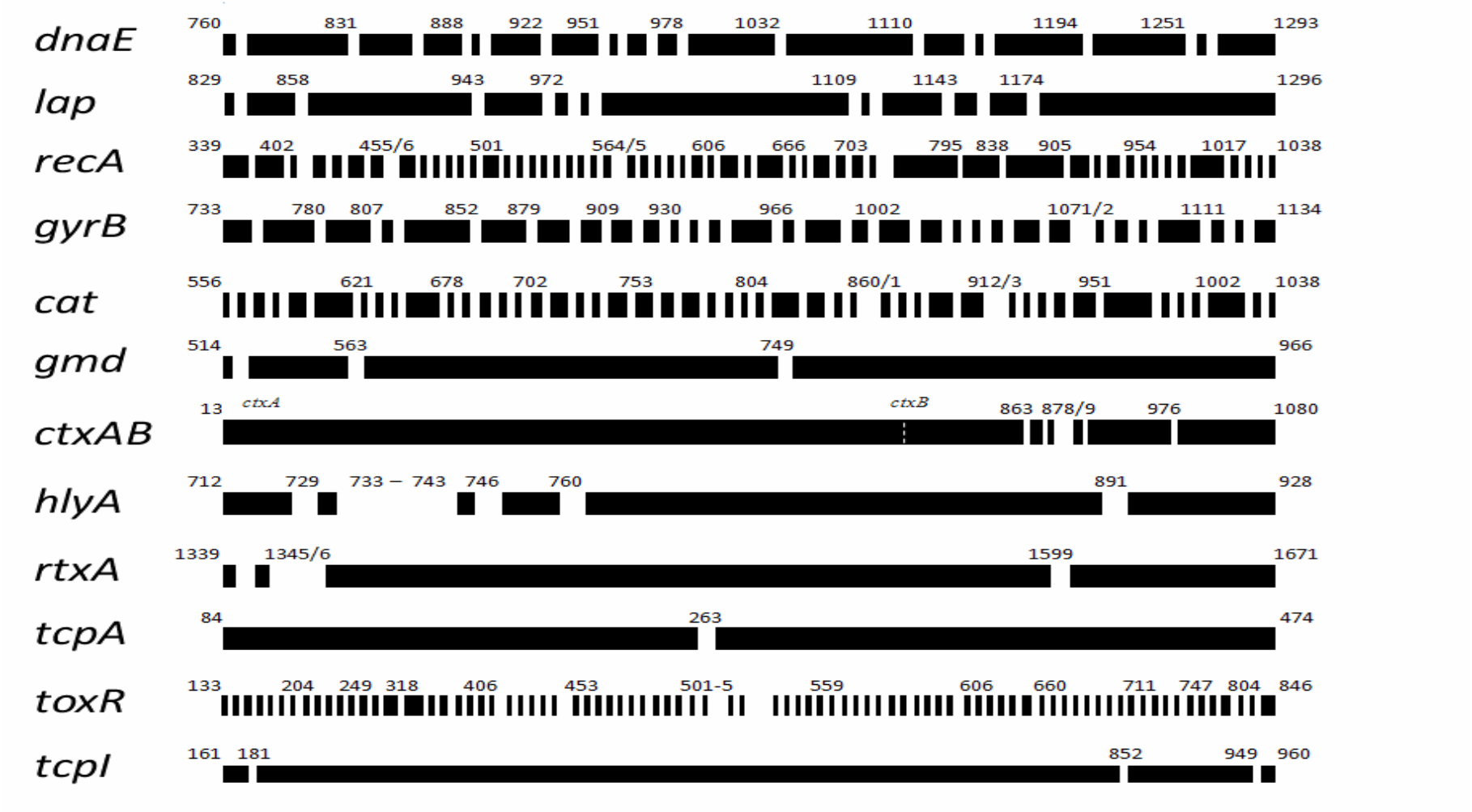


Fig. 8.1: Mutation points of the 12 genes analyzed in MLST and MVLST.

Table 8.3: Allelic Type for analyzed genes and sequence type for MLST, MVLST and combined analyses.

Strain code	Serogroup	MLST						MLST Type	MVLST						MVLST Type	Sequence Type for MLST/MVLST (ST)
		dnaE	*lap*	*recA*	*gyrB*	*cat*	*gmd*		*ctxAB*	*hlyA*	*rtxA*	*tcpA*	*toxR*	*tcpI*		
21/04	O1	1	1	1	1	1	1	A1	1	2	1	1	1	1	B2	ST2
35/04	O1	1	1	2	5	4	1	A2	0	1	1	0	2	0	B3	ST3
37/04	O1	1	1	1	1	1	1	A1	1	1	1	1	1	1	B1	ST1
38/04	O1	1	1	1	1	1	1	A1	1	2	1	2	1	1	B4	ST4
40/04	O1	1	1	1	1	1	2	A3	3	1	1	1	1	1	B5	ST5
1/08	O1	1	1	1	1	2	1	A4	1	1	1	1	1	1	B1	ST6
3/08	O1	1	1	1	3	1	1	A5	1	1	1	2	1	1	B6	ST7
4/08	O1	1	1	1	2	1	1	A6	1	1	1	1	1	1	B1	ST8
6/08	O1	1	1	1	2	2	1	A7	1	2	1	2	1	1	B4	ST9
9/08	O1	1	2	1	1	3	1	A8	3	2	1	1	2	1	B7	ST10
10/08	O1	1	1	1	1	5	1	A9	1	2	1	1	1	1	B2	ST11
11/08	O1	1	1	1	1	1	1	A1	2	1	1	1	1	1	B8	ST12
52/08	O1	1	1	1	1	1	1	A1	1	1	1	2	1	2	B9	ST13
63/08	O1	1	1	1	1	1	1	A1	1	1	1	2	1	1	B6	ST14
64/08	O1	1	1	1	1	1	1	A1	1	1	1	2	1	1	B6	ST14
65/08	O1	1	1	1	1	1	1	A1	2	2	1	2	1	1	B10	ST15
66/08	O1	1	1	1	1	1	1	A1	1	1	1	2	1	1	B6	ST14
70/08	O1	1	1	1	1	5	1	A9	1	1	1	1	1	1	B1	ST16
87/08	O1	1	1	1	1	1	1	A1	1	1	1	1	1	3	B11	ST17
88/08	O1	1	1	1	1	1	1	A1	1	1	1	1	1	1	B1	ST1
90/08	O1	1	1	1	1	1	1	A1	1	1	1	1	1	1	B1	ST1
123/08	O1	1	1	1	1	5	1	A9	1	1	1	1	1	1	B1	ST16
3P/08	O1	1	1	1	4	1	1	A10	3	2	1	1	1	1	B12	ST18
4370/08	O139	1	1	1	1	1	1	A1	2	1	1	1	1	1	B8	ST12

17/08	Non-O1/non-O139	4	1	3	2	1	1	A11	0	5	2	0	3	0	B13	ST19
77/08	Non-O1/non-O139	3	9	3	6	2	1	A12	0	4	2	0	3	0	B14	ST20
81/08	Non-O1/non-O139	2	1	4	2	2	1	A13	0	1	1	0	1	0	B15	ST21
82/08	Non-O1/non-O139	1	6	1	7	6	1	A14	0	2	1	0	5	0	B16	ST22
83/08	Non-O1/non-O139	2	1	1	8	5	1	A15	0	1	1	0	1	0	B15	ST23
84/08	Non-O1/non-O139	1	5	7	3	1	1	A16	0	1	1	0	7	0	B17	ST24
85/08	Non-O1/non-O139	5	1	1	2	1	3	A17	0	1	1	0	8	0	B18	ST25
310/08	Non-O1/non-O139	2	1	3	1	3	1	A18	0	3	1	0	1	4	B19	ST26
374/08	Non-O1/non-O139	3	10	9	6	7	1	A19	0	5	1	0	2	0	B20	ST27
933/08	Non-O1/non-O139	2	4	4	8	1	1	A20	0	1	1	0	6	0	B21	ST28
3477/08	Non-O1/non-O139	4	3	6	6	1	1	A21	0	4	1	0	4	3	B22	ST29
4933/08	Non-O1/non-O139	2	1	1	9	1	1	A22	0	2	1	0	1	0	B23	ST30
B2/08	Non-O1/non-O139	1	1	5	6	2	1	A23	0	1	1	0	1	0	B15	ST31
B4/08	Non-O1/non-O139	2	1	1	10	8	1	A24	0	5	1	0	1	0	B3	ST32
M1/08	Non-O1/non-O139	3	7	10	6	3	1	A25	0	5	1	0	1	0	B24	ST33
M2/08	Non-O1/non-	1	1	11	3	9	1	A26	0	5	1	0	4	0	B25	ST34

	O139															
GB/08	Non-O1/non-O139	1	6	8	1	10	1	A27	0	3	1	0	3	0	B26	ST35
PSW/08	Non-O1/non-O139	1	8	9	4	2	1	A28	0	1	1	0	1	0	B27	ST36
SW/08	Non-O1/non-O139	1	8	3	11	10	1	A29	0	3	1	0	2	0	B15	ST37

8.3.2 Allelic polymorphism of virulence and virulence-associated genes

Approximately 3,530 bp of DNA from each strain was assessed using MVLST analysis (48% coverage of the six complete CDS of the *V. cholera*e genome). The allelic numbers assigned for virulence and virulence-associated genes ranged from 0-3 for *ctxAB*, 1-5 for *hlyA*, 1-2 for *rtxA*, 0-2 for *tcpA*, 1-8 for *toxR* and 0-4 for *tcpI*. The virulence genes *ctxAB* and *tcpA* were absent in all of the non-O1/non-O139 strains, while *tcpI* was amplified from nearly all O1 strains (except strain 35/04). Conversely, only two non-O1/non-O139 strains possessed the *tcpI* gene. In comparison to other genes, the *hlyA* gene yielded a shorter DNA fragment (~244 bp) in two AT4 strains (77/08 and 3477/08).

Similar allelic profiles were shared among the strains: 95%, 84%, 74%, 70%, 65% and 56% for *rtxA*, *tcpI*, *ctxAB*, *toxR*, *tcpA* and *hlyA*, respectively. The *rtxA* gene was the most conserved gene in O1 strains. Overall, a total of 17 novel sequences were obtained for *ctxAB* (HM042644 and HM042645), *hlyA* (HQ452878 and HQ452879), *rtxA* (HM042636), *tcpA* (HQ452875), *toxR* (HM042637-HM042642 and HQ452872-HQ452873), and *tcpI* (HM042643, HQ452869 and HQ452870).

Based on the **Figure 8.1,** *tox*R gene was the most polymorphic with 82 polymorphic sites observed, followed by *hlyA*, *ctxAB*, *rtxA*, *tcpI* and *tcpA* gene. Three common polymorphic sites were observed in *ctxAB* (AT2 and AT3) at nucleotides 863 (TG<u>T</u> → TG<u>G</u>), 888 (<u>T</u>AT → <u>C</u>AT), and 976 (A<u>T</u>T → A<u>C</u>T). These SNPs resulted in amino acid substitutions at positions 30 (Cys → Trp), 39 (Tyr → His) and 68 (Ile → Thr). Similarly, three common polymorphic sites were also found in the *toxR* gene for AT2 through AT8 at nucleotides 510 (GT<u>T</u> → GT<u>C</u>), 684 (AA<u>C</u> → AA<u>T</u>) and 768 (AC<u>A</u> → AC<u>G</u>). No amino acid substitution was observed for these three common polymorphic sites. Except for AT2,

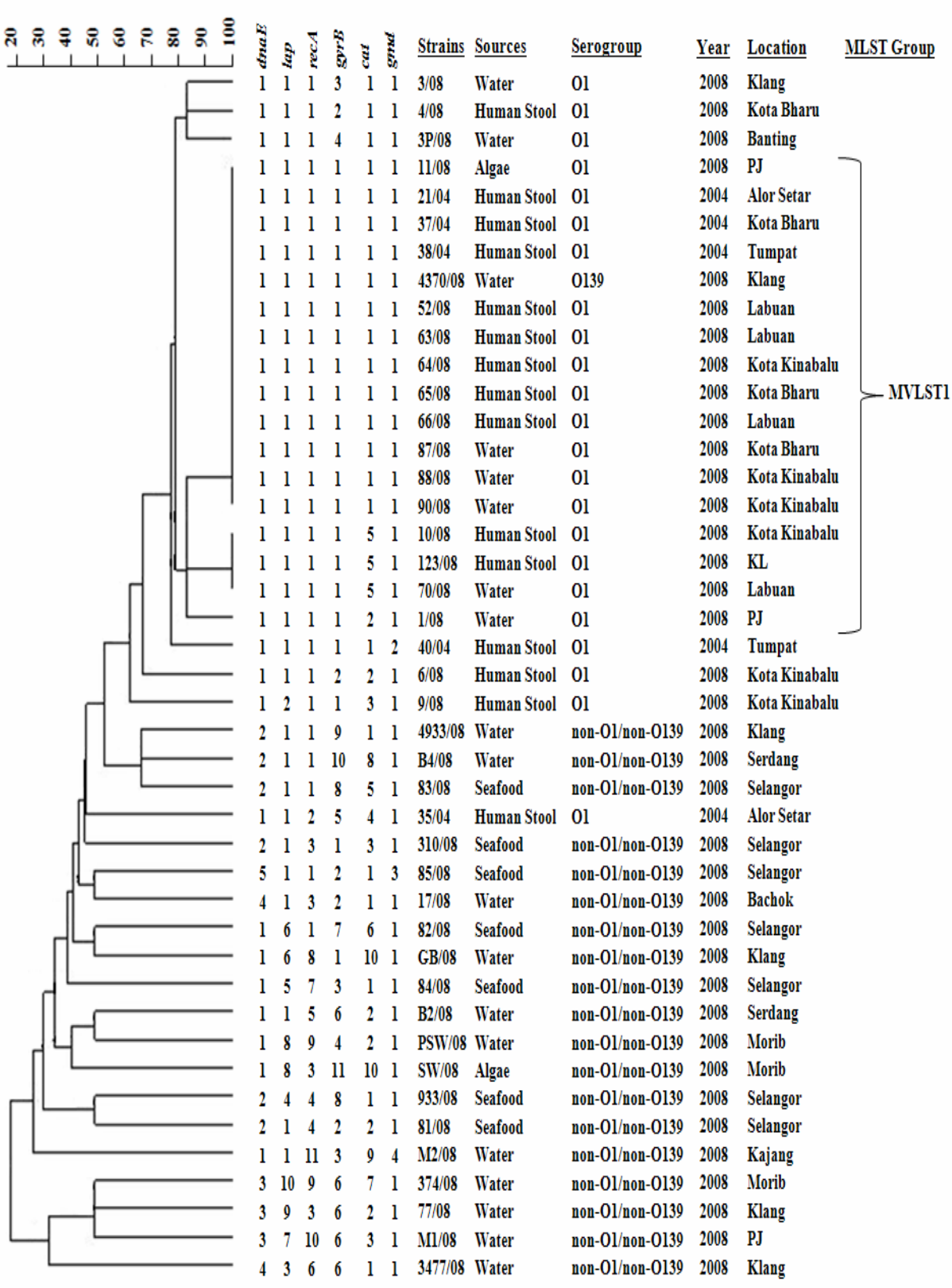
AT3 through AT8 were found in 12 non-O1/non-O139 *V. cholerae* strains. The allelic profiles and MVLST types of the strains are summarized in **Table 8.3**.

Transitional biases were observed for *ctxAB*, *rtxA*, *toxR* and *tcpI* (transition/transversion ratio, R>0.5). The transversional substitution of nucleotides occurred more frequently in *hlyA* (R=0.299) and *tcpA* (R=0) than in other genes. Non-synonymous substitutions were more commonly observed in virulence and virulence-associated genes than housekeeping genes. All of the mutations observed in the *ctxAB*, *tcpA*, and *tcpI* genes were non-synonymous. Except for the *hlyA* gene, in which deletions occurred at nucleotides 733-743 in the Classical- and El Tor-distinguishing region, a 3-bp nucleotide deletion was found at 410-412 of the *toxR* gene. This deletion was observed in one non-O1/non-O139 *V. cholerae* strain (85/08) isolated from seafood. All genes, except for *ctxAB* and *tcpA*, had positive D values based on Tajima's neutrality test, indicating diversifying selection in these two genes.

8.3.3 Cluster Analysis of *V. cholerae* based on MLST, MVLST and combined analysis

Cluster analyses were performed to determine the genetic relatedness of *V. cholerae* strains isolated in Malaysia. For MLST, a dendrogram was generated based on the character types (allelic profiles) of the 29 MLST types (F=0.20). One major cluster was observed at the similarity of 80%, which comprised 18 O1 and one O139 strains (**Figure 8.2**). In this cluster, the most common MLST type, A1 (1,1,1,1,1,1), was found in 12 O1 and one O139 *V. cholerae* strains. Three O1 *V. cholerae* strains showed the same MLST type (A9: 1,1,1,1,5,1). All of the non-O1/non-O139 strains had different MLST types and were diverse.

Discriminatory indices for each locus ranged from 0.14 for *gmd* to 0.74 for *gyrB* (**Table 8.2**) while index for the combined MLST loci was 0.91.

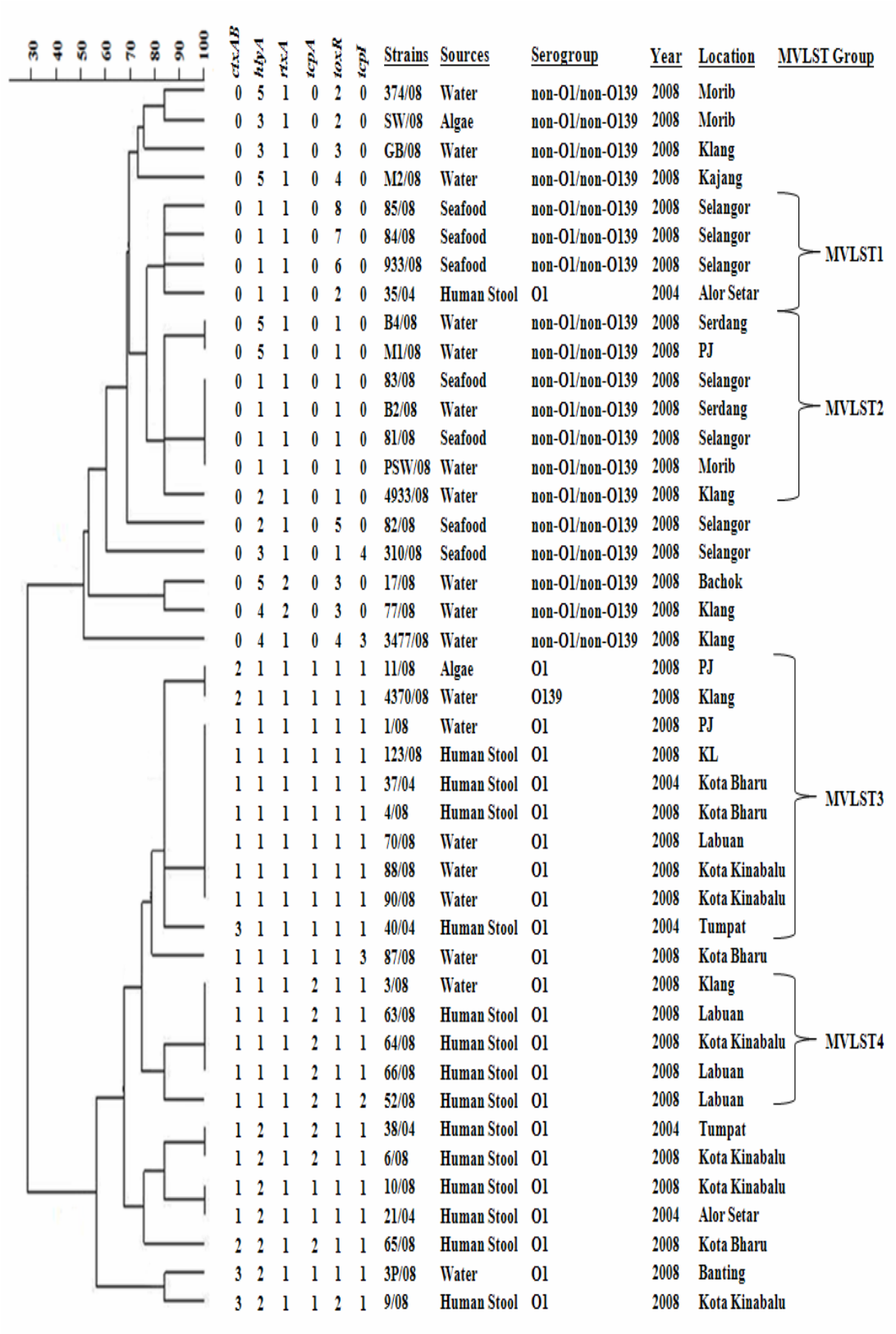


dnaE	*tap*	*recA*	*gyrB*	*cat*	*gmd*	Strains	Sources	Serogroup	Year	Location	MLST Group
1	1	1	3	1	1	3/08	Water	O1	2008	Klang	
1	1	1	2	1	1	4/08	Human Stool	O1	2008	Kota Bharu	
1	1	1	4	1	1	3P/08	Water	O1	2008	Banting	
1	1	1	1	1	1	11/08	Algae	O1	2008	PJ	
1	1	1	1	1	1	21/04	Human Stool	O1	2004	Alor Setar	
1	1	1	1	1	1	37/04	Human Stool	O1	2004	Kota Bharu	
1	1	1	1	1	1	38/04	Human Stool	O1	2004	Tumpat	
1	1	1	1	1	1	4370/08	Water	O139	2008	Klang	
1	1	1	1	1	1	52/08	Human Stool	O1	2008	Labuan	
1	1	1	1	1	1	63/08	Human Stool	O1	2008	Labuan	
1	1	1	1	1	1	64/08	Human Stool	O1	2008	Kota Kinabalu	
1	1	1	1	1	1	65/08	Human Stool	O1	2008	Kota Bharu	MVLST1
1	1	1	1	1	1	66/08	Human Stool	O1	2008	Labuan	
1	1	1	1	1	1	87/08	Water	O1	2008	Kota Bharu	
1	1	1	1	1	1	88/08	Water	O1	2008	Kota Kinabalu	
1	1	1	1	1	1	90/08	Water	O1	2008	Kota Kinabalu	
1	1	1	1	5	1	10/08	Human Stool	O1	2008	Kota Kinabalu	
1	1	1	1	5	1	123/08	Human Stool	O1	2008	KL	
1	1	1	1	5	1	70/08	Water	O1	2008	Labuan	
1	1	1	1	2	1	1/08	Water	O1	2008	PJ	
1	1	1	1	1	2	40/04	Human Stool	O1	2004	Tumpat	
1	1	1	2	2	1	6/08	Human Stool	O1	2008	Kota Kinabalu	
1	2	1	1	3	1	9/08	Human Stool	O1	2008	Kota Kinabalu	
2	1	1	9	1	1	4933/08	Water	non-O1/non-O139	2008	Klang	
2	1	1	10	8	1	B4/08	Water	non-O1/non-O139	2008	Serdang	
2	1	1	8	5	1	83/08	Seafood	non-O1/non-O139	2008	Selangor	
1	1	2	5	4	1	35/04	Human Stool	O1	2004	Alor Setar	
2	1	3	1	3	1	310/08	Seafood	non-O1/non-O139	2008	Selangor	
5	1	1	2	1	3	85/08	Seafood	non-O1/non-O139	2008	Selangor	
4	1	3	2	1	1	17/08	Water	non-O1/non-O139	2008	Bachok	
1	6	1	7	6	1	82/08	Seafood	non-O1/non-O139	2008	Selangor	
1	6	8	1	10	1	GB/08	Water	non-O1/non-O139	2008	Klang	
1	5	7	3	1	1	84/08	Seafood	non-O1/non-O139	2008	Selangor	
1	1	5	6	2	1	B2/08	Water	non-O1/non-O139	2008	Serdang	
1	8	9	4	2	1	PSW/08	Water	non-O1/non-O139	2008	Morib	
1	8	3	11	10	1	SW/08	Algae	non-O1/non-O139	2008	Morib	
2	4	4	8	1	1	933/08	Seafood	non-O1/non-O139	2008	Selangor	
2	1	4	2	2	1	81/08	Seafood	non-O1/non-O139	2008	Selangor	
1	1	11	3	9	4	M2/08	Water	non-O1/non-O139	2008	Kajang	
3	10	9	6	7	1	374/08	Water	non-O1/non-O139	2008	Morib	
3	9	3	6	2	1	77/08	Water	non-O1/non-O139	2008	Klang	
3	7	10	6	3	1	M1/08	Water	non-O1/non-O139	2008	PJ	
4	3	6	6	1	1	3477/08	Water	non-O1/non-O139	2008	Klang	

Figure 8.2 Dendrogram generated for the MLST analysis showing the relatedness of 43 *V. cholerae* strains.

Similarly, a dendrogram was generated based on the MVLST allelic profiles of the 27 types (F=0.27). The most common MVLST type of one O139 and all O1 *V. cholerae* strains was B1 (1,1,1,1,1,1), while the most common MVLST type of one O1 and all non-O1/non-O139 *V. cholerae* strains was B15 (0,1,1,0,1,0) (**Figure 8.3**). *hlyA* was the most discriminative locus (DI=0.64), followed by *toxR* (DI=0.51) and *ctxAB* (DI=0.44), while *rtxA* was the least discriminative (DI=0.09) (**Table 8.2**). The discriminatory ability of combined loci in MVLST was 0.96.

A minimum spanning tree (MST) with 37 observed STs was generated based on the combined MLST and MVLST data. With the inclusion of MVLST, the *V. cholerae* strains were distinguishable based on their toxigenicity. One distinct cluster of toxigenic O1 *V. cholerae* strains was observed in which all of the members had a close relationship, with only one to two allelic differences (**Figure 8.3**). ST1 (1,1,1,1,1,1,1,1,1,1,1,1) and ST2 (1,1,1,1,1,1,1,1,1,2,1,1) were the most common sequence type and each comprised by 3 O1 *V. cholerae* (**Table 8.3**). The discriminatory ability for the combined analysis was 0.96.



ctxAB	*hlyA*	*rtxA*	*tcpA*	*toxR*	*tcpI*	Strains	Sources	Serogroup	Year	Location	MVLST Group
0	5	1	0	2	0	374/08	Water	non-O1/non-O139	2008	Morib	
0	3	1	0	2	0	SW/08	Algae	non-O1/non-O139	2008	Morib	
0	3	1	0	3	0	GB/08	Water	non-O1/non-O139	2008	Klang	
0	5	1	0	4	0	M2/08	Water	non-O1/non-O139	2008	Kajang	
0	1	1	0	8	0	85/08	Seafood	non-O1/non-O139	2008	Selangor	MVLST1
0	1	1	0	7	0	84/08	Seafood	non-O1/non-O139	2008	Selangor	
0	1	1	0	6	0	933/08	Seafood	non-O1/non-O139	2008	Selangor	
0	1	1	0	2	0	35/04	Human Stool	O1	2004	Alor Setar	
0	5	1	0	1	0	B4/08	Water	non-O1/non-O139	2008	Serdang	MVLST2
0	5	1	0	1	0	M1/08	Water	non-O1/non-O139	2008	PJ	
0	1	1	0	1	0	83/08	Seafood	non-O1/non-O139	2008	Selangor	
0	1	1	0	1	0	B2/08	Water	non-O1/non-O139	2008	Serdang	
0	1	1	0	1	0	81/08	Seafood	non-O1/non-O139	2008	Selangor	
0	1	1	0	1	0	PSW/08	Water	non-O1/non-O139	2008	Morib	
0	2	1	0	1	0	4933/08	Water	non-O1/non-O139	2008	Klang	
0	2	1	0	5	0	82/08	Seafood	non-O1/non-O139	2008	Selangor	
0	3	1	0	1	4	310/08	Seafood	non-O1/non-O139	2008	Selangor	
0	5	2	0	3	0	17/08	Water	non-O1/non-O139	2008	Bachok	
0	4	2	0	3	0	77/08	Water	non-O1/non-O139	2008	Klang	
0	4	1	0	4	3	3477/08	Water	non-O1/non-O139	2008	Klang	
2	1	1	1	1	1	11/08	Algae	O1	2008	PJ	MVLST3
2	1	1	1	1	1	4370/08	Water	O139	2008	Klang	
1	1	1	1	1	1	1/08	Water	O1	2008	PJ	
1	1	1	1	1	1	123/08	Human Stool	O1	2008	KL	
1	1	1	1	1	1	37/04	Human Stool	O1	2004	Kota Bharu	
1	1	1	1	1	1	4/08	Human Stool	O1	2008	Kota Bharu	
1	1	1	1	1	1	70/08	Water	O1	2008	Labuan	
1	1	1	1	1	1	88/08	Water	O1	2008	Kota Kinabalu	
1	1	1	1	1	1	90/08	Water	O1	2008	Kota Kinabalu	
3	1	1	1	1	1	40/04	Human Stool	O1	2004	Tumpat	
1	1	1	1	1	3	87/08	Water	O1	2008	Kota Bharu	
1	1	1	2	1	1	3/08	Water	O1	2008	Klang	MVLST4
1	1	1	2	1	1	63/08	Human Stool	O1	2008	Labuan	
1	1	1	2	1	1	64/08	Human Stool	O1	2008	Kota Kinabalu	
1	1	1	2	1	1	66/08	Human Stool	O1	2008	Labuan	
1	1	1	2	1	2	52/08	Human Stool	O1	2008	Labuan	
1	2	1	2	1	1	38/04	Human Stool	O1	2004	Tumpat	
1	2	1	2	1	1	6/08	Human Stool	O1	2008	Kota Kinabalu	
1	2	1	1	1	1	10/08	Human Stool	O1	2008	Kota Kinabalu	
1	2	1	1	1	1	21/04	Human Stool	O1	2004	Alor Setar	
2	2	1	2	1	1	65/08	Human Stool	O1	2008	Kota Bharu	
3	2	1	1	1	1	3P/08	Water	O1	2008	Banting	
3	2	1	1	2	1	9/08	Human Stool	O1	2008	Kota Kinabalu	

Figure 8.3 Dendrogram generated for the MVLST analysis showing the relatedness of 43 *V. cholerae* strains.

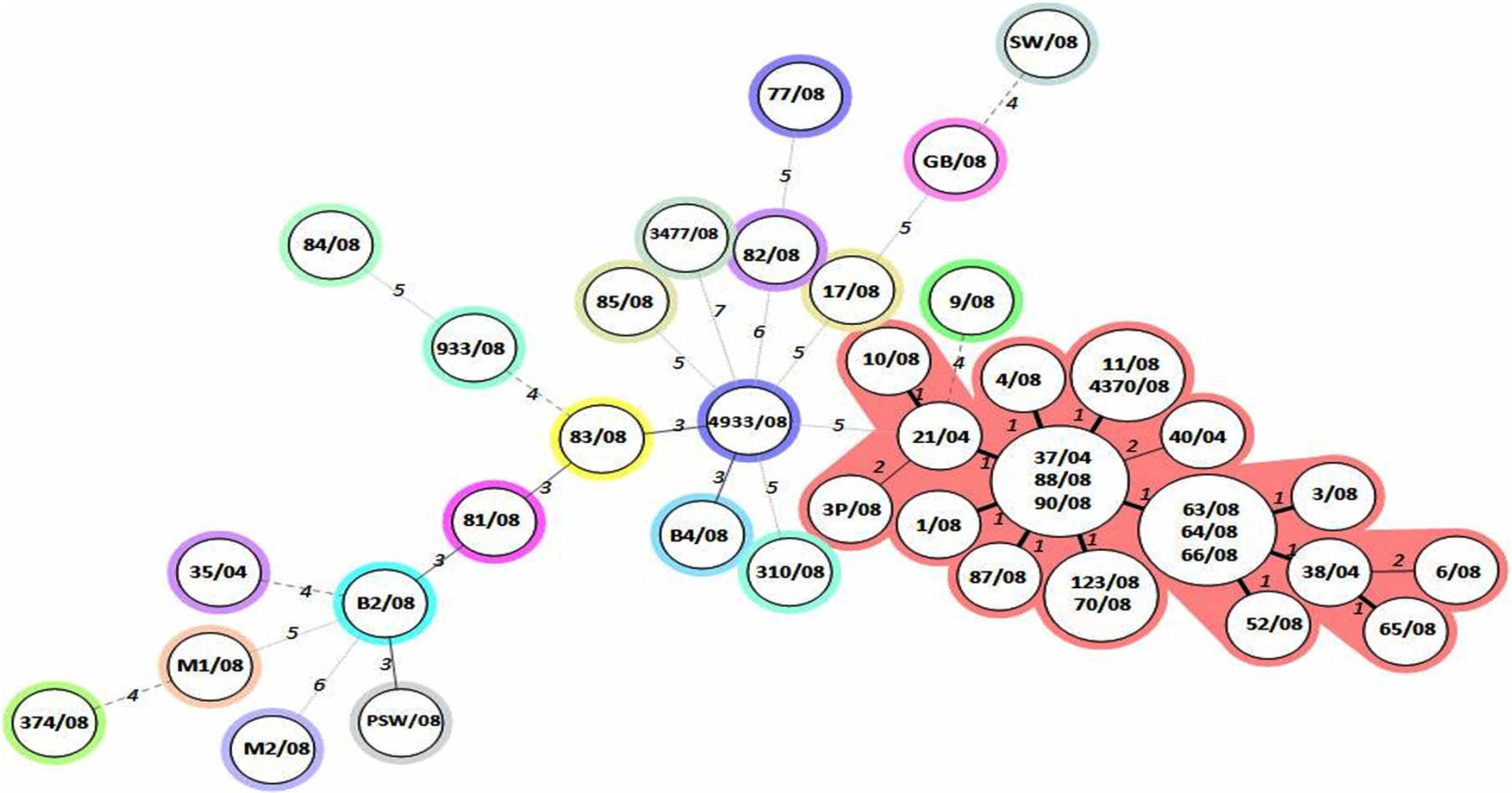


Figure 8.4 MST generated with the combine analysis of MLST and MVLST showing the relatedness of 43 *V. cholerae* strains. The closely related O1 strains were highlighted in pink while other strains were of different unique variants.

8.4 Discussion

In this study, the majority of the analyzed genes were located on the big chromosome (chromosome I), while only two genes (*lap* and *hlyA*) were located on the small chromosome II of *V. cholerae*. Although the DNA fragments analyzed in this study covered only 39% of 12 complete CDS of *V. cholerae*, the genetic variation among the strains of different serogroups (O1, O139 and non-O1/non-O139) could be determined. This result shows that sequence-based typing is a highly discriminative typing method.

The MLST of the different *V. cholerae* serogroups was based on the loci reported by Garg *et al.* (2003). Garg *et al.* (2003) reported that *lap* was the most variable gene (n=20) among the O139 isolates, followed by *gmd* (n=11) *dnaE* (n=9), *cat* (n=7), *gyrB* (n=4) and *recA* (n=2). To determine the usefulness of these genes in different serogroups, we initially evaluated the genes of varying diversity shown in Garg *et al.* (2003) in Malaysian O1, O139 and non-O1/non-O139 *V. cholerae* strains. As a result, both *recA* and *gyrB* gave the highest number of different alleles (n=11), while the *gmd* gene showed only four alleles in the O1, O139 and non-O1/non-O139 *V. cholerae* strains in our study. This result might be due to the nature of the strains used in this study, which were genetically different from the strains used in Garg *et al.* (2003) because they were from different serogroups, geographical origins and years of isolation.

Among the analyzed genes, *dnaE* and *rtxA* were highly conserved in O1 strains. Only one AT for each gene was observed for all of the O1 strains, and this suggests that no mutation or recombination occurred in the genes among the O1 *V. cholerae* strains studied. AT1 of the *dnaE* gene was the only allele for the O1 strains and was also commonly found in other non-O1 *V. cholerae* strains. The low variability of the *dnaE* gene indicates that *dnaE* is

conserved among the population and might not be a result of recombination. This theory is in agreement with the principle of balancing selection, in which an allele is actively maintained in the gene pool for the long-term stability of allelic variation (Charlesworth *et al.*, 1995).

In this study, MVLST was more useful than MLST because MVLST was able to subtype the strains into toxigenic and non-toxigenic categories. In addition, MVLST exhibited more subtypes of O1 strains than did MLST. This difference indicates that the diversity of virulence and virulence-associated genes in O1 strains is higher than the diversity of housekeeping genes. For example, MVLST could also differentiate the environmental *V. cholerae* O1 strains isolated from Kota Bharu from two other environmental *V. cholerae* O1 strains (88/08 and 90/08) from Kota Kinabalu based on the *tcpI* gene.

Virulence genes, such as *ctxAB*, *tcpA*, and *tcpI*, are associated with colonization and are always present in toxigenic O1 *V. cholerae* (Rivera *et al.*, 2001). However, the presence of virulence genes in non-toxigenic *V. cholerae* has been reported (Faruque *et al.*, 2004); therefore, it is useful to investigate the presence/absence and allelic type of *ctxAB*, *tcpA*, and *tcpI* in non-O1/non-O139 *V. cholerae*. In this study, the same allelic type (AT3) of the *tcpI* gene was found in both an environmental non-O1/non-O139 *V. cholerae* (3477/08) strain and O1 *V. cholerae* (87/08). This result is in agreement with Faruque *et al.* (2004), who showed that virulence genes could be dispersed among environmental strains of *V. cholerae* from different serogroups. Furthermore, to increase the discrimination, virulence-associated genes, such as *hlyA*, *rtxA* and *toxR*, which are present in all serogroups of *V. cholerae*, were included in MVLST analysis. However, MVLST analysis of non-O1/non-O139 strains was less satisfactory than expected because only the *tcpI* gene was found in two non-toxigenic strains (310/08 and 3477/08). Because non-O1/non-O139 strains could be distinguished using MLST

analysis, it was useful to analyse both MLST and MVLST in parallel to provide better discriminatory power.

Overall, the O1 strains and the non-O1/non-O139 strains could be discriminated. Based on the combined analysis, there were environmental and clinical strains which shared the same sequence type and suggest that clinical and environmental O1 could be originated from the same traits (van Belkum *et al.*, 2001). However, one O1 *V. cholerae* (35/04) strain isolated in 2004 was different from other O1 *V. cholerae* strains and were more closely related to non-O1/non-O139 *V. cholerae* based on MLST and MVLST analyses. This finding suggests that strain 35/04 is genetically different from the rest of the O1 *V. cholerae* strains and therefore might be a new variant of strain derived from non-O1/non-O139 *V. cholerae* as described by Chakraborty *et al.* (2001).

El Tor variant strains of *V. cholerae* O1 that produce cholera toxin of the Classical biotype have been reported since the last decade (Olsvik *et al.*, 1993). The mutation site leading to amino acid substitution at positions 39 and 68 of the gene (*ctxB*) has also been reported (Nair *et al.*, 2006). In the present study, AT2 and AT3 for *ctxAB* were found to possess the mutation at the same position of *ctxB.* This result indicates that the strains that possess AT2 (strains 11/08, 4370/08 and 65/08) and AT3 (strains 40/04, 3P/08 and 9/08) are El Tor variant strains, as previously reported by Nair *et al.* (2006). Interestingly, AT2 was observed in the O139 *V. cholerae* strain isolated from aquatic environment. This O139 strain also possessed the same sequence type as the O1 strain (11/08) isolated from algae (ST12: 1,1,1,1,1,1,2,1,1,1,1,1), hence concurred that this O139 *V. cholerae* strain was clonally related to strain 11/08 based on the combined analysis, as shown in the MST (**Figure 8.4**). This observation is in agreement with Singh *et al.* (2001), who showed that the strains of *V. cholerae* O139 originated from the horizontal gene transfer of serogroup O1 to non-O1/non-

O139. Hence, O139 *V. cholerae* is genetically related to O1 and non-O1/non-O139 *V. cholerae* strains. Li *et al.* (2002) and Kotetishvili *et al.* (2003) have also reported the occurrence of genetic recombination and transfer of O antigen-encoding genes among *V. cholerae* with a common genetic background.

The *hlyA* gene was used as a marker for differentiation of the Classical and El Tor biotypes because there is an 11-bp deletion in the Classical O1 biotype (Rader and Murphy, 1988). In addition, *rtxA* has also been used to differentiate Classical O1 and El Tor O1 biotypes because the RTX cluster is absent in the Classical O1 strains (Chow *et al.*, 2001). In this study, all O1 *V. cholerae* strains belonged to the El Tor biotype because the *rtxA* gene was amplified in these strains. Only two non-O1/non-O139 *V. cholerae* strains (77/08 and 3744/08) showed amplification of the Classical *hlyA* gene.

In conclusion, mutations in housekeeping (*dnaE*, *lap*, *recA*, *gyrB*, and *cat*) and virulence genes (*hlyA*, *rtxA*, and *toxR*) were observed in all serogroups of *V. cholerae* strains with low amino acid substitution rates. Higher genetic diversity was observed for non-O1/non-O139 strains. Regardless of the source and locatlities of isolation, clonally related O1 *V. cholerae* were found and one O1 strain was clonally related to O139 *V. cholerae*. Combination of MLST and MVLST analysis in this study was able to differentiate the toxigenic and non-toxigenic strains. In addition, sequence typing analyses provided further information on the type of gene mutations, which was not possible using other typing methods.

Chapter Nine

In-vivo Gene Expression Study for Strains of Different Serogroups and the Host Environmental Influences

9.1 Background

Environmental strains of *V. cholerae* are genetically diverse and harbour various combinations of virulence-associated genes. Hence, these environmental strains could be reservoirs for transmissible virulence genes (Faruque *et al.*, 2004). Moreover, despite sharing the same serogroups, clinical and environmental O1 *V. cholerae* are genetically heterogenous; therefore, the colonization ability of these diverse strains might be different (Chakraborty *et al.*, 2001).

The mechanism of pathogenesis of O1 *V. cholerae* is well-defined. Three major virulence-associated elements are found in *V. cholerae* such as the TCP (toxin-corregulated pilus) gene cluster, cholera toxin (CTX) genetic element and repeat in toxin (RTX) element which is associated with cytotoxicity in Hep-2 cells (Chow *et al.*, 2001; Rivera *et al.*, 2001). *tcpA, tcpI* and *acfB* are the TCP-island-specific genes necessary for intestinal colonization while *ctxA, zot,* and *ace* from CTX element are responsible for the production of cholera toxin (Reidl and Klose, 2002). The production of TCP and CT is dependent on ToxT while ToxRS and TcpPH are the key components for *toxT* induction. Meanwhile, the regulations of ToxRS and TcpPH are complex, involving additional regulators such as AphA, HapR, LuxO and others (Xu *et al.*, 2003). Other factors such as cell density, motility, chemotaxis also direct or indirectly affect the regulation of ToxT (Liu *et al.*, 2008; Matson *et al.*, 2007; Syed *et al.*, 2009). Majority of the genes in both VPI and CTX elements are not available in *V. cholerae* non-O1/non-O139 strains while the genes in RTX gene cluster are found in all

serogroups of *V. cholerae* (Chow *et al.*, 2001). Non-toxigenic O1 and non-O1/non-O139 *V. cholerae* which lack TCP and CT are also capable of colonization (Faruque *et al.*, 2004; Dalsgaard *et al.*, 1999), but strains which harbour either *tcpA* or *ctxA* were more efficient colonizer (Faruque *et al.*, 2003). In addition, *tcpI* acts as downstream regulator of *tcpA* and is used to assists in the virulence expression of TCP (Rivera *et al.*, 2001). However, the ability of colonization for non-O1/non-O139 strains which harbour only *tcpI* remains unknown.

Generally, the expression of virulence genes is significantly enhanced *in-vivo*, especially those genes which are responsible for chemotaxis, adherence, motility and regulators associated with TCP and CT production (Xu *et al.*, 2003). Pathogens must be able to regulate specific genes in order to survive in the host environment and mediate colonization during different stages of infection (Boyce *et al.*, 2004; Xu *et al.*, 2003). DNA microarray was used to monitor the expression of genes *in-vivo* for *V. cholerae* and it was reported that 80% of genes are down-regulated in human shed *V. cholerae* (Boyce *et al.*, 2004; Hinton *et al.*, 2004; Shelburne and Musser, 2004). Conversely, Faruque *et al.* (2004) had also reported the selective enrichment of potential pathogenic strains which occurred in the host, which led to the hyperinfectious stage of *V. cholerae* isolated from stools (Merrell *et al.*, 2002).

In this Chapter, the ability of colonization for two O1 *V. cholerae* strains which shared the same virulence alleles was compared. The ability of colonization for non-toxigenic O1, *tcpI*$^{+}$ non-O1/non-O139, and non-toxigenic non-O1/non-O139 *V. cholerae* strains were determined. In addition, the influence of host environment in colonization was also studied.

9.2 Materials and Methods

9.2.1 Strains selection and mouse inoculation

Five *V. cholerae* strains including one toxigenic/clinical O1 (123/08), one toxigenic/environmental O1 (1/08), one non-toxigenic/clinical O1 (35/04), and two non-toxigenic/clinical O1 (310/08 and GB/08) were selected based on MVLST analysis in **Chapter 8**. Toxigenic O1 strains, 123/08 and 1/08 shared the same allelic profiles for *ctxAB*, *hlyA*, *rtxA*, *tcpA*, *toxR* and *tcpI* genes. The inocula were prepared as previously described in Olivier *et al.*, (2007) with slight modification. Briefly, overnight cell cultures were diluted 1:1000 in fresh LB broth supplemented with 100 µg of streptomycin and allowed to grow to reach mid-log phase (A_{600}~0.5). An aliquot of 100 µl of the culture was spun down and the pellet was washed and resuspended twice with 1 X Phosphate-buffered saline (PBS). Approximately of 10^6 –10^7 CFU/ml was used for inoculation in mice.

The mouse-inoculation protocol was approved by the Faculty of Medicine, University of Malaya [PM08/06/2010/CKH(R)]. Eight weeks old healthy ICR mice were used. The mice were forced to feed with the inocula using animal feeding gauge, and labelled accordingly. One uninfected mouse was used as a control. The mice were sacrificed after 24 h. The intestines were removed and observed for fluid accumulation.

9.2.2 RNA isolation from bacterial culture and mouse intestines

RNA was isolated from both bacterial culture and mouse intestines. The RNA isolated from *in-vitro* growth was used as the reference sample of pre-infection (B1–B5) while the RNA derived from the mouse intestine was used as samples of post-infection (M1–M5).

One millilitre of bacterial culture was pelleted and resuspended with 1 ml of easy-BLUE Total RNA extraction solution (Intron Biotechnology, Gyeonggi-Do, Korea). Two hundred microlitre of chloroform was added. The mixture was centrifuged for 10 min at 4°C. Approximately 400 µl of upper aqueous layer was transferred to an empty microcentrifuge tube, followed by adding 400 µl of isopropanol into the tube. After mixing, the mixture was left at room temperature for 10 min and centrifuged. The pellet was washed with 75% ethanol, and allowed to dry. Finally, the pellet was dissolved in 30 µl of ddH_2O and kept in -20°C before use.

To isolate the total RNA from mouse intestine, 100 mg of intestine tissue was homogenized in 1ml of Easy-blue Total RNA extraction solution (Intron Biotechnology, Gyeonggi-Do, Korea). 200 ml of chloroform was added and the extraction was performed similarly as mentioned above.

One microlitre of the extracted RNA was mixed with 4 µl of TE and run on 1% (w/v) agarose gel. The total RNA was treated with DNase (Promega, Madison, USA) (incubate at 37°C for 30 mins) and quantified using a biophotometer (Eppendorf, Milan, Italy).

9.2.3 Reverse-transcription (RT)-PCR

Single-stranded cDNA was synthesized from RNA (B1-B5 and M1-M5) using the High Capacity cDNA Reverse Transcription kit according to the manufacturer's instruction (Applied Biosystems, Carlsbad, CA, USA). Briefly, 2 X RT master mix including RT buffer, dNTP mix, random primers, reverse transcriptase and ddH_2O was prepared and finally 10 ul (~2 µg) of total RNA was added. Reverse transcription was carried out in a thermal cycler with optimized condition as recommended from the manufacturer (25°C for 10 min, 37°C for 120 min, 85°C for 10 min and held at 4°C). The cDNA was directly used for real-time PCR analysis.

9.2.4 Detection of virulence genes by conventional PCR

Conventional PCR was carried out for detection of *rtxA*, *tcpI*, *hlyA*, *tcpA*, *ctxA* and *toxT* genes. cDNA of B1-B5 and M1-M5 were used as DNA template. The PCR was performed as described in **Chapter 8**, **Section 8.2.4**.

9.2.5 Comparative Expression Study

An initial standard curve experiment was carried out to quantify and validate the expression of endogenous control (*ompW*) in all samples according to the guidelines provided by the manufacturer (Applied Biosystems, Carlsbad, CA). Same concentration of cDNA for each sample was loaded.

The expressions of nine target genes (*rtxA*, *acfB*, *toxT*, *tcpI*, *hlyA*, *tcpA*, *ace*, *ctxA* and *zot*) were evaluated using Taqman probes (Applied Biosystems, Carlsbad, CA). Quantitation-Comparative Ct experiment type was used in this study. Real-time PCR was performed using Step-One Plus Real-time PCR System (Applied Biosystems, Carlsbad, CA) in a total volume of 20 µl containing 1× *Taq*Man Universal Master Mix without AmpErase UNG, 1× Assay Mix (Applied Biosystems, Carlsbad, CA) and ~10 ng cDNA template at cycle conditions: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. All reactions including negative control were run in triplicates.

B1-B5 were selected as reference while M1-M5 as samples. The expression of genes for each sample was compared in fold-changed. *RQ* value>1 indicates up-regulation of gene expression while RQ<1 indicates down-regulation of gene expression based on the manufacturer's instruction (Applied Biosystems, Carlsbad, CA).

9.3 Result

9.3.1 Colonization and observation for fluid accumulation

Severe fluid accumulation was observed in mouse infected with strains 123/08 and 1/08 along the small intestine, cecum and colon. For the mouse infected with strains 35/04 and 310/08, slight fluid accumulation was observed in the small intestines. The colonized intestines were also observed with gas accumulation. No fluid and gas accumulation was found for GB/08 (**Figure 9.1**).

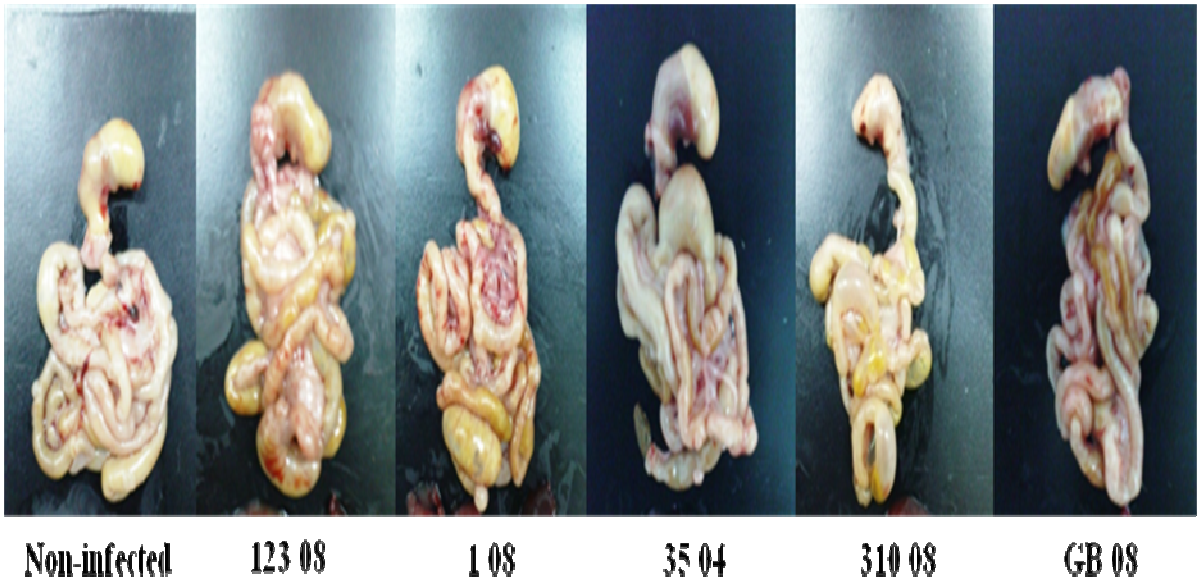


Figure 9.1 Intestines removed from the mice infected with different variants of strains showing gas and fluid accumulations compared with the non-infected mouse. Strain 123/08 was clinical O1 *V. cholerae*; Strain 1/08 was environmental O1 *V. cholerae*; Strain 35/04 was clinical O1 *V. cholerae* which did not harbour virulence genes; Strain 310/08 was non-O1/non-O1 *V. cholerae* which harboured *tcpI*; Strain GB/08 was non-O1/non0-O139 *V. cholerae* which did not harbour virulence genes.

9.3.2 Detection of *V. cholerae* from stools

Stools were collected from the mice and subjected to *ompW*-based PCR for *V. cholerae* detection. Rice watery stool was observed for the mouse infected with strain 123/08, while others were not watery. Unfortunately, no stool was collected from the mouse infected with GB/08. Overall, *V. cholerae* was detected in the stools collected from the mouse infected with strains 123/08 and 1/08 only (**Figure 9.2**).

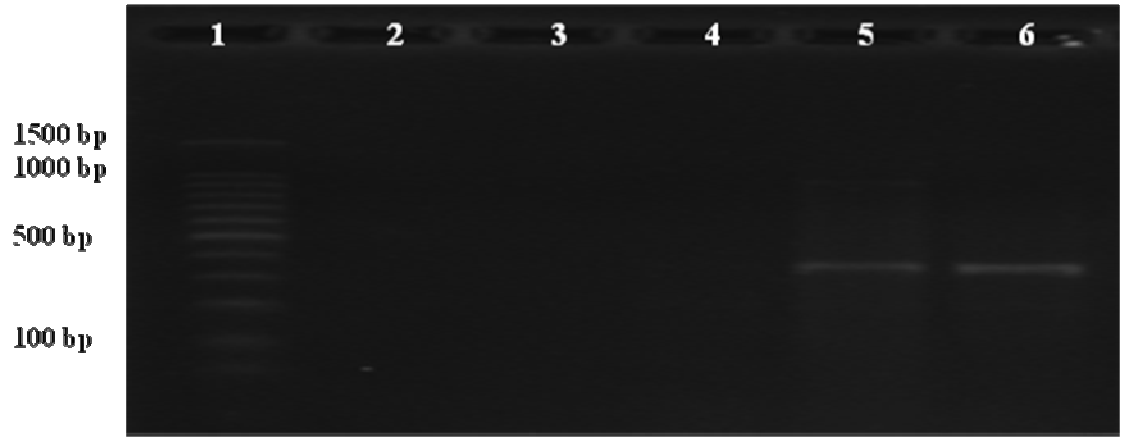


Figure 9.2: Agarose gels showing the results of PCR performed on the stools collected from the mice. Lane 1: 100 bp DNA marker; Lane 2: negative control (ddH_2O); Lane 3: 35/04 (O1); Lane 4: 310/08 (non-O1/non-O139); Lane 5: 123/08 (O1); Lane 6: 1/08 (O1).

9.3.3 PCR detection of virulence genes before and after inoculation

Before the commencement of *in-vivo* expression study, the detection of *rtxA*, *tcpI*, *hlyA*, *tcpA*, *ctxA*, and *toxT* were performed on the strains 123/08, 1/08, 35/04, 310/08 and GB/08. Strains 123/08 and 1/08 were *rtxA*$^+$, *tcpI*$^+$, *hlyA*$^+$, *tcpA*$^+$, *ctxA*$^+$ and *toxT*$^+$. Strain 310/08 harboured *rtxA*, *tcpI*, *hlyA* and *toxT* while strains 35/04 and GB/08 harboured *rtxA*, *hlyA* and *toxT*.

PCR was performed again on the cDNA which synthesized from RNA isolated from the intestines. As a result, the virulence profiles of strains 123/08, 1/08 and GB/08 remained the same. Strain 35/04 was *rtx*A$^+$, *tcp*I$^+$, *hly*A$^+$ and *toxT*$^+$ while strains 310/08 was *rtxA*$^+$, *tcpI*$^+$, *hlyA*$^+$, *tcpA*$^+$, *ctxA*$^+$ and *toxT*$^+$. PCR was carried out twice and the same results were obtained (**Figure 9.3**). The PCR results are summarized in **Table 9.1**.

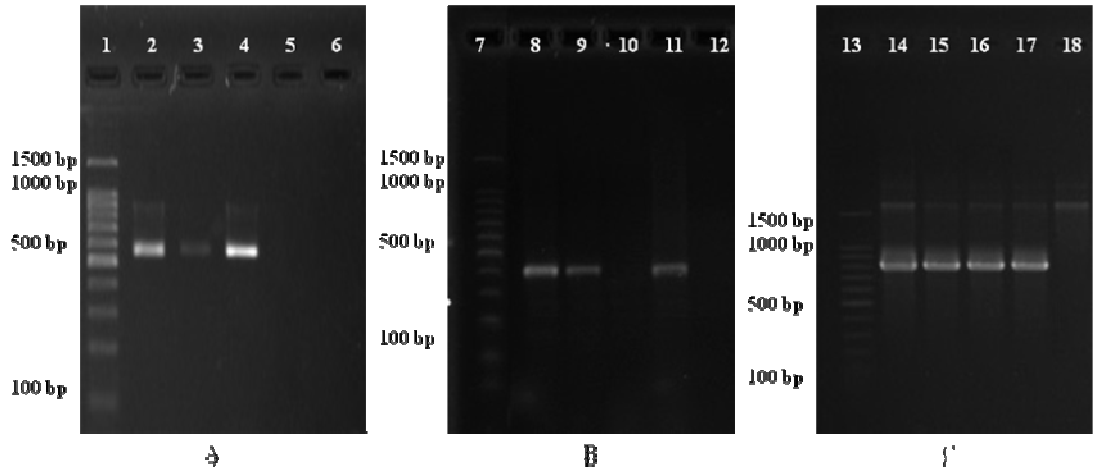


Figure 9.3: Agarose gels showing the results of PCR carried out on the cDNA (after inoculation). A) Amplification of *ctxA* gene at ~564 bp. Lane 1: 100 bp DNA marker; Lane 2: 123/08 (O1); Lane 3: 310/08 (non-O1/non-O139); Lane 4: 1/08 (O1); Lane 5: 35/04 (O1); Lane 6: GB/08 (non-O1/non-O139). B) Amplification of *tcpA* gene at ~451 bp. Lane 7: 100 bp DNA marker; Lane 8: 123/08 (O1); Lane 9: 310/08 (non-O1/non-O139); Lane 10: 35/04 (O1); Lane 11: 1/08 (O1); Lane 12: GB/08 (non-O1/non-O139). C) Amplification of *tcpI* gene at ~862 bp. Lanes 13: 100 bp DNA marker; Lane 14: 123/08 (O1); Lane 15: 35/04 (O1); Lane 16: 1/08 (O1); Lane 17: 310/08 (non-O1/non-O139); Lane 18: GB/08 (non-O1/non-O139).

Table 9.1: Strains, sources of isolation, and virulence profiles of the five *V. cholerae* strains based on conventional PCR

Strain	Source	Serogroup	Virulence Profile before infection[a]	Virulence Profile after infection[b]
123/08	Patient	O1	B1: *rtxA*$^+$, *tcpI*$^+$, *hlyA*$^+$, *tcpA*$^+$, *ctxA*$^+$, *toxT*$^+$	M1: *rtxA*$^+$, *tcpI*$^+$, *hlyA*$^+$, *tcpA*$^+$, *ctxA*$^+$, *toxT*$^+$
1/08	Water	O1	B2: *rtxA*$^+$, *tcpI*$^+$, *hlyA*$^+$, *tcpA*$^+$, *ctxA*$^+$, *toxT*$^+$	M2: *rtxA*$^+$, *tcpI*$^+$, *hlyA*$^+$, *tcpA*$^+$, *ctxA*$^+$, *toxT*$^+$
35/04	Patient	O1	B3: *rtxA*$^+$, *hlyA*$^+$, *toxT*$^+$	M3: *rtx*A$^+$, *tcp*I$^+$, *hly*A$^+$, *toxT*$^+$
310/08	Seafood	Non-O1/non-O139	B4: *rtxA*$^+$, *tcpI*$^+$, *hlyA*$^+$, *toxT*$^+$	M4: *rtxA*$^+$, *tcpI*$^+$, *hlyA*$^+$, *tcpA*$^+$, *ctxA*$^+$, *toxT*$^+$
GB/08	Water	Non-O1/non-O139	B5: *rtxA*$^+$, *hlyA*$^+$, *toxT*$^+$	M5: *rtxA*$^+$, *hlyA*$^+$, *toxT*$^+$

[a] PCR preformed on strains before inoculation.

[b] PCR performed on cDNA synthesized from RNA isolated from the intestines of the inoculated mice.

ompW was present in all the samples.

9.3.4 Genes expression via Real-time PCR

In strain 123/08, all the virulence genes were down-regulated, except for *toxT* and *tcpI* after 24 hour of inoculation. The expression of *toxT* and *tcpI* ranged from 1.2-1.45-folds. However, in strain 1/08, *acfB*, *tcpA*, *ctxA* and *zot* were down-regulated while *rtxA*, *toxT*, *tcpI*, *hlyA* and *ace* genes were expressed at the range of 1 to 2.3-folds.

Most of the expression of virulence genes in strains 35/04 and 310/08 were increased. In strain 35/04, the expression of *acfB*, *toxT*, *tcpI*, *ace*, *tcpA*, *ctxA* and *zot* genes were up-regulated and these increments ranged from 1.4 to 3.5-folds. In strain 310/08, all the expression of virulence genes were upregulated, except *rtx*A gene. Besides that, all the genes were expressed at the range of 3.2 to 6.6-folds, except for *hlyA* which only 1-fold expressed.

Only *tcpI* and *tcpA* expression were upregulated in strain GB/08. All the other genes were down-regulated. A comparative view of expressed genes for the five strains is shown in **Figure 9.4**.

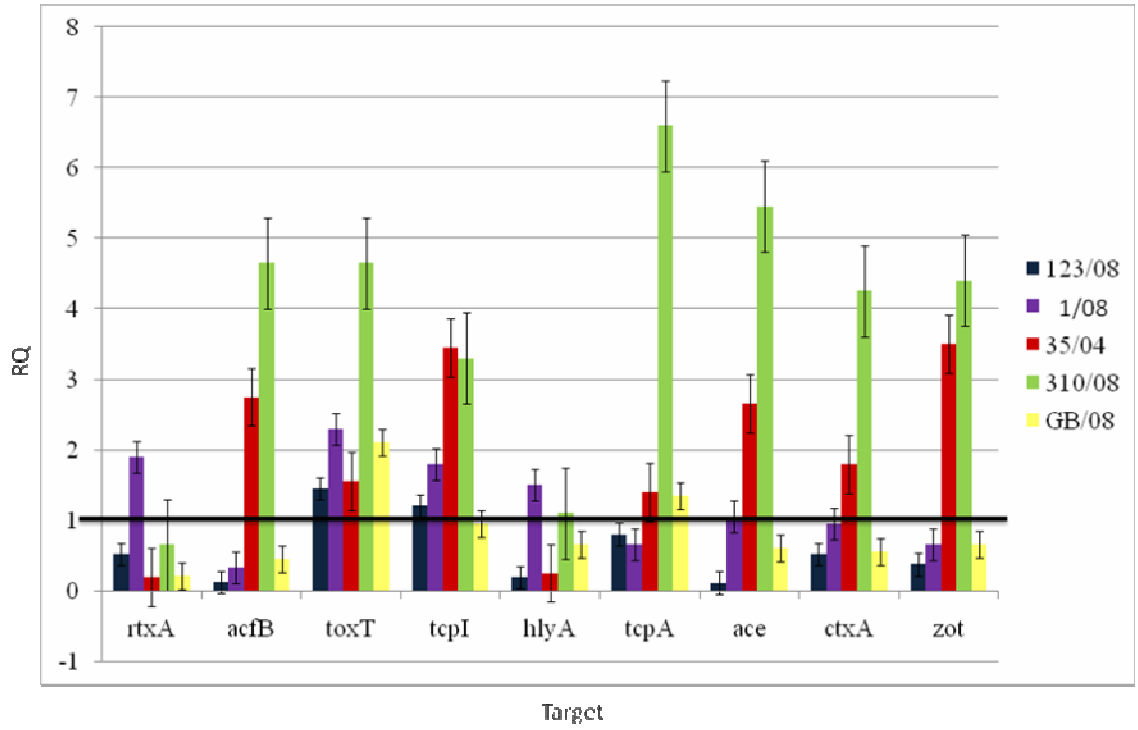


Figure 9.4 The regulation of expressed genes for the five strains. The expression of genes were up-regulated when RQ>1. Genes expression of *rtxA*, *acfB*, *toxT*, *tcpI*, *hlyA*, *tcpA*, *ace*, *ctxA* and *zot* for toxigenic/clinical O1 (123/08), toxigenic/environmental O1 (1/08), non-toxigenic/clinical O1 (35/04), *tcpI*$^{+}$ non-O1/non-O139 strain (310/08) and non-toxigenic non-O1/non-O139 strain (GB/08) were compared.

9.4 Discussion

Majority of fluid accumulation was found along the colon and cecum. This finding was in agreement with Angelichio *et al.* (1999) that *V. cholerae* are well-colonized at the cecum and large bowel with high viability. However, although the fluid accumulation was not obviously found in the small intestine, the establishment of colonization in the small intestine was still possible beyond 24 hours as long as the bacterial cells were cleared (Olivier *et al.*, 2007).

Xu *et al.* (2002) have suggested that genes for motility and chemotaxis are significantly induced in the intestinal environment. As motility is associated with the virulence of *V. cholerae*, it is suggested that the regulation of motility and virulence occurred inversely (Syed *et al.*, 2009). Flagella is used to penetrate mucin layer but is not attached to the cells after enter the mucin (Liu *et al.*, 2008). The loss of motility also represses the *hapR* and leads to induction of ToxT. Besides the motility, the effect of cell density on virulence production is also important. *V. cholerae* required 10^6 of cells to induce a symptomic infection (Salyes and Whitt, 2002). The cells continuously multiply while moving from the digestive system to intestine. However, the number of cells is decreased by barriers such as changes of temperature, osmolarity, gastric acid, and bile salt to reach the intestine. Low cell density interrupts the transcription of *hapR* and enhances the expression of *aphA*. This facilitates the activation of ToxRS and TcpPH to induce the expression of *toxT* as the initial induction of virulence factors (Liu *et al.*, 2008, Matson *et al.*, 2007). In contrast, when the cell density is high, the increased production of HapR protein can repress the transcription of *aphA* and result in the inactivation of virulence factors (Matson *et al.*, 2007). This is commonly observed at the late stage of infection as the number of cell reach a higher density. It

was suggested that virulence factors such as CT are transiently expressed for infection and repressed before being shed to the environment for transmission to the new host (Boyce *et al.*, 2004; Li *et al.*, 2008,). In our study, most of the virulence genes in the toxigenic clinical O1 *V. cholerae* (123/08) were down-regulated, indicating that the cells are in preparation or in progress of disseminating out of the host. This finding was also supported by the evidence of *V. cholerae* detected in the rice watery stool collected from the mouse infected with strain 123/08. As *tcpA* and *ctxA* were down-regulated after 24 hours of inoculation, these two genes were expected to be transiently expressed (Boyce *et al.*, 2004). The uninterrupted high expression of *toxT* and *tcpI* in the intestinal derived *V. cholerae* for this strain suggests that the acquisition of these 2 genes for cell growth and survival as *toxT* is one of the components in the cascade mechanism to regulate and express genes for cell growth and survival while *tcpI* is involved in environmental sensing and chemosensory. This observation concurred with Rivera *et al.*, (2001) that *tcpI* gene may be important in both physiology and pathogenesis of *V. cholerae*.

Olivier *et al.* (2007) have proposed the utility of accessory toxins such as *rtxA* and *hlyA* for establishment of prolonged colonization in El Tor O1 strains, where the bacteria were able to prevent clearance from the host. Two El Tor O1 strains (123/08 and 1/08) with similar MVLST profiles but different source of isolation were included in this study. Interestingly, the prolonged colonization was only observed in the toxigenic environmental O1 *V. cholerae* (1/08) which showed upregulation of *rtxA* and *hlyA* genes expression. This strain also caused asymptomic infection of the infected mouse as this mouse did not shed typical ‘rice watery’ stool. However, *V. cholerae* was detected in the stool collected from this mouse. Thus, this finding suggests that the ability of the environmental O1 *V. cholerae* to resist variety of environmental stress has

increased the fitness in intestinal environment and favourably established a prolonged colonization event. The finding supports the observation that clinical and environmental O1 *V. cholerae* strains are very different in certain genetic elements (Chakraborty *et al.*, 2001).

Merrel *et al.* (2002) suggested that the virulence genes in *V. cholerae* will be turned-off before being shed to the environment and the infectivity of *V. cholerae* does not correlate with the increased expression of virulence genes. Merrel *et al.* (2002) also found that *in-vitro* expressed virulence genes might not be expressed in the host environment. In this study, no virulence genes were detected for the non-toxigenic clinical O1 *V. cholerae* (35/04) before inoculation. However, after inoculation, *tcpI* was detected and the expression was increased by 3.45-fold. The *tcpI* gene in this strain (35/04) might be turned-on when it re-entered the intestinal environment of the mouse. Repetitive *in-vitro* experiments which have been carried out on this strain did not alter the infectivity of this strain as the virulence genes are induced in a favourable environment regardless of the period of isolation.

Harkey *et al.* (1994) reported that TcpI is the negative regulator of *tcpA* gene and is able to reduce the motility of cell. However, when the cell reaches the correct anatomical site of the intestine, signal transduction of TcpI to the environment will activate the expression of TcpA and fine-tune the components of virulence and promote TCP assembly (Harkey *et al.*, 1994). Therefore, the involvement of *tcpI* in the colonization is consequential. Rivera *et al.* (2001) have reported environmental non-O1/non-O139 *V. cholerae* that harbour *tcpI* gene. This suggests that the virulence of *V. cholerae* is associated with ecosystem (Rivera *et al.*, 2001). However, the intestinal

environment also facilitates the enrichment of strains with pathogenic potential (Faruque *et al.*, 2004).

In this study, one *tcpI*$^{+}$ non-O1/non-O139 was used to test for the efficiency for colonization. The overall result of the *tcpI*$^{+}$ non-O1/non-O139 *V. cholerae* was interesting. Firstly, the strain harboured *rtxA*, *tcpI*, *hlyA* and *toxT* before entering the host but detected with *rtxA*, *tcpI*, *hlyA*, *tcpA*, *ctxA*, *zot* and *toxT* after derived from the intestine. Except for *rtxA* and *hlyA* genes, the expression levels of genes in this strain were obviously high. The high expression level of genes (**Section 9.3.4**) in this strain had led to the occurrence of colonization. This suggests that the virulence genes were intensively enriched in the intestinal environment, thus increase the ability of colonization.

The ability of colonization by non-O1/non-O139 strains without TCP and CT has been reported (Olivier *et al.*, 2007). However, no colonization was observed for the non-toxigenic environmental non-O1/non-O139 *V. cholerae* in this study. Expressed ToxT in this strain may activate the transcription of *tcpA*, but was not able to establish colonization significantly. The repressions of *tcpI* and *acfB* lead to inappropriate circumstances for colonization and CT induction (Lee *et al.*, 1999), thus fluid accumulation was not observed. This is in agreement with Chaparro *et al.*, (2010) that *tcpI* and *acfB* have overlapping or redundant chemotactic function which lead to intestinal colonization.

Fluid accumulation caused by *V. cholerae* infection is a well-known observation. In this study, gas accumulation was also observed in addition to fluid accumulation. This happened only to the mouse infected with *tcpI* positive *V. cholerae*. It is tempting to speculate that the gas production may be associated with the TCP cluster and gas

production may be crucial with the presence of accessory toxin such as haemolysin. This speculation is outlined by the repression of *hlyA* gene in toxigenic clinical O1 *V. cholerae* in consequence of the least production of gas. Nevertheless, inflammatory responses caused by the accessory toxin mutant have been described elsewhere (Fullner *et al.*, 2002), gas production and the factors mediated has not been mentioned and could be further investigated in the future.

In conclusion, this study shows that O1 *V. cholerae* strains from different sources which shared same virulence profiles may have different performance in colonization. *tcpI*$^{+}$ non-O1/non-O139 can be more efficient colonizer than *tcpI*$^{-}$ non-O1/non-O139 *V. cholerae* as *tcpI* might be useful in regulating the expression of other colonization-associated genes in parallel with ToxT. Finally, the host environment can favourably enrich the virulence genes in non-toxigenic O1 *V. cholerae*, thus enable the colonization once the virulence genes are introduced in the intestinal environment.

Chapter Ten

General Discussion

Vibrio cholerae, the causative agent of cholera, is endemic in many parts of the world, especially in developing and less developed countries which lack of clean water supplies and inadequate public health facilities (Alam *et al.*, 2005). *V. cholerae* O1 and O139 serogroups are responsible for severe diarrhea cases which can cause mortality if no treatment is given to the patients (Faruque *et al.*, 1998).

In Malaysia, cholera outbreaks due to the *V. cholerae* O1 serotype occur periodically (Vadivelu *et al.*, 2000). Two hundred and thirty seven cases of cholera with two fatal cases were reported in 2006 by the Department of Public Health (DPH), Ministry of Health Malaysia (http://www.dph.gov.my/survelans/Statistik). Elhadi *et al.* (2004) reported that the ratio of distribution of *V. cholerae* O139 to O1 serogroups isolated from seafood from 1998-1999 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 (Elhadi *et al.*, 2004; Chen *et al.*, 2004). 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 (Rivera *et al.*, 2001; Faruque *et al.*, 2004).

Conventional identification of *V. cholerae* is time consuming and tedious. Moreover, ambiguous identification was observed due to the high similarity of biochemical properties among *V. cholerae* and other *Vibrio* species as well as *Aeromonas* species. Molecular identification of *V. cholerae* based on 16S rRNA, *ctxAB* and *tcpA* were not satisfactory because 16S rRNA was conserved among the Vibrionaceae family and some of the *V. cholerae* strains do not harbor virulence

markers (Kita-Tsukamoto *et al.,* 1993; Ruimy *et al.,* 1994; Kaper *et al.,* 1995). Therefore, Nandi *et al.* (2000) had developed a PCR detection of *V. cholera*e based on *ompW* gene and this PCR had shown high specificity in *V. cholerae* detection.

On the other hand, *V. cholerae* is autochthonous to the aquatic environments, hence it is important to monitor this bacterium in water sources for control of cholera (Choopun *et al.,* 2002). Besides *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* are another two important water-borne pathogens which primarily cause seafood-associated bacterial illness. *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* are associated as principal pathogenic *Vibrio* spp which are involved in gastrointestinal illness or septicemia (Panicker *et al.,* 2004). Therefore, it is necessary and useful to develop a PCR assay which could simultaneously detect the three pathogens from environmental sources. Current available PCR assays either target the virulence genes of pathogenic *Vibri*o species (Panicker *et al.,* 2004) or require an additional step of DNA sequencing for confirmation (Tarr *et al.,* 2007; Nhung *et al.,* 2006; Thompson *et al.,* 2005). In order to explore another specific marker for *V. cholerae* detection and overcome the limitations of current methods for pathogenic differentiation, the *gyrB*/*pntA*-based PCR was developed. Besides the differentiation of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus,* other *Vibrio* species could be detected by using this PCR approach based on *gyrB* marker.

Generally, virulence genes may not be suitable markers for *V. cholerae* detection because some of the *V. cholerae* strains including O1 serogroup do not harbor cholera toxin and toxin-corregulated pillus (Nandi *et al.,* 2000). However, it would be useful to develop a multiplex PCR that could differentiate the virulotype, biotype and serogroups of *V. cholerae*. In this study, a multiplex targeting the *ompW*, *hlyA*, *orf*

complex, *ctxA*, *toxR* and *tcpI* was developed to differentiate the biotype, serogroups and virulotypes of the 43 *V. cholerae* isolated from different sources.

V. cholerae comprises of three major serogroups (O1, O139 and non-O1/non-O139) where El Tor O1 and O139 are associated with the seventh and eighth pandemic (Salim *et al.*, 2005). The derivation of O139 *V. cholerae* is believed to be the result of horizontal genes transfer from non-O1 to O1 *V. cholerae* (Elizabeth *et al.*, 1995). This serogroup conversion reflects the phenomenon that genetic recombination and genes transfer occurred among *V. cholerae* (Li *et al.* 2002; Kotetidhvili *et al.* 2003) and directly affect the level of genetic variation as well as increase the genetic variance in a population (van Belkum *et al.*, 2001). Virdi and Sachdeva (2005) had reported the different traits of O1, O139 *V. cholerae* in India and the participation of non-O1/non-O139 *V. cholerae* in the genesis of new variants of *V. cholerae*.

A variety of genotyping methods have been developed to assess the variation of genome composition, restriction endonuclease profiles, number and position of repetitive elements and nucleotide sequences (van Belkum *et al.*, 2007). Genotypic typing is important for surveillance of infectious diseases, outbreak investigation, study of pathogenesis and investigation of genetic diversity (Virdi and Sachdeva, 2005; van Belkum *et al.*, 2007). A good typing method should be able to define the relatedness of strains, should not be too costly or complicated for operation, must be rapid, and easily assessable (van Belkum *et al.*, 2001). In the present study, a total of eight genotyping methods were performed. Overall, the discriminatory power for all the methods used were high (>0.90). To have a better separation of bands, longer gel was used for RAPD-PCR, ERIC-PCR, REP-PCR and VCR-PCR; hence, longer gel running time was needed. For MLVA and PFGE, special materials included fluorescent primers,

HiDi formamide, appropriate size standard, restriction enzyme, Proteinase K, Seakem Gold agarose were needed, hence increased the cost to perform both methods. MLST, MVLST and MLVA produced the best quality of data as the data generated was less subjective compared to gel-based analysis.

In this study, each method has shown to be efficient in subtyping the 43 *V. cholerae* strains of different serogroups that isolated from different sources. Generally, non-O1/non-O139 *V. cholera*e in this study demonstrated high genetic diversity while all the O1 *V. cholerae* were genetically linked (shared same profiles among each other for at least once), except for strain 35/04 and 3P/08 based on the combined analysis (**Table 10.1**). Strain 35/04 was isolated in 2004 from a cholera patient while strain 3P/08 was isolated from water samples in 2008. Based on RAPD-PCR and MVLST analysis, strain 35/04 was more related to non-O1/non-O139 *V. cholerae* while the close relationship (>80%) between strain 3P/08 and other non-O1/non-O139 *V. cholerae* was exhibited by RAPD-PCR. Strain 35/04 was different from other O1 *V. cholerae* strains because it did not harbour virulence genes. Besides that, this strain also harboured different allelic type of *rec*A gene which is highly conserved in O1 serogroups. On the other hand, the close genetic relatedness of strain 3P/08 and other non-O1/non-O139 *V. cholerae* suggests that there are common genetic elements shared by this O1 strain and other non-O1/non-O139 strains. This is in agreement that pathogenic *V. cholerae* evolved from nonpathogenic environmental strains and the environmental sources remain a reservoir for a variety of genetically different *V. cholerae* strains (Colwell *et al.*, 1995; Rahman *et al.*, 2008).

Table 10.1 Genotype profiles for the *Vibrio cholerae* strains based on RAPD-PCR, ERIC-PCR, REP-PCR, VCR-PCR, PFGE, MLVA, MLST and MVLST.

Strain	Serogroups	Source	Location	Year	RAPD	ERIC	REP	VCR	PFGE	MLVA	MLST	MVLST
1/08	O1	Water	PJ	2008	RA3	E3	RE7	V7	N7	M3	A3	B1
3/08	O1	Water	Klang	2008	RA4	E4	RE 8	V8	N8	M4	A4	B2
3P/08	O1	Water	Banting	2008	RA5	E5	RE 9	V9	N9	M5	A5	B8
4/08	O1	Human Stool	Kota Bharu	2008	RA6	E1	RE 10	V10	N2	M1	A6	B1
6/08	O1	Human Stool	KK	2008	RA 7	E2	RE 2	V11	N3	M6	A7	B5
9/08	O1	Human Stool	KK	2008	RA 8	E1	RE 2	V12	N10	M7	A8	B9
10/08	O1	Human Stool	KK	2008	RA 9	E2	RE 11	V13	N3	M8	A2	B4
11/08	O1	Algae	PJ	2008	RA 10	E6	RE 4	V3	N11	M9	A1	B6
21/04	O1	Human Stool	Alor Setar	2004	RA 11	E7	RE 12	V1	N12	M10	A1	B4
35/04	O1	Human Stool	Alor Setar	2004	RA 12	E8	RE 13	V14	N13	M11	A9	B10
37/04	O1	Human Stool	Kota Bharu	2004	RA 13	E9	RE 14	V1	N14	M12	A1	B1

38/04	O1	Human Stool	Tumpat	2004	RA 14	E10	RE 15	V4	N15	M13	A1	B5
40/04	O1	Human Stool	Tumpat	2004	RA 1	E11	RE 3	V1	N16	M14	A10	B11
52/08	O1	Human Stool	Labuan	2008	RA 1	E12	RE 16	V1	N17	M1	A1	B12
63/08	O1	Human Stool	Labuan	2008	RA 1	E13	RE 1	V1	N18	M1	A1	B2
64/08	O1	Human Stool	KK	2008	RA 15	E14	RE 1	V4	N2	M15	A1	B2
65/08	O1	Human Stool	Kota Bharu	2008	RA 1	E15	RE 3	V1	N19	M1	A1	B13
66/08	O1	Human Stool	Labuan	2008	RA 2	E16	RE 1	V2	N1	M1	A1	B2
70/08	O1	Water	Labuan	2008	RA 16	E1	RE 17	V3	N20	M16	A2	B1
87/08	O1	Water	Kota Bharu	2008	RA 17	E17	RE 1	V15	N1	M17	A1	B14
88/08	O1	Water	KK	2008	RA 2	E18	RE 18	V2	N1	M18	A1	B1
90/08	O1	Water	KK	2008	RA 2	E19	RE 4	V2	N1	M19	A1	B1
123/08	O1	Human Stool	HUKM	2008	RA18	E20	RE 19	V3	N21	M20	A2	B1
4370/08	O139	Water	Klang	2008	RA19	E21	RE 20	V16	N22	M21	A1	B6
17/08	Non-O1/non-O139	Water	Bachok	2008	RA20	E22	RE 21	V17	N23	M22	A11	B15
77/08	Non-O1/non-O139	Water	Klang	2008	RA21	E23	RE 22	V18	N4	M23	A12	B16

81/08	Non-O1/non-O139	Seafood	Selangor	2008	RA22	E24	RE 23	V5	N24	M24	A13	B3
82/08	Non-O1/non-O139	Seafood	Selangor	2008	RA23	E25	RE 24	V5	N25	M25	A14	B17
83/08	Non-O1/non-O139	Seafood	Selangor	2008	RA24	E26	RE 25	V19	N26	M26	A15	B3
84/08	Non-O1/non-O139	Seafood	Selangor	2008	RA25	E27	RE 26	V20	N27	M27	A16	B18
85/08	Non-O1/non-O139	Seafood	Selangor	2008	RA26	E28	RE 27	V21	N28	M28	A17	B19
310/08	Non-O1/non-O139	Seafood	Selangor	2008	RA27	E29	RE 28	V22	N29	M29	A18	B20
374/08	Non-O1/non-O139	Water	Morib	2008	RA28	E30	RE 29	V23	N30	M30	A19	B21
933/08	Non-O1/non-O139	Seafood	Selangor	2008	RA29	E31	RE 30	V24	N4	M31	A20	B22
3477/08	Non-O1/non-O139	Water	Klang	2008	RA30	E32	RE 31	V25	N31	M32	A21	B23
4933/08	Non-O1/non-O139	Water	Klang	2008	RA31	E33	RE 32	V26	N5	M33	A22	B24
B2/08	Non-O1/non-O139	Water	Serdang	2008	RA32	E34	RE 33	V27	N32	M34	A23	B3
B4/08	Non-O1/non-O139	Water	Serdang	2008	RA33	E35	RE 34	V28	N33	M35	A24	B7
M1/08	Non-O1/non-O139	Water	PJ	2008	RA34	E36	RE 5	V6	N34	M36	A25	B7
M2/08	Non-O1/non-O139	Water	Kajang	2008	RA35	E37	RE 5	V6	N35	M37	A26	B25
GB/08	Non-O1/non-O139	Water	Kajang	2008	RA36	E38	RE 6	V29	N6	M2	A27	B26

PSW/08	Non-O1/non-O139	Water	Morib	2008	RA37	E39	RE 35	V30	N5	M38	A28	B3
SW/08	Non-O1/non-O139	Algae	Morib	2008	RA38	E40	RE 6	V31	N6	M2	A29	B27

The high diversity of non-O1/non-O139 *V. cholera*e is expected as the non-O1/non-O139 *V. cholerae* is composed of more than hundreds of known and unknown serogroups (Thong *et al.*, 2002). Moreover, Chakraborty *et al.* (2001) reported that in spite of sharing the same serogroup, clinical and environmental *V. cholerae* exhibit high genetic diversity. In this study, some strains which had the same profiles in the individual typing methods were further discriminative by the the combined analysis. For example, based on the MLVA and PFGE analyses (**Chapter 7)**, same pulsotype and MLVA type were obtained for two environmental non-O1/non-O139 *V. cholerae* (GB/08 and SW/08). As these two strains were isolated independently from different sources (water and algae) and different locations (Klang and Morib), both strains should be denoted 'clonally' related according to the definition of relatedness in van Belkum *et al.* (2007). However, based on the combined analysis, these two non-O1/non-O139 *V. cholerae* strains were distinguishable. Similarly, based on the MLST and MVLST analyses (**Chapter 8)**, there were four groups of strains (Group 1: 63/08, 64/08, and 66/08; Group 2: 37/08, 88/08 and 90/08; Group 3: 70/08 and 123/08; Group 4: 11/08 and 4370/08.) which should be denoted 'clonally' related as they had 100% similarity, however, they were not identical in the combined analyses. Therefore, it is important to include multiple markers to facilitate the determination of relatedness of strains, and to support or reject the hypothesis that the strains come from a single origin (van Belkum *et al.*, 2007).

Generally, MLVA is the most suitable typing method for *V. cholerae* because of its ability to differentiate serogroups of *V. cholerae*, high discriminatory power, average cost, and precise interpretation of data. Moreover, the procedure is very straight forward and rapid. However, the determination of relatedness of strains will be more reliable by including multiple loci which are scattered around the chromosome in

order to ensure that the strains are grouped based on genetic relatedness (Achtman, 2001).

The mechanism of colonization using TCP and CTX is applicable to both O1 and O139 *V. cholerae* since the virulence properties of both serogroups are similar (Faruque *et al.*, 1998). However, colonization does occur in non-O1/non-O139 *V. cholerae* which lack of virulence genes (*tcp* and *ctx*). In general, CTX and TCP remain as the most important element for colonization. However, the production of CTX and TCP are co-regulated by the ToxR regulatory system which includes the ToxT protein and other genes such as *tcpI*, *tcpP* and *tcpH* (Faruque *et al.*, 2003). Rivera *et al.* (2001) reported the environmental *V. cholerae* strains which harboured a single virulence gene such as *tcpA*, *tcpI* or *ctxA* while Faruque *et al.* (2003) suggested that strains which harbour any of the virulence genes are better colonizer. This perhaps explains why only non-O1/non-O139 *V. cholerae* with *tcpI* positive was able to colonize the mouse intestine compared to the *tcpI* negative strains in the present study.

On the other hand, host environment is also a factor which influences expression of virulence genes for colonization (Faruque *et al.*, 2004). Virulence genes are enriched in the host and expressed during colonization, motility and chemotaxis (Xu *et al.* 2002). The virulence genes in *V. cholerae* could be turned-off after a complete colonization and before being shed to the environment (Merrel *et al.*, 2002). However, this phenomenon does not affect the infectivity of the strain as the virulence genes are potentially to be induced in a favourable environment regardless of the period of isolation.

Furthermore, it has been suggested that accessory virulence genes such as *rtxA* and *hlyA* are important for establishment of prolonged colonization in O1 El Tor strains

(Olivier *et al.*, 2007). In the present study, only environmental O1 *V. cholerae* showed prolonged colonization although the same virulence allelic profiles were shared by another O1 clinical strain. This is in agreement with Chakraborty *et al.*, (2001) that the environmental O1 strains were higher in fitness level compared to clinical O1. Additionally, this shows that the theory of prolonged colonization by Olivier *et al.* (2007) may not be applicable to all the strains of O1 serogroup.

Chapter Eleven

Conclusions

A multiplex PCR for simultaneous differential detection of *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus* and other *Vibrio* species was developed based on *pntA* and *gyrB* genes. This multiplex PCR exhibited high specificity and sensitivity compared to conventional biochemical tests and API 20E. Another multiplex PCR was also developed for detection, serogrouping, biotyping and virulotyping of *V. cholerae* and could shorten the time for conventional biotyping and serogrouping to four hours. RAPD-PCR, ERIC-PCR, REP-PCR, VCR-PCR, PFGE, MLVA, MLST and MVLST were performed and subtyped the 43 *V. cholerae* strains into 38, 40, 35, 30, 35, 38, 29 and 27 profiles, respectively and the clonally relatedness among a few O1 *V. cholerae* was shown. However, these strains were later discriminated and classified as closely related but not originated from the same clone in the combined analysis of the eight methods. This finding shows the heterogeneity of Malaysian *V. cholerae* including clinical and environmental O1 strains. Among the eight methods, MLVA developed in this study performed the best because it could differentiate the strains into serogroups and was discriminative, easy to operate, inexpensive, rapid and gave precise data for interpretation. Finally, the important role of *toxT* and *tcpI* for colonization in *V. cholerae* was ascertained. Clinical and environmental O1 *V. cholerae* of same virulence profiles showed different ability in colonization. Strain which harboured *tcpI* was easier to colonize the mouse intestine. Moreover, the virulence genes were favourably enriched in the host environment, and therefore facilitate the colonization in non-toxigenic *V. cholerae* strains.

In conclusion, all the objectives in this study were achieved. The genotypic profiles of *V. cholerae* obtained in this study may be compared to other strains either from previous outbreaks in Malaysia or from other regions for genetic relatedness surveillance. DNA microarray might also be performed as an alternative methods for *in-vivo* expression study of *V. cholerae*.