



UNIVERSITI PUTRA MALAYSIA

**MOLECULAR STUDIES OF VIBRIO CHOLERAЕ
STRAINS ISOLATED FROM LOCAL OUTBREAK**

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By

WAN SOMARNY BT. WAN MD. ZAIN

**Thesis Submitted to the School of Graduate Studies,
Universiti Putra Malaysia in Fulfilment of the Requirement for the
Degree of Master of Science**

July 2003



To Daddy , Mummy, sisters, brothers, in laws, and beloved husband,
Words cannot express how much I love you all and how blessed I am to have you
in my life. Thank you for seeing me through with your loves, strengths and
prayers.

To my friends:

Norazila Kassim Shaari, Azizah Mohd. Taib, Azlina Mohd. Daniel, Nur Asma
Ariffin, Samsiah Otoi, Ruzainah Bt. Ali, Nurmawati Syahroni, Shariza Nordin
Sharizah Alimat and Norin Zamiah Kassim Shaari

We are not lovers
because of the love
we make
but the love
we have

We are not friends
because of the laughs
we spend
but the tears
we save

I don't want to be near you
for the thoughts we share
but the words we never have
to speak

I will never miss you
because of what we do
but what we are
together

“Thanks for Everything”



Abstract of thesis presented to the Senate of Universiti Putra Malaysia in
fulfilment of the requirements for the degree of Master of Science

**MOLECULAR STUDIES OF *VIBRIO CHOLERAE*
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Chairman : Associate Professor Mariana Nor Bt. Shamsudin, Ph.D.

Faculty : Medicine and Health Sciences

Cholera caused by toxigenic *Vibrio cholerae* is a major public health problem in developing countries. Epidemiology surveillance of cholera and comparative analysis of strains collected during outbreaks has demonstrated clonal diversity among epidemic strains and a continual emergence of new clones of toxigenic *V. cholerae*. In the present study, the Random Amplified polymorphic DNA (RAPD) technique was performed to study the clonal diversity on twenty isolates of *V. cholerae* and five isolates of *V. harveyi* (as a comparison). Eighteen of *V. cholerae* isolates belong to the Ogawa serotypes, while two isolates were Inaba and 0139 Bengal serotypes, respectively. Fourteen out of twenty random primers yielded clear and reproducible bands. From the RAPD banding profiles, the polymorphism rates of bands were much higher between *V. cholerae* and *V. harveyi* than those found among *V. cholerae* isolates. According to the dendrogram generated from the RAPDistance software program,



V. cholerae and *V. harveyi* isolates were distinctly separated into their own groups. The Nei and Li's genetic distance obtained in this study ranged from 0.024691-0.644860 among *V. cholerae* isolates and 0.25368 - 0.633028 among *V. harveyi* isolates. The percentage of similarity among all *V. cholerae* isolates ranged from 32.5% to 99.4% and among *V. harveyi* isolates ranged from 32.5% to 73.0%. Analysis of RAPD bands using GEL COMPAR software at 50% similarity level could distinguish these isolates as well as Inaba and Bengal serotypes.

In this study, the Accessory cholerae enterotoxin (*ace*) gene, the third toxin of the *V. cholerae* virulence cassette was successfully amplified and isolated from 15 out of 20 local outbreak isolates of *V. cholerae*. However, this gene could not be amplified from the DNA in any of the five *V. harveyi* isolates. The amplification of *ace* gene produced a single band of 314 bp. In addition, the lipopolysaccharide (LPS) biosynthesis gene, *rfaz* was also successfully amplified in all *V. harveyi* isolates and 7 of the *V. cholerae* isolates whereby a single fragment of 1.2 kb was amplified. The specificity of the amplified products, *ace* and *rfaz* genes were then confirmed by the Southern hybridization technique using Ace and ZB3 biotinylated probes. The Southern hybridization results showed that all the PCR products amplified by *AceI/AceII* and *ZPfi/ZPr* primers are specific to Ace and ZB3 probes, respectively.



In order to allow the production of large quantities of the DNA fragment for physical and biology analysis, the *ace* and *rfaZ* genes were cloned into 2.1 TOPO TA vector and were transformed into TOP10, *E. coli* host strain. From the sequencing analysis using BLASTN package, several mutations were found in the sequence, resulting frameshift mutation. Due to the mutation in the *ace* gene sequence in 2.1 TOPO TA vector, a new expression vector pBAD/Thio TOPO was chosen to clone the *ace* gene. Since the gene was found to be toxic, the positive construct, pBAD10/*ace2* (*E. coli* TOP10 as a host strain) was retransformed into another *E. coli* host strain, LMG194 which produced a new construct, pBADLMG/*ace2*. Both of the constructs, pBAD10/*ace2* and pBADLMG/*ace2* were successfully transcribed by the detection of the *ace* gene in mRNA samples by Reverse transcriptase-PCR (RT-PCR). However, the pBAD10/*ace2* construct failed to express the *Ace* protein. On the other hand, the pBADLMG/*ace2* construct successfully expressed the soluble *Ace* protein even though there was an addition of two bases, T and G after the first ATG in the *ace* gene sequence. Analysis using the Biology Workbench 3.2 showed that the start codon is GTG, encoding the amino acid valine instead of methionine. The *Ace* fusion protein with the expected size of approximately 34 kDa was expressed after 72 hours induction with 0.02% arabinose.



Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

**KAJIAN MOLEKULAR KE ATAS *VIBRIO CHOLERAE*
STRAIN PENCILAN DARI WABAK TEMPATAN**

Oleh

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Pengerusi : Profesor Madya Mariana Nor Shamsudin, Ph.D.

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Penyakit kolera yang disebabkan oleh *Vibrio cholerae* yang toksigenik merupakan masalah kesihatan yang utama di negara membangun. Kajian epidemiologi kolera dan analisa perbandingan strain yang dikumpul sepanjang wabak menunjukkan kepelbagaian klonal terhadap strain epidemic dan kewujudan klon baru *V. cholerae* yang patogenik secara berterusan. Dalam kajian ini, teknik DNA polimorfik menggunakan primer rawak (RAPD) diaplikasikan untuk mengkaji kepelbagaian klonal bagi 20 isolat *V. cholerae* dan 5 isolat *V. harveyi* (sebagai perbandingan). Lapan belas isolat *V. cholerae* merupakan serotip Ogawa, sementara dua isolat yang masing-masing merupakan serotip Inaba dan 0139 Bengal. Empat belas daripada duapuluh primer rawak menghasilkan jalur yang terang dan kebolehlungan. Dari profil jalur RAPD kadar polimorfisma adalah lebih tinggi di antara *V. cholerae* dan *V. harveyi* berbanding yang ditemui di antara isolate *V. cholerae*. Merujuk kepada dendrogram yang dihasilkan dari



program RAPDistance, isolat *V. cholerae* dan *V. harveyi* dibahagikan kepada kumpulan mereka tersendiri. Jarak genetik Nei dan Li yang diperolehi dari kajian ini, berjulat di antara 0.024691 – 0.644869 iaitu di antara isolat *V. cholerae* dan 0.025368 – 0.633028 bagi isolate *V. harveyi*. Peratus kesamaan di antara isolat *V. cholerae* adalah berjulat 32.5% hingga 99.4% manakala antara isolat *V. harveyi* adalah berjulat dari 32.5% hingga 73.0%. Analisis jalur RAPD dengan menggunakan software GEL COMPAR menunjukkan nilai kesamaan pada tahap 50% berupaya untuk membezakan isolat – isolat ini dan begitu juga serotip Inaba dan Bengal.

Dalam kajian ini, gen *ace* iaitu yang merupakan toksin ketiga bagi virulen kaset *V. cholerae* telah berjaya diamplifikasikan dan diisolat iaitu sebanyak 15 dari 20 keseluruhan isolat serangan tempatan bagi *V. cholerae*. Namun begitu, gen ini tidak diamplifikasikan dari DNA kelima – lima isolat *V. harveyi*. Amplifikasi gen *ace* menghasilkan satu jalur tunggal pada 314 bp. Di samping itu, gen biosintesis lipopolisakarida (LPS), *rfaZ* juga berjaya diamplifikasikan dalam kesemua isolat *V. harveyi* dan 7 isolat *V. cholerae* di mana satu jalur tunggal pada 1.2 kb telah diamplifikasikan. Spesifikasi terhadap produk amplifikasi, gen *ace* dan *rfaZ* kemudiannya dipastikan dengan teknik ‘Southern hybridization’ menggunakan probe Ace dan ZB3 yang dilabelkan dengan biotin. Keputusan ‘Southern hybridization’ menunjukkan kesemua produk PCR yang diamplifikasikan oleh primer *AceII* dan *ZPfZPr* adalah spesifik terhadap probe Ace dan ZB3.



Untuk membenarkan penghasilan jalur DNA dalam kuantiti yang banyak untuk analisis fizikal dan biologi, gen *ace* dan *rfaZ* telah diklonkan dalam 2.1 TOPO TA vektor dan ditransformasikan ke dalam TOP10, strain perumah *E. coli*. Daripada analisis penjujukan dengan menggunakan pakej BLASTN, terdapat beberapa mutasi telah ditemui dalam jujukan yang menghasilkan mutasi 'frameshift'. Berdasarkan kepada mutasi dalam jujukan gen *ace* di dalam vector 2.1 TOPO TA, satu vektor ekspresi baru iaitu pBAD/Thio TOPO telah dipilih untuk pengklonan gen *ace*. Memandangkan gen tersebut adalah toksik, pembentukan positif pBAD10/*ace2* telah ditransformasi sekali lagi ke dalam strain perumah *E. coli* yang lain iaitu LMG194 di mana pembentukan yang baru dihasilkan iaitu pBADLMG/*ace2*. Kedua-dua penghasilan ini pBAD10/*ace2* dan pBADLMG/*ace2* telah berjaya ditranskripsikan dengan pengenalpastian gen *ace* dalam sampel mRNA dengan menggunakan kaedah transkripsi berbalik – PCR (RT-PCR). Walaubagaimanapun, pembentukan pBAD10/*ace2* telah gagal untuk mengekspres protein *Ace*. Sebaliknya, pembentukan pBADLMG/*ace2* telah berjaya mengekspres protein *Ace* larut walaupun dengan penambahan dua bes, T dan G selepas ATG yang pertama dalam jujukan gen *ace*. Analisis dengan Biology Workbench 3.2 menunjukkan kodon permulaan ialah GTG yang mengkodkan asid amino valine selain dari methionine. Protein *Ace* yang bersaiz kira-kira 34 kDa diekspresikan setelah 72 jam selepas menginduksikannya dengan 0.02% arabinose.



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TABLE OF CONTENTS

	Page
DEDICATION	ii
ABSTRACT	iii
ABSTRAK	vi
ACKNOWLEDGEMENTS	ix
APPROVAL	x
DECLARATION	xii
LIST OF TABLES	xvii
LIST OF FIGURES	xviii
LIST OF ABBREVIATIONS	xxi
 CHAPTER	
1 INTRODUCTION	1
2 LITERATURE REVIEW	10
Cholera	10
<i>Vibrio cholerae</i>	13
Structure and Environment Survival	13
Classification	13
Pathogenicity	16
Epidemiology	19
CTX Genetic Element	20
Lipopolysaccharide	22
History of Vaccination Against <i>Vibrio cholerae</i>	24
DNA Vaccine	27
Advantages of DNA Vaccine	28
Polymerase Chain Reaction	29
Random Amplified Polymorphic DNA	33
The Use of RAPD	34
RAPD Markers	36
Southern Blotting	37
Cloning	38
Plasmid Vectors	39
Restriction Enzyme	40
Synthesis of Protein	41
Western Blotting	44
3 MATERIALS AND METHODS	45
Sources of Bacteria	45
Biochemical Tests	47
Growth on TCBS Agar	47
Growth on Cytophaga Agar	47



Gram Staining	48
Growth in Peptone Water With Different concentration of NaCl	49
Oxidase Test	49
Catalase Test	50
Sensitivity to Vibriostatic Compound 0/129	50
Oxidative and Fermentative Test	51
Genomic Study	52
Propagation of Bacterial Culture	52
DNA Extraction	53
Quantitation of Genomic DNA	54
Random Amplified Polymorphic DNA	55
RAPD Analysis	57
Polymerase chain reaction (PCR)	58
Detection and Amplification of <i>ace</i> and <i>rfaZ</i> genes	58
PCR Amplification	58
Agarose Gel Electrophoresis of PCR Product	60
Southern Hybridization	60
Gel Preparation	61
Capillary Transfer	61
Prehybridization Procedure	63
Hybridization Procedure	63
Detection with Chemiluminescent Kit	64
Detection of DNA	65
Development of X-ray Film	66
Cloning of <i>ace</i> Gene	67
Ligation Procedure	67
Transformation of Competent TOP10 Cells	67
Small Scale Preparation Plasmid DNA	69
Harvesting	69
Plasmid Extraction	69
Restriction Enzyme Analysis of 2.1-TOPO Clones	70
DNA Sequencing	70
Subcloning of <i>ace</i> Gene	71
Ligation of <i>ace</i> Gene	71
Transformation of Competent TOP10 Cells	71
Analysis of Positive Clones	73
Confirmatory PCR	73
Restriction Enzyme Digestion of Recombinant Plasmid	73
Preparation of Competent Cells	74
Transformation of Competent LMG194 Cells	75
Messenger RNA (mRNA) Analysis	76
Isolation of mRNA	76
Washing Oligo (dT) Cellulose Procedure	77
Elution of mRNA Procedure	77



Reverse Transcription-PCR (RT-PCR)	78
Expression of Fusion Protein	79
Protein Analysis	80
Preparation of Protein Sample	80
Sodium Dodecyl Sulphate-Polyacrylamide	
Gel Electrophoresis (SDS-PAGE)	80
Silver Staining Procedure	81
Western Blotting	82
Preparation for Blotting	82
Detection of Fusion Protein Using Anti V5-HRP	83
4 RESULTS	85
Growth on TCBS and Cytophaga Agar	85
Biochemical Tests	85
Genomic Extraction	88
Random Amplified Polymorphic DNA (RAPD)	90
RAPD Analysis	99
Genetic Distance and Percentage of Similarity	99
Dendrogram	100
RAPD Analysis by GEL Compar Ver 4.1	103
Amplification of <i>ace</i> and <i>rfaZ</i> Genes by PCR	107
Confirmation of <i>Ace</i> and <i>rfaZ</i> Genes by Southern Hybridization	110
Cloning of <i>ace</i> and <i>rfaZ</i> Genes into 2.1-TOPO vector	113
Analysis of clones	114
Confirmatory Analysis of 2.1TOPO/ <i>ace</i> and	
2.1TOPO/ <i>rfaZ</i> clones	117
Sequencing of 2.1TOPO/ <i>ace</i> Clone	122
Sequence Analysis	123
Subcloning of <i>ace</i> Gene into pBAD/Thio-TOPO Vector	124
Analysis of Recombinant pBAD/TOP10 Clones by	
Confirmative PCR and Restriction Enzymes Digestion	124
Sequence Analysis of pBAD10/ <i>ace2</i> Clone	128
CLUSTAL W Multiple Sequence Alignment	129
Amino Acid Alignment	130
CLUSTAL W Dendrogram	131
Retransformation of Recombinant pBAD10/ <i>ace2</i> in	
LMG194 Host Strain	132
Analysis of LMG/ <i>ace</i> Clones	133
Restriction Enzyme Digestion	133
Confirmative PCR (cPCR) Analysis	134
Sequence Analysis of pBADLMG/ <i>ace2</i>	137
CLUSTAL W Multiple Sequence Alignments	138
Amino Acid Alignment	139
Clustal W Dendrogram	140
Reverse Transcription-PCR (RT-PCR)	141



	Expression of <i>ace</i> Gene in TOP10 Cell	142
	Expression of <i>ace</i> Gene in LMG194 Cell	143
5	DISCUSSION	148
	Growth on TCBS Media	148
	Biochemical Tests	148
	Genomic DNA Extraction	151
	RAPD Analysis	152
	Amplification of <i>ace</i> and <i>rfaZ</i> Genes	160
	Confirmation of <i>ace</i> and <i>rfaZ</i> Genes by Southern hybridization	163
	Cloning of Accessory Cholera Enterotoxin (<i>ace</i>) Gene	165
	Amplification of <i>ace</i> Gene by Reverse Transcription –PCR (RT-PCR)	169
	Expression of <i>Ace</i> Protein	171
6	CONCLUSIONS	178
	REFERENCES	181
	APPENDICES	202
	BIODATA OF THE AUTHOR	216
	PUBLICATIONS	217



LIST OF TABLES

Table		Page
1.	Identity of <i>V. cholerae</i> and <i>V. harveyi</i> isolates	46
2.	Sequences of primers screened for random amplification, RAPD procedure (Kit AE)	56
3.	The sequence of primers and biotin labeled probes used in detection and amplification of <i>ace</i> and <i>rfaZ</i> genes	59
4.	Biochemical characterizations of <i>V. cholerae</i> and <i>V. harveyi</i> isolates	87
5.	The concentrations and purities of the genomic DNA for <i>V. cholerae</i> and <i>V. harveyi</i> isolates	89
6.	Value of genetic distance and percentage of similarity between 20 <i>V. cholerae</i> and <i>V. harveyi</i> isolates based on RAPD data	101



LIST OF FIGURES

Figure		Page
1.	Net movement of ions and water across a normal intestinal a mucosa affected by cholera toxin	12
2.	Subunit of cholera toxin (cholera toxin)	16
3.	Mechanism of action of cholera enterotoxin	18
4.	Schematic diagram of cholera toxin genes on a 4.5 kb core region with flanking RSI element	22
5.	Polymerase chain reaction principle	32
6.	Schematic diagram of the capillary transfer	62
7.	Schematic diagram of 2.1-TOPO TA vector	68
8.	Schematic diagram of pBAD/Thio-TOPO vector	72
9.	Schematic diagram of Western blotting	84
10.	Yellow colonies of <i>V. cholerae</i> isolates on TCBS	86
11.	Total genomic DNA extracted using QIAGEN Tissue kit	88
12.	RAPD patterns obtained with primer OPAE-1	92
13.	RAPD patterns obtained with primer OPAE-2	92
14.	RAPD patterns obtained with primer OPAE-4	93
15.	RAPD patterns obtained with primer OPAE-6	93
16.	RAPD patterns obtained with primer OPAE-7	94
17.	RAPD patterns obtained with primer OPAE-8	94
18.	RAPD patterns obtained with primer OPAE-10	95
19.	RAPD patterns obtained with primer OPAE-12	95



20.	RAPD patterns obtained with primer OPAE-13	96
21.	RAPD patterns obtained with primer OPAE-14	96
22.	RAPD patterns obtained with primer OPAE-15	97
23.	RAPD patterns obtained with primer OPAE-16	97
24.	RAPD patterns obtained with primer OPAE-17	98
25.	RAPD patterns obtained with primer OPAE-18	98
26.	Clustering between <i>V. cholerae</i> and <i>V. harveyi</i> species	102
27.	Dendrogram generated by Gel Compar analysis by OPAE-1/OPAE-4	104
28.	Dendrogram generated by Gel Compar analysis by OPAE-7/OPAE-13	105
29.	Dendrogram generated by Gel Compar analysis by OPAE-18	106
30.	Amplification of <i>ace</i> gene by PCR	108
31.	Amplification of <i>rfaZ</i> gene by PCR	109
32.	Confirmation of <i>ace</i> gene by Southern hybridization	111
33.	Confirmation of <i>rfaZ</i> gene by Southern hybridization	112
34.	The <i>ace</i> clones on the LB agar/amp	115
35.	The <i>rfaZ</i> clones on the LB agar/amp	116
36.	Screening of <i>ace/2.1</i> TOPO clones by PCR	118
37.	Restriction endonuclease digestion of <i>ace/2.1</i> TOPO clones	119
38.	Screening of <i>rfaZ/2.1</i> TOPO clones by PCR	120
39.	Restriction endonuclease digestion of <i>rfaZ/2.1</i> TOPO clones	121
40.	Analysis of <i>ace</i> gene sequence by BLAST	123
41.	Screening of <i>ace</i> genes from the pBAD/TOP10 clones by the PCR	126



42.	Restriction endonuclease digestion of pBAD/TOP10-positive clones	127
43.	Sequence analysis of <i>ace</i> gene by CLUSTAL W program	129
44.	Analysis of amino acid on pBAD10/ <i>ace2</i>	130
45.	Cluster of recombinant pBAD10/ <i>ace2</i> (<i>ace</i> /pBAD10)	131
46.	The LMG/ <i>ace</i> cloned colonies on the RM/amp agar	133
47.	Analysis of LMG/ <i>ace</i> constructs by enzymatic digestions, <i>Pme</i> I and <i>Nco</i> I	135
48.	Confirmative PCR (cPCR) on LMG/ <i>ace</i> clones	136
49.	Sequence analysis of <i>ace</i> gene by CLUSTAL W program	138
50.	Amino acid analysis on pBADLMG/ <i>ace2</i> clone	139
51.	Cluster of recombinant pBADLMG/ <i>ace2</i> (ACE gene)	140
52.	RT-PCR amplification	142
53.	Analysis of <i>Ace</i> protein from pBADLMG/ <i>ace2</i> clone in supernatant by Western blot technique	145
54.	Analysis of <i>Ace</i> protein from pBADLMG/ <i>ace2</i> clone in pellet by Western blot technique	146
55.	Western blot analysis on TOP10 clones	147

LIST OF ABBREVIATIONS

<i>ace</i>	accessory cholera enterotoxin
AP-PCR	arbitrary primed polymerase chain reaction
APS	ammonium persulphate
ASW	artificial sea water
ATCC	American type culture collection
bp	base pair
CA	cytophaga agar
cAMP	cyclic adenosine 5'-monophosphate
cDNA	complementary DNA
CT	cholera toxin
CTX ϕ	cholera-toxin phage
<i>ctxA</i>	cholera toxin A
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetraacetatic acid
GM1	monosialosyl ganglioside
GTP	guanosine 5'-triphosphate
H ₂ O ₂	hydrogen peroxide
H ₂ O	water
HCl	hydrochloride acid
HRP	horseradish peroxidase
IF	initiation factors
IgA	immunoglobulin A
kb	kilo base pair
KCl	potassium chloride
kDa	kilo daltons
KDO	2-keto-3-deoxyoctonate
LB	luria bertani
LPS	lipopolysaccharide
MgCl ₂	magnesium chloride

mRNA	messenger Ribonucleic Acid
NaCl	sodium chloride
NJTREE	neighbour joining Tree
OD	optical density
OMPs	outer membrane proteins
PCR	polymerase chain reaction
PEG	polyethylene glycol
PFGE	pulsed field gel electrophoresis
pmoles	picomoles
RAPD	Random Amplified Polymorphic DNA
RFLPs	Restriction Fragment Length Polymorphisms
RNA	ribonucleic acid
RS1	repetitive sequence
RT-PCR	reverse transcription-PCR
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate – polyacrylamide gel electrophoresis
TBE	tris–borate-EDTA
TCBS	thiosulphate citrate bile salt sucrose
UV	ultraviolet
V	volt
WHO	World Health Organization
<i>zot</i>	zonula occludens toxin
µg	microgram
µl	microliter



CHAPTER ONE

INTRODUCTION

Vibrio cholerae is a highly motile gram-negative bacterium and is the causative agent of epidemic cholera. Cholera, a highly epidemic diarrheal disease, is caused by toxin-producing strains of *Vibrio cholerae* 01. *V. cholerae* of serogroup 01 produces a potent heat-labile enterotoxin, termed cholera toxin (CT). Cholera is caused by the action of cholera toxin, CT which through its stimulation of intestinal adenylate cyclase activity gives rise to the characteristic excessive electrolyte secretion and fluid loss from the small intestine (Osek *et al.*, 1992). In its severe form, the disease can lead to fatal diarrhea dehydration and typically occurs in explosive epidemic whereby the small quantities of purified cholera toxin at 5 µg, can cause copious purging when fed to volunteers (Levine *et al.*, 1983). In individuals infected with *V. cholerae*, cholera toxin is responsible for the drastic intestinal electrolyte secretion and fluid loss leading to the clinical state of cholera (Holmgren, 1981 ;Finkelstein, 1984). *V. cholerae* 01 are classified into two biotypes, classical and *El Tor*, and into two major serotypes, *Inaba* and *Ogawa* (Ghosh *et al*, 1996) while the Hikojima serotype has been rarely reported (Faruque *et al.*, 1998). *V. cholerae* 01 is defined by agglutination in 0 group 1 in which 0 antigens (somatic antigens) is the target for the specific antiserum directed against the lipopolysaccharide component of the cell wall.



In 1992, cholera was caused by a new serotype 0139 synonym Bengal (Shimada *et al.*, 1993). This serotype is the latest serogroup of *V. cholerae* that emerged in epidemic proportions in India and Bangladesh and is also known as a first non-01 group. *V. cholerae* non-01 serotypes are widely distributed in the aquatic environment and are free-living in nature. This serovar is identified by the absence of agglutination in 0 group 1 specific antiserum (Albert *et al.*, 1993) but agglutinated in 0 group 139 specific antiserum and also by the presence of a capsule (Jonson *et al.*, 1999). *V. cholerae* non-01, were not known to be associated with such a large outbreak of diarrhea before the present of epidemic (Faruque *et al.*, 1994). Moreover, this serotype was known to produce cholera toxin at a very low frequency (Janda *et al.*, 1988). It has often been identified as the causative agent of sporadic cases (Karaolis *et al.*, 1998 ;Russell *et al.*, 1992) and localized outbreaks (Craig *et al.*, 1981). However, in 1992 a 0139 serogroup strain emerged and caused epidemic disease throughout India and Bangladesh which replaced 01 strains of *V. cholerae* as the predominant cause of cholera on the Indian subcontinent (Albert *et al.*, 1993 and Bhattacharya *et al.*, 1993).

Strains of *V. cholerae* 01 impaired in their ability to colonize the gut are able to produce their symptoms of the disease and elaborate a powerful exotoxin, CT. Cholera toxin (84 kDa) is a fairly complicated molecule which is composed of one A subunit of 27 kDa and five B subunits, each of 11 kDa (Ghosh *et al.*, 1996). Although CT is responsible for severe dehydrating diarrhea associated with *V. cholerae*, the search for the additional enterotoxin produced by

