CONSTRUCTION AND EVALUATION OF MULTIGENE MUTANTS OF *VIBRIO CHOLERAE* O139 AS VACCINE CANDIDATES

by

CHANDRIKA MURUGAIAH

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DEDICATIONS

This thesis is dedicated to my beloved parents

This thesis is also dedicated to all those who believe

Science is simply common sense at its best

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LIST OF SYMBOLS AND ABBREVIATIONS

-	nil
%	percent
~	approximately
+	positive
+ve	positive
−	negative/ minus
-ve	negative
<	less than
>	more than
±	plus / minus
→	to
Δ	point / frame shift / deletion mutation
::	mutation by insertion
°C	degree Celsius
µg	microgram
µl	microliter
µM	micromolar
X	times
A	Absorbance
A_{600}	Absorbance at 600 nm wavelength
AB	A and B subunits
$A^{-} B^{+}$	negative A and positive B subunits
$A^{-}B^{-}$	negative A and B subunits
ace	accessory cholera enterotoxin
ADP	adenosine diphosphate
ALA	δ-aminolevulinic acid
Amp	ampicillin
APW	alkaline peptone water
ATP	adenosine 5’-triphosphate
AWD	acute watery diarrhoea
BC	before Christ
bp	basepair
BSA	bovine serum albumin
cAMP	adenosine 3’,5’-cyclic monophosphate
CDC	Centers for Disease Control and Prevention
CFU	colony forming unit
*ctx*A	cholera toxin A subunit
Cl^{-}	chloride ion
cm	centimeter
CT	cholera toxin
CT A1	cholera toxin A1 subunit
CT-2*	two-codon mutant of CT
CTX^{-}	negative cholera toxin
CTXφ	filamentous bacteriophage
DAB	diaminobenzidine
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ENSO	El Niño Southern Oscillation
EtOH	ethanol
g	gram
GalA	galacturonic acid
GM1	monosialotetrahexosylganglioside
GMT	Geometric Mean Titer

H&E	haematoxylin-and-eosin
H_2O	water
H_2O_2	hydrogen peroxide
hap	haemagglutinin/protease
HCl	hydrogen chloride
HCO_3^-	Bicarbonate ion
HEp2	human larynx carcinoma cell line
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
*Hly*A	hemolysin
HMDS	hexamethlydisilazane
HRP	horseradish peroxidase
HUSM	Hospital Universiti Sains Malaysia
i.e.	*id est*
IgA	immunoglobulin A
IgG	immunoglobulin G
IHC	immunohistochemistry
K^+	potassium ion
Kan	kanamycin
KCl	potassium chloride
kDa	kilodalton
KRT	Kreb's Ringer Tris
LB	Luria Bertani
LPS	lipopolysaccharides
M	molar / molarity
$MARTX_{Vc}$	multifunctional autoprocessing RTX toxin
Mbar	millibar
MCS	multiple cloning site
MDCK-I	Mardin-Darby canine kidney epithelial cell line I
mg	milligram
$MgCl_2$	magnesium chloride
min	minute
ml	milliliter
mm	millimeter
mM	millimolar
n	sample size
n/s	not seen
NS	normal saline
Na^+	sodium ion
NaOH	sodium hydroxide
O_2	oxygen
OD	optical density
OD_{600}	optical density at 600 nm wavelength
OH	hydroxyl group
*omp*U	outer membrane protein
ORFs	open reading frames
PBS	phosphate buffered saline
PBST	phosphate buffered saline-Tween 20
PCR	polymerase chain reaction
pmol	picomole
Poly	polymyxin B
QuiNAc	N-acetylquinovosamine
RBCs	red blood cells
RITARD	Removable Intestinal Tie Adult Rabbit Diarrhoea
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute
RT	room temperature
rtx	repeats-in-toxin
SPSS	Statistical Package for the Social Sciences
SD	standard deviation
SDS	sodium dodecyl sulphate

Ta	annealing temperature
TBE	Tris-borate-EDTA
TCBS	thiosulphate citrate bilesalt sucrose
TCP^{-}	negative toxin coregulated pilus
tcp	toxin coregulated pili
TE	Tris-EDTA
TIGR	The Institute for Genomic Research
TISS	Type I Secretion System
Tm	melting temperature
*tox*R	transmembrane transcriptional activator protein
Tris	tris(hydroxymethyl)aminomethane
U	units
UV	ultraviolet
V	volts
V. cholerae	*Vibrio cholerae*
v/v	volume/volume
VBNC	viable but non-culturable
w/v	weight/volume
WC	whole cells
WHO	World Health Organization
WT	Wild Type
www	world wide web
x *g*	relative centrifugal force
Xylhex	3,6-dideoxyxylohexose
zot	zonula occludens toxin

PEMBANGUNAN DAN PENILAIAN PELBAGAI GEN MUTAN *VIBRIO CHOLERAE* O139 SEBAGAI CALON VAKSIN

ABSTRAK

Kolera merupakan isu kesihatan yang memberi kesan ke atas jutaan nyawa setiap tahun. Berdasarkan wabak penyakit kolera yang sentiasa berulang, penghasilan vaksin yang membolehkan pengvaksinan dilakukan secara meluas adalah amat perlu. Dalam kajian ini, calon vaksin yang diubahsuai secara genetik, VCUSM21P dan VCUSM22P telah dihasilkan. VCUSM21P merupakan vaksin prototrof yang mengkodkan 'cholera toxin A' (*ctx*A) tidak bertoksik dan mempunyai mutasi pada gen-gen 'accessory cholera enterotoxin (*ace*)', 'zonula occludens toxin (*zot*)' dan 'repeats-in-toxin C/A (*rtx*C/A)'. Manakala calon vaksin VCUSM22P mempunyai mutasi pada *ace*, *zot*, *ctx*A, *rtx*C/A dan 'haemagglutinin/protease (*hap*)'. Kedua-dua mutan ini didapati tidak menjejaskan penyusunan aktin pada sel-sel HEp2. Kajian kolonisasi dalam usus mencit ('infant mouse') digunakan bagi menentukan kebolehan VCUSM21P dan VCUSM22P mengkoloni secara *in vivo*. Kajian usus arnab ('rabbit ileal loop') dilakukan untuk menilai reaktogenisiti yang disebabkan oleh kedua-dua strain mutan tersebut. Gerak balas imunisasi dijana oleh mutan serta kerintangan terhadap kolera telah dinilai melalui model arnab. Kedua-dua calon mutan didapati mengkoloni usus mencit dengan baik. Dalam kajian model usus menggunakan arnab yang tidak diimunisasi, cecair didapati berkumpul dalam gelungan usus yang disuntik dengan 'Wild Type' (WT) *V. cholerae* pada konsentrasi $1x10^6$ and $1x10^8$ unit formasi koloni 'colony forming unit (CFU)'. Sebaliknya, gelungan usus yang disuntik dengan kedua-dua strain mutan pada konsentrasi yang sama didapati tidak menyebabkan sebarang reaktogenisiti pada arnab yang tidak imun. Imunisasi menggunakan strain kedua-dua mutan pada konsentrasi $1x10^{10}$ CFU telah meningkatkan penghasilan antibodi IgA dan IgG terhadap toksin kolera dan lipopolisakarida (LPS) O139 serta meningkatkan antibodi 'vibriocidal'. Reaktogenisiti yang disebabkan oleh WT *V. cholerae* pada arnab yang telah diimun dengan $1x10^{10}$ CFU strain-strain mutan

didapati berkurangan berdasarkan ketiadaan cecair terkumpul dalam gelung usus arnab yang disuntik dengan WT *V. cholerae* pada konsentrasi $1x10^2$ - $1x10^7$ CFU. Kajian cirit-birit pada model usus arnab dewasa 'Removable Intestinal Tie Adult Rabbit Diarrhoea (RITARD)' mendapati arnab yang tidak diimun tidak dilingungi terhadap infeksi WT *V. cholerae* pada dos $1x10^9$ CFU. Walaubagaimanapun, 100% arnab yang telah diimun didapati rintang terhadap WT *V. cholerae*. Pemeriksaan secara imunohistokimia, histopatologi dan ultrastruktur pada usus arnab yang tidak diimun dan telah diinfeksi dengan WT *V. cholerae* menunjukkan kerosakan yang teruk berlaku pada usus kecil. Sebaliknya, tiada kerosakan berlaku pada bahagian tersebut apabila arnab yang telah diimun diuji dengan WT *V. cholerae*. Strain VCUSM21P dan VCUSM22P boleh digunakan dalam vaksinasi terhadap kolera yang disebabkan oleh *V. cholerae* O139.

CONSTRUCTION AND EVALUATION OF MULTIGENE MUTANTS OF *VIBRIO CHOLERAE* O139 AS VACCINE CANDIDATES

ABSTRACT

Cholera is a major health issue, affecting millions of lives annually. In light of the recurrent outbreaks of cholera, there is a pressing need for the development of vaccines that allow rapid mass vaccination. In this study, genetically modified vaccine candidates, VCUSM21P and VCUSM22P, were designed. VCUSM21P is a prototrophic vaccine which encodes non-toxic cholera toxin A (*ctx*A) subunit immunogen and has accessory cholera enterotoxin (*ace*), zonula occludens toxin (*zot*) and repeats-in-toxin C/A (*rtx*C/A) mutations. On the other hand, VCUSM22P is *ace*, *zot*, *ctx*A, *rtx*C/A and haemagglutinin/protease (*hap*) mutant. Both mutants were found not to disassemble the actin of HEp2 cells. Mouse colonization assay was used to determine VCUSM21P and VCUSM22P colonization ability *in vivo*. Rabbit ileal loop assay was performed to evaluate the reactogenicity caused by them. The immune responses provoked by the two vaccine candidates and their protective function against cholera were evaluated in a rabbit model. The mutants were found to be good colonizer of the mouse intestine. In the ileal loop assay using non-immunized rabbits, fluid accumulation was found in loops injected with $1x10^6$ and $1x10^8$ colony forming unit (CFU) of Wild Type (WT) *V. cholerae*. Unlike the WT *V. cholerae* challenge, $1x10^6$ and $1x10^8$ colony forming unit (CFU) of the mutants did not cause any reactogenicity in non-immunized rabbits. Immunization using $1x10^{10}$ CFU of the mutants induced both IgA and IgG antibodies production against cholera toxin (CT) and O139 lipopolysaccharides (LPS), as well as elevated vibriocidal antibody. The reactogenicity caused by the WT *V. cholerae* in rabbits immunized with $1x10^{10}$ CFU of the mutants was found to be reduced as evidenced by absence of fluid in loops administered with $1x10^2$-$1x10^7$ CFU of WT *V. cholerae*. In the Removable Intestinal Tie Adult Rabbit Diarrhoea (RITARD) experiment, the non-

immunized rabbits were found unprotected against a lethal challenge with $1x10^9$ CFU WT *V. cholerae*. However, 100% of rabbits immunized with the mutants survived the WT *V. cholerae* challenge. Immunohistochemical, histopathological and ultrastructural examination of non-immunized rabbits' ileum challenged with WT *V. cholerae* revealed severe ileal damages. But less severe damages were noted following the WT *V. cholerae* challenge in the ileum of rabbits immunized with VCUSM21P and VCUSM22P. The multigene mutants could be used for vaccination against potentially fatal *V. cholerae* O139.

CHAPTER 1 – INTRODUCTION

1.1 *Vibrio cholerae* and cholera

Cholera is the most feared disease in history (Seas and Gotuzzo, 1996). The hallmark of cholera is a profuse secretory diarrhoea, with an output rate of as much as 1 liter per hour (Phillips, 1966). Thus, cholera is known as an acute secretory diarrhoeal disease. This highly liquid diarrhoea, colloquially referred to as “rice-water stool” as it looks like water with flecks of rice in it, is loaded with bacteria and has a "fishy" odor (Gorbach *et al.*, 1970; Kaper *et al.*, 1995).

Vibrio cholerae (also referred to as *Kommabacillus*) is a non-invasive Gram-negative comma shaped bacteria with a single polar flagellum (Heidelberg *et al.*, 2000). It belongs to the γ subdivision of the family Proteobacteriaceae. The *V. cholerae* species encompasses more than 200 serogroups (Chatterjee and Chaudhuri, 2003). Among these serogroups, only two toxigenic strains of *V. cholerae* O1 and O139 were identified to cause cholera and are associated with epidemic and pandemic forms of the disease (Chatterjee and Chaudhuri, 2003). The principal virulence factor of *V. cholerae* is cholera toxin (CT) (Kaper and Srivastava, 1992; Kaper *et al.*, 1995).

Cholera can influence negatively to a country’s economy (Seas and Gotuzzo, 1996; Cash and Narasimhan, 2000; Kirigia *et al.*, 2009) and is one of those diseases that can brand a country as backwards (Dajer, 1992). The disease is perceived to be a highly contagious threat that can spread through international trade in food (WHO, 1993).

1.2 Prevalence of cholera

According to the World Health Organization (WHO), the intestinal disease cholera poses serious health risks in developing countries, notably in the most underprivileged regions of the world (WHO, 2005). Sporadic outbreaks and epidemics occur when large populations are displaced following natural disasters or political crisis and conflict in war zones, where there is disturbed living conditions, no clean water or adequate sewage disposal

(Siddique *et al.*, 1989; Gabastou *et al.*, 2002; Chaignat *et al.*, 2008; Ekra *et al.*, 2009; Ganin, 2009; Bhunia and Ghosh, 2011). Cholera had a tragic impact on the personal as well as social life of people living in endemic areas (Ghose, 2011).

The true burden of cholera disease is grossly underestimated as more than 90% of the cases went unreported to WHO (Sanchez and Holmgren, 2005). Every year, more than 100,000 cholera cases and 2,000-3,000 deaths are officially reported to WHO (Fournier and Quilici, 2007). In 2006 alone, 52 countries officially reported a total of 236,896 cholera cases including 6,311 deaths to the WHO (WHO, 2007). Table 1.1 shows the global update reports on a number of verified cholera outbreaks in 2009. Cholera is endemic in the East Africa corridor, large estuarine deltas in Asia (Ganges, Mekong) and countries in Northwest Africa (Lopez *et al.*, 2008). Imported cases of cholera are reported sporadically around the world from travellers to endemic countries (WHO, 1995; Steffen *et al.*, 2003; Tarantola *et al.*, 2005; Ajzenman *et al.*, 2006).

According to WHO, despite efforts taken at country level there has been no concrete global improvement to limit transmission rate (Chaignat and Monti, 2007). This disease has been rare in industrialized nations but remains as a major public health problem in the developing countries, particularly in low-income countries (Kaper *et al.*, 1995; Chaignat and Monti, 2007; Lopez *et al.*, 2008; Talavera and Perez, 2009). In endemic areas, children are affected the most (Deen *et al.*, 2008). Notification of cholera is compulsory, yet countries are reluctant to report cases or seek support from WHO due to fear of possible commercial sanction (Chaignat and Monti, 2007; WHO, 2007). Because of the lack of data on trends and patterns of cholera outbreaks, predicting potential outbreaks could not be possibly achieved (Chaignat and Monti, 2007).

Table 1.1: Global update reports on a number of verified cholera outbreaks in 2009*

Country		Report	References
Africa	Angola	Since January 2009, 1,250 cases with 35 deaths have been reported countrywide. As of the week of 12 July 2009 15 new cases, with no deaths had been reported from the provinces of Bengo, Benguela, Huila, and Malange.	(NaTHNaC, 2009)
	DR Congo	As of 20 July 2009, 4,326 cases with 63 d eaths have been reported in South Kivu, and 2,342 cases with 27 deaths in North Kivu.	(NaTHNaC, 2009)
	Kenya	As of 20 July 2009, 4,269 suspected cases with 94 deaths have been reported from 32 districts countrywide.	(NaTHNaC, 2009)
	Malawi	Between 15 November 2008 and 20 July 2009, 5,269 cases were reported. The most recent reports of new cases were during the period of 18 to 24 May, with 17 cases reported from Machinga district.	(NaTHNaC, 2009)
	Mozambique	As of 16 May 2009, 17,761 cases with 140 deaths were reported from 54 districts.	(NaTHNaC, 2009)
	South Africa	As of 1 June 2009, 12,752 cases with 65 deaths have been reported. Cases were from all 9 provinces with most cases from Limpopo (5,520), Mpumalanga (6,855), and Gauteng (286).	(NaTHNaC, 2009)
	Zambia	Between 10 September 2008 and 7 May 2009, the cumulative number of cases reported was 8,219 with 173 deaths.	(NaTHNaC, 2009)

* This is not a comprehensive listing

Table 1.1: Continued

Country		Report	References
Africa	Zimbabwe	The cholera outbreak that started in August 2008 continues to diminish. Since 13 June 2009, only one case has been reported (UMP district), bringing the cumulative number of cases to 98,592 with 4,288 deaths.	(NaTHNaC, 2009)
Europe	Swaziland	Between December 2008 and 1 June 2009, 17,448 cases of Acute Watery Diarrhoea (AWD) were reported. Two of the AWD cases have been confirmed as cholera.	(NaTHNaC, 2009)
South Asia	Nepal	As of 30 July 2009, there have been 27,456 cases of diarrhoeal illness in the remote Jajarkot district, with 197 deaths. The infection has spread to adjoining districts including Rukum, Salyan, Surkhet, Rolpa, Dailekh, and Dang (mid-western region), and to Bajura and Dadeldhura (far-west region). This outbreak of diarrhoea is being treated as cholera following the isolation of *V. cholerae* in some samples examined by the National Public Health Laboratory of Nepal.	(NaTHNaC, 2009)
South East Asia	Malaysia	In Nov 2009, a total of 98 people from state of Terengganu were suspected of having cholera after showing symptoms like vomiting and acute diarrhoea, with 11 patients being tested positive. 187 cases were reported since the outbreak started on Nov 11.	(BERNAMA, 2009; thestar, 2010)
Europe	Swaziland	Between December 2008 and 1 June 2009, 17,448 cases of Acute Watery Diarrhoea (AWD) were reported. Two of the AWD cases have been confirmed as cholera.	(NaTHNaC, 2009)

* This is not a comprehensive listing

1.3 Discovery and Historical Background of *V. cholerae*

In 1854, the Italian physician Filippo Pacini (1812 to 1883), professor of anatomy at the University of Florence, first discovered *V. cholerae* (Bentivoglio and Pacini, 1995). Records of a cholera-like disease have already been mentioned in ancient Sanskrit writings on the Indian subcontinent roughly 2,500 years ago (Colwell, 1996) and at the times of Hippocrates, 460 to 377 BC (Thompson *et al.*, 2004). Before Pacini's discovery, scientists and physicians widely believed in the miasmatic theory of disease, a theory of disease causation by miasma (ancient Greek: "pollution"), a noxious form of "bad air" or pestilential airs (Thompson *et al.*, 2004). Pacini pointed out that cholera was a contagious disease caused by *V. cholerae*, a conclusion he drew after examining the intestinal mucosa of victims of cholera (Bentivoglio and Pacini, 1995).

In the same period, John Snow (1813 to 1858), a doctor known as the father of epidemiology, studied cholera (Hare, 1955). Snow studied the epidemiology of cholera in several cities of England (Cameron and Jones, 1983). Between the 1830s and 1850s, and during the London cholera epidemic of 1854, cholera had killed tens of thousands of people in England (Hare, 1955). According to Snow, cholera was not passed by bad air (Thompson *et al.*, 2004). He felt that the outbreak that occurred in the Soho District of London and in and around Broad Street was due to contaminated water. Looking in his microscope, Snow found the water from a water pump located on Broad Street contained bacteria which he had not seen before and concluded them as "morbid poison entering the alimentary canal" (Thompson *et al.*, 2004). Snow's theory worked when the epidemic began to subside a short time after the neighbourhood's source of contaminated water was blocked by removing the pump handle (Bentivoglio and Pacini, 1995; Buechner *et al.*, 2004).

Nearly 30 years later, Robert Koch characterized *V. cholerae* as comma shaped, highly motile and concluded that it was indeed the causative agent of cholera (Thompson *et al.*, 2004). He realized that many *V. cholerae* strains were non-pathogenic for humans. Koch found that these vibrios were ubiquitous in aquatic settings and proposed that filtration of

drinking water should be incorporated to remove the bacteria from water supply systems (Thompson *et al.*, 2004).

1.4 Classification

The primary basis of classification of strains of *V. cholerae* is a serotyping scheme, which depends on the properties of somatic (O) antigen. *V. cholerae* O1 is classified into two biotypes, namely 'classical' and 'El Tor'. Each biotype is further sub-classified into three serotypes, called Inaba, Ogawa and Hikojima (is very rare) based on agglutination in antiserum. The antigenic determinants of Ogawa are type A and B antigens, determinants of Inaba are type A and C antigens, and A, B and C antigens for Hikojima. Strains other than O1 are called non-O1 (Nair *et al.*, 1994b).

1.5 Cholera Pandemic

Since 1817, seven cholera pandemics have spread to the world (Faruque *et al.*, 1998; Boutin *et al.*, 2001). It is believed that the first six of these pandemics were caused by the ancient infectious classical biotype originated from the Gangetic delta of Bengal (Sanyal, 2000). The dominant cause of cholera epidemic by the classical biotype until 1961 were replaced by El Tor strains (Faruque *et al.*, 1998; Sack *et al.*, 2004). The seventh was caused by the El Tor biotype (Faruque *et al.*, 1998). The seventh pandemic started from the Sulawesi Island and is still ongoing (Kaper *et al.*, 1995; Faruque *et al.*, 1998). *V. cholerae* serotype O1 El Tor N16961 strain harbouring the classical CTX prophage (CTXφ) had caused the most cholera pandemic (Faruque *et al.*, 1998).

In late 1992, *V. cholerae* serogroup O139 appeared in India (Albert *et al.*, 1993; Ramamurthy *et al.*, 1993). It spread rapidly to Bangladesh and other Asian countries, thus appears to be confined to Asia (Sack *et al.*, 1996; WHO, 2008). *V. cholerae* O139 is believed to be derived from and closely related to O1 El Tor strain (Berche *et al.*, 1994; Jiang *et al.*, 2000). How O139 serogroup established successful pandemics and overtook its predecessor remains a mystery (MMWR, 1993; Nair *et al.*, 1994a; Siddique *et al.*, 1996). Uniquely, *V. cholerae* serogroup O139 does have a polysaccharide capsule which is not found in its

predecessor *V. cholerae* O1 strain. The capsule is made up of N-acetylglucosamine, N-acetylquinovosamine (QuiNAc), galacturonic acid (GalA), galactose, and two residues of 3,6-dideoxyxylohexose (Xylhex) (Preston *et al.*, 1995).

1.6 Epidemiology of Cholera

The burden of cholera is predominantly borne by the developing countries of Africa and Asia (Miller *et al.*, 1985; Griffith *et al.*, 2006). Usually, *V. cholerae* is transmitted through the faeces of an infected person, due to poor sanitation practice and inadequate sanitary facilities (Misch, 1991; Seas and Gotuzzo, 1996). Highly contagious form of the disease happens in densely populated and economically reduced areas (Oeding, 1990; Misch, 1991; Dajer, 1992; Falade and Lawoyin, 1999). Foodborne transmission of cholera has been well documented by epidemiologic investigations in nearly every continent (Estrada-Garcia and Mintz, 1996), mainly due to consuming raw or partially cooked seafood such as fish, shellfish, crabs, oysters and clams (Dutt *et al.*, 1971; Goh and Lam, 1981; Desenclos, 1996; Eberhart-Phillips *et al.*, 1996; Rabbani and Greenough, 1999; Morris, 2003).

An individual's susceptibility to cholera is affected by one's blood group (Glass *et al.*, 1985; Anstee, 2010). Individuals with group AB are the most resistant to cholera disease and group A being more resistant than group B. Individuals with the most severe diarrhoea compared with those with asymptomatic infection were more often of blood group O and less often of AB, indicating that those with group O blood are the most susceptible (Glass *et al.*, 1985). In individuals with a weakened immune system or decreased gastric acidity or those who are malnourished, increased susceptibility to disease may be observed.

Worldwide there has been an increase in the number of cholera cases and outbreaks with changing profiles (WHO, 2007). Cholera cases are being reported from novel communities or in communities where the disease has been absent for many years (WHO, 2007). This has signified that the geographical distribution of cholera is changing (WHO, 2007), The reason for the changes is not known but has been associated with the weather conditions that were favourable for transmission.

Cholera is a climate-related infectious disease (Islam *et al.*, 2009; Paz, 2009; Hashizume *et al.*, 2010). Epidemiologic research suggests that the changes in the environment or climate and the El Nino phenomenon has made conditions favourable for cholera worldwide (Lipp *et al.*, 2002; Constantin de Magny and Colwell, 2009). The term El Niño is used to refer to the periods of strong and prolonged warm weather. It influences the climate worldwide and brings natural disasters such as storms, floods and droughts and famine in far-flung parts of the world. The periods of the warm waters in eastern Pacific (El Niño) and periods of cooler waters (La Niña) are accompanied by changes of air pressure in the east and west Pacific. The whole cycle is now referred to as El Niño Southern Oscillation (ENSO) (WHO, 1998). Cholera dynamics is influenced by climate, and in endemic regions cholera display regular seasonal cycles and pronounced interannual variability (Pascual *et al.*, 2002).

Few evidences are now showing that in an epidemic, cholera outbreaks are connected to climate change and immunity level in a population (Koelle *et al.*, 2005; King *et al.*, 2008). Koelle and colleagues presented a study based on climate force and temporary immunity to explain the role of climate variability in interannual disease cycles present in a four-decade cholera time series from Matlab, Bangladesh (Koelle, 2009). They used a nonlinear population model that takes into account immunity and disease transmission to show a strong correspondence between cholera transmission and climate variability, expanding the view of the pivotal climatological and immunological influences in cholera transmission. By analyzing the temporal variability of the predominant strain, El Tor, the authors showed that cholera transmission was promoted when the host population had low levels of naturally acquired immunity to reinfection with cholera of the El Tor biotype, especially when they were not in a refractory period from previous disease outbreaks. They also discovered that high transmission rates fell most heavily when both floods and droughts occurred.

1.7 Genome structure

The genome of *V. cholerae* El Tor N16961 strain was mapped by the team at The Institute for Genomic Research (TIGR) in Rockville, Maryland, which completed the sequencing using the shotgun procedure (Heidelberg *et al.*, 2000). *V. cholerae* chromosome consists of two unique and circular replicons. The larger first chromosome, chromosome 1, has 2,961,149 base pairs and chromosome 2 has 1,072,315 base pairs. Together the chromosomes encode 3,885 open reading frames (ORFs), the first has 2,770 ORFs and second has 1,115 ORFs (Heidelberg *et al.*, 2000).

Unlike the smaller second chromosome, chromosome 1 contains the crucial genes for toxicity (for example, toxins, surface antigens and adhesins), regulation of toxicity and important cellular functions, such as DNA replication, transcription, translation and cell-wall biosynthesis. The genes required to produce cholera toxin (CT) are located on it (Heidelberg *et al.*, 2000).

Chromosome 2 contains many more genes that appear to have origins other than the gamma-Proteobacteria. The second chromosome may have originally been a megaplasmid that was captured by an ancestral Vibrio species. This chromosome gives the organism a more competitive advantage in diverse environments. The authors suggest that gene exchange between chromosome 1 and 2 occurred over evolutionary time and both chromosomes became crucial for the organism to survive (Heidelberg *et al.*, 2000).

1.8 Horizontal gene transfer

The ability of the O1 and O139 biotypes to produce CT appears to be the result of horizontal gene transfer. The DNA that codes for CT is thought to be the genome of a filamentous bacteriophage (CTXφ) that lysogenizes *V. cholerae* (Lipp *et al.*, 2002). The CTXφ genome also includes genes required for virion assembly and secretion, replication (*rst*A), regulation of phage gene expression (*rst*R) and phage integration (*rst*B) (Davis and Waldor, 2003). It is unclear what role *rst*B plays in CTXφ integration (Sarah and Matthew, 2004). The CTXφ genome also contains two intergenic regions, ig-1 and ig-2. The former,

lies between *ctx*B and *rst*R, contains the phage origin of replication and the sequences required for chromosomal integration. The latter contains the transcriptional regulatory regions for *rst*A and *rst*R (Kimsey and Waldor, 2004).

CTXφ integrates site-specifically into the larger of the two *V. cholerae* chromosomes (Pearson *et al.*, 1993; Huber and Waldor, 2002), converting non-pathogenic isolates of *V. cholerae* to toxigenic strain (Waldor and Mekalanos, 1996). Its integration depends on the chromosome-encoded tyrosine recombinases XerC and XerD of *V. cholerae*, as its genome lacks an integrase. During integration, recombination occurs between regions of homology in CTXφ genome (*attP*) and the *V. cholerae* chromosome (*attB*) (Sarah and Matthew, 2004).

1.9 Ecology

Water connects humans with *V. cholerae* (Reidl and Klose, 2002). *V. cholerae* thrives in water ecology and finds favourable conditions in waters characterized by moderate salinity, high nutrient content, warm temperature, neutral or slightly alkaline pH (Borroto, 1997). They are highly abundant in aquatic environments and are virtually always detected in tropical environments (Shears, 1994). They are found in estuaries, marine coastal waters and sediments, and aquaculture settings worldwide (Khan *et al.*, 1984; Borroto, 1997; Faruque *et al.*, 1998; Hervio-Heath *et al.*, 2002; Vezzulli *et al.*, 2009). They are a commensal of abalones, bivalves, copepods, corals, fish, molluscs, seagrass, sponges, shrimp, seaweed and zooplankton (Borroto, 1997; Faruque *et al.*, 1998; Gallardo *et al.*, 2004; Thompson *et al.*, 2004; Gopal *et al.*, 2005; Aguirre-Macedo *et al.*, 2008). Cholera outbreaks are linked with algae and planktonic blooms and the sea surface temperature (Epstein, 1993; Lobitz *et al.*, 2000). It is thought that seasonal outbreaks are triggered by seasonal blooming of aquatic planktons (Epstein, 1993). This is why in endemic regions, such as South Asia, cholera is seasonal. Thus, potential outbreaks may be predicted by monitoring these parameters by remote sensing (Lobitz *et al.*, 2000).

The bacterium enters a quiescent state in water ecology known as viable but non-culturable (VBNC) state, where it is alive but fails to multiply unless triggered by specific

environmental conditions (Colwell and Huq, 1994). They are identified as VBNC form of *V. cholerae* in a wide range of marine life, including cyanobacteria (*Anabaena variabilis*), diatoms (*Skeletonema costatum*), phaeophytes (*Ascophyllum nodosum*), in copepod molts, and in freshwater vascular aquatic plants (water hyacinths and duckweed) (Epstein, 1993).

In nature, *V. cholerae* grows as matrix-enclosed surface-associated communities known as biofilms that are critical for the environmental survival and transmission of *V. cholerae* (Thompson *et al.*, 2004). In biofilms, *V. cholerae* can resist antibiotics, and establish favourable partnerships with other bacteria or hosts. Biofilms also help them in trapping and absorbing nutrients. Biofilm formation is linked to the predominant cause of cholera as the strong ability of *V. cholerae* to form densely packed biofilms in the environment gives a survival advantage to this organism (Thompson *et al.*, 2004).

1.10 Antibiotic Resistance

The Centers for Disease Control and Prevention (CDC) has pointed out an increase in the incidence of antibiotic resistant strains of *V. cholerae*, resulting in further challenges for the antimicrobial therapy of cholera (Mahon *et al.*, 1996). Some cholera outbreaks show sporadic cases of antibiotic resistance (Kitaoka *et al*., 2011; Taneja *et al*., 2011). *V. cholerae* resistance to antimicrobials is due to the injudicious use of antimicrobials. Therefore, changes in current methods of prevention and treatment using antimicrobials should be considered (Weber *et al.*, 1994; Ghosh and Ramamurthy, 2011).

1.11 Pathophysiology of cholera

After oral ingestion of contaminated food or water, the comma shaped vibrios lead their way to the acid rich stomach. As much as 10^8 CFU of inoculum doses can be infective and cause the disease symptoms (Sack *et al.*, 2004). The bacteria that have overcome the normal defences of acidic stomach infect the intestine by penetrating the mucus layer and colonizing the small intestinal mucosa. Specific adherence of *V. cholerae* to the intestinal mucosa is mediated by toxin coregulated pilus (Tcp), a long filamentous fimbriae that form bundles at the poles of the cells. These fimbriae have been termed as Tcp because expression

of these pili genes is coregulated with expression of the CT genes. For its adherence to the intestinal mucosa, *V. cholerae* might also use nonfimbrial adhesins to mediate a tighter binding to host cells than is attainable with fimbriae alone (Ghose, 1996). Other colonization factors include mannose-fucose hemagglutinin, regulatory proteins, outer membrane porins, biotin and purine biosynthetic genes, iron-regulated outer membrane proteins, the O antigen of the lipopolysaccharide and accessory colonization factors (Thompson *et al.*, 2004).

In cholera disease, fluid accumulation is greater in the upper intestine, duodenum, and jejunum than in the ileum. On the fourth to eighth day of illness, the number of vibrio decreases in the small bowel as the diarrhoea decreases and disappear. In rare cases, patients continue to have vibrios in the small bowel even though the diarrhoea has ceased (Thomas *et al.*, 1974).

1.12 Toxins

The CT produced by *V. cholerae* during the organism's colonization of its host's small intestine accounts for the characteristic diarrhea of the disease cholera (Sanchez and Holmgren, 2011). It is an enterotoxin made up of five B subunits (a pentameric ring of 11,500 daltons) and an active A subunit (Zhang *et al.*, 1995). The A subunit contains A1 subunit of 23,500 daltons, and an A2 of 5,500 daltons that links A1 to the 5B subunits (De Haan and Hirst, 2004). B subunits bind to oligosaccharide of monosialosyl ganglioside (GM1 ganglioside) and internalize A subunit. Binding of B subunits to GM1 induces a conformational change in the CT molecule (Kaper *et al.*, 1995). This is followed by insertion of the A subunit into the cell via receptor-mediated endocytosis. Once inside the cell, the Gs alpha subunit of the heterotrimeric G protein is permanently ribosylated by it (Kaper *et al.*, 1995; De Haan and Hirst, 2004). This causes an increase in adenylate cyclase activity as well as the level of cyclic adenosine 3’,5’-monophosphate (cAMP) in the cells (Kaper *et al.*, 1995). Elevated production of cAMP leads to secretion of H_2O, Na^+, K^+, Cl^-, and HCO_3^- into the lumen of the small intestine; this account for the life-threatening cholera disease (Thomas *et al.*, 1974; De Haan and Hirst, 2004).

Besides CT, there are various other toxigenic and pathogenic genes, such as zonula occludens toxin (*zot*), accessory cholera enterotoxin (*ace*), repeats-in-toxin (*rtx*), haemagglutinin/protease (*hap*), hemolysin (*Hly*A), outer membrane protein (*omp*U) and transmembrane transcriptional activator protein (*tox*R) (Fasano *et al.*, 1991; Trucksis *et al.*, 1993; Kaper *et al.*, 1995; Menzl *et al.*, 1996; Fullner and Mekalanos, 2000; Silva *et al.*, 2003; Childers and Klose, 2007; Chomvarin *et al.*, 2008).

The *ctx*, *zot*, and *ace* genes comprise a *V. cholerae* "virulence cassette" (Trucksis *et al.*, 1993), shown in Figure 1.1. Both *zot* and *ace* toxins are located immediately upstream of the *ctx* operon encoding CT, and influence the pathogenesis and contribute to diarrhoea in cholera (Waldor and Mekalanos, 1996). *Zot* toxigenic gene consists of a 1.3-kb open reading frame, which potentially encodes a 44.8-kDa polypeptide that affects the intercellular tight junctions and subsequently decreases intestinal tissue resistance (Fasano *et al.*, 1991; Johnson *et al.*, 1993). The third toxin of a *V. cholerae* virulence cassette, *ace*, is a hydrophobic protein that increases the transcellular ion transport across the epithelial layer (Trucksis *et al.*, 1997).

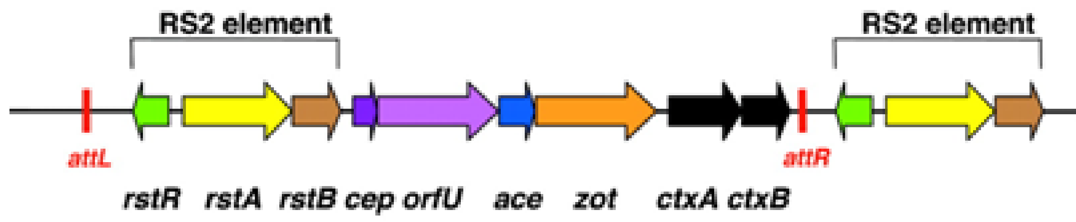

Figure 1.1 Virulence cassettes within *V. cholerae* CTXφ locus (Ruby *et al.*, 2005)

The repeats-in-toxin (RTX) exoproteins are a diverse collection of proteins exported via type I secretion by gram-negative bacteria (Satchell, 2007). RTX of *V. cholerae*, predicted to be 484,000 Da in size, is secreted by a four-component type I secretion system (TISS) (Boardman *et al.*, 2007). TISS is encoded by *rtx*B, *rtx*D, *rtx*E, and *tol*C. The T1SS consists of three components: a homodimer of an inner membrane transport ATPase, a trimer of a transmembrane linker protein, and an outer membrane porin that is either a specialized porin or the common porin *tol*C (Boardman and Satchell, 2004; Lee *et al.*, 2007).

It has been shown that the *V. cholerae* toxin is a novel toxin distinct from the other RTX toxins of gram-negative bacteria and was renamed as multifunctional autoprocessing RTX toxin, $MARTX_{Vc}$ (Satchell, 2007). $MARTX_{Vc}$ is not a pore-forming toxin; the majority of RTX toxins are pore-forming toxins that lead to alteration of membrane permeability and cell lysis (Seshadri *et al.*, 2006).

In *V. cholerae*, the *rtx*A toxin genes encode the $MARTX_{Vc}$ toxins, as shown in Figure 1.2. The *rtx*A protein that contains a glycoprotein rich repeated motif is the largest single polypeptide toxin in *V. cholerae*. It is a 4,545 amino acid in length with a predicted molecular mass of 484 kDa (Lin *et al.*, 1999).

*Rtx*A causes the depolymerization of actin stress fibers resulting in the rapid rounding of cells in culture (Lin *et al.*, 1999; Fullner and Mekalanos, 2000). This toxin-induced depolymerization of actin, causes actin monomers to be covalently cross-linked into dimers, trimers, and higher multimers (Fullner and Mekalanos, 2000). The integrity of the polarized T84 intestinal epithelial cell monolayer was found to be destroyed by it (Fullner *et al.*, 2001).

The order of the *rtx* genes is CABD, where gene A encodes the toxin, genes B and D encode two secretory proteins, and gene C encodes the toxin activator (Lin *et al.*, 1999). The organization of *rtx* gene clusters is shown in Figure 1.2. *Rtx*B, *rtx*E and *rtx*D are essential for *V. cholerae* to secrete the RTX toxin (Boardman and Satchell, 2004), its activity depends on an activator, *rtx*C, and an associated ABC transporter system, *rtx*B and *rtx*D.

Figure 1.2 Generalized organization of *rtx* gene clusters (Satchell, 2007)

V. cholerae produces haemagglutinin/protease (Hap), a soluble Zn^{2+}-dependent metalloprotease (mucinase), encoded by *hap*A (Finkelstein and Hanne, 1982; Booth *et al.*, 1983). The extracellular Hap consists of 414 amino acids representing a major protease secreted by *V. cholerae* (Hase and Finkelstein, 1991). It displays mucolytic activity that degrades different types of protein including fibronectin and lactoferrin (Finkelstein *et al.*, 1983). It also nicks the CT; when unnicked cholera enterotoxin was incubated with purified Hap, the unnicked A subunit was converted to a molecular weight consistent with that of the A1 subunit (Booth *et al.*, 1984a). Hap of *V. cholerae* also produces CTXφ -destroying factor that potentially destroys the CTXφ infectivity to generate *V. cholerae* CTXφ lysogens, indirectly indicating Hap may be a factor in preventing CTXφ reinfection in natural environments and in the human host (Kimsey and Waldor, 1998).

Hap perturbs the barrier function of Mardin-Darby canine kidney epithelial cell line I (MDCK-I) by affecting the intercellular tight junctions and the F-actin cytoskeleton (Wu *et al.*, 1998). Hap digested 66-85 kDa bands of occludin, a tight junction-associated protein, to two predominant bands of around 50 kDa and 35 kDa. This also suggests that the intercellular tight junctions is affected by Hap (Wu *et al.*, 2000). Its cytotoxic activity is likely to play an important role in the pathogenesis of cholera and the reactogenicity of attenuated vaccine strains (Robert *et al.*, 1996; Silva *et al.*, 2003). Its role in virulence is clearly not known but it is identified to have a major role in cholera pathogenesis by promoting mucin gel penetration, detachment and spreading of infection along the gastrointestinal tract (Finkelstein *et al.*, 1992; Silva *et al.*, 2003). Hap is thought to contribute to detachment rather than attachment of vibrios (Finkelstein *et al.*, 1992) and has a positive effect on colonization (Robert *et al.*, 1996).

1.13 Diagnosis of cholera

Cholera is confirmed through culture of a stool specimen or rectal swab from faeces of patients with gastroenteritis. Cary Blair media is ideal for transport (Ciufecu and Nacescu, 1974). Direct culturing on the selective thiosulfate–citrate–bile salts agar (TCBS) or alkaline peptone water (APW) enrichment prior to direct culturing on TCBS is ideal for

isolation and identification of *V. cholerae* (Lesmana *et al.*, 1985; Sood *et al.*, 1996). Diagnosis is confirmed by identification of *V. cholerae* in sample (Keen and Bujalski, 1992). Colonies identification is made by biochemical, serological tests and Polymerase Chain Reaction (PCR) (Grujic *et al.*, 1991; Varela *et al.*, 1994; Albert *et al.*, 1997).

1.14 Clinical manifestations of cholera

Cholera occurs as a result of the loss of salt and water through diarrhoea (Seas and Gotuzzo, 1996). It begins with a sudden onset of profuse, watery diarrhoea (Lopez *et al.*, 2008). In most severe cases, patient experiences nausea and vomiting with extreme loss of water and electrolytes, mainly sodium, chloride and bicarbonate (Phillips, 1964; Seas and Gotuzzo, 1996). These symptoms could happen within a short time, resulting in hypovolemic shock and acidosis (Kaper *et al.*, 1995; Lopez *et al.*, 2008). Table 1.2 shows the clinical symptoms and signs according to the degree of dehydration. If left untreated, the disease can rapidly lead to serious dehydration, electrolyte imbalance, tachycardia, hypotension and circulatory collapse leading to death (Kaper *et al.*, 1995; Seas and Gotuzzo, 1996; Lopez *et al.*, 2008).

Table 1.2: Clinical Findings in Patients with Cholera according to Degree of Dehydration (Seas and Gotuzzo, 1996)

Finding	Mild Dehydration	Moderate Dehydration	Severe Dehydration
Loss of fluid	< 5%	5-10%	> 10%
Mentation	Alert	Restless	Drowsy or comatose
Radial pulse			
Rate	Normal	Rapid	Very rapid
Intensity	Normal	Weak	Feeble or impalpable
Respiration	Normal	Deep	Deep and rapid
Systolic blood pressure	Normal	Low	Very low or unrecordable
Skin elasticity	Retracts rapidly	Retracts slowly	Retracts very slowly
Eyes	Normal	Sunken	Very sunken
Voice	Normal	Hoarse	Not audible
Urine production	Normal	Scant	Absent

1.15 Treatment of cholera

Cholera patient can be treated effectively with oral rehydration therapy, developed in 1960s (Bhattacharya, 1995; Rabbani, 2000; Schultz, 2007; Nalin, 2009). According to WHO, 80-90% of cholera victims during an epidemic can use oral rehydration salts (WHO, 1991). With this therapy, patients are given oral rehydration solution to make up the salt and water deficits and to replace ongoing losses (Bhattacharya, 1995; Rabbani, 2000). Oral rehydration solution is formulated based on the concentrations of salts that are lost in the stool in patients with cholera. The electrolyte composition of oral rehydration solution are 90 mmol of sodium, 20 mmol of potassium, 80 mmol of chloride, 10 mmol of citrate, and 111 mmol of glucose per litre. This electrolyte composition is recommended by the WHO and the United Nations Children's Fund for universal application as it proved to be effective and safe in treating all types of diarrhoea in children and adults (Rabbani, 2000).

For effective medical care, administration of oral rehydration salts to replace lost fluid and electrolytes should be started right away (Carpenter, 1992; Kaper *et al.*, 1995; Rabbani, 2000). If administered in a timely manner and in adequate volumes, prompt oral rehydration therapy nearly results in cure and reduces case–fatality rates (Carpenter, 1992; Bhattacharya, 1995; Seas *et al.*, 1996; Rabbani, 2000). This may be done orally for ordinary case or by intravenous administration for severely dehydrated cases (Seas *et al.*, 1996). The solution can not reduce the duration of illness or the volume of watery diarrhoea but limits dehydration and prevents acidosis (Scarpignato and Rampal, 1995; Rabbani, 2000).

Another possible way to produce a better rehydration solution is rice-cereal-based to reduce diarrhoea by adding more substrate to the gut lumen without increasing osmolality (Molla *et al.*, 1982; Khin Maung and Greenough, 1991). Moreover rice-based solution has the advantage of being more culturally acceptable and it is readily available even in rural homes in developing countries (Nathavitharana and Booth, 1992). Also, combined standard solution with nutrients, amino acids and vitamins (such as vitamin A, zinc, magnesium, selenium, and micronutrients) definitely will be a better oral solution (Pillai *et al.*, 1994;

Rabbani, 2000). This is due to the ability of the solution to increase absorption in the small intestine, thus reduce the watery diarrhoea in cholera, and increase antioxidative actions.

The use of antimicrobials is an adjunctive to rehydration therapy. Antimicrobial agents have been shown to reduce the volume of stool in patients and stop excretion of vibrios in the stool (Seas and Gotuzzo, 1996; Bhattacharya, 2003). Tetracycline, erythromycin, luoroquinolones, norfloxacin, lomefloxacin, ciprofloxacin and doxycycline, are the drugs of choice in treatment of cholera (Seas and Gotuzzo, 1996). Drugs that inhibit CT binding to receptors in the intestine provide an attractive strategy for managing severe diarrhoea in cholera. These drugs are currently not available for cholera treatment but are expected to be available soon (Thiagarajah and Verkman, 2005).

1.16 Immunology of cholera

In the small intestine, *V. cholerae* secretes mucinases and attach themselves to the villi of the cells. On the luminal surface, *V. cholerae* multiply and secrete cholera toxin (CT) (Svennerholm *et al.,* 1994). Bacteria and secreted CT is sampled through the M cells and presented to immature dendritic cells. A few vibrio cells and CT may be sampled directly by dendritic cells. Dendritic cells process the antigens in endosomes and present the specific antigens on their surface with MHC class II proteins. Dendritic cells with MHC class II-associated antigen attracts CD4+ T cells and IgA-B cells. Primed CD4+ T cells also interact with dendritic associated-B cells to stimulate them. Stimulated B cells leave the Peyer's patches and migrate to the mesenteric lymph nodes where they mature into IgA secreting plasma cells. IgA secreting plasma cells 'home back' to the lamina propria and secrete antibodies. While IgA is being transported through the mucosal epithelial cells, IgA is bound by a secretory component and the complex is secreted into lumen as sIgA (Svennerholm, 1994). It has been observed that although CT can itself elicit immunity, this immunity is short lived and lasts only six months (Fica *et al.,* 1993).

1.17 Prevention of cholera

Cholera was eliminated from the industrialized world through safe water and sanitation facilities and better hygiene (von Seidlein, 2007). On the other hand in resource poor countries with huge population burden, lack of safe water and proper sanitation facilities cause sudden cholera outbreaks. This necessitated the invention of safe and effective cholera vaccines for prevention of disease. The advent of safe and effective oral vaccines against cholera has created renewed interest in the cholera-prevention efforts (Shin *et al.,* 2011). Before 2002, cholera vaccines were recommended only for travellers to areas where the disease is endemic (Lopez *et al.*, 2008). In 2002, WHO for the first time recommended the potential use of oral cholera vaccines in endemic and epidemic countries as an additional public health tool (WHO, 2002). Mass oral cholera vaccinations have been conducted in Beira, Mozambique, in Darfur, Sudan, and in Aceh, Indonesia after WHO experts recommended vaccinations for cholera control. These projects help experts to achieve more experience with vaccination under actual public health conditions (Lucas *et al.*, 2005).

There are currently four licensed oral cholera vaccines, namely Dukoral™, CVD 103HgR or Orochol™, heat-killed oral cholera vaccine by Vabiotech produced in Vietnam and Shanchol by Shantha Biotechnics produced in India (Table 1.3) (Lopez *et al.*, 2008). Dukoral™ is produced by Crucell (formerly SBL). It contains the recombinant cholera B-subunit and heat or formalin killed *V. cholerae* organisms. Dukoral™ is licensed for persons two years and older. It requires the administration of two doses of Dukoral given one to six weeks apart. CVD 103HgR or Orochol™ is also produced by Crucell (formerly Berna Biotech). Orochol is given in a single-dose and it was shown to be safe and immunogenic in various trials in North America (Kotloff *et al.*, 1992), Switzerland (Cryz *et al.*, 1992), Peru (Gotuzzo *et al.*, 1993), Indonesia (Suharyono *et al.*, 1992; Simanjuntak *et al.*, 1993), in HIV seropositive individuals in Mali (Perry *et al.*, 1998), and in challenge studies in the US (Tacket *et al.*, 1999). Orochol™ has been licensed in the US and Europe for use by travellers

to cholera-endemic settings. Its production was halted several years ago. This is because there was limited market potential for Orochol™ (Lopez *et al.*, 2008).

Killed oral whole cell vaccine partly modeled on the Swedish vaccine has been developed in Vietnam by the local manufacturer Vabiotech. This vaccine contains killed whole cells (WC) of *V. cholerae* O1 and O139 without the CT B subunit. This vaccine was licensed in Vietnam in 1997 and it was appropriate for use in public health programs in developing countries for its low production cost (Thiem *et al.*, 2006; Lopez *et al.*, 2008). Vietnam is the only country in the world to have introduced the oral cholera vaccine (OCV) in their immunization schedule. In an open trial in Hue, Vietnam, involving half of the communes of Hue that is more than 50,000 subjects older than 1 year of age, this vaccine was shown to have 50% long term effectiveness (Thiem *et al.*, 2006).

Shanchol™, an oral vaccine for cholera is manufactured by Shantha Biotechnics, Hyderabad, India. It consists of killed whole cells from a mixture of pathogenic strains of *V. cholerae* O1 and O139. Shanchol vaccine is recommended for children above one year old and adults. Two doses, one to six weeks apart is recommended. Shanchol is the first new vaccine to be developed and licensed with funding from the Gates Foundation (International Vaccine Institute, 2009). It is recommended as a supplement to conventional tools against cholera like safe drinking water and sanitation. It does not require dilution in a buffer (Paho, 2010). In a study conducted in more than 65,000 persons of an endemic region of India, Shanchol showed 67% efficacy against cholera. Efficacy in children aged 1-5 years was 49%, but dropped to 40% when only one dose was administered or doses were incompletely ingested (Sur *et al.,* 2009).

Table 1.3: Licensed oral cholera vaccines (Lopez *et al.*, 2008)

No.	Vaccine	Contents	Availability
1.	Dukoral (Crucell)	1 mg of Recombinant CT B Plus 2.5 x 10^{10} of the following *V. cholerae* O1 organisms: Formalin-Killed El Tor Inaba (Phil 6973) Heat-Killed Classical Inaba (Cairo 48) Heat-Killed Classical Ogawa (Cairo 50) Formalin-Killed Classical Ogawa (Cairo 50)	Licensed in more than 20 countries, including the European Union
2.	Oral vaccine (Vabiotech)	600 EU [a] LPS [b] of Formalin-Killed El Tor Inaba (Phil 6973) 300 EU LPS of the following *V. cholerae* O1 organisms: Heat-Killed Classical Inaba (Cairo 48) Heat-Killed Classical Ogawa (Cairo 50) Formalin-Killed Classical Ogawa (Cairo 50) 600 EU LPS of *V. cholerae* O139 (4260B)	Licensed only in Vietnam, new formulation undergoing Phase III trial in Kolkata, India
3.	Orochol (Crucell)	Live attenuated CVD 103 HgR	Licensed in some countries but no longer produced
4.	Shanchol (Shantha Biotechnics)	Killed whole cells from a mixture of pathogenic strains of *V. cholerae* O1 and O139	Licensed in India

[a]EU, ELISA units
[b]LPS, lipopolysaccharide