GENETIC AND PHENOTYPIC CHARACTERISATION OF CLINICAL *Vibrio cholerae* O1 STRAINS FROM MALAYSIA

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FACULTY OF SCIENCE
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KUALA LUMPUR

2018

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DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF BIOTECHNOLOGY

INSTITUTE OF BIOLOGICAL SCIENCES
FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2018

UNIVERSITY OF MALAYA
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Matric No: SGF150007

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GENETIC AND PHENOTYPIC CHARACTERISATION OF CLINICAL *Vibrio cholerae* O1 STRAINS FROM MALAYSIA

ABSTRACT

Vibrio cholerae serogroup O1 and O139 cause cholera outbreaks globally. The O1 serogroup consists of classical and El Tor biotypes, which cause the fifth, sixth and seventh cholera pandemic. More recently, the sporadic Matlab variant of O1 strain that possess both the classical and El Tor biotype characteristics was found to be emerging. The hybrid and variant strains have been reported to cause cholera outbreaks in India, Pakistan, and Bangladesh, then recently in Kelantan, Malaysia. These hybrid and variant biotypes could be misidentified as El Tor biotype due to their combined characteristics of both the El Tor and classical biotypes. Therefore, this study aimed to characterise and re-identify the biotypes of forty-four *V. cholerae* O1 El Tor clinical isolates from different outbreaks in Sabah(n=27), Sarawak (n=15) and Klang (n=2) from year 2009 to 2012. The isolates were subjected to phenotypic and genotypic tests for determination of their biotype and genetic characteristics. In order to identify the phenotypic characteristics of these clinical *V. cholerae* O1 isolates, polymyxin B susceptibility test, Voges-Proskauer test, haemolysis test, and chicken blood cell agglutination test were performed. The presence of genes of *tcpA*, *ctxA*, *ctxB*, *hlyA*, *rtxC*, and *rstC* in the isolates were detected using PCR approach. The results showed that 42 out of the 44 (95%) *V. cholerae* O1 clinical isolates tested in this study possess all the six genes tested. Both of the isolates from Klang lacked the *rstC* gene. Notably, based on the phenotypic characteristics and *ctxB* gene sequence, all of the *V. cholerae* O1 outbreak clinical isolates examined in this study, which were previously identified as El Tor biotype, was re-identified as either belongs to hybrid or variant biotype. Of the 44 isolates examined, 13 (30%) isolates were classified as the hybrid biotype; while 31

(70%) isolates were identified to be the variant biotype. DNA fingerprinting using REP-PCR was also carried out to determine the relationship among the *V. cholerae* O1 clinical isolates in this study. Based on the results, four REP profiles (REP 1, REP 2, REP 3 and REP 4) were observed based on the dendrogram generated. REP 1 and REP 2 groups were predominant while the two strains of O139 serogroup demonstrated a distinct profile (REP 4). In conclusion, all of the forty-four *V. cholerae* clinical isolates from cholera outbreaks occurred in Sarawak, Sabah and Klang in year 2009 to 2012 were not of the typical 7th pandemic El Tor biotype but demonstrated the characteristics of the variant or hybrid biotype, which were reported to be emerging. The isolates causing cholera outbreaks in Sarawak and Sabah were found to be genetically close and demonstrated limited genetic variation based on the REP-PCR genotyping.

Keywords: *V. cholerae* O1, El Tor variant biotype, hybrid biotype

PENCIRIAN GENETIK DAN PHENOTYPIC STRAIN KLINIKAL *Vibrio cholerae* O1 DARI MALAYSIA

ABSTRAK

Vibrio cholerae serogroup O1 dan O139 menyebabkan wabak taun secara global. Serogroup O1 terdiri daripada biotip klasikal yang bertanggungjawab untuk pandemik penyakit taun (kelima dan keenam) dan El Tor yang bertanggungjawab untuk pandemik ketujuh. Baru-baru ini, varian Matlab yang sporadikal dari strain O1 yang mempunyai ciri-ciri biotip klasikal dan El Tor didapati telah muncul. Strain hibrid dan varian telah dilaporkan menyebabkan wabak taun di India, Pakistan, Bangladesh dan yang baru di Kelantan, Malaysia. Biotip hibrid dan varian mungkin disalah anggap sebagai biotip El Tor kerana ciri-ciri mereka yang bergabungan kedua-dua biotip El Tor dan klasikal. Oleh itu, kajian ini bertujuan untuk mencirikan dan mengenalpasti semula biotip empat puluh empat *V. cholerae* O1 El Tor isolat klinikal dari wabak yang berlainan di Sabah (n = 27), Sarawak (n = 15) dan Klang (n = 2) dari tahun 2009 hingga 2012. Isolat telah tertakluk kepada ujian fenotip dan genotip untuk menentukan biotip dan ciri-ciri genetik. Untuk mengidentifikasikan ciri-ciri fenotip strain klinikal *V. cholerae* O1, ujian kecenderungan polymyxin B, Voges-Proskauer, hemolisis, dan ujian agglutination sel darah ayam telah dilakukan. Kewujudan gen virulensi *tcpA*, *ctxA*, *ctxB*, *hlyA*, *rtxC*, dan *rstC* dalam isolat dikesan melalui cara PCR. Keputusan menunjukkan bahawa 42 daripada 44 (95%) *V. cholerae* O1 isolat klinikal yang diuji dalam kajian ini mempunyai kesemua enam gen virulensi yang diuji. Kedua-dua isolat dari Klang tidak mempunyai gen *rstC*. Terutamanya, berdasarkan ciri-ciri fenotip dan jujukan gen *ctxB*, kesemua isolat klinikal wabak *V. cholerae* O1 yang termasuk dalam kajian ini, yang sebelum ini dikenal sebagai biotip El Tor, telah dikenal pasti semula sama ada sebagai biotip hybrid atau varian. Daripada 44 isolat yang diperiksa, 13 (30%) isolat dikelaskan

sebagai biotip hibrid; manakala 31 (70%) isolat dikenalpasti sebagai biotip varian. DNA fingerprinting menggunakan REP-PCR juga dilakukan untuk menentukan hubungan antara *V. cholerae* O1 isolat klinikal dalam kajian ini. Berdasarkan keputusan, empat profil REP (REP 1, REP 2, REP 3 dan REP 4) diperhatikan berdasarkan dendrogram yang dihasilkan. Jenis REP 1 dan REP 2 adalah utama manakala dua strain O139 serogroup menunjukkan profil yang berbeza (REP 4). Kesimpulannya, semua isolat klinikal *V. cholerae* dari wabak taun berlaku di Sarawak, Sabah dan Klang pada tahun 2009 hingga 2012 bukan biotip tipikal El Tor pandemik ketujuh tetapi menunjukkan ciri-ciri biotip varian atau hibrid, yang dilaporkan muncul. Isolat yang menyebabkan wabak taun di Sarawak dan Sabah didapati secara genetik dan menunjukkan variasi genetik terhad berdasarkan genotip REP-PCR.

Kata kunci: *V. cholerae* O1, El Tor varian biotip, hibrid biotip

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my supervisors, Dr Chai Lay Ching and Dr Cindy Teh Shuan Ju for guiding and encouraging me throughout this project. Their advices by means of knowledge in the related field of study have solidified my understanding and point of view towards the overall project. Their patience in explaining methods and knowledge involved have helped in the accomplishment of this project. There is also the mental support provided by them that assisted me to overcome difficulties in this project.

It is also my pleasure to thank all the lab mates. Their valuable peer review and encouragement in numerous discussions carried out have enabled me to gain more information in the field of study. Furthermore, I would also like to thank laboratory personnel and department staffs from Faculty of Science and Faculty of Medicine that assisted in the laboratory instruments operation and application paperwork of this project. A special thanks to personnel from UM Animal Science Center, whom helped in preparation of certain material during the project.

I am especially grateful to my family and friends for their encouragements during this project. Their supportive attitude and behaviour have aided me in maintaining a positive mentality throughout the project.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

Ace	: Accessory cholera enterotoxin
ADP	: Adenosine diphosphate
APW	: Alkaline peptone water
bp	: Base pair
CDC	: *Centers for Disease Control and Prevention*
Cl^-	: Chloride ion
CT	: Cholera toxin
cDNA	: Complementary deoxyribonucleic acid
DNA	: Deoxyribonucleic acid
dNTP	: Deoxynucleoside triphosphate
dsDNA	: Double stranded deoxyribonucleic acid
ERIC	: Enterobacterial repetitive intergenic consensus
et al.	: et alii (and others)
G+C	: Guanine+cytosine
G_{M1}	: Monosialotetrahexosylganglioside
Hep-2	: Human epithelial type 2
H_2O_2	: Hydrogen peroxide
HRS1	: Hyper-reiterated sequences
IHF	: Integration host factor
kb	: Kilo base
KDO	: Keto-3-deoxy-D-mannose-octulosonic acid
KOH	: Potassium hydroxide
LB	: Luria-Bertani
LPS	: Lipopolysaccharide

MARTX : Multifunctional autoprocessing RTX toxins

Mg^{2+} : Magnesium ion

$MgCl_2$: Magnesium chloride

mRNA : Messenger ribonucleic acid

MRVP : Methyl red Voges-Proskauer

n : Number of isolate

NaCl : Sodium chloride

PBS : Phosphate buffered saline

PCR : Polymerase chain reaction

Pfu : *Pyrococcus furiosus*

pH : Potential of hydrogen

ppt : Parts per thousand

rDNA : Ribosomal deoxyribonucleic acid

REP : Repetitive extragenic palindromic

rep-PCR : Repetitive-element polymerase chain reaction

RLEP : Mycobacterium leprae-specific repetitive element

RNA : Ribonucleic acid

rpm : Revolutions per minute

RTX : Repeat in toxin

SNP : Single nucleotide polymorphism

TA : Thymine, adenine

Taq : *Thermus aquaticus*

TBE : Tris-Borate-EDTA

TCBS : Thiosulphate citrate bile salts sucrose

TCP : Toxin-coregulated pilus

TLC : Toxic-linked cryptic

Tm	: Melting temperature
VBNC	: Viable but non-culturable
Vent	: *Thermococcus litoralis*
VP	: Voges-Proskauer
WHO	: World Health Organization
Zot	: Zonula occludens toxin

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 Introduction

Vibrio cholerae is a gram-negative, comma-shaped bacterium. They are facultative anaerobes that are highly motile. *V. cholerae* is a pathogen that causes cholera, which is a gastrointestinal infection. Severe cholera may leads to watery diarrhea, vomiting, and low blood pressure. Dehydration and hypovolemic shock due to rapid body fluid loss in patient will lead to death. Widespread epidemic is usually caused by two toxigenic serogroups of *V. cholerae*, which are O1 and O139. Under *V. cholerae* O1 strain there are classical and El Tor biotypes. Each of the two biotypes has different serotypes, namely Inaba, Ogawa and Hikojima. The serotypes could be differentiated through agglutination test based on their type-specific antigen.

The classical and El Tor biotypes are comprised of a number of phenotypic traits: 1) The classical strains are susceptible to Polymyxin B antibiotic, while El Tor strains are resistant. 2) El Tor strains are able to agglutinate chicken erythrocytes, but classical strains do not have that ability. 3) El Tor strains give a positive reaction to VP test while classical strains show negative reaction. 4) El Tor strains are susceptible to El Tor specific bacteriophage V only and classical strains are susceptible to classical bacteriophage IV. 5) The El Tor strains were originally reported being haemolytic to sheep erythrocytes and classical strains as non-haemolytic. However, El Tor strains become non-haemolytic since 1972 and now only those isolated from US Gulf Coast and Australia could haemolyse sheep erythrocytes (Barrett & Blake, 1981). The cholera toxin (CT) for classical and El Tor strains are different. Specific monoclonal antibody could bind to different CT of classical or El Tor. Few genes are used as genetic markers to distinguish El Tor and classical biotype, such as the toxin-coregulated pilus (TCP),

tcpA gene, haemolysin, *hlyA* gene, repeat-sequence transcriptional regulatory, *rstR* gene, repeat in toxin (RTX), *rtx* gene, and cholera toxin, *ctx* gene.

The classical biotype of *V. cholerae* O1 strain was mostly responsible for the earlier epidemic diseases such as the sixth cholera pandemic. Classical strain was replaced by the strains of El Tor biotype, which is the predominant cause of the seventh cholera pandemic. In recent years, there has been identification of new *V. cholerae* O1 variant strains from patients with acute diarrhea (Nair et al., 2002). There are two newly emerging biotypes, namely hybrid and El Tor variant, they have combined characteristics of classical and El Tor biotypes. The existence of these hybrids leads to difficulty in differentiation of their phenotypic traits and pose problem for public health and epidemiological studies. Cholera is classified as a pandemic disease. There is a higher occurrence of cholera in certain areas where the source of clean water is limited. Rapid treatment is needed for severe cholera. Early detection will be of significance to the recovery of patients. Isolates that do not show El Tor biotype characteristic may be misidentified as other strains, due to the common perception that the current pandemic is mainly caused by El Tor biotype of O1 strain and O139 strain.

Cholera is still a serious public health issue, as it is endemic in certain parts of the world. Biotyping of O1 strain plays important role in epidemiological study of cholera infection (Raychoudhuri et al., 2008). Subtyping of hybrid and variant biotypes is also important for finding its source and distribution. DNA fingerprinting methods have been used to determine the relatedness of isolates and provide better understanding of the derivation of new biotypes and their evolution.

In Malaysia, cholera outbreaks occurred more frequent in places where natural disaster occurs such as areas with flood cases. Most of the strains isolated from these outbreaks were of O1 serogroup. Variant strain was detected in the 2009 cholera outbreak in Kelantan (Ang et al., 2010). However, there are no further characterisations

conducted to detect the intrusion of hybrid and variant biotypes in Malaysia, as well as to profile the strains for epidemiological understanding.

Therefore, this research project was carried out with the aim to detect the presence of hybrid and variant strains from selected cholera outbreaks in Sabah, Sarawak and Klang from 2009 to 2012.

1.2 Research objectives

The objectives of this research project are:

a) to identify and characterise the biotypes of selected *V. cholerae* O1 isolates originated from different cholera outbreaks in Sabah, Sarawak and Klang from 2009 to 2012 using phenotypic and genotypic assays;

b) to determine the genetic relationship of the *V. cholerae* O1 outbreak strains using REP-PCR typing method.

CHAPTER 2: LITERATURE REVIEW

2.1 Cholera

Cholera is an acute illness caused by the *V. cholerae* bacterium. The symptoms of cholera ranged from mild to severe depending on virulence characteristics of strains. Symptoms for severe cholera include profuse watery diarrhea with voluminous rice water stools, accompanied by vomiting and muscle cramps at stomach and leg (Centers for Disease Control and Prevention [CDC], 2015). The loss of body fluids will lead to severe dehydration and electrolyte imbalance (World Health Organization, 2010). The infected person will enter a shock state because of the sudden decrease in blood pressure. If patient is left untreated after onset of symptoms, severe cholera can lead to fatality.

Transmission of cholera is through faecal-oral route. People are infected by drinking water or eating food that are contaminated with the *V. cholerae* bacterium. Cholera usually occurs in developing countries that have poor water and sanitation systems. Travellers have higher risk in contracting cholera when they travel to places with inadequate water treatment systems and poor hygiene.

Cholera patients usually develop diarrhea symptoms that lead to mild or severe loss of fluids in the body. Oral rehydration therapy is an easy and effective treatment to restore the loss of fluids through diarrhae and vomiting. The oral rehydration solution, a combination of salt, sugar and water is commonly utilised throughout the world for diarrhea treatment (CDC, 2014). Intravenous rehydration is used when the patient have a high severity of dehydration. Antibiotic treatment is able to decrease the duration of illness and reduces the need for fluid restoration. Only severe cases of cholera will require the use of antibiotic, or else sufficient rehydration is capable to recover the infected person. Micronutrients that contribute to mucosal immunity like vitamin A and

zinc is associated with *V. cholerae* infection (Harris et al., 2008). Zinc supplementation is applied to reduce severity of diarrhea in cholera affected children (Roy et al., 2008). Zinc supplemented children have reduced duration of illness and diarrheal stool volume decreases by 10%.

During the epidemic cholera in London in 1854, John Snow was the first to demonstrate that contaminated water is the likely source of the cholera transmission (CDC, 2004). An Italian anatomist named Filippo Pacini has isolated the cholera bacterium for the first time in 1854 (Bentivoglio & Pacini, 1995). The *V. cholerae* bacterium as a causative agent of cholera was identified by Robert Koch, a German bacteriologist in 1883 during an outbreak in Egypt (Handa et al., 2016).

2.1.1 Pandemic history

There have been seven cholera pandemics since the infection started spreading in 1817. The first pandemic, also known as Asiatic cholera started near Calcutta and it spread to other places of the Indian subcontinent within a year. In 1817 to 1824, the pandemic already started to spread throughout Southeast Asia, Middle East, eastern Africa and the Mediterranean coast (Hays, 2005). The second cholera pandemic was an infection that began to expand from India across western Asia to Europe and America. In the span of the second pandemic from 1827 to 1835, the cholera broke out in Russia and followed human traffic from western Russia and began to move into Europe. In between 1839-1856, the third pandemic was a major cholera outbreak compared to the first two pandemics as it was originated from India and spread towards Latin America, China and Japan. The fourth cholera pandemic started in 1863 until 1875. This epidemic claimed many Mecca pilgrims' lives as they travelled from their country to Mecca. The cholera was passed along as the pilgrims moved back to their home in areas of Egypt, Syria and Northern Africa.

The confirmed etiological agent for the fifth to seventh pandemics was the *V. cholerae* O1 serogroup. The fifth cholera pandemic occurred during 1881-1896, the outbreak in Asia reached the Borneo and Philippine islands. In Europe, the outbreak moved across seaports in Italy and France. At the end of 19th century and the beginning of 20th century, the sixth pandemic broke out. As the spread of cholera outward from India to other places, these places involved in cholera may have merged and become endemic. The outbreak of seventh cholera pandemic started in 1961, in Celebes, Indonesia and continued to the present day. It began in Indonesia and rapidly advanced throughout Southeast Asia (Hays, 2005). The worldwide spread of the seventh pandemic is believe to be caused by the advancement of transportation, where travelling by airplane has become more common. The spread of cholera can be travel-associated.

2.1.2 Cholera outbreaks in Malaysia

The earliest records of cholera in Malaysia appeared from year 1823 to 1830 in Durian Daun Hospital, Malacca (Yadav & Chee, 1990). In 1873, there was a cholera epidemic in Sarawak where many people died. Another major epidemic occurred during 1902 when severe drought was recorded in Sarawak, with an estimate of over 1500 deaths. There were a few more outbreaks took place after that, most of them occurred after drought season. There was a confirmed cholera outbreak in Sarawak during 1961 that lasted for 100 days (Yadav & Chee, 1990).

Sporadic cholera outbreaks occurred in areas of inadequate water supply and sanitation, especially during dry weathers. During 1996 to 1997 in peninsula Malaysia, more cholera cases were recorded in urban areas. This reflects the shift of high risk outbreak from rural to urban population due to rural-urban migration and food sanitation (Meftahuddin, 2002). Unhygienic food handling practices were proven to be the cause of the cholera outbreaks in Penang (1996) and Klang (end of 1997). There

were 17 cholera cases in Kelantan linked to the Penang outbreak. From February to August 1998 in Kelantan, cholera outbreak had affected eight districts in the area. Tetracycline resistant strains were detected in this outbreak (Ranjit & Nurahan, 2000). The widespread of multiple *V. cholerae* O1 clones during 1997-1998 might have caused the Miri outbreak in Sarawak (Radu et al., 2002). Between 1998 and 1999, PFGE results showed that *V. cholerae* O139 and rough strains (strains that express only the rough antigen, but not the smooth 'O' antigen of *V. cholerae* polysaccharides) isolated from seafood samples in Malaysia were originated from Bengal and Thailand-Malaysia-Laos, respectively (Chen et al., 2004).

A large scale cholera outbreaks took place during 2001, two thirds of the total national cases were recorded in Sabah state. Two separate cholera outbreaks occurred in Petaling district and Klang district located in Selangor the same year. In year 2002, two distinctive cholera outbreaks occurred in Malaysia. Occurrence of cholera outbreak among semi-boarding school students in Selangor was the first and followed by the cholera outbreak caused by the newly mutated strain of *V. cholerae* O139 strain in Penang (Patrick et al., 2012). Cholera spreads in Terengganu in 2009 where it began in Kuala Terengganu that was caused by contaminated fish crackers. Ice factories, cracker factories and eatery suspected of contamination was ordered closed (Teh et al., 2012). Meanwhile in Semporna, Sabah, cholera cases were also reported (Bernama, 2009). Sporadic and small scale outbreaks usually occurred throughout the country.

2.2 *Vibrio cholerae*

V. cholerae is the causative agent for cholera. The bacterium belongs to the Vibrionaceae family of the Gammaproteobacteria. *V. cholerae* is a Gram-negative, curved or comma-shaped rods and highly motile facultative anaerobe. They are capable

of utilising both respiratory and fermentative mechanism. The motility of this bacterium is due to the presence of a single polar flagellum. The extension of the outer membrane of its cell wall formed an enclosed sheath on the flagellum.

V. cholerae can be separated into three serogroups, namely *V. cholerae* O1, *V. cholerae* O139 and *V. cholerae* non-O1. The strains that cause the current cholera pandemic are from *V. cholerae* O1 serogroup and O139 serogroup. The *V. cholerae* non-O1 strains were known to cause sporadic cases of extraintestinal infections (Janda et al., 1988). Strains from O1 and O139 serogroups that are toxigenic, based on the production of cholera toxin, have the ability to cause cholera when ingested by human host. *V. cholerae* O1 and O139 strain is usually transmitted to human host via contaminated water or food consumption. In the environment, the *V. cholerae* non-O1 strains are more abundant than the O1 strains. Most of the non-O1 strains do not possess virulence factors that are found in O1 and O139 strains that causes infection.

V. cholerae requires certain condition for optimal growth, such as the salt requirement and the pH condition. Some *Vibrio* species need a moderate amount of salt to stimulate their growth. *V. cholerae* can grow in salt concentration in the range of 0-3.5% NaCl. In acidic condition, *V. cholerae* is unable to grow as it is sensitive to low pH, but it is considerably tolerant in alkaline condition (Finkelstein, 1996). *V. cholerae* is capable of growing in temperature within the range of 10-43°C under nutrient sufficient condition. The optimal temperature for *V. cholerae* growth is 37°C. They are commonly found in environment with temperate or tropical climate.

V. cholerae can be found in both freshwater and saline environment, example sewage, brackish water, estuaries and polluted water environments, in a “free living” state. This state corresponds to the “viable but non-culturable” (VBNC) state as it usually occurs because of the deprivation of nutrient. The bacterial cells in VBNC state still maintain their capacity for basic metabolic processes (Oliver, 2005). When nutrient

concentration is relatively low, the bacteria tend to exist in epibiotic form associated with microorganisms like cyanobacteria, phytoplankton and zooplankton (Chakraborty et al., 1997). These bacteria are able to attach itself onto the exoskeleton of crustaceans. The ability of *V. cholerae* to survive in a wide range of environmental conditions contributes to the continuous environmental source for their maintenance and spreading throughout geographically.

2.2.1 *V. cholerae* O1 strains

The *V. cholerae* O1 serogroup is the vibrio strain that have O group 1 antigen. Within this serogroup, there are the classical and El Tor biotype. The *V. cholerae* classical biotype was first identified in 1883 by Koch. In early 1900s, *V. cholerae* that resembles classical biotype but it lyses goat erythrocytes was isolated at El Tor, in Sinai Peninsula. The bacteria were isolated from a person that was not diagnosed with cholera, so it was considered as relatively insignificant at that time. Relatively restricted outbreaks of diarrheal disease known as paracholera caused by similar bacteria occurred in Celebs. Cholera that was due to *V. cholerae* El Tor erupted in Hong Kong and started to spread across the world during 1961 (Finkelstein, 1996).

Both of the classical and El Tor biotype consist of three serotypes, which are the Inaba, Ogawa and Hikojima. These serotypes are differentiated by their dominant heat-stable lipopolysaccharide (LPS) type-specific somatic antigens. All the three serotype have a common antigen, A. Inaba serotype contains C type-specific antigen, while Ogawa have B antigen. Hikojima is a rare serotype that has both B and C antigen (Finkelstein, 1996). The LPS of O1 strain consists of lipid A, core polysaccharide and O polysaccharide side chain. A single keto-3-deoxy-D-mannose-octulosonic acid (KDO) molecule was found in the core polysaccharide region of *V. cholerae* O1. They have a

homopolymer of perosamine as the main component of its side chain (Hisatsune et al., 1993).

V. cholerae O1 classical strains are mostly responsible for the fifth and sixth cholera pandemic. The seventh pandemic that began in 1961 in Indonesia was mainly caused by El Tor biotype. *V. cholerae* O1 strains that are non-toxigenic were isolated from environmental sources. These atypical O1 strains are usually not enteropathogenic in humans. They also do not contain genes that involve in cholera enterotoxin production (Finkelstein, 1996).

O1 serogroup bacteria ferment sucrose and mannose. They do not ferment arabinose, and produces acid. This causes them to be categorised under Heiberg I fermentation (Finkelstein, 1996). The El Tor biotype strains produces less cholera enterotoxin compare to the classical biotype (Ghosh-Banerjee et al., 2010). Classical strain usually causes more severe cholera symptoms, while El Tor strain causes milder cholera cases. However, the El Tor biotype is able to colonise intestinal epithelium better than the classical ones. They also show a higher infection-to-case ratio and have higher resistance towards environmental factors than the classical biotype. This leads to a higher tendency for El Tor biotype to become endemic (Finkelstein, 1996).

2.2.2 *V. cholerae* O139 strains

In 1992, *V. cholerae* O139 was first isolated during an epidemic in Bangladesh (Albert, 1994). The *V. cholerae* O139 strains have some common morphological and biochemical properties with *V. cholerae* O1 El Tor biotype. It is also genetically similar to El Tor biotype. Cholera toxin produce by *V. cholerae* O139 and O1 El Tor biotype is identical. Genes that encode for other enterotoxin such as zonula occludens toxin (Zot) and accessory cholera enterotoxin (Ace) have been shown to be present in O139 strains (Albert, 1994). Furthermore, the *ctxAB* gene and 16S rDNA gene sequences of this

strain are identical to that of El Tor *V. cholerae*. Therefore, it is believed that the O139 derived from El Tor biotype through mutation.

The *V. cholerae* O139 serogroup have a different O antigen component on their outer membrane compare to O1 serogroup. The other difference between these two serogroups is the present of capsule on O139 serogroup bacterium, while O1 serogroup bacterium does not have capsule surrounding their outer membrane (Nelson et al., 2009). The capsule on *V. cholerae* O139 may increase the virulence of the microorganism through resistance against serum killing and ability to produce bacteremia. *V. cholerae* O139 has a short O polysaccharide side chains in its LPS in comparison to the long side chains of O1 strains (Knirel et al., 1997). The side chain of O139 strains is made up of a unique sugar, called colitose (Hisatsune et al., 1993). Genes related to synthesis of O-antigen and modification of Ogawa serotype found in O1 are absent in O139 *V. cholerae* (Manning et al., 1994). The production of heat-stable toxin in *V. cholerae* non-O1 strains was not identified in the strains. The present of a variety of fimbrial antigens for colonisation leads to the strong *in vitro* adhesion of O139 strains to HEp-2 cell monolayers. Islam et al. (1993) found that water samples that contain *V. cholerae* O139 was higher in percentage compare to samples that contain *V. cholerae* O1, indicating that O139 may be able to survive better in the environment.

2.2.3 *V. cholerae* variant strains

There have been three types of El Tor variants described since they first emerged, namely Matlab variant, Mozambique variant and altered El Tor variant. In 2002, *V. cholerae* O1 sporadic strains that contain phenotypic characteristics of classical and El Tor biotype, named Matlab variants was identified in Bangladesh (Nair et al., 2002). After the first isolation of variant strain in Bangladesh, other variants were also isolated in several Asia and Africa countries (Ansaruzzaman et al., 2004; Nguyen et al.,

2009). Matlab variants were hybrids that have a combination of classical and El Tor phenotypic traits. These variants can be classified into type I, II and III. Matlab type I is of Inaba serotype. They are resistant to both bacteriophage IV and V, negative for agglutination and Voges-Proskauer (VP) test, and resistant to polymyxin B. Type II belongs to Ogawa serotype. The variant in this group is only susceptible to bacteriophage IV, negative in agglutination and VP test, and sensitive to polymyxin B. Type III is another Ogawa strain as well, that is susceptible to bacteriophage IV. They are negative for agglutination and VP test, and polymyxin B resistant (Nair et al., 2002). The *tcpA* gene for all Matlab variant type is of the classical biotype.

During January to March 2004, a *V. cholerae* El Tor biotype variant from Mozambique was reported (Ansaruzzaman et al., 2004). This Mozambique variant has most of the genes of El Tor strain with two copies of classical type CTX prophage tandem repeat in its small chromosome, also called chromosome 2. Its CTX prophage is of classical type that expresses classical type cholera toxin. The variant is susceptible to El Tor specific bacteriophage IV, resistance to polymyxin B, produces El Tor type haemolysin and agglutinate chicken blood cell. They demonstrated most of the El Tor biotype characteristics (Faruque et al., 2007). The tandem arrays of two copies of CTX prophage enable prophage excision, but the excised prophage cannot replicate. The RS1 element, a satellite phage genome that promotes phage production gene expression is absent in the Mozambique variant same as that of classical biotype (Davis et al., 2002). Faruque et al. (2007) proposed that the variant was derived from El Tor biotype, where the El Tor CTX prophage, toxic-linked cryptic (TLC) element and RS1 element was lost due to the occurrence of loop-out recombination event that obstruct the future acquiring of El Tor CTX phage. This left the attachment site on chromosome 2 that can only be used by classical strain to insert their CTX prohage into the chromosome 2 of vibrio genome.

The altered El Tor variant has most of its genome same as El Tor biotype and contain an El Tor CTX prophage, but the cholera toxin produced is of classical type. This was shown in a study from Nair et al. (2006) where El Tor biotype of *V. cholerae* O1 isolated in Bangladesh since 2001 produces classical biotype cholera toxin. El Tor strains associated with cholera in Bangladesh yield cholera toxin with epitype and genotype of classical type, which is epitype CT1 and genotype 1 cholera toxin.

An addition to the existing biotyping scheme for the classification of different *V. cholerae* O1 variant biotypes was proposed by Raychoudhuri et al. (2008). Strains that have phenotypic characteristics of either positive or negative for VP test, polymyxin B sensitivity, agglutination test, and bacteriophage IV and V lysis which implies classical or El Tor biotype traits are categorise as 'Hybrid biotype'. The hybrids also contain molecular characteristics of both classical and El Tor type, such as the *ctxB* genotype, *tcpA* gene, the presence of *rtxC* gene and presence of different RS element. An 'El Tor variant biotype' was designated where this strain have the typical El Tor phenotypic traits, but expresses the cholera toxin of classical biotype. This means that the strains will have classical cholera toxin epitype and *ctxB* genotype of classical type.

Interaction and genetic material exchange between the El Tor and classical biotypes may have occurred in the natural environment or in their host. It is hypothesised that variant strains derived from El Tor strains which contain the traits of classical strain is more prevalent than classical strains that harbour El Tor characteristics. It is still not known whether the genetic variation in these hybrid strains will affect the genetic stability, pathogenicity or the potential of causing cholera epidemic.

2.3 Phenotypic characteristic of O1 strains

V. cholerae possesses cytochrome c oxidase, where this enzyme catalyses the transport of electrons from donor to the electron acceptor. They are positive in oxidase test as vibrios are facultative anaerobic in nature. These bacteria use oxygen as the terminal electron acceptor in their respiratory electron transport chain. Vibrios ferment glucose and sucrose (CDC, 2016). There is no gas production in the fermentation process, only production of acid. *V. cholerae* O1 produces lysine and ornithine decarboxylase (Finkelstein, 1996). The enzyme lysine and ornithine decarboxylase will catalyse the removal of carboxyl group from lysine and ornithine respectively. This suggests that *V. cholerae* uses both lysine and ornithine as carbon source and energy. *V. cholerae* typically does not use arginine as source of carbon, because they do not produce arginine dihydrolase. *V. cholerae* can utilise iron for their growth through producing siderophores, an iron chelating molecules. These catechol siderophores from *V. cholerae* that helps the microbe acquires iron was named vibriobactin.

The classical and El Tor biotype within the O1 serogroup can be differentiated on the basis of their phenotypic properties. There are a number of test used for examining the characteristic of O1 vibrios. Biotypes of *V. cholerae* O1 have dissimilar sensitivity to selected bacteriophage. Classical biotype are susceptible to bacteriophage IV and resistant to bacteriophage V, while the El Tor biotype are vice versa. The susceptibility of the bacterial cell against polymyxin B is that classical biotype is sensitive to the antimicrobial agent, whereas El Tor biotype is resistant to it. Different O1 biotypes give different reaction in the Voges-Proskauer (VP) test for acetoin. Classical biotype yields a negative reaction in the test, where they produce organic acid as their end product for fermentation process. El Tor biotype produces a positive reaction, implying the utilisation of butylenes glycol pathway to produce the neutral end products. The classical O1 strain does not have the capability to produce hemagglutinin

that is active toward chicken or goat erythrocytes, while El Tor strains do have active hemagglutinin. The ability to express haemolysin is absent in classical biotype, while the haemolytic activity in El Tor biotype varied depending on the strain.

2.3.1 Polymyxin B

Polymyxin B is an antibiotic mainly utilised for infection by Gram-negative bacterium. It is made up of a mixture of major components polymyxins B1 and B2. These components are similar in structure with a variable fatty acid group on each fraction. This antibiotic is derived from *Bacillus polymyxa* strains. The components are basic polypeptides that contain around eight amino acids. The antibiotic use of polymyxin B has side effects as it is relatively neurotoxic and nephrotoxic.

Polymyxins are cationic peptides that act like detergent on the cell membrane. They bind to a negatively charged site in lipopolysaccharide layer of the outer membrane of bacteria through the positively charged peptide portion of polymyxins (Cardoso et al., 2007). Once bound it changes the structure of the membrane to make it more permeable leading to excess water uptake. Membrane integrity of target bacteria is disrupted when fatty acid portion of polymyxins dissolves in hydrophobic part of the membrane. The outer membrane break apart, causes cellular components to leak out and leads to bacterial cell death.

2.3.2 Acetyl methyl carbinol production

Acetyl methyl carbinol, also known as acetoin is a four carbon neutral molecule. Certain fermentative bacteria use acetoin as a source of external energy. The decarboxylation of alpha-acetolactate will produce the acetoin. During exponential growth of bacteria, acetoin is secreted to prevent over acidification of the surrounding of bacteria created by the accumulation of acidic products. Acetoin is utilised as a

supplementary carbon source to maintain bacterial growth when the main carbon source was exhausted (Xiao & Xu, 2007).

Different pathway is use by different bacteria to change glucose and dextrose to pyruvate. There are two different pathways that carry out the reaction. First, the butylene glycol pathway converts the unstable acidic metabolic products to neutral end products such as acetoin and 2,3-butanediol. Second pathway is the mixed acid pathway, where stable organic acidic end products like acetic acid and lactic acid are produced. The stable acidic end products will not be converted to neutral molecules and remain acidic. Voges-Proskauer test is to detect bacteria that undergo butylene glycol pathway to neutralise their acidic products into acetoin. In the presence of potassium hydroxide, acetoin will be oxidise and produce diacetyl. Diacetyl will react with guanidine containing compound to give red colouration (MacFaddin, 1980).

2.3.3 Haemolysis

Haemolysis is the process when red blood cells are broken down. Haemolytic bacteria are capable of breaking down the red blood cells when it is grown in the blood agar. Haemolysis is categorised into three types, alpha haemolysis, beta haemolysis and gamma haemolysis. Alpha haemolysis is the partial haemolysis as the bacterium produces hydrogen peroxide that oxidises haemoglobin into green methemoglobin. Beta haemolysis is when the bacterium completely lyse the red blood cells in the media surrounding their growth. The area around the bacterial colonies will become transparent. Bacteria that produce haemolysin will cause complete lysis of the red blood cell. Gamma haemolysis is when the bacterium is non-haemolytic and does not induce haemolysis in the blood agar.

V. cholerae O1 classical strain is non-haemolytic, while El Tor strains are originally defined as haemolytic. Most El Tor biotypes had lost their ability to produce

haemolysin in the recent pandemic (Finkelstein, 1996). The Australia and US Gulf Coast clones of El Tor biotype are haemolytic, while the Latin America and Asia, Africa, Europe, Pacific clones are non-haemolytic (CDC, 2016).

2.4 Virulence factors

V. cholerae O1 and O139 serogroup are known to cause diarrhea. This prominent ability is due to the presence of virulence genes in their bacterial chromosome. The cholera enterotoxin gene, *ctx* gene encodes the cholera toxin that is responsible for the diarrhea symptom during infection. Cholera toxin is made up of subunit A and subunit B. Subunit A is the enzymatic part of the toxin protein, while subunit B is a receptor binding unit. The subunit A is responsible for the intracellular toxicgenic activity when it is proteolytically cleaved into 2 peptides, A_1 and A_2. The A_1 peptide will undergo an internal activation. The activation causes ADP-ribosyltransferase activity that leads to alteration in ion transport and hypersecretion of water and chloride ion (Cl^-) into the intestinal lumen. The subunit B binds cholera holotoxin to the oligosaccharides of G_{M1} ganglioside on the cell membrane. This triggers the changes of holotoxin conformation that releases subunit A to the cell surface and enters the target cell. The cholera toxin gene is within a pathogenicity island, named CTXΦ that is situated on chromosome 1 of *V. cholerae* genome (Waldor & Mekalanos, 1996). Subunit A and subunit B of cholera toxin is produced by *ctxA* and *ctxB* genes respectively. Based on the subunit B, there are epitypes that are related immunologically but not identical for the different biotype of O1 vibrios. Cholera toxins produced by classical biotypes have CT1 epitype, while El Tor biotypes as well as O139 strains have CT2 epitype. Cholera toxin subunit B have another type of classification based on the three nucleic acid base changes on the *ctxB* genes that leads to alteration in

their amino acid sequence. There is genotype 1 found in classical biotype worldwide and in US Gulf Coast El Tor strains; genotype 2 in Australia El Tor strains; genotype 3 found in El Tor from seventh pandemic and Latin America El Tor strains. For O139 strains isolated between 1993 and 1997, they contained type 3 *ctxB* gene. In a study by Bhuiyan et al. (2009), three new *ctxB* genotypes (genotype 4-6) had been identified in *V. cholerae* O139 strains isolated within the period of 1998 to 2005 in Bangladesh : 1) Genotype 4 have amino acid sequence similar to genotype 3 except at position 47 (I→T). 2) Genotype 5 is similar to genotype 1 with a substitution at position 28 (D→A). 3) Genotype 6 is similar to genotype 4, but has an amino acid substitution at position 34 (H→P). In 2010, strain from Haitian cholera outbreak with a new *ctxB* genotype (genotype 7) was reported. Genotype 7 have *ctxB* gene similar to classical type with a single nucleotide polymorphism (SNP) at position 58 (Chin et al., 2011).

In one of the pathogenicity island in VPIΦ filamentous bacteriophage of *V. cholerae,* known as VPI contains a toxin coregulated pilus (TCP) gene. The TCP enables the bacterium to attach to the gastrointestinal epithelium (Faruque et al., 1998). It has an important function for *in vivo* intestinal colonisation. The toxin coregulated pilus is a type of fimbriae that consist of TcpA protein subunit. This protein subunit is encoded by the *tcpA* gene. Formation of the TcpA protein is coregulated with the expression of cholera toxin. TcpA also serves as a coat protein of VPIΦ that are able to be transfer between certain *V. cholerae* strains. Thus, TCP acts as a surface receptor for the CTXΦ phage entry into the bacteria when it is a VPIΦ coat protein. The TCP gene cluster is located on chromosome 1 of the bacteria. This gene cluster consists of several open reading frames, including *tcpA* and *toxT* genes. The expression of TCP gene is regulated by a *toxR* regulatory gene as well as the cholera toxin expression (Lee et al., 1999).

V. cholerae O1 classical biotype is non-haemolytic because of the presence of base pair deletion in their *hlyA* gene for encoding haemolysin that shows enterotoxic activity (Rader & Murphy, 1988). El Tor biotype does not have this base pair deletion, therefore it does not affect haemolytic capability of the bacteria. The cholera toxin prophage region, CTXΦ is flanked by RS1 and RS2 elements. RS1 element is only present in O1 El Tor and O139 *V. cholerae* strains. The *rstC* gene is located in the RS1 element, but absent in the RS2 element (Waldor et al., 1997). Classical biotypes only have RS2 elements approximate to their CTX prophage, so *rstC* gene is absent in classical biotype.

Repeat in toxin, RTX is a virulence associated protein synthesised by a cluster of genes consist of *rtxABCDE*. This toxin gene cluster is associated with the prophage of cholera toxin in El Tor biotype, but it does not depend on the CTX element enzymatically. The RtxA toxin is encoded by the *rtxA* gene, which shows cytotoxic activity toward HEp-2 cells *in vitro*. The *rtxC* gene produces an RtxC acyltransferase activator that is essential for RTX toxin activity. The *rtxB, rtxD* and *rtxE* gene in the cluster encode for ATP-binding transporter protein, membrane fusion protein and a second ATPase transporter protein respectively (Boardman & Satchell, 2004). The RTX gene cluster of El Tor biotype has the complete set of genes, while classical biotype has a deletion within its cluster that removes the *rtxC* gene (Lin et al., 1999). RTX toxin of *V. cholerae* is a multifunctional autoprocessing RTX toxins (MARTX) protein with multiple activities. This RTX protein catalyses the cellular actin covalent cross-linking and leads to the rounding of epithelial cells. MARTX protein with the absence of RtxC activator reduces actin cross-linking activity, but does not affect the MARTX function (Cheong et al., 2010). This enables the differentiation between El Tor and classical biotype.

2.5 Polymerase chain reaction (PCR)

Since the early 1980s, the development of polymerase chain reaction has been a very essential and useful molecular biology method. It is a technique use for specific amplification of the targeted sequence within the larger DNA molecule. A synthesised oligonucleotide, which is the primer, will direct the target-specific synthesis that produces multiple DNA copies and the synthesis of nucleotide will be perform by DNA polymerase. There is a wide application for PCR, such as the use of it in the cloning of cDNA and genomic DNA, DNA sequencing, and mutation detection. There may have modification in the process of these applications, but they still abide to the fundamental principle of PCR.

In the PCR process, there are three major steps involved. These steps are the denaturation, annealing and extension step. The steps come in accordance, where any error in one of the step will affect the DNA amplification. First is the denaturation of double stranded DNA template as this allows the primer to hybridise to the specific sequence within the DNA strand. Denaturation occurs approximately at the temperature of 95°C. The denaturation temperature is based on the G+C content of the template DNA. When *Taq* polymerase is utilised in the PCR reaction, denaturation temperature cannot exceed 95°C as it is the maximum tolerable temperature for the *Taq* polymerase. In practice, there is an additional initial denaturation step to enable the DNA template to fully denature. At the annealing step, the primers will hybridise to the denatured single stranded DNA template, the temperature for this process is crucial. The specificity of the DNA sequence amplification will be determined by the annealing temperature. The annealing temperature is usually based on the melting temperature (Tm) of both the forward and reverse primers. It is commonly set at 3-5°C lower than the Tm of the primers. The range for annealing temperature is usually between 55°C and 70°C, as it cannot be too low or too high for the specificity of the reaction. During the extension

process, DNA polymerase begins to synthesise new strand of the targeted DNA sequence. The temperature is commonly at 72°C, because it is the optimal temperature for *Taq* polymerase to catalyse the synthesis of DNA. There are different thermostable DNA polymerases available to be used in the PCR amplification, each of them will have a different extension temperature. Usually there is another cycle of final extension to ensure the completion of all amplification of PCR product.

During the PCR amplification, a few components are required which are the DNA template, DNA polymerase, primers, deoxynucleoside triphosphate (dNTP), divalent ion and buffer. The template DNA that contains the targeted sequence can be in either single or double stranded form. Thermostabe capability of DNA polymerase involves in PCR is important due to the denaturing process of dsDNA molecule. There are a few types of thermostable polymerase, e.g. *Taq*, *Pfu* and *Vent* polymerase. They are chosen depending on the fidelity and productivity need of the PCR, where different polymerases have different productivity and fidelity rate. *Taq* polymerase has a higher productivity rate, but it does not have a 3'→5' proofreading ability. *Taq* polymerase is favourable when TA cloning is followed after the PCR was performed. This is because of the A overhang produced in the PCR product when using *Taq* polymerase. Primer is a very important component in the PCR amplification reaction. The specificity and efficiency of the amplification process is determined by the primers. There are factors that need to be considered when carrying out primer design, e.g., length (usually 18-25bp), base composition, melting temperature, self complement ability of the primer sequence and ability of the primers to form dimer. Each of the four dNTP will be present at equal amount in the PCR mixture. The amount of dNTP added into the PCR mixture should be sufficient depending on the expected size of the PCR product, as it act as a limiting factor for the DNA synthesis. There is a common divalent ion use as a cofactor for DNA polymerase in PCR, the magnesium ion (Mg^{2+}). Mg^{2+} can also

stabilise the primer-template complexes. The PCR buffer usually comes with the commercial DNA polymerase. The buffer functions to maintain the pH of the reaction mixture.

2.6 Molecular fingerprinting

Outbreak of an infection is caused by an etiologic agent. The agent will be a source organism where its progeny is identical or closely related in their genetic content. Therefore, the source organism of an outbreak will have a common origin which is clonally related. Clonally related bacteria shares common virulence factors, biochemical and genomic characteristics. In spite of that, there is still sufficient diversity at the level of species to classified bacteria into subtypes or strain. Subtypes can be differentiated based on the different source of bacterial isolation in different geographical area and at different time (Olive & Bean, 1999).

Molecular typing is a method used to differentiate different type of bacterial isolates within the same species based on their genotype or DNA sequences. Typing is an essential epidemiological tool in investigation, prevention and control of bacterial pathogen infection. Subtyping enables the recognition of infection outbreak, determination for source of infection, recognition of particular virulent strains and detection for cross transmission of infectious pathogen (Olive & Bean, 1999). Traditionally, typing methods are phenotypic, examples serotyping based on presence of serological marker that reaction with certain antibody, phage typing based on presence of a bacteriophage receptor and antibiogram based on susceptibility for different antimicrobial agents. Molecular typing method development have allowed enhancement in outbreak detection and surveillance.

There are a number of different methods available for subtyping. Although there are different methods, but all must be able to subtype microbes within a species. There are few criteria required for any molecular method to be use for typing. Unrelated strains, like bacteria isolated from a distinct geographical area than that of the source organism must be able to differentiate clearly. Hence, method with high discriminatory power that separate epidemiologically unrelated strains, but still shows relatedness of strains from individuals infected through the same source (Arbeit, 1995). Subtyping methods must be able to yield reproducible results. The technique has to be capable of producing the same result when a selected strain is tested repeatedly. When establishing a reliable database that contains known strains of a species, reproducibility of a typing method is crucial as results from unknown strains can be compared with the available database for classification purpose. Molecular basis of typing depend on the separation of different length DNA fragment using electrophoretic method.

Microbes usually have a variety of repetitive DNA sequences in their genome. Most of these repetitive sequences are non-coding sequences that dispersed across the genome either in intergenic or extragenic regions. Some repetitive elements such as the hyper-reiterated sequences (HRS1) and *Mycobacterium leprae*-specific repetitive element (RLEP) are only present in specific group of bacteria, whereas other repetitive elements like repetitive extragenic palindromic (REP) DNA sequences are widely distributed among phylogenetically diverse bacteria (Sadowsky & Hur, 1998). Repetitive-element PCR (rep-PCR) is a bacterial genomic fingerprinting method based on the examination of repetitive DNA elements found in the genomes (Versalovic et al., 1991). The repetitive DNA elements in the bacterial genomes will be amplified through PCR. Amplicons with various lengths depending on the distribution of repetitive element within the genome will be produced. These amplicons will be separated electrophoretically and generate band patterns unique for different strains to determine

the relatedness between analysed isolates genetically. There are a few repeat sequences commonly used for molecular typing, example enterobacterial repetitive intergenic consensus (ERIC) sequences and REP element. ERIC sequences, otherwise known as intergenic repeat units is a 126 bp elements made up of a highly conserved central inverted repeat and positioned in intergenic regions of polycistronic operons within the bacterial genome (Hulton et al., 1991; Sharples & Lloyd, 1990). The REP element, also known as palindromic units is a 38 bp consensus sequence that contains six degenerate positions and a 5 bp variable loop between each side of the conserved palindromic stem (Stern et al., 1984). These elements are highly conserved and disperse within the genomes. REP elements are mostly located between convergent genes as their frequency will be higher in such location (Tobes & Ramos, 2005). The functions of these REPs are still unclear, but it is believed that they involve in RNA and DNA physiology related functions. When transcription of REP element occurred, the REP transcript is associated with mRNA stabilisation due to its stem-loop secondary structure (Newbury et al., 1987). The presence of REP sequence in the mRNA act as a barrier against 3'-5' exonuclease digestion and prevent degradation of the 5'-proximal cistrons. This stabilises the mRNA, thus increases the upstream gene expression (Khemici & Carpousis, 2004). DNA polymerase and the integration host factor (IHF) protein for DNA bending that are needed for site-specific recombination, DNA replication and transcription will bind to REP sequence (Engelhorn et al., 1995; Gilson et al., 1990).

CHAPTER 3: MATERIALS AND METHODS

3.1 Maintenance and revival of bacterial isolates

The *V. cholerae* O1 isolates were revived and enriched in APW (Merck KGaA, Germany). After overnight incubation, the cultures were streaked on TCBS agar (Merck KGaA, Germany) to check for purity. Once the purity is confirmed, a single bacterial colony were transferred onto nutrient agar (Merck KGaA, Germany) with 3% (w/v) NaCl and incubated at 35°C±2°C overnight. The *V. cholerae* isolates was maintained as stab culture for short-term storage. Glycerol stock of the revived bacterial isolates was prepared for long-term storage.

3.2 Phenotypic characterisation

Biochemical tests that differentiate the biotypes of the 44 revived *V. cholerae* isolates (27 isolates from Sabah, 15 isolates from Sarawak and 2 isolates from Klang) were performed. The O139 USM and O139 IMR were used as control samples. The characteristics of the *V. cholerae* O1 isolates was determined using Voges-Proskauer test, polymyxin B antibiotic susceptibility test, haemolysis test and blood cell agglutination test. Biotype of the 44 *V. cholerae* O1 isolates based on the examined phenotype was determined using a tree diagram shown in Figure 3.1.

3.2.1 Voges-Proskauer test

All the *V. cholerae* O1 isolates were grown in tryptic soy broth. Then, two loopful of pure culture was inoculated into 10 ml of 1% (w/v) NaCl methyl red VP (MR-VP) broth (Oxoid, UK). The inoculated MR-VP broths were incubated at

35°C±2°C for 48 hours. After incubation, 1 ml of MR-VP bacterial culture was aliquot into a clean test tube. A 0.6 ml of 5% (w/v) alpha-naphthol solution was added into the test tube, followed by adding 0.2 ml 40% (w/v) KOH with 0.3% (w/v) creatine (CDC, 2016). The test tube was shaken gently to mix the reagents and allowed it to remain undisturbed for 15 minutes on the bench. After 15 minutes, a colour change in the mixture was observed. The VP test was performed three times for each of the 44 *V. cholerae* O1 isolates and O139 strains.

The development of pinkish red colour at the surface of the medium indicated a positive reaction for the VP test. VP negative result was demonstrated when the surface of the medium turns yellow or light brown in colour.

3.2.2 Antimicrobial susceptibility test

The polymyxin B susceptibility test was carried out using the Kirby-Bauer disk diffusion method. All 44 *V. cholerae* isolates were examined for their resistance toward polymyxin B (PB, 300 units).

The *V. cholerae* isolates was cultured onto tryptic soy agar (Merck KGaA, Germany) and incubated at 35°C±2°C overnight. After incubation, 4-5 single colonies of *V. cholerae* isolates were inoculated into tryptic soy broth. The broth cultures were incubated at 35°C±2°C overnight. The tryptic soy broth bacterial culture was vortex and its turbidity was adjusted to that of 0.5 McFarland standards.

The cell suspension was spread evenly onto the Mueller-Hinton agar (BD, USA) by dipping a sterile swab into the suspension and swabbed the entire surface of agar several times, each time rotating the plate at an angle. The inoculated agar plate was left aside covered with the lid for 3 minutes to allow the absorption of the bacterial cell suspension. Polymyxin B antimicrobial disk (Oxoid, UK) was placed gently onto the agar surface using a sterile forceps. The agar plate was inverted and incubated at

35°C±2°C for 16-18 hours. The susceptibility test was repeated for each of the *V. cholerae* O1 isolates.

3.2.3 Plate haemolysis test

The *V. cholerae* isolates was cultured on pre-poured Columbia agar with 5% sheep blood plate (Isolac, Malaysia). The cultured blood agar plates were incubated at 35°C±2°C for 18 hours (CDC, 2016). The test was performed twice for each of the isolates. The results were recorded.

Incubated blood agar plates were observed for the presence of clear zones around haemolytic bacterial colonies. If complete haemolysis occurred, the blood agar medium where the bacteria colonies grow appeared transparent and yellow in colour. When bacteria are non-haemolytic, there are no clearing and changes of colour in the agar medium surrounding their colonies. Partial haemolysis may also occur, where the area of medium surrounding the colonies grown turns greenish yellow, but there is no transparent clearing around the colonies.

3.2.4 Chicken blood cell agglutination test

The agglutination test was carried out according to CDC guideline (2016) with a slight modification. The 3% (v/v) packed chicken blood cell suspension was prepared in normal saline.

Two separate drops of blood cell suspension were placed on a clean glass slide. A small portion of bacterial culture was then added to one of the blood cell suspension droplet on the glass slide using an inoculation loop. The bacterial culture and blood cell suspension was mixed well by moving the slide in a circular motion. Changes in blood suspension were observed after 1 minute. The steps were repeated by replacing the bacterial culture with PBS (Merck KGaA, Germany) on another droplet of blood cell

suspension as negative control (CDC, 2016). The procedure was repeated two times for each of the *V. cholerae* isolates. The bacterial isolate that agglutinate red blood cells have clumps present in their blood cell suspension after the bacterial pure culture was added.

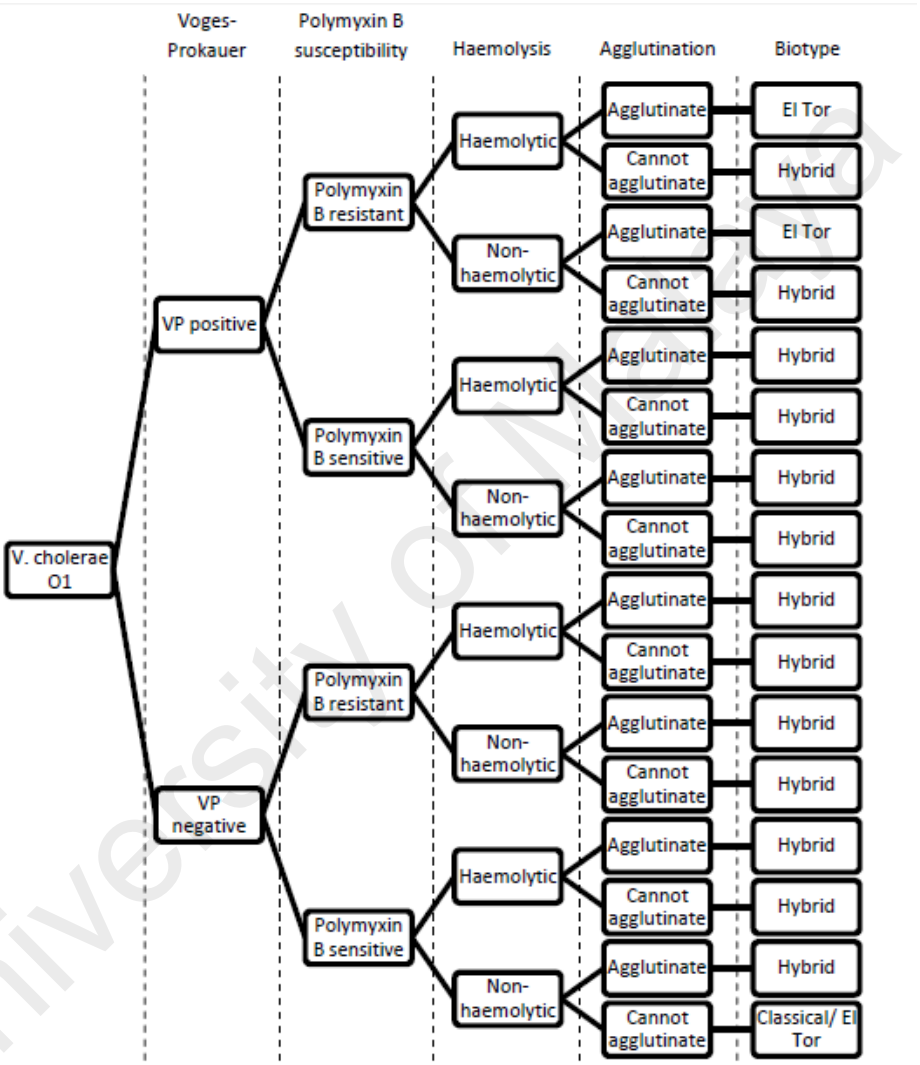


Figure 3.1: Decision tree diagram for phenotype characterisation of the *V. cholerae* O1 isolates.

3.3 Bacterial DNA extraction

The crude DNA of the 44 *V. cholerae* O1 isolates was extracted using the rapid boiling method. Briefly, 1 ml of LB broth (Merck KGaA, Germany) bacterial culture

was transferred into a 1.5 ml microcentrifuge tube. The tube was centrifuged at 134000 rpm for 2 minutes and supernatant was discarded. A 500 µl of sterile distilled water was added to the cell pellet and the tube was vortex to mix properly.

The cell suspension was heated at 100°C for 10 minutes in a heating block (Thermo Fisher Scientific, USA). Lysed cell suspension was chilled at -20°C for 10 minutes. The cell lysate was centrifuged at 134000 rpm for 1 minute and the supernatant was collected to be used in PCR analysis.

3.4 Genetic characterisation

Molecular techniques for characterising the 44 *V. cholerae* O1 isolates genetically were carried out using the extracted DNA. This is to determine the genetic variation of the *V. cholerae* isolates. Two *V. cholerae* O139 strains (O139 USM and O139 IMR), one reference strain of *V. cholerae* O1 El Tor biotype (N16961) and one clinical hybrid strain from Terengganu, 2009 (1761) (Teh et al., 2012) were used for comparison purposes.

3.4.1 PCR detection of virulence genes

PCR targeting *O1rfb* gene, *O139rfb* gene, *tcpA* gene, *ctxA* gene, *ctxB* gene, *hlyA* gene, *rtxC* gene and *rstC* gene were performed using specific primers as shown in Table 3.1. Optimisation was performed to obtain the optimal PCR condition for target sequence amplification.

A total of seven PCR amplification reactions were performed on forty-four *V. cholerae* O1 isolates. One of the PCR reactions is a duplex PCR for *O1rfb* gene and *O139rfb* gene, while the other six were carried out through monoplex PCR. The O139 USM and O139 IMR were used as O139 control strains in the duplex PCR for *O1rfb*

gene and *O139rfb* gene. For the six monoplex PCR, N16961 El Tor strain and 1761 hybrid strain were used as control strains. The PCR master mixture for each reaction was prepared by mixing 5× PCR buffer, 25mM $MgCl_2$, 10mM dNTPs mix (Promega, USA), 10 µM of each primer involved in individual reaction, 5 U Taq polymerase (Promega, USA), and ultra pure water in a 1.5 ml microcentrifuge tube. The mixture was then aliquot into 0.5 ml microcentrifuge tube and DNA template was added. The PCR was performed in aliquots of 25 µl total volume for the individual reaction as shown in Table 3.2 to Table 3.4.

Table 3.1: The sequence of primers used for genetic characterisation of *V. cholerae* O1 isolates.

Target genes	Primers	Sequence (5'→3')	Primer length (bp)	Size of PCR product (bp)
O1rfb	*O1rfb*-F	GTTTCACTGAACAGATGGG	19	192
	O1rfb-R	GGTCATCTGTAAGTACAAC	19	
O139rfb	*O139rfb*-F	AGCCTCTTTATTACGGGTGG	20	449
	O139rfb-R	GTCAAACCCGATCGTAAAGG	20	
tcpA	*tcpA*-F (El tor/ Classical)	CACGATAAGAAAACCGGTCAAGAG	24	620
	tcpA-R (Classical)	TTACCAAATGCAACGCCGAATG	22	
	tcpA-R El tor2 (El tor)	CGAAAGCACCTTCTTTCACACGTTG	25	453
rstC	*rstC1*	AACAGCTACGGGCTTATTC	19	238
	rstC2	TGAGTTGCGGATTTAGGC	18	
rtxC	*rtxC*-F	CGACGAAGATCATTGACGAC	20	263
	rtxC-R	CATCGTCGTTATGTGGTTGC	20	
hlyA	*hlyA*-F*(non-classical)*	GAGCCGGCATTCATCTGAAT	20	481
	hlyA-R	CTCAGCGGGCTAATACGGTTTA	22	
ctxA	*ctxA*-F	CGGGCAGATTCTAGACCTCCTG	22	564
	ctxA-R	CGATGATCTTGGAGCATTCCCAC	23	
ctxB	*ctxB2*	GATACACATAATAGAATTAAGGATG	25	460
	ctxB3	GGTTGCTTCTCATCATCGAACCAC	24	

Table 3.2: PCR reaction mixture for *O1rfb* and *O139rfb* gene.

Component	Volume (µl)	Final concentration
5×PCR buffer	5	1×
25mM $MgCl_2$	2.5	2.5mM
10mM dNTPs mix	0.5	0.2mM
10µM O1rfb-F	0.5	0.2µM
10µM O1rfb-R	0.5	0.2µM
10µM O139rfb-F	0.5	0.2µM
10µM O139rfb-R	0.5	0.2µM
5U Taq polymerase	0.2	1U
DNA template	3	~10ng/ µl
ddH_2O	11.8	-
Total	25	

Table 3.3: PCR reaction mixture for *tcpA* gene.

Component	Volume (µl)	Final concentration
5×PCR buffer	5	1×
25mM $MgCl_2$	2.5	2.5mM
10mM dNTPs mix	0.5	0.2mM
10µM tcpA –F Cla.	0.5	0.2µM
10µM tcpA –R Cla.	0.5	0.2µM
10µM tcpA –R El tor2	0.5	0.2µM
5U Taq polymerase	0.2	1U
DNA template	3	~10ng/ µl
ddH_2O	12.3	-
Total	25	

Table 3.4: Monoplex PCR reaction mixture for *rstC*, *rtxC*, *hlyA*, *ctxA* and *ctxB* gene.

Component	***rstC/ hlyA/ ctxA***		***rtxC/ ctxB***	
	Volume (µl)	**Final concentration**	**Volume (µl)**	**Final concentration**
5×PCR buffer	5	1×	5	1×
25mM $MgCl_2$	2.5	2.5mM	1.5	1.5mM
10mM dNTPs mix	0.5	0.2mM	0.5	0.2mM
10µM forward primer	0.5	0.2µM	0.5	0.2µM
10µM reverse primer	0.5	0.2µM	0.5	0.2µM
5U Taq polymerase	0.2	1U	0.2	1U
DNA template	3	~10ng/ µl	3	~10ng/ µl
ddH_2O	12.8	-	13.8	-
Total	25		25	

The PCR mixture was spun down using a quick spin mini centrifuge (ExtraGene, Taiwan). The PCR reaction tubes were placed in a thermal cycler (Applied Biosystems, USA). Each of the PCR reaction was performed using parameters shown in Table 3.5 to Table 3.11.

Table 3.5: Parameters of PCR cycle for *O1rfb* and *O139rfb* gene.

Stage	Temperature (°C)	Time (min.)	Cycle
Initial denaturation	95	5	1
Denaturation	95	0.5	
Annealing	54	0.5	35
Extension	72	1	
Final extension	72	7	1
Hold	4	∞	-

Table 3.6: Parameters of PCR cycle for *tcpA* gene.

Stage	Temperature (°C)	Time (min.)	Cycle
Initial denaturation	94	2	1
Denaturation	94	2	
Annealing	60	1	30
Extension	72	1	
Final extension	72	10	1
Hold	4	∞	-

Table 3.7: Parameters of PCR cycle for *rstC* gene.

Stage	Temperature (°C)	Time (min.)	Cycle
Initial denaturation	94	5	1
Denaturation	94	0.5	
Annealing	52.4	0.5	30
Extension	72	1	
Final extension	72	7	1
Hold	4	∞	-

Table 3.8: Parameters of PCR cycle for *rtxC* gene.

Stage	Temperature (°C)	Time (min.)	Cycle
Initial denaturation	94	12	1
Denaturation	94	1	
Annealing	55	1	30
Extension	72	1	
Final extension	72	10	1
Hold	4	∞	-

Table 3.9: Parameters of PCR cycle for *hlyA* gene.

Stage	Tempersture (°C)	Time (min.)	Cycle
Initial denaturation	94	2	1
Denaturation	94	1	
Annealing	60	1	30
Extension	72	1	
Final extension	72	10	1
Hold	4	∞	-

Table 3.10: Parameters of PCR cycle for *ctxA* gene.

Stage	Temperature (°C)	Time (min.)	Cycle
Initial denaturation	95	5	1
Denaturation	95	0.5	
Annealing	60	0.5	35
Extension	72	1	
Final extension	72	7	1
Hold	4	∞	-

Table 3.11: Parameters of PCR cycle for *ctxB* gene.

Stage	Temperature (°C)	Time (min.)	Cycle
Initial denaturation	95	5	1
Denaturation	95	1	
Annealing	55	1	25
Extension	72	1	
Final extension	72	7	1
Hold	4	∞	-

After PCR amplifications, the presence of PCR product was detected through gel electrophoresis. A 1% (w/v) TBE agarose gel with the addition of SYBR™ Safe DNA gel stain was prepared (Invitrogen, USA). A 3µl of PCR reaction mixture was loaded into the well of the agarose gel. The 100bp DNA ladder (Promega, USA) was

used as the marker in all gel electrophoresis. The gel electrophoresis was run at 90V for 85 minutes. After electrophoresis, the gel was visualised under a UV illuminator (Syngene, UK) with CCD camera.

3.4.2 *ctxB* gene sequencing

The PCR products of *ctxB* gene detection PCR were purified and sequenced. The sequenced PCR product sequences were analysed using BioEdit software. The PCR product nucleotide sequences were translated into amino acid sequences. The *ctxB* gene amino acid sequence of 569B classical (AAC34728) and N16961 El Tor (NP_231099) biotype was obtained from GenBank. Multiple sequence alignment of the amino acid sequence of isolates' PCR product with the classical and El Tor reference strain was performed.

3.5 Repetitive extragenic palindromic (REP)-PCR for DNA fingerprinting

The genetic relationship of the 44 *V. cholerae* isolates and the control strains in this study was examined by means of DNA fingerprinting. REP-PCR was used to determine the relatedness among the isolates.

The PCR reaction master mixture consist of 1× PCR buffer, 2.5mM $MgCl_2$, 0.05mM dNTPs mix, 0.6 µM of REP primer (5'-GCG CCG ICA TGC GGC ATT-3'), 1 U Taq polymerase, and ultra pure water. The PCR was carried out in a total reaction volume of 25 µl as shown in Table 3.12. The PCR mixture was mixed and placed into the thermal cycler. The PCR amplification was performed following the program as described by Teh et al. (2011), shown in Table 3.13.

Table 3.12: REP-PCR reaction mixture.

Component	Volume (µl)	Final concentration
5×PCR buffer	5	1×
25mM $MgCl_2$	2.5	2.5mM
10mM dNTPs mix	0.2	0.05mM
10µM REP primer	1.5	0.6µM
5U Taq polymerase	0.2	1U
DNA template	5	~50ng
ddH_2O	10.6	-
Total	25	

Table 3.13: Parameters of REP-PCR cycle.

Stage	Temperature (°C)	Time (min.)	Cycle
Initial denaturation	94	4	1
Denaturation	94	1	35
Annealing	42	1	
Extension	68	8	
Final extension	72	8	1
Hold	4	∞	-

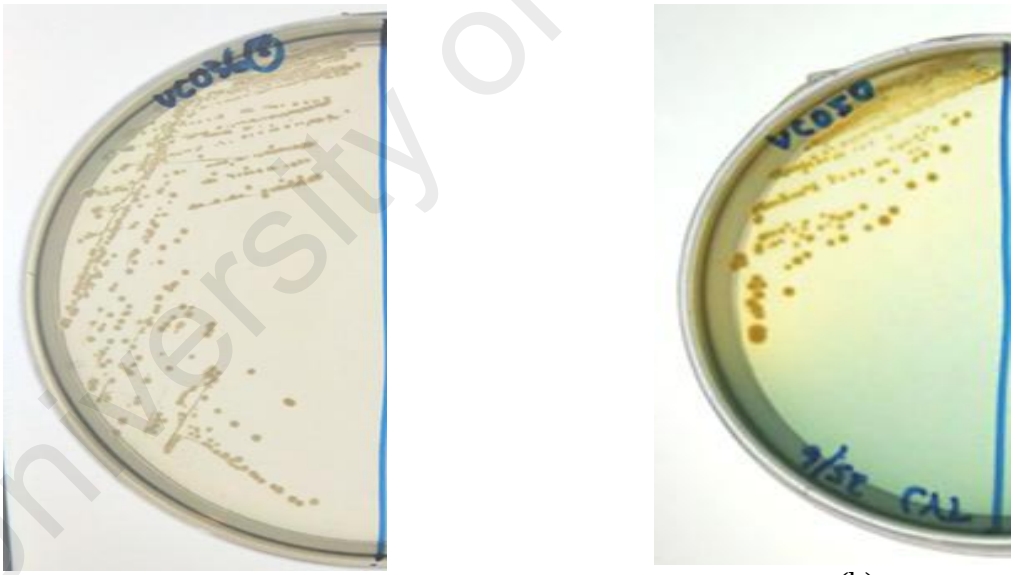
The PCR product of REP-PCR was analysed using 1.2% (w/v) agarose gel electrophoresis in TBE. 10µl of PCR mixture was loaded to the agarose gel, as well as the 1kb DNA ladder (Promega, USA). The gel was electrophoreses at 90V for approximately 5 hours and visualised through the UV illuminator. The gel images of REP-PCR were analysed using BioNumerics software.

CHAPTER 4: RESULTS

4.1 Bacterial isolates revival

Pure culture that produced opaque yellow colonies on TCBS agar were picked and inoculated in 3% NaCl nutrient agar slant. Revivable *V. cholerae* isolates were grown on 3% NaCl nutrient agar plate and TCBS agar plate shown in Figure 4.1.

The total number of revivable strains involved in this study is 44 strains. There are 27 strains from Sabah, 15 strains from Sarawak and 2 strains from Klang, Selangor. These strains are isolated in different geographical area and at different time as shown in Figure 4.2.



(a) (b)

Figure 4.1: Bacterial isolate plated on 3% NaCl nutrient agar and TCBS agar plate. (a) Growth of VC036 pure culture on 3% NaCl nutrient agar plate. (b) Yellow colonies produced by VC050 isolate on TCBS agar.

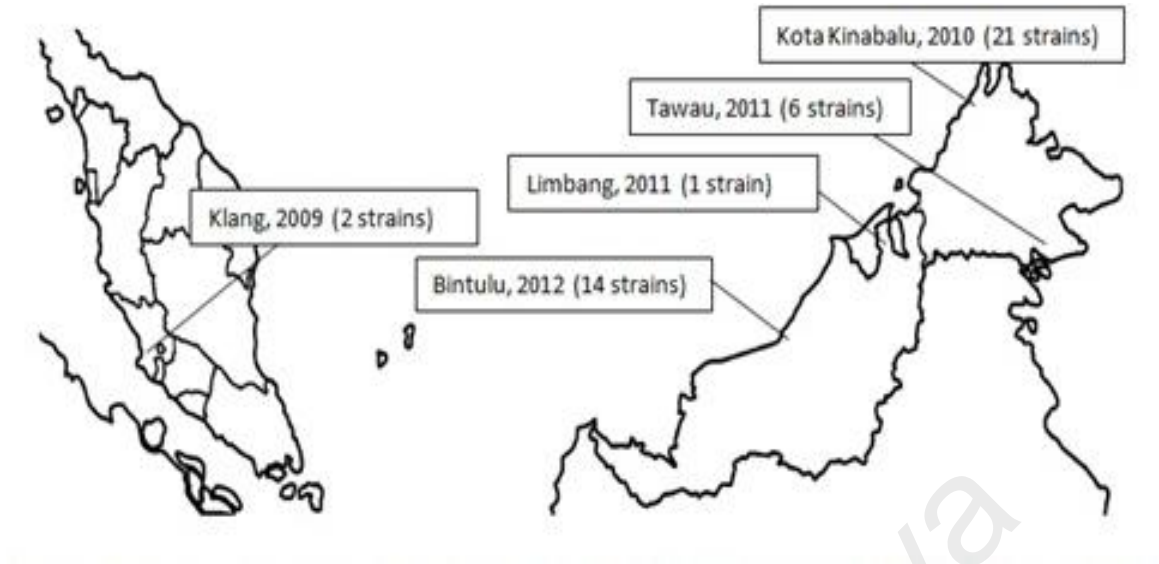


Figure 4.2: Distribution of revived bacterial strains from different area in Malaysia and their year of isolation.

4.2 Phenotypic characterisation

4.2.1 Voges-Proskauer test

Out of the 21 *V. cholerae* isolates from Sabah 2010, 15 isolates were VP positive while 6 isolates were VP negative. All 6 of Sabah 2011 isolates were VP positive. One isolate from Sarawak 2011 was VP positive. For Sarawak 2012 isolates, 11 of the 14 isolates were VP positive and 3 isolates were VP negative. The Klang 2009 isolates were all VP positive. The overall VP test results of all the 44 *V. cholerae* isolates was 35 VP positive (80%) isolates and 9 VP negative (20%) isolates. The change of the colouration of pure bacterial isolate culture medium in VP test is shown in Figure 4.3.

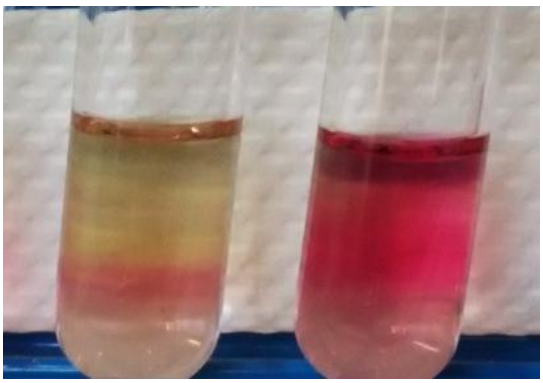


Figure 4.3: Voges-Proskauer test. The VP negative results for isolate V295 as light brown colour was developed at the surface of the reaction medium (left). The presence of pinkish red colour in the reaction medium for isolate V294 indicates VP positive results (right).

4.2.2 Antimicrobial susceptibility test

The diameter of inhibition zone was measured. The zone diameter breakpoints are those established by CLSI for the disk diffusion for *Pseudomonas aeruginosa* in Table 2B-1 of M100S, 26th ed. Performance Standards for Antimicrobial Susceptibility Testing Informational Supplement. The inhibition zone diameter that was ≥12 mm indicate bacterial isolate is susceptible to polymyxin B, while diameter ≤11 mm indicate isolate is resistance to polymyxin B. The 44 *V. cholerae* isolates examined, all were polymyxin B resistant.

(a)

(b)

Figure 4.4: Polymyxin B antibiotic susceptibility test. Inhibition zone diameter ≤11 mm is sensitive and ≥12 mm is resistant, based on M100S, 26th ed. CLSI Performance Standards. (a) Zone of inhibition diameter viewed on a dark surface. (b) Inhibition zone of VC030 isolate in a Mueller-Hinton agar plate.

4.2.3 Plate haemolysis test

Within the 21 Sabah 2010 *V. cholerae* isolates, 19 of them were haemolytic and 2 were non-haemolytic. Four of the 6 Sabah 2011 isolates were haemolytic while 2 isolates were non-haemolytic. The one isolate from Sarawak 2011 was haemolytic. Within the 14 Sarawak 2012 isolates, 12 isolates were haemolytic and 2 isolates were non-haemolytic. For the Klang 2009 isolates, one was haemolytic while the other was non-haemolytic. Out of the total 44 *V. cholerae* isolates, 37 isolates (84%) were haemolytic while 7 isolates (16%) were non-haemolytic.

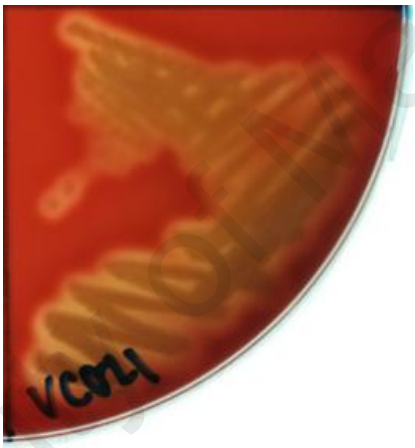


Figure 4.5: Haemolysis test. The presences of clear transparent zone around the growth of VC021 isolate colonies.

4.2.4 Chicken blood cell agglutination test

For the agglutination test, 17 isolates out of the 21 *V. cholerae* isolates from Sabah 2010 were able to agglutinate chicken blood cell while 4 isolates were unable to agglutinate. All 6 of Sabah 2011 isolates were able to agglutinate blood cell. The one isolate from Sarawak 2011 was able to agglutinate. The 14 Sarawak 2012 isolates, 13 isolates were able to agglutinate chicken blood cell and one was unable to agglutinate. For the Klang 2009 isolates, one isolates was able to agglutinate while the other isolate was unable to agglutinate chicken blood cell. Overall agglutination results of the 44 *V. cholerae* isolates was 38 isolates (86%) were able to agglutinate chicken blood cell and

6 isolates (14%) were unable to agglutinate chicken blood cell. Results of the agglutination test for all bacterial isolates were tabulated in Table 4.1.

All the results of phenotypic tests performed on the bacterial isolates were summarised in Table 4.1. There are 31 out of the 44 isolates examined, where their phenotype was VP positive, resistant to polymyxin B, either haemolytic or non-haemolytic, and agglutinate chicken blood cell. In the remaining 13 isolates, all of them were polymyxin B resistant. Two of the 13 isolates were VP negative, haemolytic, and unable to agglutinate chicken blood cell; 3 were VP positive, haemolytic, and unable to agglutinate chicken blood cell; 7 isolates were VP negative, haemolytic, and able to agglutinate; and 1 isolate that was VP positive, non-haemolytic, and unable to agglutinate.

Table 4.1: Phenotypic tests result of bacterial isolates from different geographical area.

Source	Bacterial isolate	Voges-Proskauer	Polymyxin B susceptibility*	Haemolysis	Agglutination
Kota Kinabalu, Sabah 2010 (n=21)	V264	-	R	+	-
	V266	+	R	+	+
	V273	+	R	+	+
	V275	+	R	+	-
	V276	+	R	-	+
	V279	+	R	+	+
	V283	-	R	+	+
	V285	+	R	+	+
	V286	+	R	+	+
	V293	+	R	+	+
	V294	+	R	+	+
	V295	-	R	+	+
	V299	+	R	+	+
	V301	+	R	+	+
	V302	-	R	+	+
	V303	-	R	+	-
	V311	+	R	-	+
	V312	+	R	+	-
	V313	-	R	+	+
	V314	+	R	+	+
	V319	+	R	+	+

+, positive; -, negative
* R, resistant; S, sensitive

Table 4.1, continued.

Source	Bacterial isolate	Voges-Proskauer	Polymyxin B susceptibility*	Haemolysis	Agglutination
Tawau, Sabah 2011 (n=6)	V349	+	R	+	+
	V352	+	R	+	+
	V353	+	R	+	+
	V355	+	R	+	+
	V357	+	R	-	+
	V360	+	R	-	+
Limbang, Sarawak 2011 (n=1)	V329	+	R	+	+
Bintulu, Sarawak 2012 (n=14)	VC012	+	R	+	+
	VC015	-	R	+	+
	VC016	+	R	+	+
	VC021	+	R	+	+
	VC024	+	R	+	+
	VC027	-	R	+	+
	VC028	+	R	-	+
	VC030	+	R	+	+
	VC031	+	R	+	+
	VC032	-	R	+	+
	VC033	+	R	+	+
	VC037	+	R	+	+
	VC050	+	R	-	-
	VC062	+	R	+	+
Klang 2009 (n=2)	213/09	+	R	-	+
	214/09	+	R	+	-
O139 Control (n=2)	O139 USM	-	R	+	-
	O139 IMR	+	R	+	+

+, positive; -, negative
* R, resistant; S, sensitive

4.3 Genotypic characterisation

The PCR for *O1rfb* gene, *O139rfb* gene, *tcpA* gene, *ctxA* gene, *ctxB* gene, *hlyA* gene, *rtxC* gene and *rstC* gene were done to detect the presence of these genes among the isolates. The 44 isolates in this study were positive for *O1rfb* gene, but negative for *O139rfb* gene. All isolates from Sabah 2010 (n=21), Sabah 2011 (n=6), Sarawak 2011 (n=1), and Sarawak 2012 (n=14) contained *ctxA*, *tcpA* El Tor type, *rtxC*, *rstC* and *hlyA*

El Tor type genes. The *ctxA*, *tcpA* El Tor type, *rtxC*, and *hlyA* El Tor type genes were present in the two isolates from Klang 2009, while *rstC* gene was absent from these isolate. Figure 4.6 shows the gel electrophoresis image of PCR products for *O1rfb*, *O139rfb* and *hlyA* gene. Table 4.2 shows the presence of different genes of all the isolates in this study.

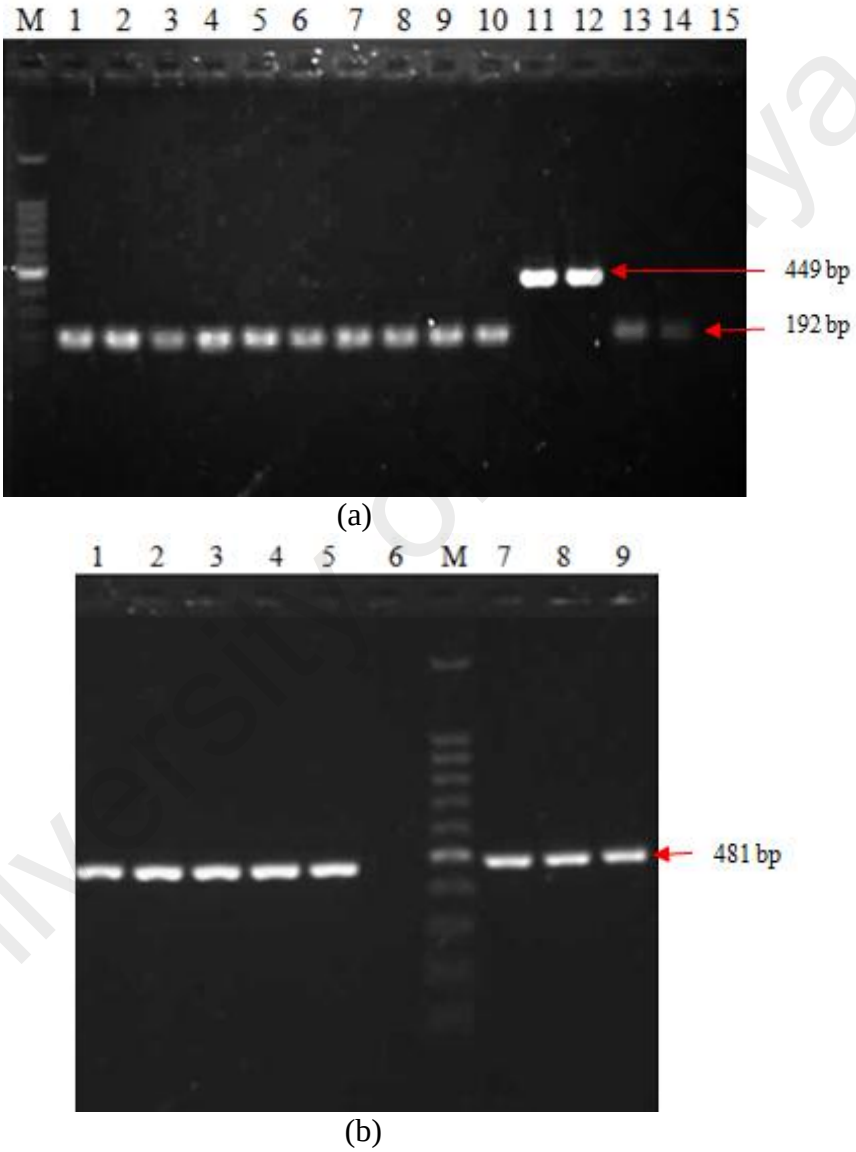


Figure 4.6: Representative gel electrophoresis of PCR products for gene detection. (a) Agarose electrophoresis of duplex PCR products for *O1rfb* and *O139rfb* genes. Lane M: 100bp DNA ladder; Lane 1-10: isolate VC028, VC030, VC031, VC032, VC033, VC037, VC050, VC062, 213/09 and 214/09 respectively; Lane 11-14: positive control O139USM, O139IMR, N16961 El Tor and 1761 hybrid respectively; Lane 15: negative control. (b) Agarose electrophoresis of PCR products for *hlyA* gene. Lane 1-3: isolate VC062, 213/09 and 214/09 respectively; Lane 4-6: positive control N16961 El Tor, 1761 hybrid and negative control respectively; Lane M: 100bp DNA ladder; Lane 7-9: isolate V264, V266 and V273 respectively.

Table 4.2: Presence of different genes in bacterial isolates using PCR.

Source	Bacterial isolate	PCR gene detection of different genes						
		O1rfb	***O139rfb***	***ctxA***	***tcpA*** *	***rtxC***	***rstC***	***hlyA***
Kota Kinabalu, Sabah 2010 (n=21)	V264	+	-	+	E	+	+	+
	V266	+	-	+	E	+	+	+
	V273	+	-	+	E	+	+	+
	V275	+	-	+	E	+	+	+
	V276	+	-	+	E	+	+	+
	V279	+	-	+	E	+	+	+
	V283	+	-	+	E	+	+	+
	V285	+	-	+	E	+	+	+
	V286	+	-	+	E	+	+	+
	V293	+	-	+	E	+	+	+
	V294	+	-	+	E	+	+	+
	V295	+	-	+	E	+	+	+
	V299	+	-	+	E	+	+	+
	V301	+	-	+	E	+	+	+
	V302	+	-	+	E	+	+	+
	V303	+	-	+	E	+	+	+
	V311	+	-	+	E	+	+	+
	V312	+	-	+	E	+	+	+
	V313	+	-	+	E	+	+	+
	V314	+	-	+	E	+	+	+
	V319	+	-	+	E	+	+	+
Tawau, Sabah 2011 (n=6)	V349	+	-	+	E	+	+	+
	V352	+	-	+	E	+	+	+
	V353	+	-	+	E	+	+	+
	V355	+	-	+	E	+	+	+
	V357	+	-	+	E	+	+	+
	V360	+	-	+	E	+	+	+
Limbang, Sarawak 2011 (n=1)	V329	+	-	+	E	+	+	+
Bintulu, Sarawak 2012 (n=14)	VC012	+	-	+	E	+	+	+
	VC015	+	-	+	E	+	+	+
	VC016	+	-	+	E	+	+	+
	VC021	+	-	+	E	+	+	+
	VC024	+	-	+	E	+	+	+
	VC027	+	-	+	E	+	+	+
	VC028	+	-	+	E	+	+	+
	VC030	+	-	+	E	+	+	+
	VC031	+	-	+	E	+	+	+
	VC032	+	-	+	E	+	+	+
	VC033	+	-	+	E	+	+	+
	VC037	+	-	+	E	+	+	+
	VC050	+	-	+	E	+	+	+
	VC062	+	-	+	E	+	+	+

+, present; -, absent.

* C, classical type; E, El Tor type.

Table 4.2, continued.

Source	Bacterial isolate	PCR gene detection of different genes						
		O1rfb	***O139rfb***	***ctxA***	***tcpA*** *	***rtxC***	***rstC***	***hlyA***
Klang 2009 (n=2)	213/09	+	-	+	E	+	-	+
	214/09	+	-	+	E	+	-	+
O139 Control (n=2)	O139 USM	-	+	NT**	NT	NT	NT	NT
	O139 IMR	-	+	NT	NT	NT	NT	NT
Positive control (n=2)	El Tor N16961	+	-	+	E	+	+	+
	Hybrid 1761	+	-	+	E	+	+	+

+, present; -, absent.
* C, classical type; E, El Tor type.
** not tested

Multiple sequence alignment of the amino acid sequence of *ctxB* was carried out in order to identify their *ctxB* genotype showed in Figure 4.7. The different genotype of *ctxB* was differentiated based on amino acid residue changes at specific position in the *ctxB* polypeptide sequence. Genotype 1 which is found in classical biotype worldwide and El Tor biotype from U.S. Gulf Coast have histidine at position 39, phenylalanine at position 46, and tyrosine at position 68 of the *ctxB* amino acid sequence. Genotype 2 which is found in El Tor biotype from Australia have histidine at position 39, leucine at position 46, and tyrosine at position 68 of the amino acid sequence. Genotype 3 found in El Tor biotype of the seventh cholera pandemic and Latin American epidemic have tyrosine at position 39, phenylalanine at position 46, and isoleucine at position 68. Table 4.3 shows the *ctxB* genotype of all the isolates from Sabah 2010, Sabah 2011, Sarawak 2011, Sarawak 2012 and Klang 2009 in this study was of genotype 1. The two strains of O139 control had *ctxB* genotype 3.

The biotype of 44 *V. cholerae* isolates in this study were summarised in Table 4.4, based on the phenotypic characterisation and the genetic characterisation results. Out of the 44 isolates, 13 (30%) isolates were classified as hybrid biotype. The remaining 31 (70%) isolates were identified to be variant biotype.

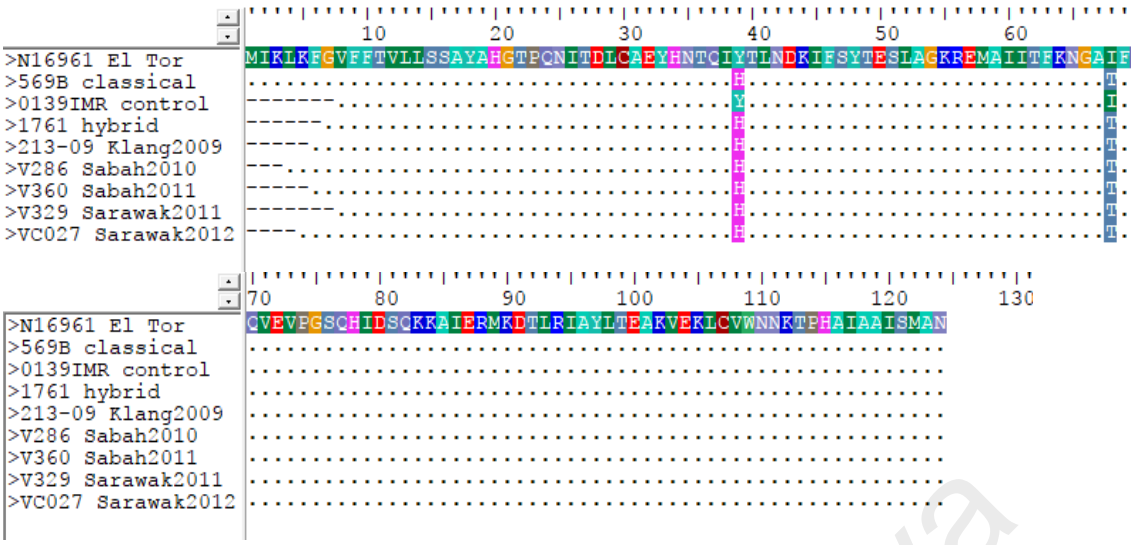


Figure 4.7: Multiple sequence alignment of *ctxB* amino acid sequence. Multiple sequence alignment of representative *V. cholerae* isolates from year 2009 to 2012 in Klang, Sabah and Sarawak with control strains (O139IMR and 1761 hybrid) and reference strains (N16961 El Tor and 569B classical). Identical amino acid residues are indicated by dots.

Table 4.3: Genotype of *ctxB* gene for different bacterial isolates.

Source	Bacterial isolate	*ctxB* genotype
Kota Kinabalu, Sabah 2010 (n=21)	V264	1
	V266	1
	V273	1
	V275	1
	V276	1
	V279	1
	V283	1
	V285	1
	V286	1
	V293	1
	V294	1
	V295	1
	V299	1
	V301	1
	V302	1
	V303	1
	V311	1
	V312	1
	V313	1
	V314	1
	V319	1

1, genotype 1 (classical & U.S. Gulf Coast El Tor biotype); 2, genotype 2 (Australia El Tor biotype); 3, genotype 3 (7^{th} pandemic El Tor & Latin American El Tor biotype)

Table 4.3, continued.

Source	Bacterial isolate	*ctxB* genotype
Tawau, Sabah 2011 (n=6)	V349	1
	V352	1
	V353	1
	V355	1
	V357	1
	V360	1
Limbang, Sarawak 2011 (n=1)	V329	1
Bintulu, Sarawak 2012 (n=14)	VC012	1
	VC015	1
	VC016	1
	VC021	1
	VC024	1
	VC027	1
	VC028	1
	VC030	1
	VC031	1
	VC032	1
	VC033	1
	VC037	1
	VC050	1
	VC062	1
Klang 2009 (n=2)	213/09	1
	214/09	1
O139 Control (n=2)	O139 USM	3
	O139 IMR	3
Positive control (n=2)	El Tor N16961	3
	Hybrid 1761	1

1, genotype 1 (classical & U.S. Gulf Coast El Tor biotype); 2, genotype 2 (Australia El Tor biotype); 3, genotype 3 (7^{th} pandemic El Tor & Latin American El Tor biotype)

Table 4.4: Biotype of *V. cholerae* isolates from different sources.

Source	Number of isolate	
	Hybrid biotype	**Variant biotype**
Sabah 2010 (n=21)	8	13
Sabah 2011 (n=6)	0	6
Sarawak 2011 (n=1)	0	1
Sarawak 2012 (n=14)	4	10
Klang 2009 (n=2)	1	1
Total	13	31

4.4 DNA fingerprinting

REP-PCR was carried out to examine the relationship between all the *V. cholerae* isolates studied. The number of the bands produced by REP-PCR for the *V. cholerae* isolates ranged from 7 to 9 bands. The band size of these bands was in the range of below 250 bp to 2000 bp.

Dendrogram derived from the REP-PCR band patterns of the 44 *V. cholerae* isolates and the control strains in Figure 4.9 shows the grouping of isolates into four profiles (REP 1, REP 2, REP 3 and REP 4). REP 1 contained 15 isolates from Sabah outbreak in 2010, 5 isolates from Sabah outbreak in 2011 and 5 isolates from Sarawak outbreak in 2012. REP 1 cluster comprised of 8 hybrid and 17 variant biotypes. Isolates under REP 2 were made up of 2 isolates from Klang outbreak in 2009, 6 isolates from Sabah outbreak in 2010, 1 isolate from Sabah outbreak in 2011, 1 isolate from Sarawak outbreak in 2011, 9 isolates from Sarawak outbreak in 2012 and the N16961 El Tor control strain. There are 5 hybrid and 14 variant biotypes in REP 2 cluster. These two profiles consisted of all of the 44 *V. cholerae* isolates and the N16961, the El Tor control strain; while REP 3 profile contained only the 1761 hybrid strain from Terengganu in 2009. Two strains of *V. cholerae* O139 (O139IMR and O139USM) are within REP 4 profile. REP 1 and REP 2 have a 92% similarity, while REP 1 and REP 2 have 82.9% similarity with REP 3.

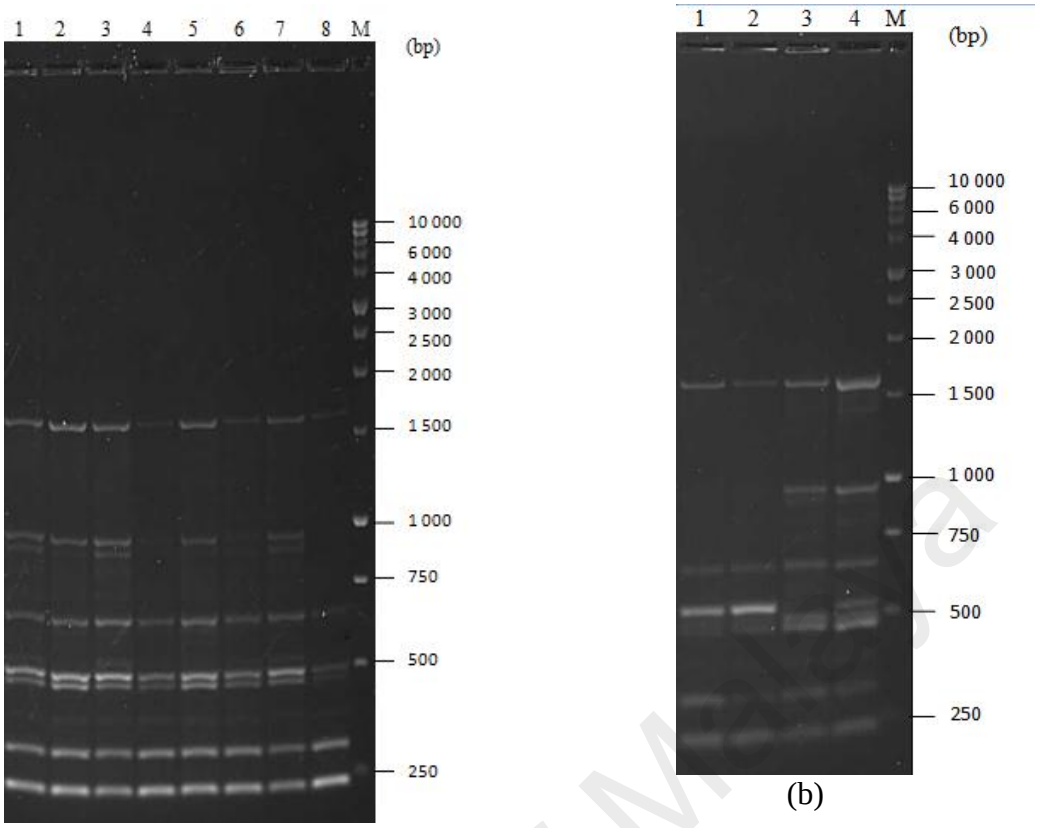


Figure 4.8: Representative REP-PCR electrophoretic band patterns of *V. cholerae* O1 isolates. (a) 1.2% agarose gel electrophoresis of REP-PCR products. Lane 1-8: V286, V293, V294, V295, V299, V301, V302 and V303 respectively; Lane M: 1 kb DNA ladder. (b) 1.2% agarose gel electrophoresis of REP-PCR products. Lane 1-4: O139USM, O139IMR, N16961 and 1761 respectively; Lane M: 1 kb DNA ladder.

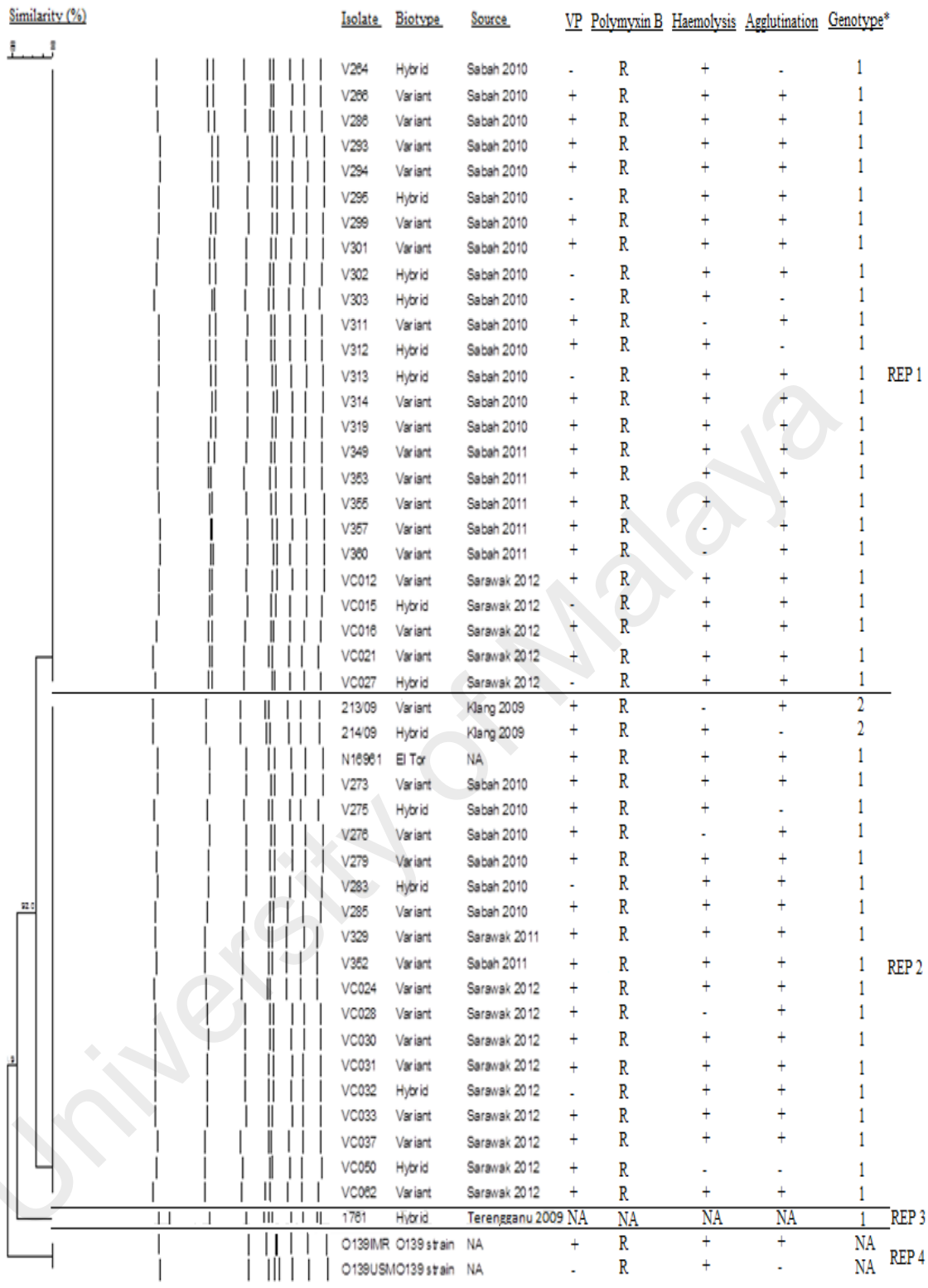


Figure 4.9: Dendrogram of REP-PCR. *Genotype 1- present of *ctxA*, El Tor *tcpA*, *rtxC*, *rstC*, and *hlyA* gene; Genotype 2- present of *ctxA*, El Tor *tcpA*, *rtxC*, and *hlyA* gene, while absent of *rstC* gene.

CHAPTER 5: DISCUSSION

5.1 Growth of *V. cholerae* isolates on TCBS agar

Stab culture of all the bacterial isolates was streaked on 3% NaCl nutrient agar to obtain single colonies of bacteria. Single bacterial colony grown on nutrient agar with 3% NaCl was subculture onto TCBS agar. TCBS agar is a selective media mainly for *Vibrio* species (Pfeffer & Oliver, 2003). Gram-positive bacteria will not be able to grow on the TCBS medium. The medium is also differential as different species within Vibrio produces colonies with different colouration. *V. cholerae* produces large, flattened, yellow colonies with opaque centre and translucent peripheries on TCBS agar. *V. cholerae* ferments sucrose that is present in the medium (Donovan & van Netten, 1995; Lotz, Tamplin, & Rodrick, 1983). Sucrose fermentation by the bacteria leads to production of acid as a by-product. In the presence of acid, pH of the medium was lowered. The low pH would be detected through the colour change of pH indicators, namely thymol blue and bromothymol blue. Thymol blue displayed yellow colouration when the TCBS medium is under acidic conditions. Similar for bromothymol blue, it changes to yellow colour in acidic pH. Therefore, the yellow bacterial colonies formation indicates the capability of isolates to utilise sucrose as a carbon source. The various ingredients in the composition of TCBS medium serve different functions to allow the growth of *V. cholerae*. Bile salts and sodium cholate act as an inhibitor for gram-positive bacterial growth (Gomez-Gil & Roque, 2006). Sodium thiosulphate and sodium citrate in the medium inhibit the growth of Enterobacteriaceae as these chemicals increases the pH of TCBS. Sodium thiosulphate is also a source of sulphur, it will combines with ferric citrate to detect hydrogen sulphide production. The alkaline

condition of TCBS medium enhances the growth of *V. cholerae*, as they are usually sensitive to acidic environment.

5.2 Phenotypic characterisation

The phenotypic tests were carried out to determine phenotype of the 44 *V. cholerae* isolates, whether it is of classical or El Tor type. The VP test was used to detect the production of acetoin by the bacterial isolates. Typical classical strain has a negative response to VP; while the El Tor strain is VP positive. Out of the 44 isolates, 35 isolates were found to be VP positive, while 9 isolates are VP negative. These 9 VP negative isolates were the outbreak strains from Sabah 2010 (n=6) and Sarawak 2012 (n=3). Therefore, these 9 VP negative strains demonstrated the response of the classical strain, not the El Tor strain.

For polymyxin B susceptibility, the typical classical strain is sensitive to polymyxin B while the El Tor strain is resistant to polymyxin B. Based on the susceptibility test result, all the isolates were resistant to polymyxin B (Table 4.1), which were a characteristic of the El Tor biotype. Polymyxin B is a membrane-specific antibiotic. Their bactericidal activity toward the classical biotype *V. cholerae* is through interaction with the negatively charged site on the LPS and altering the outer membrane permeability (Cardoso et al., 2007). The outer membrane is disrupted and its fatty acid portion dissolves with cytoplasmic membrane lipid portion. This affects membrane integrity, leading to leakage of intracellular component that kills the non-resistant vibrios (Yeaman & Yount, 2003). The polymyxin B resistant isolates in this study may have modification on their outer membrane composition that contributed to the susceptibility that is different from the classical strain. Outer membrane modification may prevent polymyxin B from binding to the membrane and detain its action towards

the isolates. According to Moffatt et al. (2010), bacteria change their LPS that have a net negative charge through means of lipid A covalent modification. This is a strategy to decrease affinity of the cationic polymyxin B to the membrane. There are other strategies that bacteria employ to overcome the antimicrobial activity of polymyxin B, example the utilisation of efflux system that pump out the polymyxin that enter the cytoplasm, production of polymyxin degrading protease and formation of capsule (Campos et al., 2004; Padilla et al., 2010).

Thirty-seven *V. cholerae* isolates demonstrated haemolytic properties, which is mainly a trait of El Tor strains. There are 7 isolates which were non-haemolytic, but it could not be concluded as classical type characteristic. This is due to the presence of non-haemolytic El Tor strains from certain geographical area such as Latin America, Asia, Africa, Europe and the Pacific (CDC, 2016). On the chicken blood cell agglutination test, among the studied isolates, 6 were negative for the agglutination test, depicting the classical phenotype. Isolates that agglutinate the blood cell (n=38) demonstrated characteristic of El Tor biotype.

According to the phenotypic test results in Table 4.1, the *V. cholerae* isolates tested in this study demonstrated six different phenotypes. Among the six phenotypes, the predominant phenotype that was observed in 57% (25 out of 44 isolates) of the *V. cholerae* isolates. These isolate show characteristics of VP positive, polymyxin B resistant, haemolytic and able to agglutinate red blood cells. Then, follow by a total of 14% of isolates (6 out of 44 isolates) demonstrated phenotypic characteristics of VP positive, polymyxin B resistant, non-haemolytic and able to agglutinate red blood cells. These two phenotypes depicted characteristics that belong to the typical El Tor biotype. The remaining 29% (13 out of 44 isolates) of the tested *V. cholerae* isolates were found to demonstrate overall phenotypic characteristics that were combination of both the El Tor and classical biotypes. The four different phenotypes of the 29% *V. cholerae*

isolates were: 1) Phenotype with VP negative, polymyxin B resistant, haemolytic and able to agglutinate red blood cell. 2) Phenotype with VP negative, polymyxin B resistant, haemolytic and cannot agglutinate red blood cell. 3) Phenotype with VP positive, polymyxin B resistant, haemolytic and cannot agglutinate blood cell. 4) Phenotype with VP positive, polymyxin B resistant, non-haemolytic and cannot agglutinate red blood cell. These 4 phenotypes were categorised under phenotype of hybrid strain based on the definition of hybrid biotype.

Within the 44 *V. cholerae* O1 isolates in this study, isolates with phenotypic traits similar to that of hybrid and variant strains identified from previous studies were present. There are 2 isolates that displayed phenotype identical to Matlab type I and type II variant strains based on the VP, polymyxin B resistance and chicken cell agglutination test. Both Matlab type I and type II variant strains have phenotypic characteristics of VP negative, resistant to polymyxin B and unable to agglutinate chicken blood cell (Nair et al., 2002). The phenotype of 31 isolates out of the total 44 isolates were the same as Mozambique variant and altered El Tor variant strains for VP, polymyxin B resistance and chicken cell agglutination test. These variant strains have been described to be VP positive, resistant to polymyxin B and able to agglutinate chicken blood cell (Ansaruzzaman et al., 2004; Nair et al., 2006; Safa, Nair, & Kong, 2010).

Phenotypic test is a conventional method for characterising the *V. cholerae* isolates. The phenotypic tests carried out were able to differentiate El Tor and classical strains. In VP test, the result is according to the observations of changes in reagent's colouration. There is a problem noticed when carrying out the test in this study. The VP reagents, KOH and alpha-naphthol may react with each other to produce copper-like colour. This will mask the results of weak positive reaction and causes a false negative result interpretation (Johnson & Case, 2015). Biochemical test results determined

through colour development can be vague sometimes, because the colour changes may be recorded differently by different observer. The situation is similar for the agglutination and haemolysis test. Misinterpretation of the phenotypic test observation may occur, thus having different results or grey area depending on the observer that recorded the results. It is difficult to conclude the differentiation of isolates solely based on the phenotypic characterisation results. Therefore, genotypic characterisation was performed to have a more accurate El Tor and classical biotype differentiation of the isolates.

5.3 Genotypic characterisation

Genotypic characterisation of the *V. cholerae* isolates using PCR gene detection was able to identify the presence of different genes examined in the isolates' genomic DNA. The *ctxA* gene is a gene possessed by both classical and El Tor strain. This gene encodes the enzymatic subunit of cholera toxin which is commonly present in *V. cholerae* biotypes that produces cholera toxin. The *tcpA* gene has two genotypes, one is of the classical type and another is the El Tor type. The *O1rfb* and *O139rfb* gene detection was done to confirm the isolates as O1 strains. The *rtxC* and *rstC* genes are only present in El Tor strain, while absent in classical strain (Lin et al., 1999; Waldor et al., 1997).

Haemolysin A, *hlyA* gene have different genotype for classical and El Tor biotype. There is a deletion in *hlyA* gene of classical strains which leads to a non-functional end product (Rader & Murphy, 1988). In this study, the 44 *V. cholerae* isolates contains *hlyA* gene of El Tor type that produces functional haemolysin shown in Table 4.2. The plate haemolysis phenotypic test result for all the bacterial cultures of the 44 *V. cholerae* isolates should have been haemolytic, since their *hlyA* genotype is that of

El Tor type. However, there were 7 *V. cholerae* isolates that have non-haemolytic trait when the plate haemolysis was performed. All isolates have same genotype for *hlyA*, but produces a varied phenotype. The explanation for this phenomenon may be the occurrence of posttranslational modification for the haemolysin protein. It was mentioned by Hall and Drasar (1990) in their study that product of *hlyA* structural gene undergo proteolytic cleavage to produce haemolysin with different relative molecular mass. Haemolysin is an accessory toxin that is associated with enterocolitis cause by El Tor biotype and non-O1 serogroup that are cholera toxin negative (Olivier et al., 2007). The effect of *V. cholerae* isolates' haemolytic ability toward their capability to cause disease symptoms will not be discussed in this study due to that our study's isolates produces cholera toxin, as indicated by the presence of *ctxA* and *ctxB* genes. Whereas in the study by Olivier et al. (2007), it focused on the role of accessory toxins as virulence factor in the absence of CT production.

Cholera toxin is one of the two major virulence factors of *V. cholerae* that are responsible for the characteristic symptoms of cholera. The receptor binding subunit of cholera toxin is encoded by the *ctxB* gene (van Heyningen, 1976). There are a number of different genotypes for the *ctxB* gene that is grouped on the basis of amino acid changes in the polypeptide chain. All 44 of the *V. cholerae* isolates in this study have genotype 1 *ctxB* gene, while the O139 control strain and N16961 El Tor strain have genotype 3 *ctxB* gene. The difference between these two genotypes is the amino acid at position 39 and 68 of cholera toxin subunit B polypeptide. Based on Figure 4.7, genotype 1 is that of classical *ctxB* gene, whereas genotype 3 is that of El Tor *ctxB* gene. The two O139 control strains had genotype 3 *ctxB*. This shows the CT of O139 strains in our study were more closely related to CT of seventh pandemic El Tor strain and O139 strains isolated from 1993 to 1997. None of the *ctxB* genotype of *V. cholerae* isolates in this study was that of genotype 4 to 6. This may indicate that the variant and

hybrid strains identified in this study have cholera toxin gene with higher similarity to El Tor type, while diverge from the cluster of O139 strains isolated during 1998 to 2005.

Even though both classical and El Tor biotype produces cholera toxin, the disease symptoms cause by these biotypes varies (Ghosh-Banerjee et al., 2010). The speculated explanation for this is that the different in cholera toxin subunit B amino acid sequence will lead to differences in binding affinity of cholera toxin to the target receptor on the host cell's plasma membrane. Changes of binding affinity occur due to the different amino acid in the polypeptide of cholera toxin subunit B. Histidine and tyrosine have side chain with different properties, positively charged and polar R group respectively, similar for threonine and isoleucine. Polar and charged side chains are hydrophilic, non-polar side chains are hydrophobic. These side chains will involve in interactions that determine the conformation of the cholera toxin subunit B protein. The conformation will play an important role for the function of cholera toxin subunit B to bind their targets.

The *V. cholerae* isolates from Sabah and Sarawak have El Tor genotype based on the few specific genes examined in this study, except their *ctxB* gene is that of classical type. Sabah and Sarawak isolates probably have CTXΦ structure and RS1 associated with altered El Tor variant strains. The isolates from Klang have most of the examined genes in El Tor type and also harbour classical *ctxB* gene. Klang isolates are slightly different from Sabah and Sarawak isolates as they do not have *rstC* gene. The rstC antirepressor is encoded by sequences in the RS1 element that inhibit the rstR CTX phage repressor activity (Davis et al., 2002). This enable gene needed for phage production to be expressed. The Klang isolates may not have RS1 element that flanked the CTX prophage in their chromosome 1, similar to the classical biotype. The RS1 element and El Tor type CTX prophage that contains *ctxB* gene of El Tor type may have been removed through loop-out recombination, allows classical CTX prophage and RS2

element to be inserted into the remaining attachment site (Faruque et al., 2007). The other possibility is that their RS1 element is truncated, like the RS1 and CTX prophage structure of hybrid El Tor strains. Hybrid El Tor strains were isolated from countries in Asia and Africa during 1991 to 2004 that contained classical type *ctxB* (Safa, Sultana, Cam, Mwansa, & Kong, 2008). All Sabah, Sarawak and Klang isolates have *ctxB* genotype 1, this generally correspond to the present of classical type CTX prophage. It is also possible that these isolate have mosaic-like structure of CTX prophage, which is a combination of genetic structure from different type of CTX phage (Kim et al., 2014; Choi et al., 2016). This cannot be concluded in this study, because the analysis of SNPs in *rstR*, *rstA* and *rstB* gene with *ctxB* genotyping is necessary to identify variation in CTX phage.

The Sabah, Sarawak and Klang *V. cholerae* isolates were characterised as hybrid and variant biotype. 70% of the isolates were El Tor variant biotype, while the remaining 30% were hybrid biotype. It is hypothesised that El Tor strains are able to gain cholera toxin gene from classical strain through horizontal gene transfer (Raychoudhuri et al., 2009). Classical strain is more virulence as it causes more severe cholera, while El Tor strain survive better in the environment. This may enable hybrid and variant strains to have a survival advantage as environment other than the human host can act as reservoir for *V. cholerae* bacteria to survive and disperse during interepidemic period.

5.4 DNA fingerprinting

Based on the dendrogram generated by banding patterns of REP-PCR in Figure 4.9, REP 1 to REP 4 profiles were observed. Generally, the isolates in this study could be clustered into REP 1 and REP 2 profiles. The 1761 hybrid strain and O139 control

strains demonstrated distinctive profiles from the 44 isolates in this study. The 1761 hybrid strain was isolated from Terengganu in 2009. In Figure 4.9, the Klang isolates were grouped together under the same REP 2 profile with Sabah (2010 and 2011) and Sarawak (2011 and 2012) isolates.

REP 1 as well as REP 2 group contained isolates with typical El Tor phenotypes and isolates with hybrid phenotypes. There was no isolate in REP 1 with one of the four hybrid phenotype (VP positive, polymyxin B resistant, non-haemolytic and unable to agglutinate chicken blood cell) identified in this study. Another hybrid phenotype (VP negative, polymyxin B resistant, haemolytic and unable to agglutinate chicken blood cell) was absent among the isolates in REP 2. However, both REP 1 and REP 2 have isolates with the two identified typical El Tor phenotype (68% in REP 1 and 73.7% in REP 2). The El Tor phenotype may allow the studied isolates to have more advantages for inhabitation in environments like the human host.

The 42 Sabah and Sarawak isolates in this study have the *ctxA* gene, El Tor *tcpA* gene, *rtxC* gene, *rstC* gene and El Tor *hlyA* gene. The Klang isolates also have all these genes, except *rstC* gene. All Sabah and Sarawak isolates have the same genotype for examined genes, but the dendrogram shows these isolate separated into two profiles. The isolates in REP 1 may have different CTX array with repeats of region or element in their genome compare to REP 2 isolates (Kim et al., 2014). These isolate may also have variable in genes and genome region that was not analysed in this study. Within each group of REP 1 and REP 2, isolates with typical El Tor and hybrid phenotype were present. It shows that the REP-PCR fingerprinting was unable to segregate the isolates based on the phenotype of isolate examined. Other justification for isolates demonstrating different phenotypic traits as their genomic structure was similar may be mechanism that inhibit the expression of certain gene or posttranslational modification that alter function of protein.

In REP 2 profile, there are 53% Sarawak isolates and 37% Sabah isolates. REP 1 has more Sabah isolates (80% of the 25 isolates in REP 1) than Sarawak isolates (20%). The majority of the isolates from Sabah have genomic pattern of REP 1 profile, whereas genomic structure of Sarawak isolates was mainly of REP 2. Within REP 1 group, Sarawak isolates were present. It is the same for Sabah isolates under REP 2 group. The occurrence of genetic elements exchange between Sabah and Sarawak isolates or mutation of these isolate may result in the closely related REP 1 and REP 2 profile. The assumption that all 44 *V. cholerae* isolates in this study may have derived from a common strain is feasible. N16961 El Tor strain has the same genetic profile as isolates in REP 2. This suggests that variant and hybrid strains in REP 1 and REP 2 probably have a same common ancestral strain with the N16961 strain; or isolates in REP 1 may have been the derivative strains from isolates in REP 2, which is clonally related to N16961 El Tor strain. *V. cholerae* strains have high genome plasticity that causes emergence of strains with different phenotype and genotype combination. A suggestion of the mechanism for this outcome was that genetic recombination of *V. cholerae* genome happened through lateral gene transfer (Chun et al., 2009).

Klang and Terengganu are both located at west peninsular of Malaysia, but Klang isolates does not have the same profile with the 1761 hybrid strain which indicated that they are not clonally related. There are a few possible explanations for this result. Klang is situated at west coast of the peninsular, which is next to the Strait of Malacca under the Indian Ocean. The location of Terengganu is at the east coast, which is a state in proximity to the South China Sea that is a part of the Pacific Ocean. The *V. cholerae* strains from different oceanic reservoir, Indian and Pacific Ocean may have slight genetic diversity, thus the different in REP profile. Nevertheless, with this explanation there will be argument that Sabah and Sarawak isolates should have profile related with Terengganu strain since both places have coastal area next to South China

Sea. The 1761 hybrid should be more closely related to the Sabah and Sarawak *V. cholerae* isolates rather than the Klang isolates. The plausible reason for the higher similarity of Klang isolates with Sabah and Sarawak isolates could be due to higher rate of air travel between Sabah, Sarawak and Klang valley. The moving of individuals with asymptomatic infection or undiagnosed cases may spread the *V. cholerae* bacteria between East peninsular, Kuala Lumpur and Selangor through domestic travels. The population shift from Sabah and Sarawak towards area around Kuala Lumpur due to economic and employment opportunities may have contributed to the dispersal of *V. cholerae* strains (Department of Statistics Malaysia, 2015). Individuals from East peninsular whom worked in Kuala Lumpur may be infected by *V. cholerae* asymptomatically and spread the strain between Klang, Sabah and Sarawak. There is a limitation for the result interpretation as the sample size of isolates from Klang and Terengganu is insufficient to allow proper epidemiologic explanation for the results. It will be difficult to have a definite conclusion based on small sample size of isolates.

The band patterns generated from PCR-based fingerprinting such as REP-PCR can be difficult to differentiate bands that are located very close to each other. There will be confusion of whether such band pattern is made up of two bands with very close proximity or it is a thick band with high PCR product copy number. Multi-locus sequence typing (MLST) and multi-virulence locus sequencing typing are sequencing-based fingerprinting methods. These fingerprinting methods uses a unique sequence type (ST) obtain by sequencing a set of housekeeping genes or virulence genes to study evolutionary relationship of different bacterial isolates (Maiden et al., 1998). MLST will be able to overcome the banding issue observed in the REP-PCR of this study as it does not require observation of gel electrophoresis band patterns. REP-PCR fingerprinting was able to differentiate *V. cholerae* based on their serogroup. This can be seen in the dendrogram, O139 control strains have a different REP profile (REP 4) compare to the

profile of *V. cholerae* O1 strains. Within REP 1 and REP 2 profiles, there is a mixture of hybrid and variant biotype isolates. Therefore, REP-PCR was unable to differentiate the *V. cholerae* isolates in this study according to biotypes. In order to study the epidemiology of *V. cholerae* hybrid and variant strains, DNA fingerprinting method that enable the discrimination of classical and El Tor biotype may be necessary. The fingerprinting result from this study only allows us to discuss the relationship of isolates according to geographical area and serogroup. However, the biotype of isolates was unable to be examined from the REP-PCR dendrogram.

5.5 Further studies

REP-PCR fingerprinting alone does not allow a thorough epidemiologic investigation on the *V. cholerae* isolates. Additional molecular fingerprinting of these isolate with different method is necessary to obtain a better understanding of the relatednesss between the *V. cholerae* isolates. The *V. cholerae* isolates in this study are either El Tor variant or hybrid biotypes. The next step may be to re-examine the *V. cholerae* isolates within these few years that may have been identified as typical El Tor strains previously. There will be a need to study the epidemiology of cholera caused by hybrid and variant biotypes in Malaysia. The shift of seventh pandemic El Tor strain toward variant and hybrid strains in causing diseases can be examine through epidemiologic studies of these emerging biotypes through phylogenetic analysis (Azarian et al., 2014; Choi et al., 2016). In addition, the pathogenicity and adaptation of variant and hybrid strains under different conditions can be examined. This will help us to further understand the changes in virulence and adaptability of *V. cholerae*. Study of the molecular mechanisms behind the genomic diversity, like the CTX prophage

variation in these variant strains may allow us to achieve the comprehension of their survivability.

CHAPTER 6: CONCLUSION

The *V. cholerae* isolates from Sabah and Sarawak have a combination of phenotypic traits from classical and El Tor biotype. All of these isolate were showing same allele types for *ctxA, ctxB, tcpA, hlyA, rstC* and *rtxC* genes. All the isolates from East Malaysia have the genes of El Tor type except for the *ctxB* where genotype 1 *ctxB* was detected.

Biotypes of the isolates were identified using the results from phenotypic and genetic characteristics. Among the forty-four *V. cholerae* isolates from Sarawak, Sabah and Klang isolated from year 2009 to 2012, 13 (30%) isolates were classified as hybrid biotype; while 31 (70%) isolates were identified to be El Tor variant biotype based on their phenotypic and genetic characteristics. All the *V. cholerae* isolates are either hybrid or variant biotype and none of them are typical El Tor biotype.

All the *V. cholerae* isolates from Sarawak, Sabah and Klang were genetically close based on the dendrogram REP profiles (REP 1 and REP 2). Strains in REP 2 are more clonally related to N16961 El Tor strains than the strains in REP 1. There are hybrid and variant biotype strains clustered under the group of REP 1 and REP 2. This indicates strains from Sarawak, Sabah and Klang are closely related genetically whether it is hybrid or variant biotypes. This study revealed the emergence of *V. cholerae* O1 hybrid and El Tor variants in East Malaysia.

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