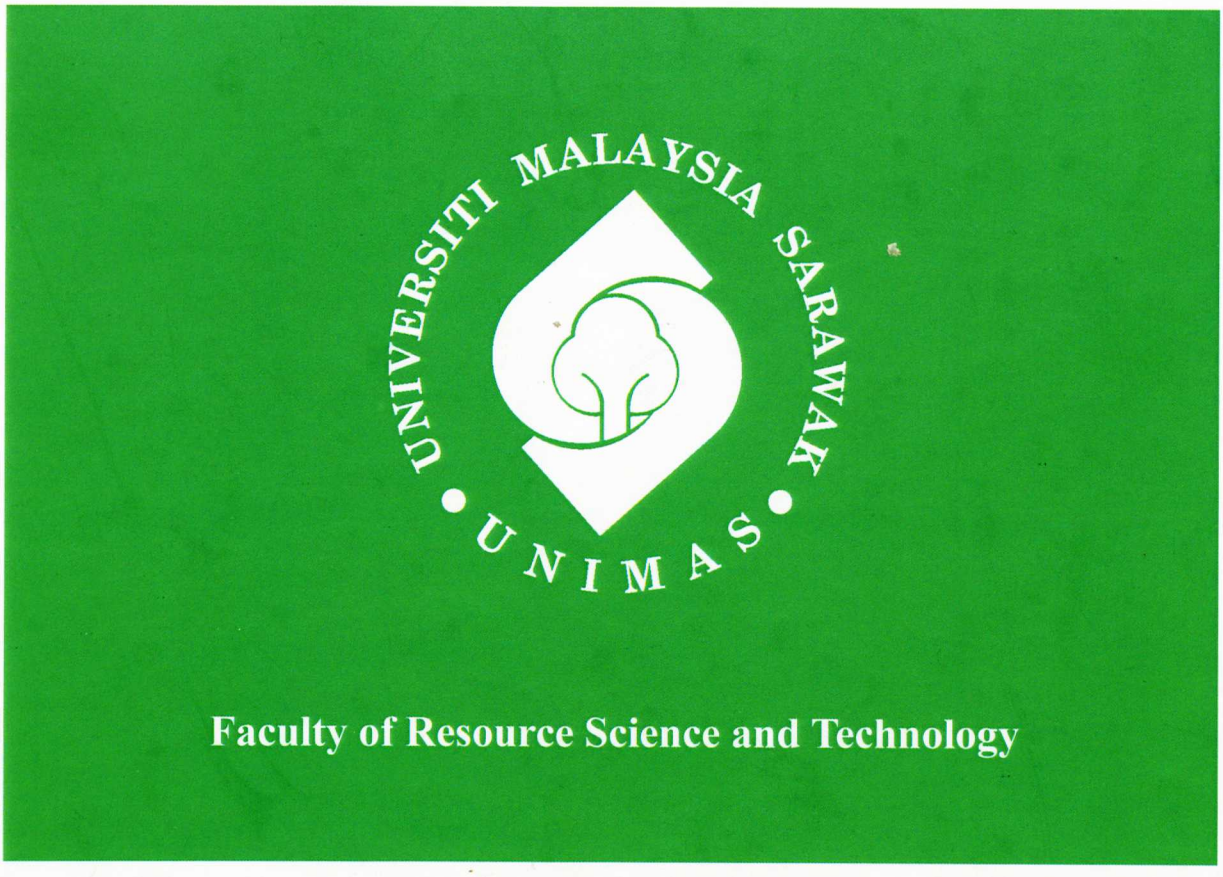

Isolation and Enumeration of *Vibrio cholerae ctx*A gene from Street Vendor Drink

Nur Sabrina Binti Habib (50807)

Bachelors of Science with Honours
(Resource Biotechnology)
2018

Isolation and Enumeration of *Vibrio cholerae ctx*A from Street Vendor Drink

Nur Sabrina Binti Habib (50807)

A project is submitted in partial fulfilment of the Final Year project (STF3013) for
The degree of Bachelor of Science with Honours
(Resource Biotechnology)

Supervisor: **Dr. Elexson Nillian**

Resource Biotechnology Programme
Faculty of Resource Science and Technology
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UNIVERSITI MALAYSIA SARAWAK

Grade: _____

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Final Year Project Report	☑
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This declaration is made on the 5th day of June year 2018

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Isolation and Enumeration of *Vibrio cholerae ctx*A from Street Vendor Drink

Nur Sabrina Binti Habib

Biotechnology Resource
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ABSTRACT

Cholera remains a global threat to the public's health in Malaysia because the outbreaks occur sporadically. Knowledge on the rate of outbreak and pattern distribution is needed as well as its prevalence in food in order to control the foodborne outbreak of cholera. The study aims to isolate and enumerate Vibrio cholerae and to detect the presence of pathogenic or virulence strain of V. cholerae in drink samples from street vendor in Kuching. A total of 30 (n=30) drink samples was collected from two types of drinks, drinks containing milk and drinks without milk. MPN-Plate method was conducted to estimate the concentration of V. cholerae and PCR was done with the ctxA gene to verify the pathogenicity of V. cholerae. In this project, it was shown that the occurrence of V. cholerae in drinks with milk is higher compared to drinks without milk. Drink samples with milk had an MPN/g estimation with a minimum value of <3 MPN/g and a maximum value of >1100 MPN/g. Whereas, drink samples without milk had an MPN/g estimation with a minimum value of <3 MPN/g and a maximum value of 460 MPN/g. However, none of the isolates were found to contain the ctxA gene. This study could highlights the importance of public education in the proper handling of food and be adequately informed about potential microbiological hazards of food handling.

Key words: *Vibrio cholerae,* MPN-Plate, PCR, *ctx*A

ABSTRAK

Penyakit taun kekal sebagai ancaman global kepada kesihatan masyarakat kerana wabak taun berlaku secara berkala. Pengetahuan mengenai kadar wabak dan juga corak pengedaran diperlukan serta kelazimanya dalam makanan bagi mengawal wabak taun bawaan makanan. Matlamat kajian ini adalah untuk mengasing dan menghitung Vibrio cholerae dan juga untuk mengesan kehadiran V. cholerae yang patogenik dalam sampel air dari gerai tepi jalan di dalam kawasan Kuching. Sejumlah 30 (n=30) sample air telah dikumpul daripada dua jenis air, iaitu air mengandungi susu dan air yang tidak mengandungi susu. Teknik MPN-Plate telah digunakan untuk membuat angaran konsentrasi V. cholerae dan teknik PCR telah dilakukan menggunakan ctxA gene untuk mengesahkan V. cholerae yang patogenik. Dalam projek ini, ia didapadi bahawa kadar kejadian V. cholerae dalam air mengandungi susu lebih tinggi dibandingkan dengan air yang tidak mengandungi susu. Sampel air yang mengandungi susu mempunyai nilai angaran MPN/g minimum <3 MPN/g dan nilai maksimum >1100 MPN/g. Manakalah, sampel air yang tidak mengandungi susu mempunyai nilai angaran MPN/g minimum <3 MPN/g dan nilai maksimum 460 MPN/g. Walaubagaimanapun, antara kesemua sampel, tiada satu pun yang mengandungi gen ctxA. Kajian ini dapat menitikberatkan kepentingan pendidikan bagi orang awam terhadap cara yang betul dalam mengendali makanan dan dimaklumkan secukupnya tentang potensi bahaya mikrobiologi dalam pengendalian makanan.

Kata kunci: *Vibrio cholerae,* MPN-Plate, PCR, *ctx*A

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LIST OF ABBREVIATIONS

ADP	:	Adenosine diphosphate
AGE	:	Agarose gel electrophoresis
APW	:	Alkaline peptone water
cAMP	:	Cyclic adenosine 5'-monophosphate
ChiRP	:	Chitin-regulated pili
CT	:	Cholera toxin
Ctx	:	Cholera toxin gene
CTXΦ	:	Cholera toxin phage
ddH_2O	:	Doubled distilled water
dH_2O	:	Distilled water
DNA	:	Deoxyribonucleic acid
dNTP	:	Deoxynucleotide Triphosphate
g	:	Gram
GTP	:	Guanosine 5'-triphosphate
HlyA	:	Hemolysin
LB	:	Luria-Bertani
$MgCl_2$	:	Magnesium chloride
mL	:	Millilitre
mM	:	Millimolar
MSHA	:	Mannose-sensitive hemagglutinin
MPN	:	Most probable number
NaCl	:	Sodium chloride
NAD	:	Nicotinamide adenine dinucleotide
PBS	:	Phosphate buffered saline
PCR	:	Polymerase chain reaction
rpm	:	Revolutions per minute
RTX	:	Repeat in toxin
Stn	:	Heat-stable enterotoxin
Taq	:	*Thermus aquaticus*
TE	:	Tris/EDTA
TBE	:	Tris/Borate/EDTA
TCBS	:	Thiosulfate citrate bile-salt sucrose
TCP	:	Toxin co-regulated pili
TcpA	:	Toxin co-regulated pili protein A
TFP	:	Type IV pili
TTSS	:	Type 3 secretion system
V	:	Voltage
VCT1	:	Forward primer
VCT2	:	Reverse primer
WHO	:	World Health Organization
°C	:	Degree Celsius
µm	:	Micrometre
µL	:	Microlitre

1.0 INTRODUCTION

Vibrio cholerae is a gram-negative bacteria with a curved rod or coma-shaped morphology between the length of 1.5 to 2 μm and a width of 0.5 μm. It is covered with pili and a single polar flagellum. The strains of *V. cholerae* are divided into different serotypes based on the lipopolysaccharides, which is known as the O-antigens. The most significant strain of *V. cholerae* is *V. cholerae* O1 and O139 which is known as the causative agent of cholera. The toxigenic O1 consist of three serotypes, Inaba, Ogawa and Hikojima, and two biotype which are classical and El Tor (Finkelstein, 1996). The more recent strain of cholera which is the *V. cholerae* O139 caused a large epidemics of cholera in Bangladesh, India and neighbouring countries. Other serotypes of this bacterium are known to cause only diarrheal disease and other infection.

Cholera is an intestinal infection that causes acute watery diarrhoea. The symptoms include watery diarrhoea and vomiting with severe dehydration in more serious cases and if it is left untreated, it can lead to shock and eventually be fatal (Blahd, 2017). The symptoms can appear as early as a few hours or as long as five days of infection. However some infected people are known to be asymptomatic or only mild symptom of cholera but the bacteria can still be transmitted in the faeces, 1 to 10 days after infection (World Health Organization (WHO), 2017) Cholera is transmitted through the faecal-oral route via food or water contaminated with the bacteria and the disease is more apparent in countries with inadequate water treatment, hygiene and sanitation.

V. cholerae are acid sensitive and the natural acidic environment of the gastric juices in the stomach would normally kill the bacteria, however, some of the *vibrio* might prevail and adhere to the intestinal wall. The bacteria are known to tolerate alkaline media which would kill most intestinal commensal (Finkelstein, 1996). Another factor that

contributes to the pathogenesis of *V. cholerae* is the cholera toxin gene (*ctx*). The production of the exotoxin causes the development of the acute-profuse diarrhoea in the infected person

Cholera remains a global threat to the public's health with an estimation of 3 to 5 million cases and over 100,000 deaths worldwide (Center for Disease Control and Prevention (CDC), 2016). In the year 1817, the cholera outbreak has crossed the Indian border and caused the first out of seven recorded cholera pandemics to date. The estimation of the death toll would reach millions. The pandemic outbreak up to the sixth pandemic was caused by a single lineage of the classical biotype, while the seventh pandemic was caused by the El Tor biotype (Hu, et al., 2016). Cholera poses as a health threat in Malaysia because the outbreaks occur sporadically (Vadivelu, et al., 2000).

Cholera has been a problem worldwide and in Malaysia, it occurs periodically with epidemic cases only. However, the disease occur almost every year with an incidence rate estimate by the Ministry of Health Malaysia is 2.02% and 0.04% mortality rate per a population of 100,000 in 2011. The major mode of transmission of *V. cholerae* in Malaysia is mainly foodborne and several cases had been reported to be linked to foods and drinks sold by street vendor. A study conducted by Anyi, et al. (2011), Serdang, Selangor analysed the prevalence of *Vibrio* species and *V. cholerae* from four types of drinks from street vendor and restaurants. Three of the type of samples which contained milk had a higher prevalence compared to the fruit juices. Knowledge on the rate of outbreak and pattern distribution is needed as well as its prevalence in drinks in order to control the foodborne outbreak of cholera. It is hypothesize that the occurrence of *V. cholerae* in drink samples with milk would be higher than drink samples without milk.

The main method used in the study was most probable number (MPN) method, with polymerase chain reaction (PCR) analysis for rapid analysis of the sample. It is found

that the maximum percentage for both sample type have the MPN value of <3 MPN/g, which was deemed clean to drink. Nevertheless, a few of the samples was found unfit to drink, indicated by the high MPN/g value. The PCR result however, indicates that none of the samples have the *ctx*A gene which confers the bacteria's toxicity. Although no pathogenic *V. cholerae* was found, the level of water quality is not satisfactory based on the estimation of *V. cholerae* concentration in the samples.

The objective of this research is:

1. To isolate and enumerate *V. cholerae* in drink samples from selected street vendor in Kuching.
2. To compare the level of contamination in terms of *V. cholerae* concentration between drinks with milk and drinks without milk.
3. To detect the presence of pathogenic or virulence strain of *V. cholerae* using PCR in drink samples from street vendor by targeting the *ctx*A gene of 308 bp.

2.0 LITERATURE REVIEW

2.1 Characteristic of *Vibrio cholerae*

Vibrios are a group of bacteria belonging to the family *Vibrionaceae*. The family *Vibrionaceae* comprise of seven genera which are *Vibrio, Photobacterium, Allomonas, Listonella, Enhydrobacter, Salinivibrio and Enterovibrio,* in which the genus *Vibrio* contain the most number of species (Nair et al., 2006). *Vibrios* are in the phylum proteobacteria, under the class γ-proteobacteria. Proteobacteria is the major phylum of gram-negative bacteria, with a number of significantly important pathogens. In terms of foodborne infections, *Vibrio cholerae* is the most prominent species within the genus. The bacteria *V. cholerae* is known to be the causative agent of cholera which is linked to various epidemics and pandemics outbreak cases worldwide. *V. cholerae* is a halophilic, non-spore forming, facultative anaerobe, curved rod with the size between 1.5 to 2 μm in length and a width of 0.5 μm. It can undergo both respiratory and fermentative metabolism.

A single polar flagellum and numerous pili covering the bacterial body aid the bacterium as a mode of locomotion. Both the polar flagellum and pili work synergistically, in which the rotation of the flagellum will cause the *vibrio* to counter-rotate along its major axis which will allow the pili to have periodical mechanical contact with the surface to scan the surface before attachment (Utada et al., 2014). *V. cholerae* is abundant in marine, estuarine and freshwater environments. In the environment, it can either exist as a single floating planktatonic cell, or attached in a commensal interaction with zooplankton or shellfish or to other surfaces in the ocean, while others are able to colonize the human gut and cause an infection (Nair et al., 2006). The basic morphology of *V. cholerae* was shown in Figure 2.1

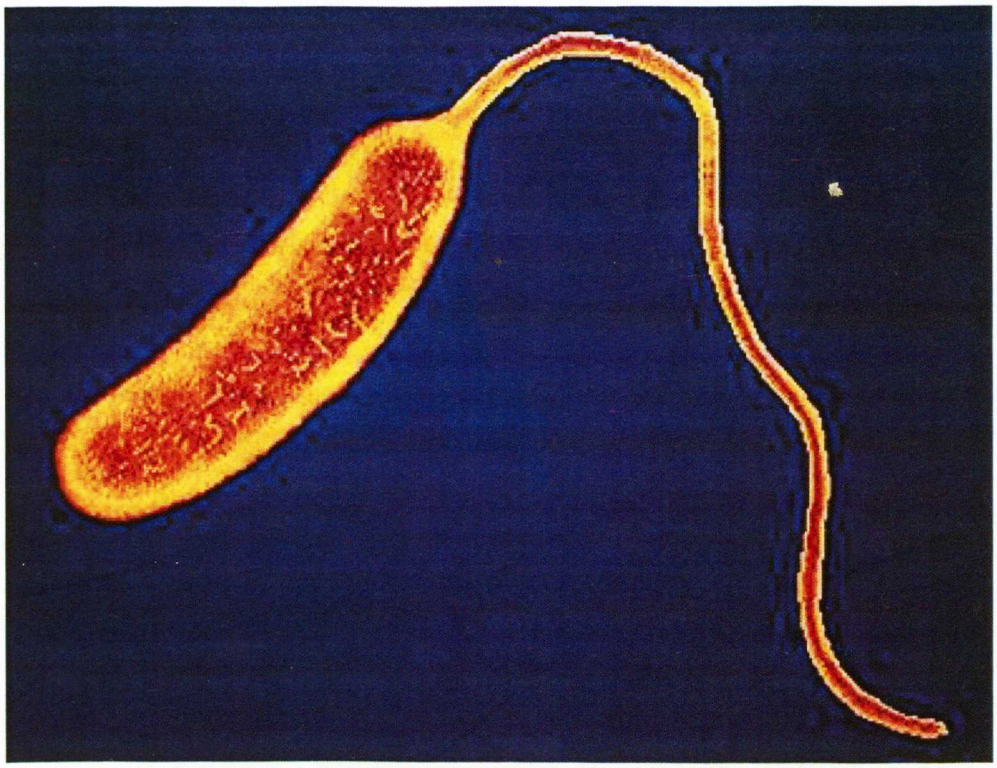

Figure 2.1.1: Morphology of *V. cholera* (Acharya, 2013).

2.2 *Vibrio cholerae* O1 and O139

Altogether, there are more than 200 serogroups based on somatic O antigen of *V. cholerae,* however, only two serogroups are known to cause cholera epidemics which are serogroup O1 and O139. Other serogroups of *V. cholerae* that does not cause epidemics or pandemics are termed as non-O1 and non-O139 serogroups. These serogroups are strains that did not agglutinate with both of the O1 or O139 antisera. The non-O1 and non-O139 serogroups are more commonly isolated from the environment and can only cause periodic cases of a cholera-like disease. Non-O1 and non-O139 serogroup are not able to cause cholera because of the lack of cholera toxin (CT), but is still able to produce a cholera-like disease through the presence of heat-stable enterotoxin (Stn), hemolysin (HlyA), repeat in toxin (RTX) and type III secretion system (TTSS) (Dutta et al., 2013).

The O1 serogroup is identified by the agglutination in the O1 antiserum directed against the liposaccharide of the cell wall and also by the demonstration of enterotoxigenicity (Finkelstein, 1996). It exist as two biotypes which is the classical and the El Tor, which is further classified by antigenic factor into two major serotype, Ogawa and Inaba and a rare and unstable Hikojima. The three serotypes differ in antigen A, B and C expression, at which the Ogawa serotype express the A and B antigens and a small amount of C antigen , the Inaba serotype express the A and C antigens, while the Hikojima serotype express all three antigens (Miller et al., 1972). The O1 serogroup is responsible for all the seventh pandemic, where the first six pandemics were caused by the classical biotype and the seventh was caused by the El Tor biotype. Both of these two major biotypes of the O1 serogroup is differentiated by the presence of HlyA and the acetoin fermentation pathway, which both are characteristic of the El Tor strain (Hu et al., 2016),

In the year 1992, a new non-O1 serogroup was isolated from an epidemic outbreak of cholera in Bangladesh. The strain did not agglutinate with the O1 antiserum and it did not match to any of the previously describe O serogroup. This new serogroup was henceforth called O139 serogroup because of agglutination in O group 139 antiserum. The O139 serogroup also produce the same cholera toxin as the O1 serogroup. In addition, it has the ability to produce a polysaccharide capsule which helps in distinguishing between the two serogroups (Finkelstein, 1996). The evolution of the O139 serogroup was the result of horizontal gene transfer between the O1 and the non-O1 strains. Evidence suggest the O139 serogroup arose from the O1 El Tor biotype by a deletion of the gene responsible for O1 antigen biosynthesis and DNA obtained from another non-pathogenic serogroup (Nair, n.d.).

2.3 Virulence factor

V. cholerae have several virulence factors that contribute to its pathogenesis which allows the colonization of the small intestine and cause cholera. *V. cholerae* have type IV pili (TFP) which allow attachment and biofilm production. *V. cholerae* have three different types of TFP which are mannose-sensitive hemagglutinin (MSHA) pili, virulence-associated toxin co-regulated pili (TCP), and chitin-regulated pili (ChiRP) (Utada et al., 2014). The MSHA pili are present in both O1 El Tor biotype and the O139 serogroup, but are absence in the O1 classical biotype (Chiavelli et al., 2001). The MSHA pili work synergistically with the polar flagellum to scan the surface of the small intestine by having periodic contact with the surface. This help in surface selection and attachment. The TCP are long thin, flexible homopolymers of the toxin co-regulated pili protein A (TcpA) subunit. It associates together into microcolonies to protect the bacteria from the host defence system and also to concentrate the secretion of cholera toxin. TCP are highly specific receptor to the cholera toxin phage (CTXΦ), which can infect non-pathogenic *Vibrio* species and add the genes for CT subunits which causes it to be pathogenic (Li et al., 2008). Another function of TCP is the secretion of soluble colonization factor which enhances the formation of microcolony (Almagro-Moreno et al., 2015).

V. cholerae also produce enterotoxin called CT as a mean to cause cholera. The toxin is a polymeric protein with two domains, the A and B region. The A region controls the biological activity of the toxin, while the B region or choleragenoid binds the toxin to the receptor on the host cell (Finkelstein, 1996). The cholera toxin subunit was shown in Figure 2.3.1. There are two antigenically related but distinct form of CT which is the CT-1 and CT-2. The CT-1 is produced by the O1 classical biotype and also the O1 El Tor biotype isolated from U.S. Gulf Coast, whereas the CT-2 is produced by *V. cholerae* of

other O1 El Tor biotype and the O139 serogroup (Nguyen et al., 2009). The mechanism of action of the cholera toxin starts with the binding of region B to a glycolipid, the G_{M1} ganglioside in the cell membrane and then the A region penetrates the host cell and enzymatically transfer adenosine diphosphate-ribose (ADP-ribose) from nicotinamide adenine dinucleotide (NAD) to guanosine 5'-triphosphate (GTP)-binding regulatory protein and cause the activation of adenylate cyclase which produces an excessive amount of cyclic adenosine 5'-monophosphate (cAMP) (Finkelstein, 1996). This lead to an excessive secretion of chloride, bicarbonate, potassium and water in enterocytes and cause the isotonic voluminous cholera stool (vibrio-cholerae.org, 2013).

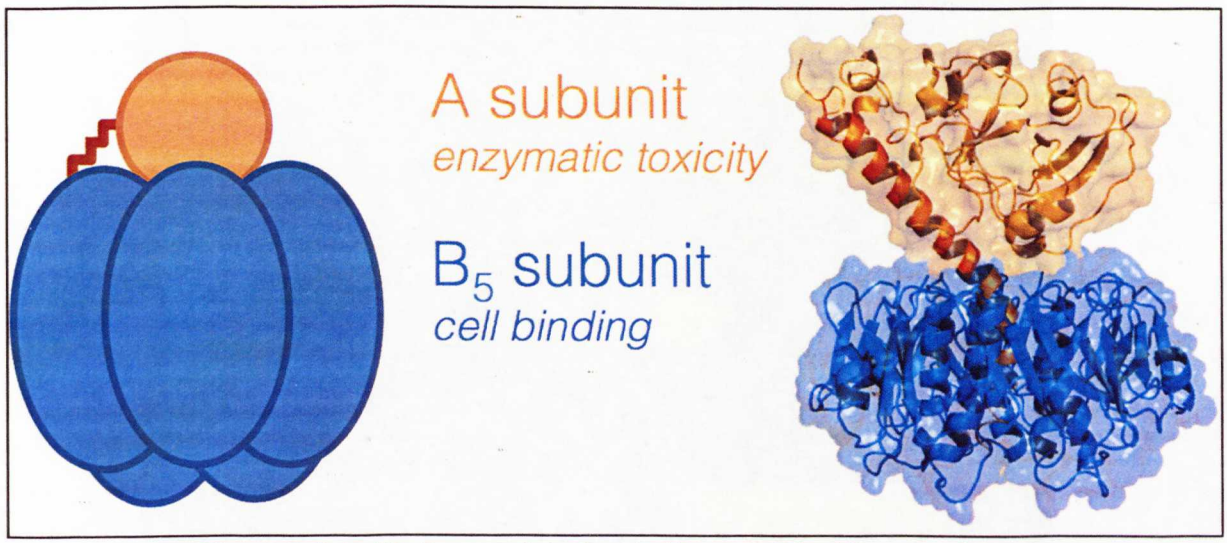

Figure 2.3.1: Cholera toxin subunit (Ryan & Washburn, n.d.).

Another factor that contributes to the pathogenicity of *V. cholerae* is its ability to constantly change its shape. This ability of the bacterium allows it to enter and escape from the mucus lining the intestine, which allows it to invade the human gut more easily. The expression of a shape-shifting protein named CrvA allows the bacteria to twist its shape into a spiral or corkscrew morphology. The CvrA protein accumulates at one side of the bacterium, forming a hard polymer delaying the growth on one side (Bartlett et al., 2017).

The location of the CrvA protein on the bacterium was shown in Figure 2.3.2. While the other side grow at a normal rate thus, resulting in the curved shape of *V. cholerae*. *V. cholerae* uses quoram sensing to alert other bacteria of the change in environment, from water to the intestine, in which the curved shape would be more advantageous. Quoram sensing is a regulation mechanism in gene expression of a bacteria in response to a change in cell population density (Miller & Bassler, 2001).

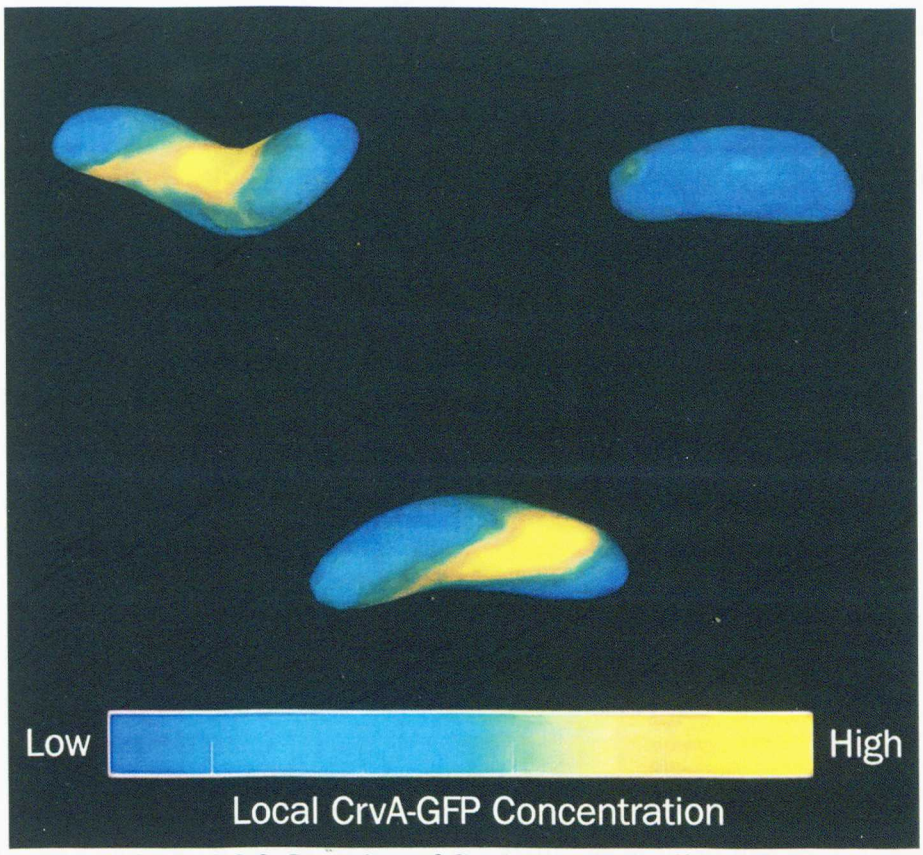

Figure 2.3.2: Location of CrvA protein (Kelly, 2017).

2.4 Disease Caused by *Vibrio cholerae*

The most prominent disease caused by the *Vibrio* species is cholera, caused by the species *V. cholerae*. It follows a range of incubation period from 6 hours to 5 days and causes an acute diarrheal disease that if left untreated will lead to death. The diarrheal

symptom is often accompanied by vomiting. The most severe form of the disease is termed as cholera gravis with rapid dehydration and potentiality of death. One of the sure factor of cholera gravis is the production of CT by *V. cholerae.* It is estimated that 5% of patient infected with *V. cholerae* develop cholera gravis (Handa, 2017). It has a rapid rate of diarrhoea about 500 to 1000 mL/h, leading to tachycardia, hypotension, and vascular collapse due to dehydration (Kaper et al., 1995). This will eventually lead to hypovolemic shock. In rare cases, cholera can also manifest as cholera sicca or also known as dry cholera. This form of cholera causes fluid to be secreted into the intestine but not excreted as stool. Rather it accumulates in the intestine for several hours before being excreted. Cases of cholera usually originate from *V. cholerae* serogroup O1 and O139, which were able to produce the CT. Few of the strains from the two serogroups, however do not produce cholera disease and are not involve in any epidemic cases due to the absence of the CT. Strains isolated from the environment are usually CT negative, which were regarded as non-pathogenic. However, some of the CT negative *V. cholerae* O1 have been isolated from cases of diarrhoea.

2.5 Outbreaks of Cholera

Throughout history, seven recorded pandemic of cholera has occurred worldwide which has caused hundreds of thousands of death. The first six pandemics originated from the Indian subcontinent, the Ganges Delta, reaching other continents and affecting many countries for a prolonged period of time. Before the onset of the first pandemic outbreak in the year 1817, evidence of the disease dates back to the 5th century B.C. and existed on the Indian subcontinent for centuries. The first pandemic originated from the Ganges River during a festival where people all over India gathered at the river and bathed, drank and

defecate in the river. By the second pandemic in the year 1829, the disease manage to reach the British Isles and Canada and by the third pandemic in the year 1852 cholera was widespread in the United States until the end of the fourth pandemic (Faruque et al., 1998). The fifth pandemic affected South America and it was only during the fifth pandemic that the causative agent of cholera was isolated from the stool of an infected person by Robert Koch. The sixth pandemic was able to reach the Middle East. Strains from the fifth pandemic came from the O1 classical biotype and a second pandemic genome sequence from a preserved human intestine grouped the fifth and sixth pandemic strains in a phylogenetic tree, was evident that the first to sixth pandemic were caused by a single lineage of classical biotype (Hu et al., 2016).

The seventh pandemic was different from the others as it originated in Indonesia. The disease was apparent across Asia and the Middle East, and finally making its way to Africa in 1971 and Italy by 1973. In January 1991, the disease re-emerges in Peru, South America after more than a century of silence. The disease spread rapidly and caused an estimation of 750,000 cases and 6,500 death in 16 countries of the Americas from 1991 to 1992 (Faruque et al., 1998). The seventh pandemic was the first pandemic caused by the O1 El Tor biotype. In 1992, a new serotype emerged and cause a major epidemic outbreak in the Indian subcontinent caused by a non-O1 serogroup which was later called the O139 serogroup. It is derived from the El Tor biotype by lateral transfer of genomic island replacing the O1 antigen with O139 antigen (Harris et al., 2012).

During the seventh pandemic that originated from the Sulawesi, Indonesia, the outbreak spread to Borneo, infecting Sabah and Sarawak. By 1963 to 1969, the disease spread to the mainland of Asia, infecting Peninsular Malaysia as well. Malaysia is also affected during the O139 epidemic in the 1990s. Other than these major events in cholera outbreak, the disease only occur as periodic epidemic cases only and are often associated

with endemic El Tor biotype (Ang et al., 2010). In November 2009, an outbreak of cholera occurred in both Terengganu and Kelantan. The outbreak in Terengganu was caused by two variants of the El Tor biotype. Whereas, in Kelantan the outbreak was caused by the Ogawa serogroup of the El Tor biotype and the sequencing of the cholera toxin gene (*ctx*) B gene showed that the outbreak strain confer a classical cholera toxin which belong to a new variant of the El Tor biotype (Bharati & Bhattacharya, 2014). The major mode of transmission of *V. cholerae* in Malaysia is through drinking water, cooked food and raw or undercooked seafood (Bilung et al., 2014). The number of reported cases of cholera worldwide was shown in Figure 2.5.1.

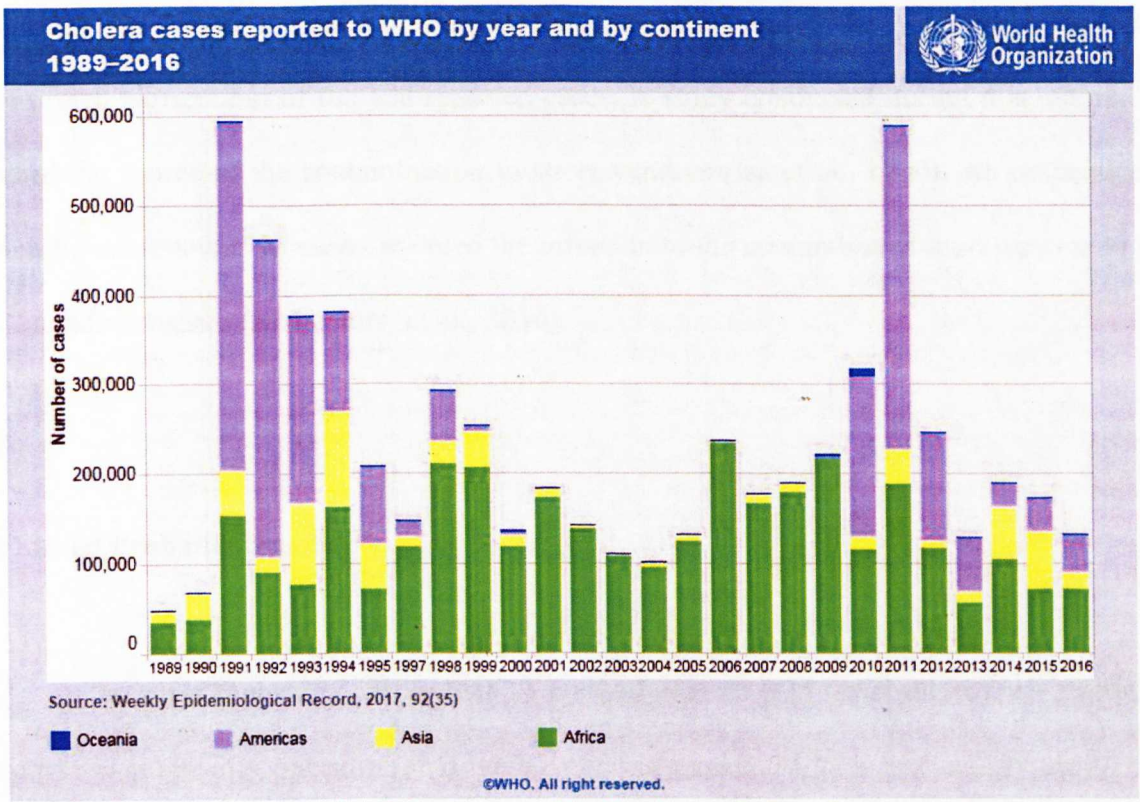

Figure 2.5.1: Outbreak of cholera worldwide from 1989 to 2016 (World Health Organization, 2018).

2.6 Outbreak of Cholera from Street Vendor

Cholera occur when a person ingest food or water contaminated with faecal matter from an individual contaminated with *V. cholerae.* The disease is more prevalent in undeveloped or developing countries, due to inadequate water treatment. For developed countries with safe water sources and proper sewage disposal, the source of cholera might be through contaminated food and beverages sold by street vendor. A study conducted on the cholera outbreak in Guatemala, 1993 revealed that a significant number of cholera cases were related to consumption of food and beverages from street vendor (Koo et al., 1996). There were 234 confirmed cases of cholera with 17 deaths reported during this outbreak. In Malaysia, an outbreak in Tumpat, Kelantan in the year 1990, had 85 confirmed carriers out of the 108 reported cases. A study conducted during this outbreak, traced the source of the contamination to street vendors (Isa et al., 1990). An outbreak in Bintulu had reported 33 cases, sourced the infection to the contaminated food water sold at a Ramadhan bazaar (Mahapatra et al., 2014).

2.7 Most Probable Number

The most probable number (MPN) method uses 10-fold serial dilution of replicate broth to estimate the concentration of viable microorganism. MPN is particularly useful for samples with particulate material that will interfere with plate count enumeration methods. This method assumes that the preparation of the sample will produce bacteria that have a random distribution, separated and does not repel one another, and the growth medium and the incubation conditions will produce a detectable growth for an inoculum that contains at