CLONING AND MUTATION OF CHINESE HAMSTER OVARY CELL ELONGATION FACTOR (*cef*) OF *Vibrio cholerae*

by

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CERTIFICATE

This is to certify that the dissertation entitled

"CLONING AND MUTATION OF CHINESE HAMSTER OVARY CELL ELONGATION FACTOR (*cef*) OF *Vibrio cholerae*"

is the bona fide record of research work done by

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Jaime Jacqueline . J

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LIST OF ABBREVIATIONS

Ace	Accessory toxin
Acf	Accessory colonization factor
bp	Base pair
cAMP	Cyclic adenosine monophocphate
CDC	Center for Disease Control
cef	Cell elongation factor
CFU	Colony forming units
CHO	Chinese hamster ovary cells
CT	Cholera toxin
EtBr	Ethidium bromide
GTP	Guanosine 5'-triphosphate
IEF	Isoelectric focusing gel
Kb	Kilo base pair
kDa	Kilo Dalton
LB	Luria bertani medium
MW	Molecular weight
NAD	Nicotinamide adenine dinucleotide
nm	Nanometer
OD	Optical density
ORS	Oral rehydration salt
PCR	Polymerase chain reaction
PGE_2	Prostaglandin
SDS – PAGE	Sodium dodecyl sulphate – Polyacrylamide gel electrophoresis

Tcp	Toxin coregulated pili
WHO	World Health Organization
Zot	Zonula occludens toxin

ABSTRACT

Cholera caused by toxigenic *Vibrio cholerae* is a major public health problem confronting developing countries, where outbreaks occur in a regular seasonal pattern and are particularly associated with poverty and poor sanitation. The disease is characterized by a devastating watery diarrhea which leads to rapid dehydration, and death occurs in 50 to 70% of untreated patients. Prophylactic vaccination for cholera was initially thought as a means of control programme. Although cholera vaccine strains have been constructed with all known toxin (El Tor hemolysin, CT, Zot, and Ace) genes deleted or inactivated, some volunteers fed with these strain still develop diarrhea probably due to the presence of cell – associated CHO cell – elongating factor, *cef.*

In this study we therefore, have tried to clone and mutate this *cef* gene with the hope of eliminating its residual diarrheagenic effect, thus the development of improved VCUSM2 and VCUSM4 vaccine candidates. Accordingly, the *cef* factor was amplified from wild type *Vibrio cholerae* which was subsequently cloned into a cloning vector, pTZ57R/T at *Eco321* site. The *cef* gene in pTZ57R was digested with *Psyl* enzyme, to allowing insertional mutational ligation to occur at this site. The PCR amplified *kan* resistance gene cassette from pTOPO2.1 was used to insertionally mutate the cef gene as well as a marker for selection following transformation. The selected clones with *kan* marker were screened by PCR for confirmation and subsequently verified by DNA sequencing

1.0 INTRODUCTION

1.1 Cholera – The Disease

Cholera (8, 13, 14) is characterized by a severe watery diarrhea caused by toxigenic *Vibrio*[1] *cholerae*, which colonizes the small intestine and produces an enterotoxin, cholera toxin (CT). Cholera (or Asiatic cholerae) is generally a disease frequently associated with poverty and poor sanitation. Cholera infection is seen as a result of consumption of contaminated water supplies, consumption of shellfish harvested from feacally polluted coastal waters and also, from non-polluted waters since *Vibrio cholerae* O1 is part of the autochthonous microbiota of these waters. Hence, oral – fecal route of transmission of infection.

Symptoms of Asiatic cholera may vary from a mild, watery diarrhea to an acute diarrhea, with characteristic rice water stools, reflecting the conversion of the intestinal contents to a thin material like barley soup. Onset of the illness is generally sudden, with incubation periods varying from 6 hours to 5 days. The patient's eyes become gray and sink into their orbit (Figure 1.1). The skin is wrinkled, dry, and cold, and muscular cramps occur in the arms and legs. Despite the continuous thirst, sufferers cannot hold fluids. The blood thickens, urine production ceases, and the sluggish blood flow to the brain lead to shock and coma. In untreated cases, the mortality rate may reach 70 percent.

All people are believed to be susceptible to infection, but individuals with damaged or undeveloped immunity, reduced gastric acidity, or malnutrition may suffer more severe forms of the illness.

[1] Vibrio, derived from the Latin which means 'to vibrate'.

1.1.1 *Vibrio cholerae* – The causative organism

The bacterium responsible for Asiatic or epidemic cholera is *Vibrio cholerae* (Figure 1.2). It is first isolated by Robert Koch in 1883. *Vibrio cholerae* are Gram negative (Figure 1.3), usually curved rods (vibrios), measuring 3 – 4 μm with a single flagellum at one end. *Vibrio cholerae* are highly motile organism with a distinctive rapid to - and – fro (darting) movement and best seen using dark – field microscopy.

These organisms are sensitive to acid pH but tolerate alkaline pH very well. Alkaline peptone water (pH 8.6) containing 10g/l sodium chloride is a good enrichment medium for *Vibrio cholerae* (typically marine organisms) and other vibrio species growth. *Vibrio cholerae* is a sucrose fermenting organism, therefore it appears as large colonies of 2-3 mm in diameter, smoothly convex, yellow in color with a fine consistency on TCBS (Figure 1.4) culture plate. Definite and rapid growth (6-8 hours) in nutrient agar is another cultural feature of *Vibrio cholerae*. *Vibrio cholerae* grow over the temperature ranging from 16 – 42°C with optimum growth at 37°C. The organism is aerobic in nature and gives a very sparse growth appearance under anaerobic condition. (15)

The cholera vibrios cause many distinctive reactions. They are oxidase positive. They ferment sucrose and mannose but not arabinose, and they produce acid but not gas. *Vibrio cholerae* also possesses lysine and ornithine decarboxylase, but not arginine dihydrolase.

Vibrio species possess O (somatic) and H (flagellar) antigens. The flagellar antigens of *Vibrio. cholerae* are shared with many water vibrios and therefore are of no use in distinguishing strains causing epidemic cholera. O antigens, however, do distinguish strains of *Vibrio cholerae* into 139 known serotypes. Almost all of these strains of *Vibrio cholerae* are non-virulent. Until the emergence of the Bengal strain

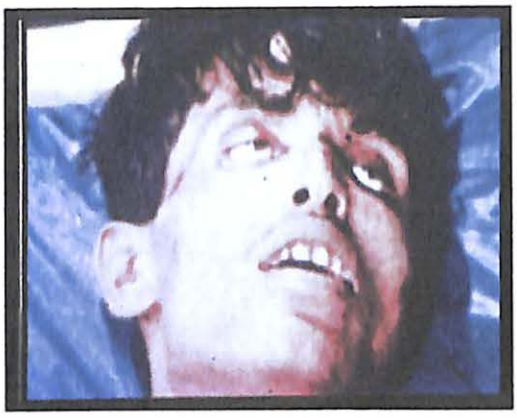


Figure 1.1

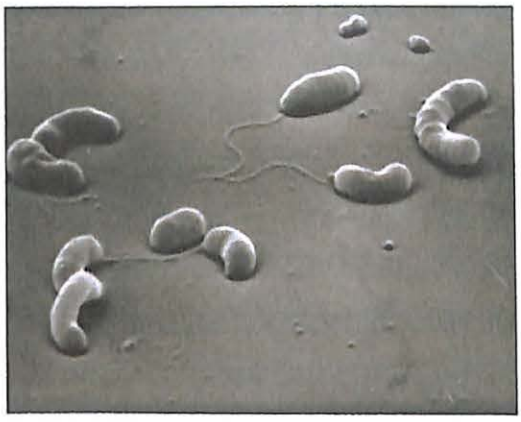


Figure 1.2

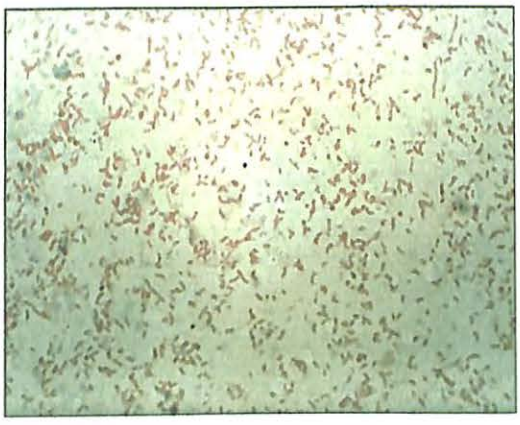
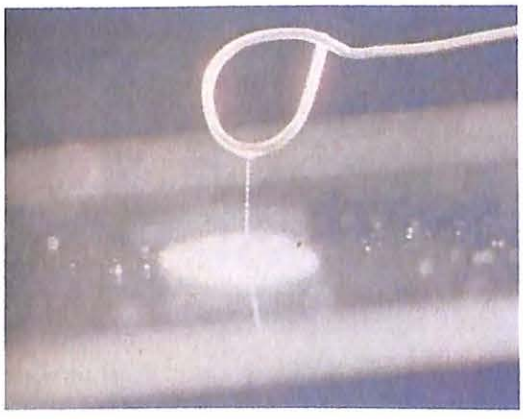


Figure 1.3

Figure 1.4



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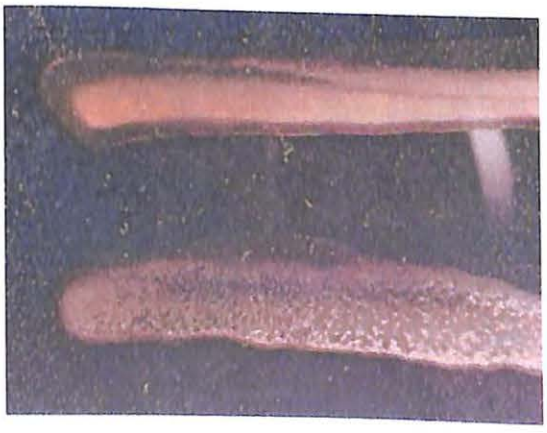

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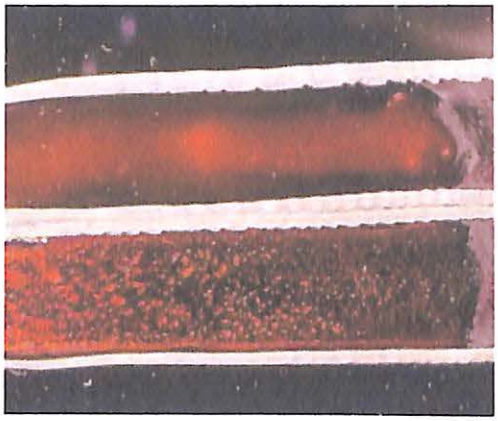

Figure 1.7

Figure 1.1 Patient's condition following diarrhea caused by *Vibrio cholerae*.

Figure 1.2 Electron micrograph of cholera causing organism – *Vibrio cholerae*.

Figure 1.3 Microscopic morphology of *Vibrio cholerae*; Gram stain.

Figure 1.4 *Vibrio cholerae* growth on TCBS culture plate. The yellow colonies results from citrate utilization and the formation of acid from utilization of the sucrose in the medium.(1)

Figure 1.5 *Vibrio cholerae* - Positive string test. *Vibrio cholerae* when mixed in a drop of 0.5% sodium deoxycholate, produce an extremely viscid suspension that can be drawn into a string with an inoculating loop. (1)

Figure 1.6 Positive slide agglutination test for *Vibrio cholerae* using polyvalent O anti – serum. (1)

Figure 1.7 Chicken erythrocyte agglutination test. Classic strains of *Vibrio cholerae* do not agglutinate chicken erythrocytes (top), in contrast to the El Tor biotype (bottom) which is capable of agglutinating the red cells. (1)

(which is "non-O1") a single serotype, designated O1, has been responsible for epidemic cholera.

Serogroup O1 *Vibrio cholerae* (13) has three distinct subtypes, named Ogawa, Inaba and Hikojima, and each biotype may display the "classical" or El Tor biotype. Test types employed in differentiating the 'Classical' and 'El Tor' biotype of *Vibrio cholerae*, includes production of a hemolysin, sensitivity to selected bacteriophages, sensitivity to polymyxin, the Voges-Proskauer test for acetoin and hemagglutination (Figure 1.7).

As mentioned earlier, both biotypes (El Tor and classical) are further sub – typed into Ogawa, Inaba and Hikojima (Figure 1.8). These serotypes are differentiated in agglutination and vibriocidal antibody tests on the basis of their dominant heat-stable lipopolysaccharide somatic antigens (Figure 1.6). The cholera group has a common antigen, A, and the serotypes are differentiated by the type-specific antigens, B (Ogawa) and C (Inaba). An additional serotype, Hikojima, which has both specific antigens, is rare.

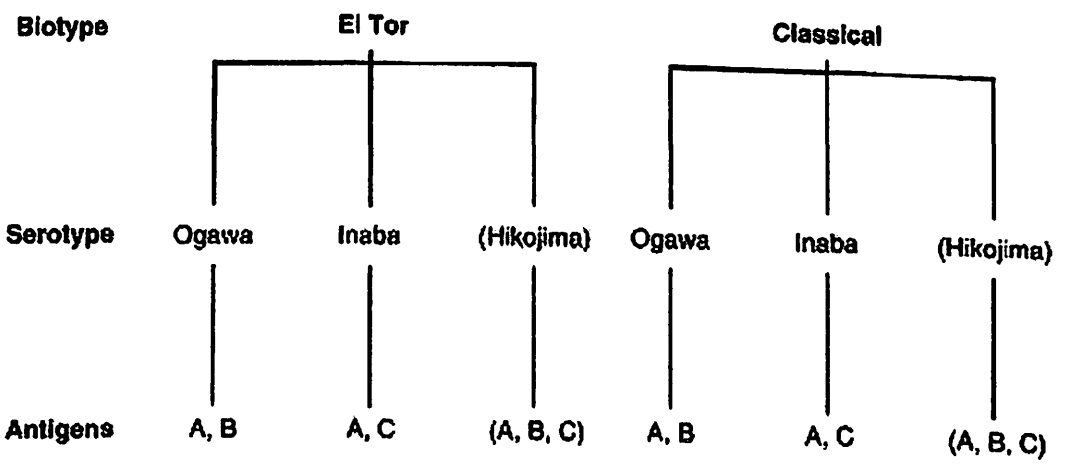

Figure 1.8 Biotype and sub-serotype classification of Serogroup O1 *Vibrio cholerae*.

1.1.2 Epidemiology (25)

Outbreaks of cholera cause deaths estimated at 120,000 annually worldwide and many more cases each year, of which the vast majority occurs in children. Hallmarks of the epidemiology of cholera include (i) a high degree of clustering of cases by location and season, (ii) highest rates of infection in children 1 to 5 years of age in areas of endemic infection, (iii) antibiotic resistance patterns that frequently change from year to year, (iv) clonal diversity of epidemic strains, and (v) protection against the disease by improved sanitation and hygiene and preexisting immunity. Cholera has been categorized as one of the "emerging and reemerging infections" threatening many developing countries.

Cholera appears to exhibit three major epidemiologic patterns: heavily endemic, neoepidemic (newly invaded, cholera-receptive areas), and, in developed countries with good sanitation, occasional limited outbreaks. These patterns probably depend largely on environmental factors (including sanitary and cultural aspects), the prior immune status or antigenic experience of the population at risk, and the inherent properties of the vibrios themselves, such as their resistance to gastric acidity, ability to colonize, and toxigenicity. Recent studies, however, have suggested that cholera vibrios can persist for some time in shellfish, algae or plankton in coastal regions of infected areas and it has been claimed that they can exist in "a viable but non-culturable state."

There have been eight great pandemics. Epidemic cholera was first described in 1563 by Garcia del Huerto, a Portuguese physician at Goa, India. The mode of transmission of cholera by water was proven in 1849 by John Snow, a London physician. In 1883, Robert Koch successfully isolated the cholera vibrio ("Kommabacillus") from the intestinal discharges of cholera patients and proved

conclusively that it was the agent of the disease during the fifth pandemics. The sixth pandemic and presumably the fifth pandemic were caused by *Vibrio cholerae* of the classical biotype. The seventh pandemic (Figure 1.9) is the most extensive of the pandemics in geographic spread and in duration, and the causative agent is *V.ibrio cholerae* O1 of the El Tor biotype. However, in 1992, *Vibrio cholerae* belonging to a non-O1 serogroup (now referred to as O139) caused large epidemics of cholera in India and Bangladesh and spread to some other countries; this may represent the beginning of the eighth pandemic.

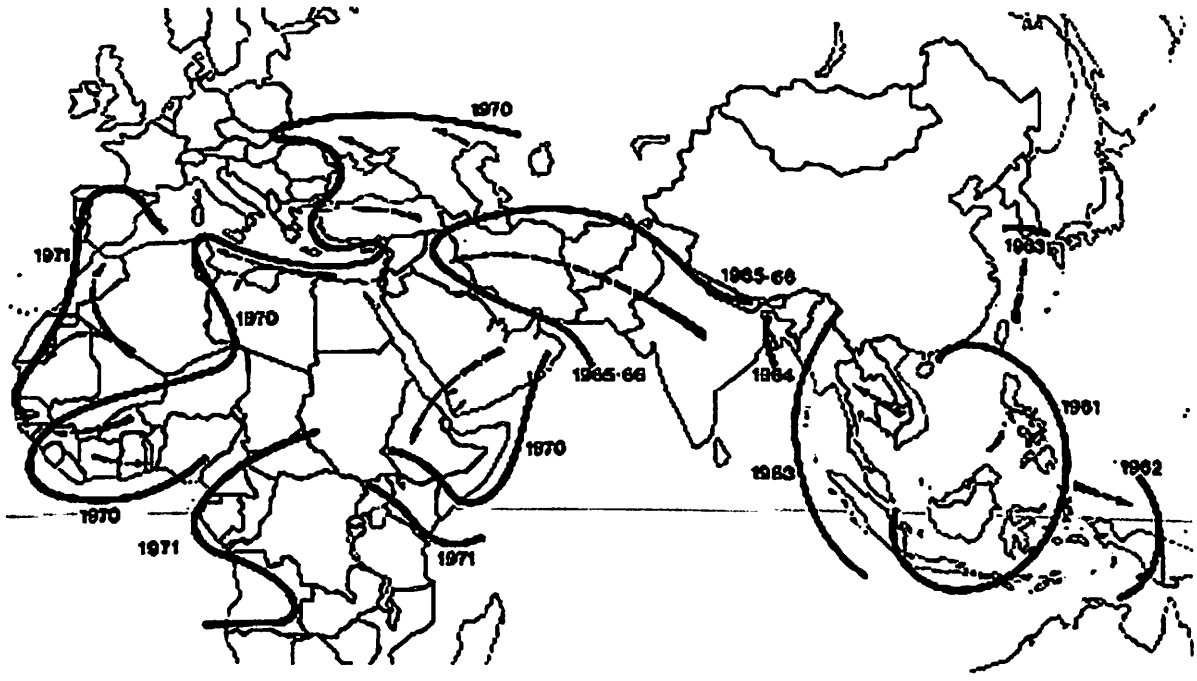

Figure 1.9 The global spread of cholera during the seventh pandemic, 1961-1971. (CDC) (28)

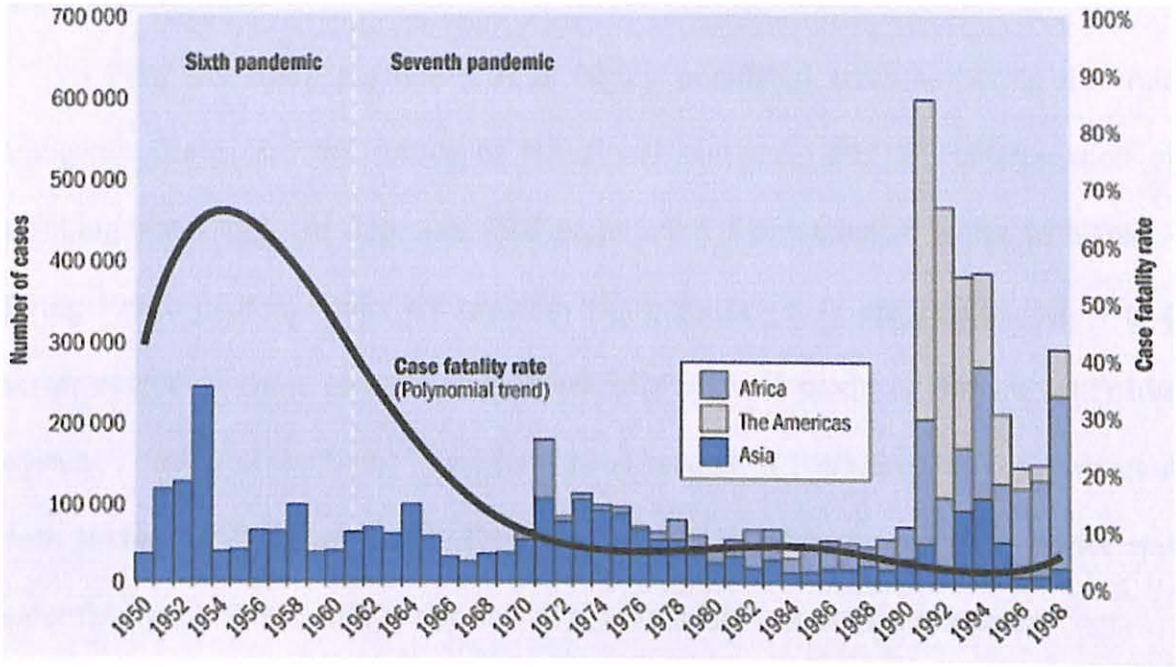

Figure 1.10 Cholera, reported number of cases and case fatality rates, 1950-1998.

Adapted from WHO Report on Global Surveillance of Epidemic; 2001 (28)

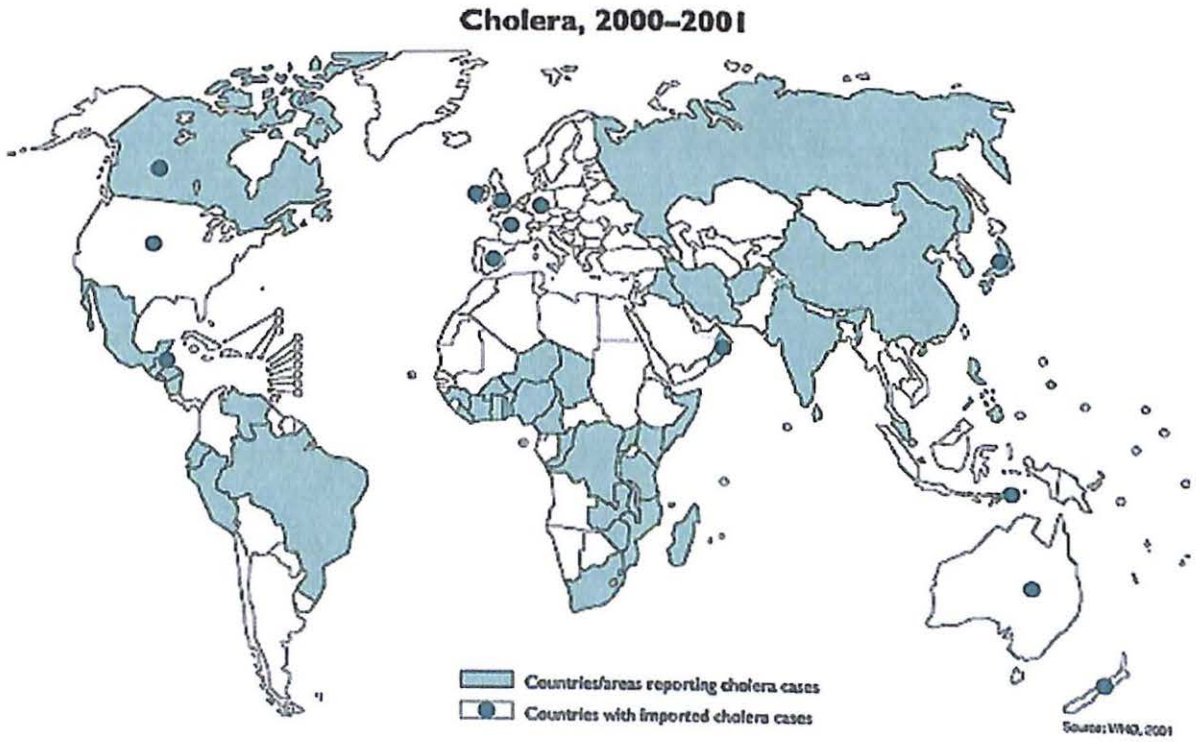

Figure 1.11 Cholera cases reported over the year 2000 – 2001.

1.1.3 Transmission

Poor sanitation practice (20) in highly populated areas harboring endemic toxigenic strains are the source of occasional outbreaks due to contamination of drinking water and / or improper food preparation. Contaminated water with free – living *Vibrio cholerae* cells are probably the main origin of epidemics, follow to a lesser extent by contamination food, especially seafood products (Figure 1.12) like oysters, crabs, and shellfish. There have been reports of bottled water contamination with toxigenic *Vibrio cholerae*. The evidence suggested that carbonated water was safer than non – carbonated water, due to the organism's poor acid tolerance.

Figure 1.12 Cholera causing contaminated seafood products.

Vibrio cholerae infections normally starts with the oral ingestion of food or water contaminated with *Vibrio cholerae* (Figure 1.13) (20). Subsequently, the bacteria must pass through and survive the gastric acid barrier of the stomach, then penetrate the mucus lining that coats the intestinal epithelia. The infectious dose in human is determined to be fairly high, ranging from 10^6 to 10^{11} colony forming units (c.f.u). The surviving bacterium adheres to and colonizes the intestinal epithelial cells, eventually producing the toxin substances and causing cholera symptoms. The

primary site of *Vibrio cholerae* colonization is the small intestine. During the transition from the environment to the human body the bacterial cells are exposed to series of changes, such as the temperature, acidity, and osmolarity. They must also survive the intestinal environment, which contains growth inhibitory substances like bile salts and organic acids, and also factors of the innate immune system, such as complement secreted by intestinal epithelial cells and defensins produced by Paneth cells. Therefore, *Vibrio cholerae* has developed the ability to survive, colonize, and express virulence factors in spite of, and possibly in response to, harsh environmental conditions.

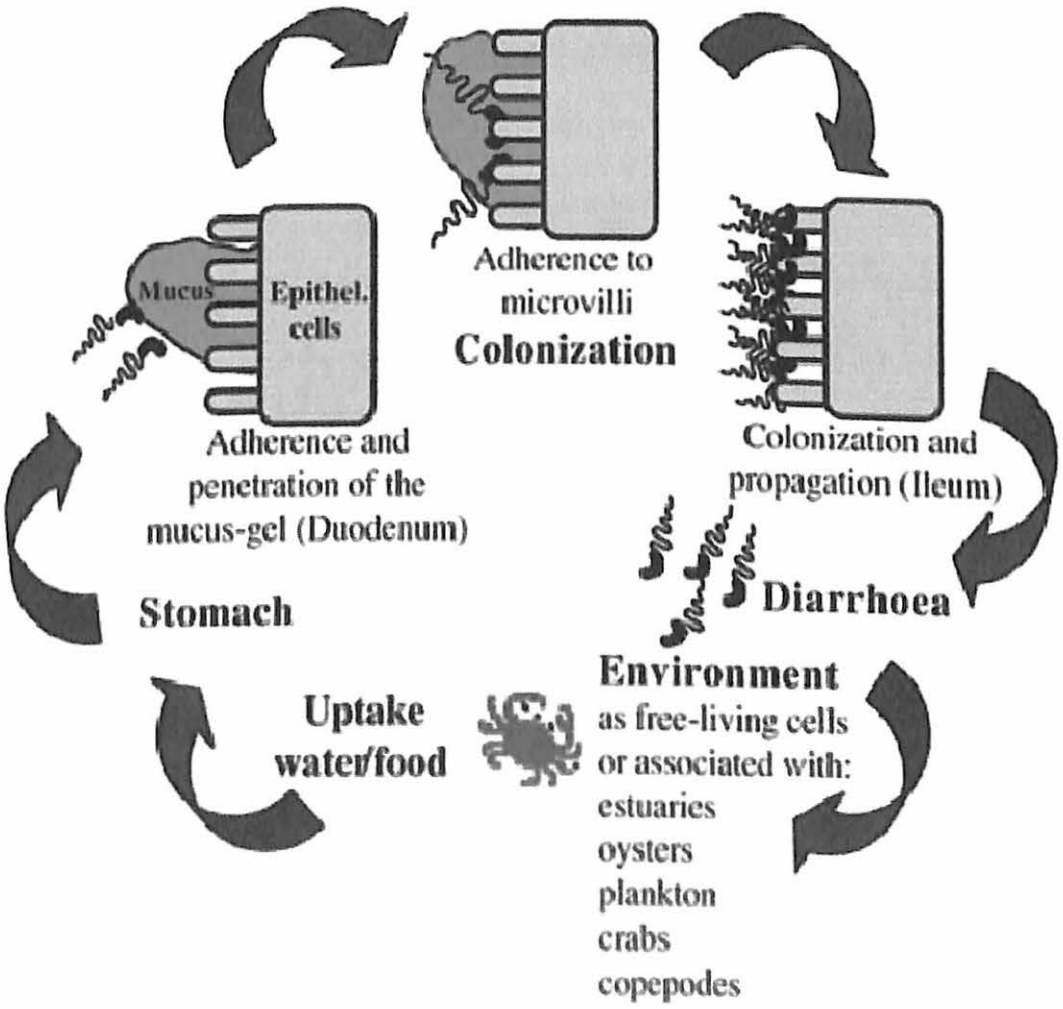

Figure 1.13 Infection cycle of *Vibrio cholerae*. (20)

1.1.4 Pathogenesis

Cholera toxin (26)

The growing cholera vibrios elaborate the cholera enterotoxin (CT or choleragen), a polymeric protein (MW 84,000) consisting of two major domains or regions. (21) The A region (MW 28,000), responsible for biologic activity of the enterotoxin, is linked by noncovalent interactions with the B region (MW 56,000), which is composed of five identical noncovalently associated peptide chains of MW 11,500. The B region, also known as choleragenoid, binds the toxin to its receptors on host cell membranes. It is also the immunologically dominant portion of the holotoxin. The structural genes that encode the synthesis of CT reside on a transposon-like element in the *Vibrio cholerae* chromosome. The amino acid sequences of these structurally, functionally, and immunologically related enterotoxins are very similar. Their differences account for the differences in physicochemical behavior and the antigenic distinctions that have been noted. There are at least two antigenically related but distinct forms of cholera enterotoxin, called CT-1 and CT-2. Classical O1 *Vibrio cholerae* and the Gulf Coast El Tor strains produce CT-1 whereas most other El Tor strains and O139 produce CT-2. Synthesis of CT and other virulence-associated factors such as toxin-coregulated pili are believed to be regulated by a transcriptional activator, Tox R, a transmembrane DNA-binding protein.

The molecular events in these diarrheal diseases involve an interaction between the enterotoxins and intestinal epithelial cell membranes. The toxins bind through region B to a glycolipid, the GM1 ganglioside, which is practically ubiquitous in eukaryotic cell membranes. Following this binding, the A region, or a major

portion of it known as the A1 peptide (MW 21,000), penetrates the host cell and enzymatically transfers ADP-ribose from nicotinamide adenine dinucleotide (NAD) to a target protein, the guanosine 5'-triphosphate (GTP)-binding regulatory protein associated with membrane-bound adenylate cyclase. The ADP-ribosylation reaction essentially locks adenylate cyclase in its "on mode" and leads to excessive production of cyclic adenosine 51-monophosphate (cAMP). The final effect is hypersecretion of chloride and bicarbonate followed by water, resulting in the characteristic isotonic voluminous cholera stool.

Intestinal colonization

There are several characteristics of pathogenic *Vibrio cholerae* that are important determinants of the colonization process. These include adhesins, neuraminidase, motility, chemotaxis and toxin production. If the bacteria are able to survive the gastric secretions and low pH of the stomach, they are well adapted to survival in the small intestine. *Vibrio cholerae* is resistant to bile salts and can penetrate the mucus layer of the small intestine, possibly aided by secretion of neuraminidase and proteases. They withstand propulsive gut motility by their own swimming ability and chemotaxis directed against the gut mucosa.

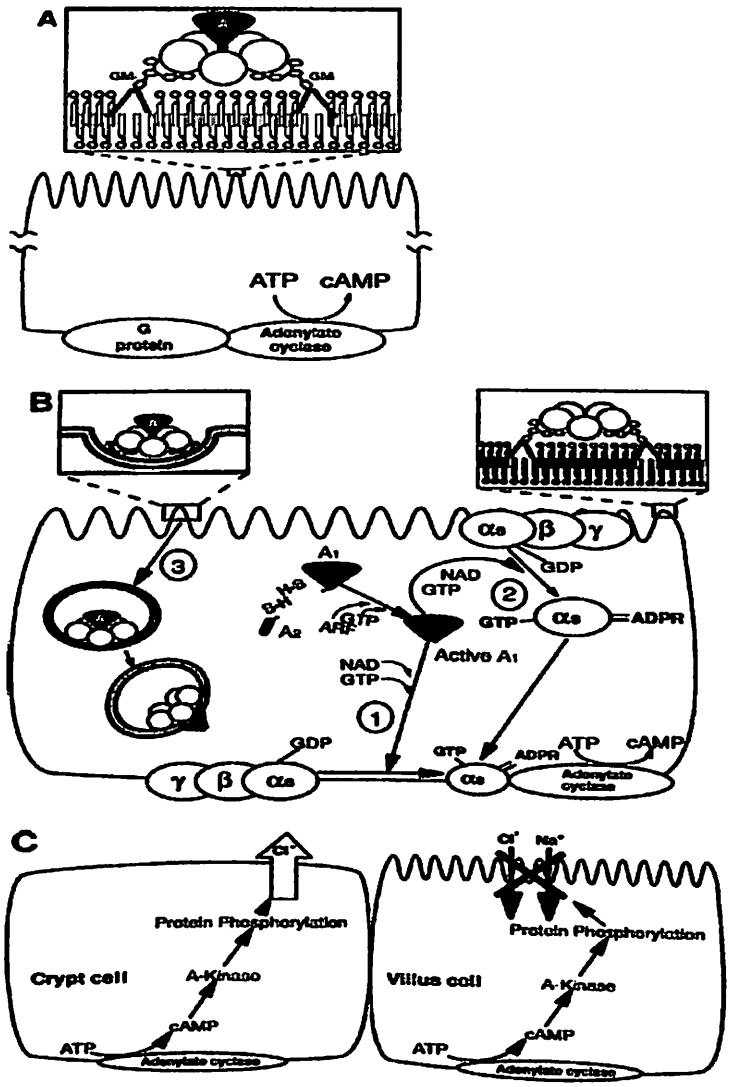

Figure 1.14 Mode of action of cholera toxin (CT) (12).

(A) Adenylate cyclase, located in the basolateral membrane of intestinal epithelial cells, is regulated by G proteins. CT binds via the B – subunit pentamer to the G_{M1} ganglioside receptor inserted into the lipid bilayer. (B) The A subunit enters the cell, probably via endosomes, and is proteolytically cleaved, with subsequent reduction of the disulfide bond to yield A_1 and A_2 peptides. The A_1 peptides is activated by ARFs and transfers an ADP – moiety (ADPR) from NAD to the α subunit of the Gs protein. The ADP ribosylated α subunit dissociates from the other subunits of Gsα and activates adenylate cyclase, thereby increasing the intracellular cAMP concentration. (C) Increased cAMP activates protein kinase A, leading to protein phosphorylation. In crypt cells, the protein phosphorylation leads to increase Cl^- secretion, and in villous cells, it leads to decreased $NaCl^-$ coupled absorption.

Specific adherence of *Vibrio cholerae* to the intestinal mucosa is probably mediated by long filamentous fimbriae that form bundles at the poles of the cells. These fimbriae have been termed Tcp pili (for toxin coregulated pili), because expression of these pili genes is co-regulated with expression of the cholera toxin genes. Not much is known about the interaction of Tcp pili with host cells, and the host cell receptor for these fimbriae has not been identified. Tcp pili share amino acid sequence similarity with N-methylphenylalanine pili of *Pseudomonas* and *Neisseria*.

Two other possible adhesins in *Vibrio cholerae* are a surface protein that agglutinates red blood cells (hemagglutinin) and a group of outer membrane proteins which are products of the *acf* (accessory colonization factor) genes. (13) Acf mutants have been shown to have reduced ability to colonize the intestinal tract. It has been suggested that *Vibrio cholerae* might use these non-fimbrial adhesins to mediate a tighter binding to host cells than is attainable with fimbriae alone.

Vibrio cholerae produces a protease originally called mucinase that degrades different types of protein including fibronectin, lactoferrin and cholera toxin itself. Its role in virulence is not known but it probably is not involved in colonization since mutations in the mucinase gene (designated hap for hemagglutinin protease) do not exhibit reduced virulence. It has been suggested that the mucinase might contribute to detachment rather than attachment. Possibly the vibrios would need to detach from cells that are being sloughed off of the mucosa in order to reattach to newly formed mucosal cells.

1.1.5 Treatment (12)

The key to therapy is provision of adequate rehydration until the disease has run its course (usually 1 – 5 days in the absence of antimicrobial therapy). Rehydration can be accomplished by, (a) intravenous infusion of fluids (in severe cases), (b) oral rehydration (in mild or moderate dehydration) and, (c) antimicrobial agents.

For both adults and childrens intravenous replacement solution should be infused as soon (rapidly) as possible. The precise rates of fluid administration should be adjusted according to monitoring of the patient's state of hydration and continuing stool losses. The intravenous fluid chosen for rehydration should be adequate to replace the isotonic fluid and electrolyte losses of cholera. The World Health Organization (WHO) recommends Ringer's lactate as the best commercial solution. Isotonic saline corrects hypovolumia, but potassium, base and glucose must be supplemented.

Patients with mild or moderate dehydration can receive initial fluid replacement to repair water and electrolyte deficits exclusively by the oral route. Oral rehydration are accomplished with an oral rehydration solution namely, ORS as recommended by WHO. This solution provides adequate quantities of electrolytes to correct the deficits associated with diarrheal dehydration and an optimal amount of glucose to facilitate the absorption of sodium and water. Vomiting rarely prevents successful use of ORS and is not a contraindication to its use.

Antimicrobial agents / therapy play a secondary in decreasing the severity of illness and the duration of excretion of the organism. This rapid clearance of vibrios

from stool may help to reduce secondary transmission of cholera, especially in hospitals, treatment centres, and refugee settings. Antimicrobial therapy is immediately started after initial rehydration and correction of acidosis. Tetracycline is the drug of choice for antibiotic treatment of cholera, also, Doxycycline. In instances of tetracycline resistant isolates, alternative antimicrobial agents may be issued, namely; Erythromycin, Trimethoprim – Sulfamethoxazole, Furazolidone and Ciprofloxacin.

1.1.6 Control and prevention (12)(20)(23)

Strategies for the prevention and control of cholera are:

a) Early detection of epidemics through diarrheal disease surveillance and investigation of severe cases and cluster of illness.
b) Education to promote good personal hygiene emphasizing proper hand-washing with soap and food preparation techniques. Bathing in potentially contaminated open water should also be discouraged.
c) Construction and maintenance of sewage disposal facilities.
d) Provision and protection of safe and plentiful water and storage (e.g.: homes and restaurants). Simple and inexpensive methods of domestic water disinfection and storage have been developed and they reduce the risk of cholera and diarrheal diseases. Point-of-use disinfection and the appropriate use of safe water storage containers are important in maintaining the water supply.

Besides hygienic and sanitary control measures and cholera surveillance, one of the main efforts at combating cholera epidemics is directed towards the development and use of modern vaccine strategies. Cholera is predicted to have a high potential for successful prevention by vaccination. Efficient protection is dependent on the biotype: infection with the classical biotype shows more conserved protection against different serotypes (Inaba, Ogawa, and Hikojima) of classical strains, and El Tor – derived protection is more labile against different El Tor isolates. Vaccination strategies consists of, (a) whole cell vibrio preparations and, (b) attenuated live vaccine strain, both which delivered orally.

Whole cell toxoids an oral combination vaccine (7) consisting of 1mg of purified CT B subunit and killed *V. cholerae* cells was first developed by Svennerholm and Holmgren. The whole – cell component in a dose of vaccine contains three *Vibrio cholerae* strains totaling 10^{11} cells. The strains represent both biotypes and serotypes and killed by either heat or formalin. However, this preparation only provides short lived preparation and proved to be reactogenic.

Live attenuated vaccine strains (preparation) (7) closely mimics infection derived immunity. Therefore, live strains are more efficiently taken up M cells, the major antigen presenting cells in the gut, than are killed cells. Attenuated strains have been constructed by both chemical mutagenesis and as well as using recombinant DNA techniques to prepare mutants which are deleted several hundred nucleotide base pairs.

1.2 Chinese Hamster Ovary (CHO) cell – elongating factor (cef) (5)(16)(24)

1.2.1 History – *cef* factor

In 1988, a genetically manipulated El Tor strain (JBK70) was developed in which the *ctx A* gene coding for the A subunit of cholera toxin (CT) was deleted. But when JBK 70 was fed to volunteer, more than half developed mild diarrhea. This findings had lead for search for a possible additional virulence. This search has resulted in the identification of zonula occludens toxin (*Zot*), and accessory toxin (*Ace*).

In 1993, when CVD110 a strain (derivative of El Tor Ogawa *Vibrio cholerae* O1) with the DNA sequences coding for *Zot*, *Ace* and CT deleted and with a mercury resistance gene and *ctxB* inserted into the *hlyA* locus, was fed to volunteers, 7 out of 10 still developed mild diarrhea. This result suggests the presence of an additional virulence factor in *Vibrio cholerae*.

Henceforth, in year 2000, a research team had finally identified the factor which causes the mild diarrheagenic effect following vaccination with potential vaccine strain (JBK 70) and, named it after its remarkable elongation effect on Chinese hamster ovary cells, thus cell elongation factor (*cef*). Though studies shows that *cef* factor was unrelated to cholera toxin, but it is still identified as enterotoxin as such, since it enabled to explain the production of gastrointestinal symptoms in volunteers given to *Vibrio cholerae* strains devoid of all other known toxins.

1.2.2 Characteristic of CHO cell elongation factor

CHO cell elongation factor was released from an El Tor strain of *Vibrio cholerae* when subjected to treatment with lysozyme – EDTA or polymyxin B. Lysozyme – EDTA released material was preferred to that polymyxin B lysate for purification.

The partly purified *cef* factor by anion chromatography and elution from a pH 3 - 9 iso-electric focusing gel shows specific activity of the partially purified *cef* factor approximately 4.8 X 10^5 CHO cell units / mg of protein and 2.56 X 10^6 CHO cell units / mg of protein respectively (see Figure 1.15).

Fraction	Total volume (ml)	Total protein (mg)	Total activity units[a]	Specific activity units/mg protein[b]	Recovery (%)	Purification (fold)
Step 1. EDTA-lysozyme	30[c]	33.3	4.8×10^5	1.44×10^4	100	1
Step 2. Anion exchange pool	6	1.6	7.68×10^5	4.8×10^5	160	34
Step 3. IEF gel eluate	20	0.1	2.56×10^5	2.56×10^6	53	178

[a] Amount of CHO cell units (highest dilution showing at least 50% elongation × volume).
[b] CHO cell units per mg of protein.
[c] From 4 l of culture.

Figure 1.15 Partial purification of a CHO cell elongating factor from *Vibrio cholerae* O1.

Biochemically, *cef* factor was found to be located at a pI of approximately 3.8 on an IEF gel. IEF gel eluate of *cef* factor on 8 – 25% SDS – PAGE gel shows CHO cell activity associated with one, 85 kDa band via silver stain or Coomassie blue stain (Figure 1.16).

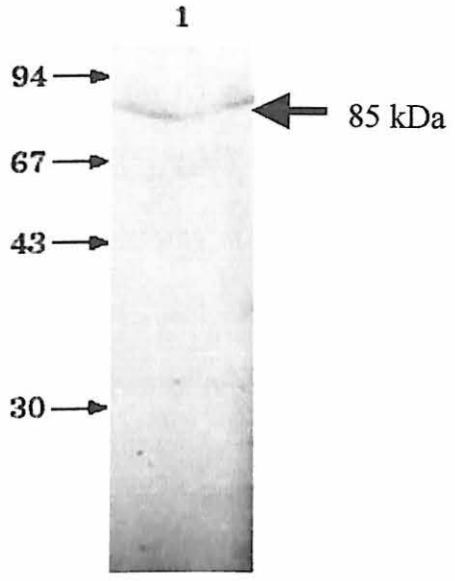

Figure 1.16 Analysis of partially purified *cef* on polyacrylamide gels. Lane 1 shows CHO cell activity at approximately 85 kDa on an 8 – 25% SDS – PAGE gel.

When blots of the 85 kDa band from the SDS – PAGE gel were subjected to amino terminal sequence analysis, the sequence was determined to be XGDETNSSGASTEVVYESYIQQ for amino acids 1 – 22. This unique amino acids sequence matched accurately the sequence data from *Vibrio cholerae* N16961 when the database from the Institute for Genomic Research (TIGR) was searched.

Physical heat treatment at 100°C completely destroys CHO cell activity of *cef* factor indicates that the factor is heat labile. Following treatment with subtilisin and proteinase K, approximately 80% and 50% of the original CHO cell activity respectively was lost suggesting susceptibility of this factor to the mentioned proteolytic enzymes.

Effects of various other treatments was also studied on CHO cell activity of *cef* toxin namely, heat treatment at 56°C for 15 min, treatment with anti – cholera toxin, ganglioside G_{M1}, etc (see Figure 1.17). However, unlike the above mentioned treatments after – effects, the CHO cell activity of *cef* toxin was retained – 100%. Activity which was not decreased by pre – incubation with anti – serum to cholera toxin suggests that *cef* is not immunologically related to cholera toxin. As for the uninhibited CHO cell activity of *cef* toxin by ganglioside G_{M1} and mixed gangliosides that was observed indicates that the cellular receptor for *cef* is not a ganglioside as seen in CT (cholera toxin) and LT (heat labile toxin).

Treatment	CHO cell activity remaining (%)
None (IEF eluate)	100
Heat 56°C 15 min	100
Heat 100°C 5 min	0
Anti-cholera toxin (1:5)	100
Normal rabbit serum (1:5)	100
Ganglioside G_{M1}	100
Mixed gangliosides	100
0.1 mg/ml subtilisin	20
0.125 mg/ml proteinase K	50

All results are based on at least three replicate experiments.

Figure 1.17 Effect of various treatments on CHO cell activity of *cef* toxin.

In the quest of studying enzymatic activity of *cef* toxin, the toxin was subjected for esterase and phospholipase assays. The toxin was assayed with a variety of esterase and phospholipase substrates. The enzymatic activity was then read at 405nm following 60 minutes at 37°C. Assay result shows that nil activity of lipase and phopholipase activity was detected. However, c*ef* activity was observed on 4 – 10 carbon ester when ester substrates were tested.

CHO treated with c*ef* toxin was found not to bear any effect on cyclic AMP (cAMP) and prostaglandin (PGE_2) in CHO cells following a 24 hours incubation. In contrast, CHO cells treated with CT shows greatly elevated cAMP levels and slightly elevated PGE_2 levels.

Cef was observed to exhibit enterotoxic activity via its effect in producing fluid accumulation in the sealed infant mice model. However, the exact mechanism by which the c*ef* causes CHO such effect remains unknown.

2.0 OBJECTIVES

- To clone the *cef* gene of *Vibrio cholerae* in pTZ57R vector.
- To obtain kanamycin resistance gene cassette from pTOPO vector by PCR amplification.
- To mutate the *cef* gene at *Psy1* site by insertion of kanamycin resistance gene cassette.

EXPERIMENTAL OVERVIEW