EVALUATION OF CHOLERA VACCINE CANDIDATES VCUSM2 (O139), VCUSM4 (El Tor) AND ITS BIVALENT VACCINE FORMULATION

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ABBREVIATIONS

ALA	Aminolevulinic acid
APW	Alkaline peptone water
BA	Blood agar
cAMP	cyclic adenosine monophosphate
CBA	Chocolate blood agar
CFU	colony forming unit
CT	Cholera Toxin
CTB	cholera toxin B subunit
CTX	cholera toxin
CTXΦ	cholera toxin phage
GM1	mono sialoganglioside
HCl	Hydrochloric acid
HRP	Horse Radish Peroxidase
Ig	Immunoglobulin
kDa	kiloDalton
KIA	Kligler iron agar
LB	Luria Bertani media
LIA	lysine iron agar
LPS	Lipopolysaccharide
ml	milli liter
mm	millimetre
mM	milli molar
NaCl	sodium chloride
NaHCO3	sodium bicarbonate
OD600	Optical density at 600nm
ORS	Oral Rehydration Solution
PBS	Phosphate Buffered Saline
PBS-Tween-20	PBS with 0.05% tween-20
RTX	Repeat-in-Toxin

TCBS	Thiosulfate Citrate Bile salt Sucrose
TSI	triple sugar iron agar
µm	Micrometer
vs	versus
VP	Voges Proskauer
v/v	volume/volume
w/v	weight/volume
WC/BS	whole cells plus B subunit
WC/rBS	whole cells plus recombinant B subunit
WHO	World Health Organization
WT	wild type
ZOT	Zonula occludens toxin
21G	21 gauge

LIST OF SYMBOLS

<	Less than
>	More than
~	Almost
%	Percent
^{o}C	Degree centigrade

PENILAIAN CALON VAKSIN KOLERA VCUSM2 (O139), VCUSM4 (El Tor) DAN FORMULASI BIVALENNYA

ABSTRAK

Kolera pernah berlaku di hampir kesemua pelusuk dunia dan dua kumpulan-sera telah dikenalpasti sebagai penyebab kolera iaitu *V. cholerae* O1 and *V. cholerae* O139. Peningkatan secara mendadak pada tahun 2006 menunjukkan keupayaan *V. cholerae* untuk menyebabkan kerosakan yang lebih serius dan tidak boleh dipandang ringan. Pemahaman terhadap *V. cholerae* sebagai organisma bukan invasif serta vaksinasi secara oral yang boleh menghasilkan gerak-balas imun yang optimum telah memberi kelebihan terhadap vaksinasi oral berbanding dengan vaksinasi melalui suntikan. Kebanyakan vaksin yang telah dihasilkan hanya bertindak terhadap kumpulan-sera O1 sahaja. Oleh kerana ketiadaan perlindungan silang di antara dua kumpulan-sera O1 dan O139, maka wujud keperluan terhadap vaksin bivalen untuk kolera. Dalam kajian ini kami cuba menghasilkan vaksin bivalen yang mampu memberi perlindungan terhadap *V. cholerae* O1 and *V. cholerae* O139. Vaksin bivalen yang terdiri daripada auksotrof VCUSM2 dan VCUSM4 telah dinilai untuk gerak-balas imun, kesan buruk, keselamatan penggunaan dan kebolehan untuk memberi perlindungan serta persamaan ciri-ciri berbanding strain induk masing-masing. VCUSM2 dan VCUSM4 telah dihasilkan melalui mutasi pada *hem*A yang menyebabkan mereka bergantung kepada amino levulinic asid (ALA) untuk hidup. Apabila tambahan ALA yang optimum diberikan, strain-strain vaksin VCUSM telah menunjukkan persamaan ciri-ciri dengan strain induk tetapi hal ini tidak berlaku tanpa kehadiran ALA. Tanpa kehadiran ALA strain-strain vaksin VCUSM menunjukan ciri-ciri hidup yang lemah. Vaksin auksotrof berfokuskan pengekalkan ciri-ciri strain

induk tetapi diatenuasi dari segi kemandirian. VCUSM2 dan VCUSM4 mempunyai kemampuan kemandirian yang terhad dalam pelbagai persekitaran air. VCUSM2 dan VCUSM4 juga tidak menyebabkan cirit-birit apabila diuji ke atas model arnab berbanding jenis virulen *V. cholerae*. Vaksin bivalen juga menghasilkan gerak-balas imun terhadap kedua-dua kumpulan-sera. Kajian perlindungan menggunakan salah satu jenis virulen *V. cholerae* berdos tinggi menunjukkan perlindungan menyeluruh kepada arnab yang telah divaksinasi dengan vaksin bivalen. Vaksin bivalen menunjukkan keputusan yang memberangsangkan dari segi keselamatan penggunaan, imunogenesiti, kesan sampingan dan kemampuan memberi perlindungan serta boleh digunakan terhadap kedua-dua kumpulan-sera O1 dan O139 *V. cholerae.*

EVALUATION OF CHOLERA VACCINE CANDIDATES VCUSM2 (O139), VCUSM4 (El Tor) AND ITS BIVALENT VACCINE FORMULATION

ABSTRACT

Cholera has been implicated in many parts of the worlds and two serogroup identified for causing epidemic cholera are the *V. cholerae* O1 and *V. cholerae* O139. Sharp increase of cholera cases and deaths in 2006 showed potential of *V. cholerae* to cause more severe damages and must not taken lightly. Better understanding of *V. cholerae* as non-invasive organism and oral route vaccination can optimally induce intestinal immune response has led to the preference of oral cholera vaccine over parenteral cholera vaccine. Vast majority of vaccine created were directed against O1 serogroup only. Since cross protection cannot be established between O1 and O139 serogroup there is need for bivalent vaccine for cholera. In this study we attempted to formulate a bivalent vaccine capable of protecting against *V. cholerae* O1 and *V. cholerae* O139. A bivalent vaccine composed of auxotrophic VCUSM2 and VCUSM4 previously developed was evaluated for immunogenicity, reactogenicity, safety and protective ability as well as their characteristic similarities to their respective parent strain. VCUSM2 and VCUSM4 were created by *hem*A mutation making them dependent on amino levulinic acid (ALA) for survival. When supplemented with optimal ALA VCUSM vaccine strains characteristically similar to parent strains but not in absence of ALA. In the absence of ALA, VCUSM vaccine strains showed weakened characteristics. Auxotrophic strains development focused on retaining characteristic features of wild types but attenuated in term of survival abilities. VCUSM2 and VCUSM4 had limited survival capabilities in various environmental waters. VCUSM2 and VCUSM4 did not cause diarrhea when

tested in rabbit models when compared with virulent wild type *V. cholerae*. Bivalent vaccine elicited immune response against both serogroups. Protection studies using high dose of virulent of either wild types showed complete protection in rabbits vaccinated with bivalent vaccine. Bivalent vaccine showed promising result in term of safety, immunogenicity, side effects and protective capabilities and can be used against both O1 and O139 serogroups of *V. cholerae*.

CHAPTER 1 - INTRODUCTION

1.1 Cholera

Cholera can be defined as sudden onset of watery diarrhea and in severe form can lead to hypovolemic shock and acidosis. Cholera is an intestinal infection caused by certain member of species of Vibrio namely *Vibrio cholerae*.

1.2 *Vibrio cholerae*

1.2.1 General characteristics

Cholera is a natural inhabitant of brackish and estuarine water (Faruque *et al.*, 1998) and associated with aquatic organisms such as zooplankton, phytoplankton, crustaceans, green algae and insects (Lipp *et al.*, 2002, Reidl and Klose, 2002, Halpern *et al.*, 2007, Rawlings *et al.*, 2007). *Vibrio cholerae* are facultative anaerobes, slightly curved or straight and highly motile Gram-negative rods belong to family vibrionaceae. It measures 1.4 -2.6 μm in length (Faruque *et al.*, 1998, Reidl and Klose, 2002). *V. cholerae* can enter viable but non culturable (VNC) state under certain conditions (Reidl and Klose, 2002, Binsztein *et al.*, 2004, Chaiyanan *et al.*, 2007, Halpern *et al.*, 2007). VNC form can revert back to pathogenic state under favourable conditions (Binsztein *et al.*, 2004). During transmission to VNC state, *V. cholerae* transform from curved rod to irregular form and finally to a small coccoid form (Chaiyanan *et al.*, 2007).

Vibrio cholerae has two distinct antigens which is the somatic (O) antigen and flagellar (H) antigen. The O antigen is thermostable polysaccharide and part of cell wall lipopolysacharide (Gustafsson, 1984). The flagellar (H) antigen is similar between all *Vibrio cholerae* strains but the somatic (O) antigen differs between each strains. The O

antigen was the basis for differentiation of *V. cholerae* strains into different groups (serogroups) (Kaper *et al.*, 1995, Chatterjee and Chaudhuri, 2003). To date more than 200 serogroups recognized. Out of 200 serogroups are recognized so far only O1 and O139 are known for causing cholera on epidemic and pandemic scale (Chatterjee and Chaudhuri, 2003). Figure 1.1 shows the LPS structure differences between *V. cholerae* O1 and *V. cholerae* O139.

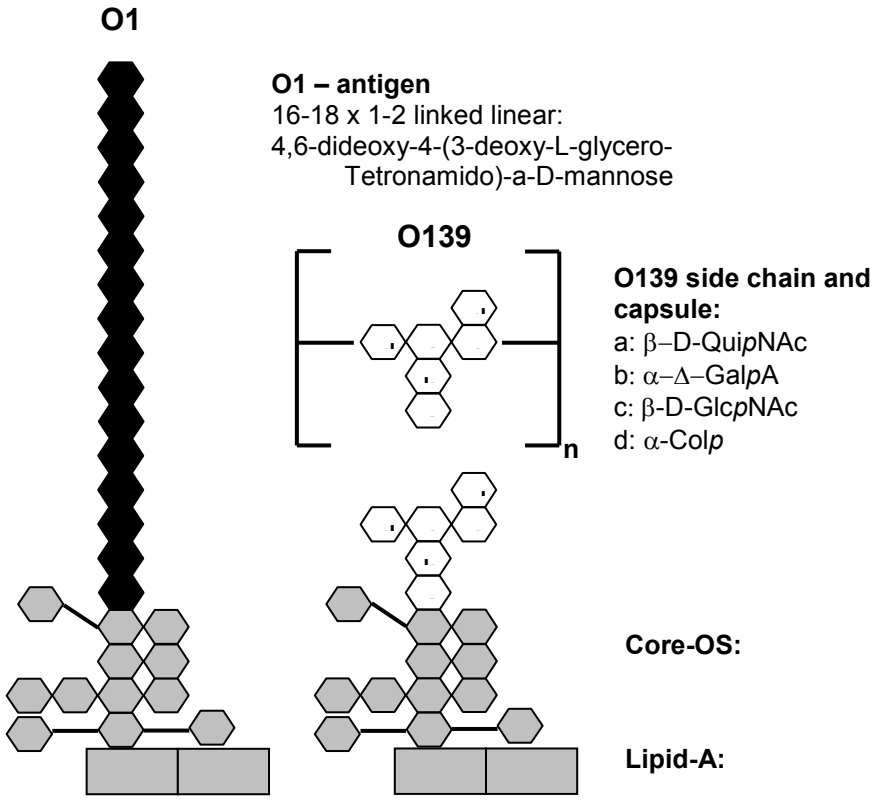


Figure 1.1. LPS structure differences between *V. cholerae* O1 and *V. cholerae* O139. Shown are different O antigen and capsule structures, and similar core-OS and Lipid-A structures. *V. cholerae* O139 O-antigen has a different carbohydrate composition, which also presented as a capsule (Reidl and Klose, 2002).

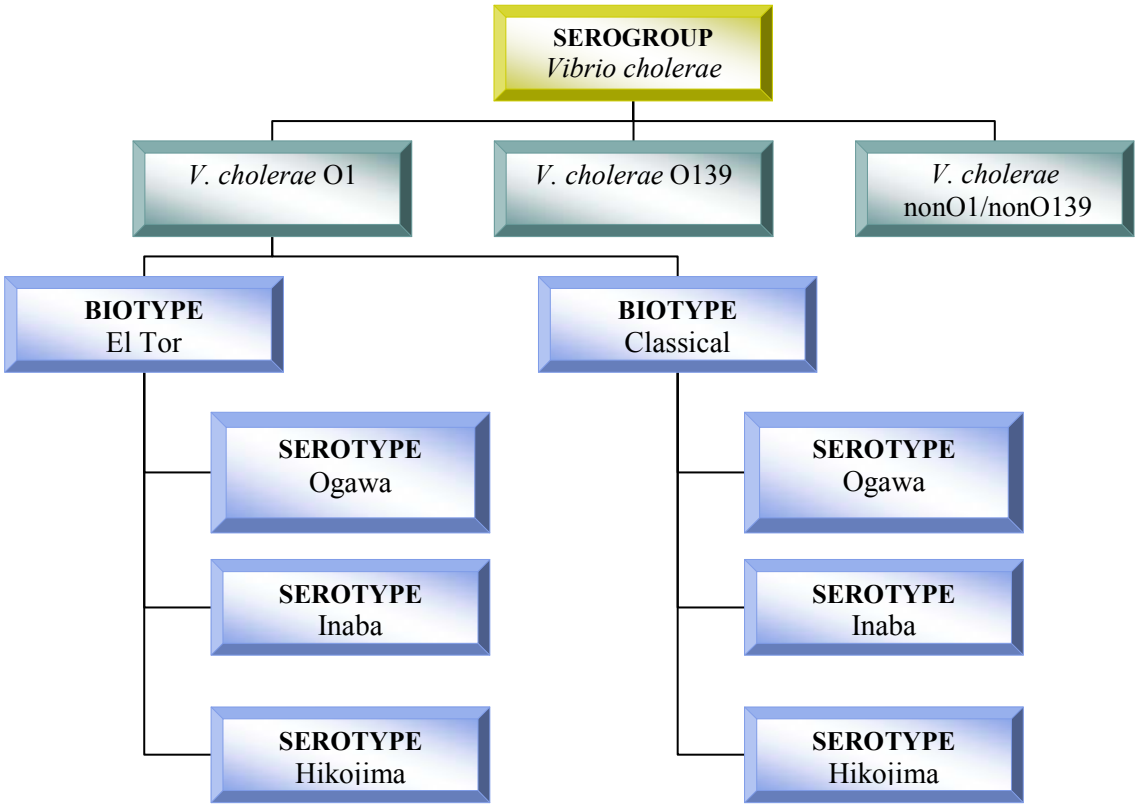
1.2.2 Nomenclature of *Vibrio cholerae*

1.2.2.1 *Vibrio cholerae* O1

Before the discovery of *V. cholerae* O139 Bengal in late 1992, cholera epidemics were caused by only O1 serogroup (Siddique *et al.*, 1994, Morris, 1995). The O1 serogroup can be further divided into two biotypes and three serotypes (based on O antigen's components). Figure 1.2A shows the serogroups, biotypes, and serotypes classification of *V. cholerae.*

1.2.2.1.1 Serotypes of *Vibrio cholerae* O1

Ogawa, Inaba and Hikojima are designated names for each serotype of *V. cholerae* O1 and A, B, and C are the antigenic determinants on O antigen used for the serotype classification. A is the major antigenic determinant for *V. cholerae* O1 and is present in all three serotypes. B is the major antigenic determinant present in Ogawa and Hikojima and C is present as the major antigenic determinant in Inaba and Hikojima. Hikojima as the rarest serotype possesses all 3 antigenic determinants (Kay *et al.*, 1994). It is also possible for Ogawa to Inaba and Inaba to Ogawa interconversion to occur (Stroeher *et al.*, 1992, Kay *et al.*, 1994, Garg *et al.*, 2000). Figure 1.2B shows hypothetical picture of major antigenic determinants in O antigen in *V. cholerae* O1 for serotypes classification.



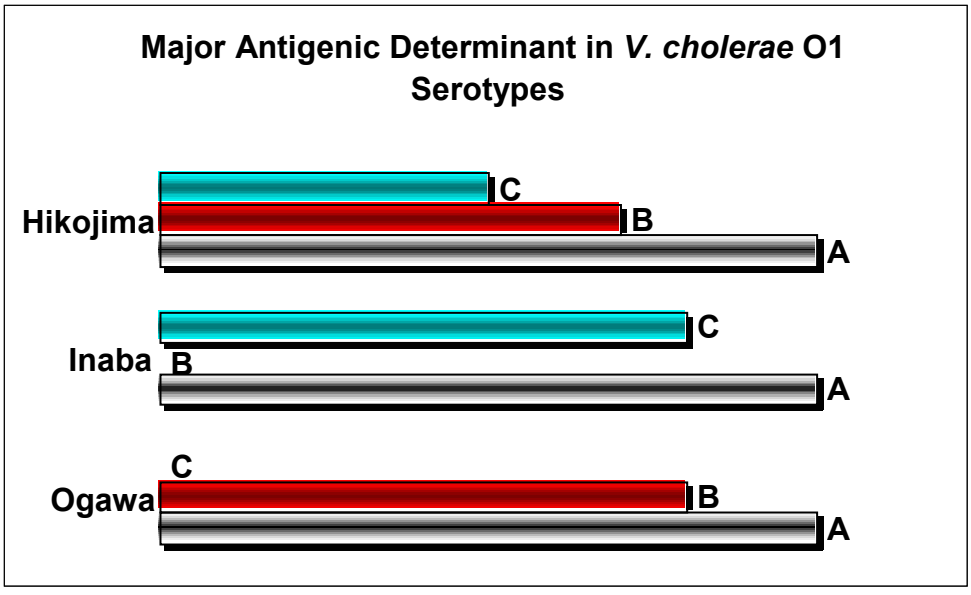


Figure 1.2. Serogroups, biotypes, and serotypes classification in *V. cholerae* (A) and major antigenic determinants in O antigen in *V. cholerae* O1 serotypes (B)

1.2.2.1.2 Biotypes of *Vibrio cholerae* O1

V. cholerae O1 is divided into two biotypes; classical and El Tor based on characteristics displayed on a number of tests. Table 1.1 shows tests and corresponding characteristic features of Classical and El Tor biotypes (Kaper *et al*, 1995).

	Biotype	
Test or property	**Classical**	**El Tor**
Hemolysis	-	+
Agglutination of chicken erythrocytes	-	+
Voges-Proskauer	-	+
Inhibition by polymyxin B (50-U disk)	+	-
Lysis by		
i) Classical IV bacteriophage of Murkejee	+	-
ii) FK bacteriophage of Takeya *et al*	+	-

Table 1.1. Tests and corresponding characteristic features of Classical and El Tor biotypes

1.2.2.2 *Vibrio cholerae* O139 Bengal

Prior to the year 1993, *V. cholerae* O1 was the only serogroup responsible for epidemic cholera but in September 1992 a new strain other than *V. cholerae* O1 was discovered to cause severe and cholera-like disease in eastern India and Bangladesh. At first, it was referred as non-O1 *V. cholerae* because it did not agglutinate with O1 antisera. Later it was designated as O139 synonym Bengal (Trucksis *et al.*, 1993). In general, the O139 strain was a hybrid of O1 and non-O1 strains. O139 Bengal strain was similar with O1 El Tor strains in its virulence characteristics, specifically, cholera toxin and toxin-coregulated pilus (TCP). But it lacks some genetic materials necessary for production of O1 antigen. Like most strains, the *V. cholerae* O139 produces polysaccharide capsules (Keasler and Hall, 1993, Kaper *et al.*, 1995).

1.3 Epidemiology of Cholera

Almost all parts of the world have reported cholera cases caused by *V. cholerae* O1 El Tor the serogroup responsible for the seventh pandemic. Latest data on cholera indicated increase in number of officially reported cases from 131 943 cases in 2005 to 236 896 cases in 2006 and number of deaths increased from 2272 to 6306 respectively. There was a 79% increase in number of cases and 3 fold increase in number of deaths. The number of countries officially reported cases during 2006 were from 52 countries around the world. The causative strains were mainly of *V. cholerae* O1 and and to lesser due to extend *V. cholerae* O139. WHO estimates that the officially reported cases represent around 5-10% of actual cases worldwide. But the actual figures are likely to be higher because of limitations of surveillance system and underreporting (WHO, 2007,

Zuckerman *et al.*, 2007). Figure 1.3A shows the countries reported cholera cases in year 2006.

1.3.1 Early Pandemic of Cholera

Cholera-like disease can be traced back to the time of Hippocrates and Buddha and may even earlier (Lipp *et al.*, 2002, Reidl and Klose, 2002). Many infectious agents were identified in 19th century and Robert Koch in year 1883 identified that cholera was caused by a comma shaped bacterium. However, modern history of cholera began in 1817 when epidemic reported in India and spread across the Indian continent and defined as first pandemic outbreak of cholera (Reidl and Klose, 2002). The fifth and sixth pandemics were believed to be caused by *V. cholerae* of classical biotype in Indonesia but earlier pandemics were not associated with a particular biotype (Reidl and Klose, 2002).

1.3.2 Seventh Pandemic

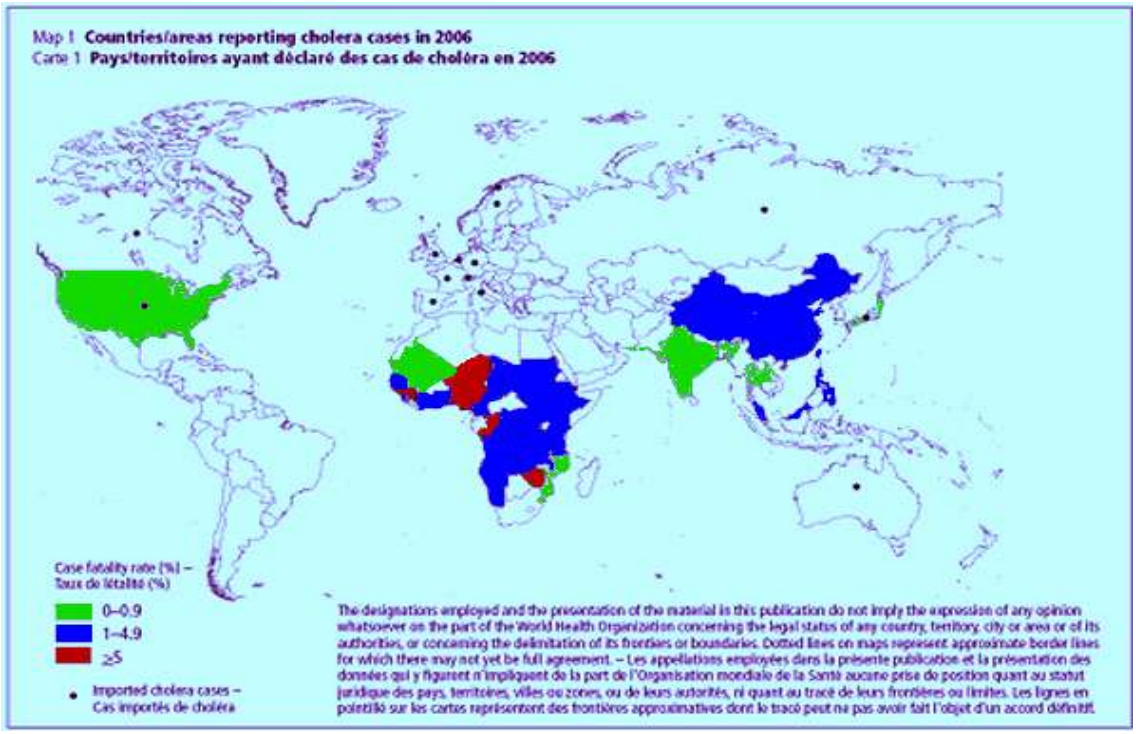
The seventh pandemic was caused by *V. cholerae* El Tor biotype was known for its extensive spread and duration of pandemic (Reidl and Klose, 2002). Starting from island of Sulawesi in 1961 and then to entire Southeast Asia at the end of 1962. By 1970 Pakistan, India, Malaysia, Iran, Iraq, Syria, West Africa and some other countries reported cholera cases (Kaper *et al.*, 1982, Kaper *et al.*, 1995). Out of 36 countries reported cholera cases in 1970, 28 were newly affected countries (Kaper *et al.*, 1995). Beginning January 1991 cholera outbreak started in Peru and spread to almost all Latin America countries within a year. This was known to be the first epidemic in America after more than a century (Olsvik, 1992, Ries *et al.*, 1992). Seasonal outbreak of cholera

cases continued in many developing countries especially Bangladesh and India. But in 1992, large epidemics *of cholera by V. cholerae* non-O1 (now referred as *V. cholerae* O139) reported in Bangladesh and India which marks the beginning of eight pandemic (Faruque *et al.*, 1998).

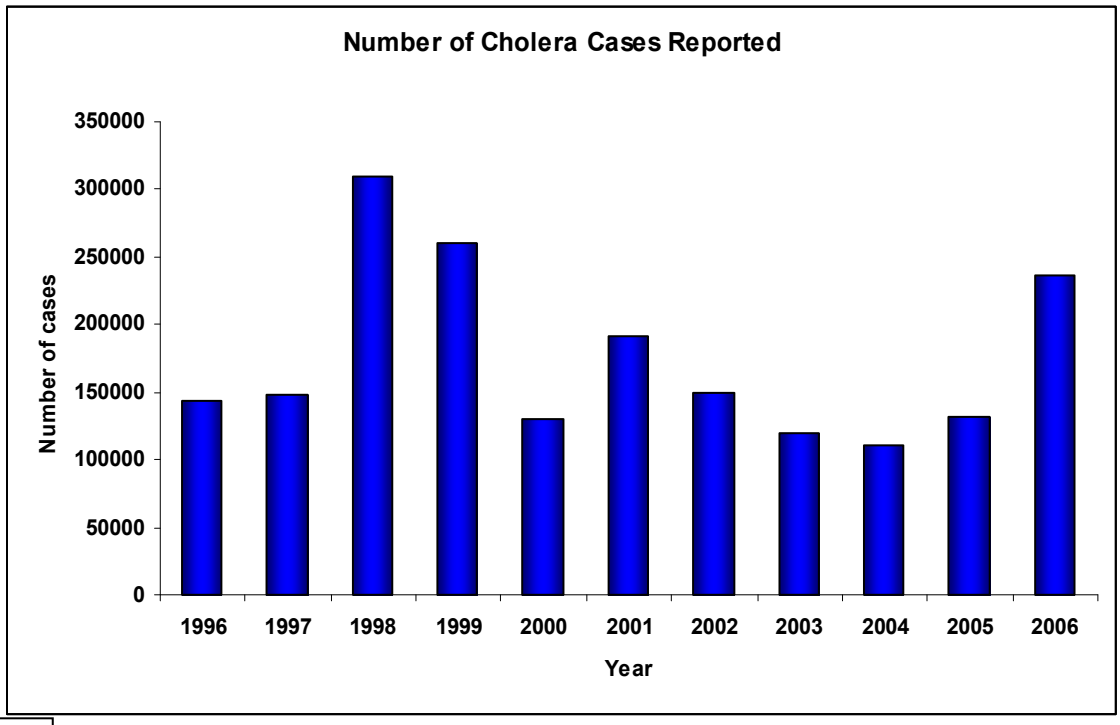
1.3.3 Eight Pandemic

In 1992, cholera-like infection in epidemic proportion was reported in India and Bangladesh. The causative agent was not *V. cholerae* O1 but by non-O1 strain that do not agglutinate with O1 antiserum (Ramamurthy *et al.*, 1993). The non-O1 strain was designated as *V. cholerae* O139 Bengal after serological studies revealed it was clonal in nature (Bhattacharya *et al.*, 1993). *V. cholerae* O139 rapidly spread to other Asian countries and imported cases were also noted in several countries around the world (Nair *et al.*, 1994, Siddique *et al.*, 1996). Beginning of pandemic, *V. cholerae* O139 completely displaced *V. cholerae* O1 but by 1994 new clone of *V. cholerae* O1 El Tor became dominant strain causing cholera in India and Bangladesh (Faruque *et al.*, 1997, Sharma *et al.*, 1997). *V. cholerae* O139 re-emerged in India and Bangladesh starting in 1996 (Mitra *et al.*, 1996). Year 2006 showed sharp increase in number of cases (79%) and number of deaths (300%) (WHO, 2007). The causative strains were mainly of *V. cholerae* O1 and to lesser extend due to *V. cholerae* O139. *V. cholerae* O139 continued to be confined only to countries in South-East Asia (WHO, 2005, WHO, 2007). In 2006, China and Thailand were the only countries reported cases by *V. cholerae* O139. Still *V. cholerae* O139 poses potential threat as the cause for next pandemic (WHO, 2007).

Safe drinking water, proper sanitation facility, and safe food for consumption can keep cholera cases to a minimum (Qadri *et al.*, 1995). But still cholera poses threat for many developing countries where safe drinking water, proper sanitation facility, and safe food for consumption cannot be ensured for all (Faruque *et al.*, 1998). Number of cases reported to WHO from 1996 to 2006 showed in Figure 1.3B.



A



B

Figure 1.3. Countries reported cholera cases in year 2006 (A) and number of cases reported to WHO from 1996 to 2006 (B) (WHO, 2007).

1.4 Ecology of *V. cholerae*

V. cholerae regarded as major inhabitant of aquatic environments and a part of normal, free-living organisms in estuarine areas and river (Chaiyanan *et al.*, 2007). Although all strains of *V. cholerae* can be isolated from environments but non-O1 and non-O139 commonly found outside of epidemic areas as compared to O1 and O139 *V. cholerae*. These non-O1 and non-O139 strains usually are cholera toxin negative compared with O1 and O139 strains that carry not only the cholera toxin but also other factors essential for colonization of mammalian cells particularly gastrointestinal tract (Pearson *et al.*, 1993, Karaolis *et al.*, 1998). Within aquatic environments *V. cholerae* multiply by means of zooplankton and phytoplankton and in certain part of the world seasonal outbreaks demonstrated that cholera highly dependent on environmental factors such as algae bloom and climate changes (Islam *et al.*, 1989, Qadri *et al.*, 1997b, Lowenhaupt *et al.*, 1998, Lipp *et al.*, 2002).

The life cycle of *V. cholerae* consists of two phases; within a host and in aquatic environment as free swimming cells or attached to surfaces provided by plants, green algae, crustaceans, copepods and egg masses of chironomids (Qadri *et al.*, 1997b, Abd *et al.*, 2007, Halpern *et al.*, 2007). Recent study on copepods species, *Acartia tonsa* and *Eurytemora affinis* revealed that *V. cholerae* O1 has better affinity to them compared with *V. cholerae* O139 suggesting reason for predominance in *V. cholerae* O1 in rural Bangladesh epidemics (Rawlings *et al.*, 2007). Deprivation of nutrients leads to formation of biofilm and lead to VNC state facilitating persistence in aquatic environment and recovered as VNC state in chironimids eggs (Chaiyanan *et al.*, 2007, Halpern *et al.*, 2007). Figure 1.4 shows the ecology of *V. cholerae*.

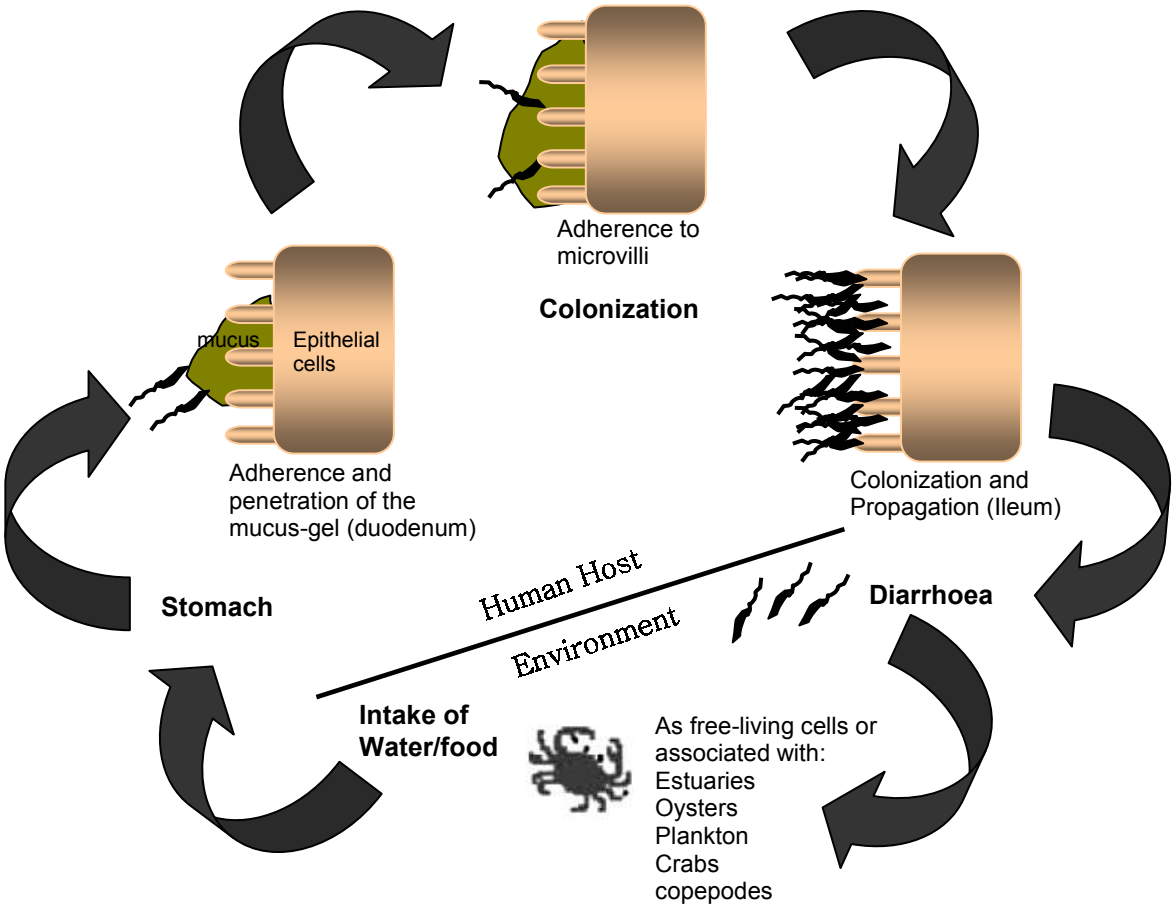


Figure 1.4. The ecology of *V. cholerae* (Reidl and Klose, 2002).

1.5 Pathogenesis of *V. cholerae*

1.5.1 Ingestion and colonization

Cholera primarily result from ingestion of *V. cholerae* contaminated food and water (Kirkpatrick and Alston, 2003). Human has been known as natural reservoirs for *V. cholerae*. But planktons, shellfish and crustaceans in certain aquatic environments are known to carry *V. cholerae* (Faruque *et al.*, 1998). *V. cholerae* is sensitive to low pH and do not survive in pH below 5.0 and in normal human gastric acid can kill most of the *V. cholerae* before reaching the small intestine thus becomes a natural barrier against *V. cholerae* (Reidl and Klose, 2002, Wang and Gu, 2005). In human studies the infectious dose was determined to start from 10^6 cfu if gastric acid was neutralized with 2g of sodium bicarbonate prior to ingestion (Kaper *et al.*, 1995, Reidl and Klose, 2002). In North American volunteers, 10^{11} cfu in buffered saline was consistently causing cholera (Kaper *et al.*, 1995). After surviving the gastric pH barrier, *V. cholerae* must attach themselves to mucosal cells of small intestine to initiate colonization. But before attaching themselves, they have to overcome mucous layer covering the surface of mucosal cells. Here a filamentous protein structure called Toxin Coregulated Pilus (TCP) takes a primary role and aided by motility penetrate the mucous layer to reach mucosal cells (Attridge and Rowley, 1983, Angelichio *et al.*, 1999). TCP, a type IV pilus is considered as most important colonization factor as demonstrated in human and animal models (Chiang and Mekalanos, 1998, Kirn *et al.*, 2005). But apparently flagellum which responsible for motility of *V. cholerae* does not directly involved in attachment onto mucosal cells (Postnova *et al.*, 1996). Additional colonization factors are shown to be important mainly in infant mouse and adult rabbit model. These include

accessory colonization factors (ACFs), mannose fucose-resistant hemagglutinin, and O-antigen of the lipopolysacharide (LPS).

1.5.2 Cholera Toxin production

Massive diarrhea induced by *V. cholerae* is caused primarily by cholera toxin (CT). But strains lacking the gene encoding for cholera toxin still induce mild to moderate diarrhea as later found out to be the cause of additional toxins present in *V. cholerae* (Fasano *et al.*, 1991, Wu *et al.*, 2000, Somarny *et al.*, 2004, Tapchaisri *et al.*, 2007).

Cholera toxin is composed of A-B subunit. Each subunit has specific functions. The B subunit is for binding to GM1 receptor of host cells and the A subunit by enzymatic reaction to induce diarrhea. The A subunit with molecular weight of 28 kDa can be cleaved into two polypeptides chain, the A_1 peptide of 21.8 kDa and A_2 peptide of 5.4 kDa. The B subunit with mass of 56 kDa composed of 5 identical peptide chain linked together by non-covalent bond (Kaper and Baldini, 1992).

Cholera toxin act through binding of GM1 receptor commonly found in eukaryotes cell membranes. Internalization of A subunit following GM1 binding and adenylate cyclase activation by A subunit increases intracellular cAMP level. Increased level of intracellular cAMP causes hypersecretion of electrolytes from cells into intestinal lumen and also inhibits active sodium chloride absorption, thus result in passive water loss from cells into intestinal lumen (Reidl and Klose, 2002) by which 'rice watery stool' name came from.

The rice watery stools contains large amounts of sodium, potassium, chloride and bicarbonate. The loss of water and electrolytes from mucosal cells were replaced from blood, which in turn reduces the blood volume followed by low blood pressure and shock. Loss of potassium and bicarbonate leads to acidosis (Qadri *et al.*, 1997b).

1.5.3 Additional Toxins

1.5.3.1 Soluble HA/Protease

HA/Protease is encoded by *hap* gene. HA/Protease is not a colonization factor but a metallprotease that nicks cholera toxin and also cleavage fibronectin, mucin and lactoferrin. In infant rabbit model, HA/Protease mutant was not less virulent than its parent strain. Ha/Protease seem to have a 'detachase' activity which allows the vibrios to detach from cultured human intestinal epithelial cells. This might aid in spread of the vibrios (Finkelstein *et al.*, 1992, Kaper *et al.*, 1995).

1.5.3.2 Accessory Cholera Enterotoxin (ACE)

ACE was identified by Truckis *et al* in 1993 as another enterotoxin of *V. cholerae* (Trucksis *et al.*, 1997). ACE, a product of 11.5kDa upstream of Zot causes an increased fluid accumulation in rabbit ileal loops compared with mutant without *ace* gene. The predicted protein sequence had similarity to eukaryotic ion transporting ATPases, including human plasma membrane calcium pump (56%) and cystic fibrosis transmembrane conductance regulator a protein of cystic fibrosis gene (42%) (Trucksis *et al.*, 1997)

1.5.3.3 Zonula Occludens Toxin (Zot)

Zot predicted to be 44.8 kDa product of *zot* gene immediately upstream of CTX locus. Zot increases permeability of small intestine by inducing actin polymerization and loosening of tight junction (Baudry *et al.*, 1992, Fasano *et al.*, 1997, Uzzau *et al.*, 1999).

1.5.3.4 Haemolysin-cytolysin

A 65kDa protein encoded by *hly*A gene by El Tor strains (Ichinose *et al.*, 1987, Alm *et al.*, 1988). It was shown to be cytolytic for human erythrocytes by causing cell swelling (Huntley *et al.*, 1997). The hemolysin is capable of causing fluid accumulation in rabbit ileal loops but it was bloody with mucus rather than watery fluid (Ichinose *et al.*, 1987).

1.5.3.5 Repeat in Toxin (RTX)

RTX toxin is encoded by *rtx*A gene and located in actin cross-linking domain (ACD). The activity of RTX toxin leads to host cell rounding and actin depolymerization by convalently cross-linking actin monomers into dimer, trimer and higher multimer protein (Sheahan *et al.*, 2004, Cordero *et al.*, 2006). Analysis of ACD reveals a 412 amino acid region is essential for cross-linking activity (Sheahan *et al.*, 2004). The toxin has been associated with pathogenesis of cholera (Fullner *et al.*, 2002).

1.5.3.6 Shiga-like toxin

V. cholerae O1 was reported to produce a Shiga-like toxin and identified on the basis of cytotoxicity in HeLA cells that was neutralized by antibody raised against Shiga toxin purified from *Shigella dysenteriae* 1(O'Brien *et al.*, 1984). CVD 103-HgR a Δ*ctx* mutant that does not produce detectable Shiga-like activity, shows little or no reactogenicity in volunteers (Levine *et al.*, 1988a, Kaper *et al.*, 1995, Viret *et al.*, 1999).

1.6 Signs, symptoms and clinical features of cholera

The distinctive features of cholera are production of voluminous watery stool and dehydration if water lost in the stools is not replaced. Initial stools may contain fecal material but later on becomes white and opalescent. The diarrhea is painless and in some instances patients complains of intestinal cramping or generalized muscle cramping. Vomiting also noticed in some cases but the cause of vomiting is not well established (Bennish, 1994). Several complications can occur with cholera such as renal failure because of hypotension and miscarriage or premature birth in pregnant women if improper/insufficient treatment was given (Qadri *et al.*, 1997b).

Onset of diarrhea may be as short as 18 hours to 5 days and symptoms are generally sudden. Clinical features may range from asymptomatic to severe diarrhea. In cases of mild diarrhea, patient shows symptoms of dehydration and vomiting. But in cases of severe diarrhea, characteristic signs and symptoms of severe dehydration such as an increase in pulse rate and a decrease in pulse volume; hypotension; sunken eyes and cheeks; weakness; decrease urine output; and thirst can be seen (Qadri *et al.*, 1997b)

1.7 Diagnosis of Cholera

1.7.1 Isolation of *V. cholerae*

Until 1992, only *V. cholerae* O1 was known for cholera cases with epidemic proportion. New *V. cholerae* O139 emerged afterwards and caused cholera outbreak similar to *V. cholerae* O1. Culture and biochemical characteristics for both *V. cholerae* O1 and O139 is similar and only method of identification is by using O-antigen-group specific antisera (Bopp *et al.*, 1999). Alkaline peptone water (APW) and thiosulfate citrate bile salts sucrose agar (TCBS) are commonly used media for identification of *V. cholerae.* But in certain instances such as early stage of illness in patient, these two media are not necessary. It is always recommended to be used with convalesant patient, environmental sample and when presence of high number of competitive organisms in specimen is suspected (Bopp *et al.*, 1999)

1.7.1.1 Enrichment in alkaline peptone water (APW)

Enrichment in APW can enhance the isolation of *V. cholerae* if small numbers of vibrios are present, such in, convalesant patients and asymptomatic carriers. In 6 to 8 hours greater number of vibrios are present compared to non-vibrios(Bopp *et al.*, 1999). However, APW is not suitable for culture older than 8 hours since other organisms outnumber vibrios (Ichinose *et al.*, 1987, Glass *et al.*, 1989). APW can be inoculated with liquid stool, fecal material, or rectal swab. After incubation, one or two loopful of APW from top portion of APW should be subcultured on TCBS (Bopp *et al.*, 1999).

1.7.1.2 Selection on TCBS plate

TCBS is highly differential and selective medium and it does not require autoclaving. TCBS contains sucrose which allows differentiation of *V. cholerae* and *V. parahemolyticus* which appear as yellow colonies (sucrose-fermenting) and blue-green colonies (sucrose-nonfermenting) respectively after incubation at 35^0C-37^0C for 18-24 hours. Yellow, shiny colonies and 2-4mm in diameter are suspected as *V. cholerae* (Lotz *et al.*, 1983, Bopp *et al.*, 1999).

1.7.1.3 Growing on nonselective medium

Suspicious colonies of *V. cholerae* on TCBS should be subcultured on nonselective medium such as heart infusion agar slant. Nutrient agar is not recommended as it does not allow optimal growth for *V. cholerae* (Bopp *et al.*, 1999).

1.7.2 Screening for *V. cholerae*

1.7.2.1 Biochemical tests

Biochemical tests for screening of suspected *V. cholerae* isolates are not necessary prior to testing with O1 and O139 antisera. Biochemical tests for *V. cholerae* include oxidase test, string test, KIA and TSI, LIA and VP test. Oxidase test is useful in differentiating genera *Vibrio*, *Aeromonas*, *Pseudomonas* and few other organisms which are oxidase positive and *Enterobacteriaceae* which is oxidase negative (Kay *et al.*, 1994, Bopp *et al.*, 1999).

String test is positive for *V. cholerae* and usually negative for *Aeromonas* strains. Other *Vibrio* spp may give positive or weak positive result. Kligler iron agar (KIA) and triple sugar iron agar (TSI) can be used to rule out *Pseudomonas* spp and certain *Enterobacteriaceae*. KIA and TSI tests are based on production of acid or alkali on the slant and butt of the tube and also production of gas and H_2S. Lysine iron agar (LIA) can be used to screen out *Aeromonas* and certain *Vibrio* spp based on decarboxylation of lysine in which *V. cholerae* is positive (Bopp *et al.*, 1999).

1.7.2.2 Gram stain and wet mount

Gram stain of overnight growth on nonselective media will reveal small Gram negative curved rod. Microscopic examination under dark field or phase contrast by wet mount (bacterial suspension in saline) will reveal small, curved rods with darting motility (Bopp *et al.*, 1999).

1.7.3 Confirmative identification of *V. cholerae*

1.7.3.1 Latex agglutination using O1 and O139 antisera

Slide agglutination using polyvalent O1 or O139 can easily confirm and distinguish between O1 and O139 from suspected *V. cholerae*. Slide agglutination should be done using fresh growth from nonselective medium and not from TCBS as it can give false-negative (Bopp *et al.*, 1999).

1.7.4 Rapid identification Using Polymerase Chain Reaction (PCR)

PCR is a sensitive and rapid technique in delivering result compared to conventional methods (Hoshino *et al.*, 1998, Miyagi *et al.*, 1999). Direct detection of fecal *V. cholerae* is also possible using PCR (Miyagi *et al.*, 1999).

1.8 Treatment of cholera

Principal treatment of any given diarrheal diseases is fluid replacement. Administration of fluid and electrolytes by oral or intravenous is essential in treatment of cholera. Fluid therapy at early stage of illness is essential in replacing lost fluid and preventing dehydration and at a later stage of illness is essential in restoring fluid balance and preventing death (Bennish, 1994). With proper treatment, mortality is less than 1% from reported cases (Bopp *et al.*, 1999). Without treatment mortality rate for severe cholera is about 50% (Qadri *et al.*, 1997b). Antimicrobial therapy in other hand is useful, but not essential in treatment of cholera (Bennish, 1994, Bopp *et al.*, 1999).

1.8.1 Intravenous fluid therapy

Intravenous fluid therapy is important in rehydration treatment of severely dehydrated patients and patients with persistent vomiting and high rates of stool losses. Initially intravenous fluid therapy given to replace already lost fluid to restore blood volume and then to maintain ongoing losses. The volume of fluid required for rehydration depends on the severity of dehydration. Electrolytes composition of replacement fluid must be similar to that of lost fluid (Bennish, 1994, Qadri *et al.*, 1997a) (Table 1.2).

1.8.2 Oral fluid therapy

Oral rehydration solution (ORS) is preferred therapy in patients with little or no detectable dehydration and in severely dehydrated patients. The volume of ORS administrated must be sufficient to replace undetectable fluid and ongoing stool losses. Patients on ORS should be carefully monitored for first 24 hours as high purging rate, insufficient ORS intake and persistent vomiting can't replace fluid lost sufficiently (Bennish, 1994, Qadri *et al.*, 1997a).

1.8.3 Antimicrobial therapy

Antimicrobial therapy is not essential in treatment of cholera. But in cases of high purging rates it can shorten the duration of symptoms and decrease the volume of purged stool (Islam, 1987, Rabbani *et al.*, 1989) (Table 1.3).

1.8.4 Drug therapy

Research in drug usage as therapy for cholera is a new attractive approach in combating cholera. The approach includes inhibitions of cholera toxin binding to receptor and use of anti-secrectory agents such as cystic fibrosis transmembrane conductance regulator (CFTR) in managing severe diarrhoea in cholera. These drugs can be useful in addition to existing options of treatments for cholera (Thiagarajah and Verkman, 2005).

Stool or solution	Electrolyte				
	Sodium	Potassium	Chlorine	Base	Glucose
Cholera stool, [a] child	105	25	90	30	
Cholera stool, [a] adult	135	15	100	45	
Lactated Ringer's solution	130	4	109	28	
Isotonic saline (0.9%)	154		154		
Oral glucose-electrolyte solution	90	20	80	30	111

[a] When rate of stool output is 50ml/kg per hour or more

Table 1.2 Electrolyte concentrations of cholera stool and of solutions used for intravenous and oral fluid therapy (Kaper *et al*, 1995)

Antimicrobial agents	Dose	
	Adult	Pediatric
Tetracycline	500mg four times daily for 3 days or 1g as single dose	50mg/kg of body wt/day divided into four doses for 3 days
Doxycycline	300mg as single dose	Not evaluated
Furazoline	100mg four times daily for 3 days	5mg/kg of body wt/day divided into four doses for 3 days or 7mg of body wt as a single dose
Trimethoprim-sulfamethonazole	320mg trimethoprim-1600mg sulfamethonazole twice daily for 3 days	8mg Trimethoprim- 40mg sulfamethonazole/kg of body wt/day divided into two doses for 3 days
Norfloxacin	400mg twice daily for 3 days	Not recommended for use in children

Table 1.3. Choices of antimicrobial agents in therapy for cholera patients (Bennish, 1994)