

**UNDERSTANDING THE MOLECULAR
ENTEROPATHOGENESIS OF MARTX TOXIN GENES
AND CHOLERA TOXIN GENE MUTATIONS OF
VIBRIO CHOLERAE O139 SEROGROUP TOWARDS
CHOLERA VACCINE DEVELOPMENT**

by

TAN GIM CHEONG

**Thesis submitted in fulfillment of the requirements
for the degree of
Doctor of Philosophy**

2010

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my supervisor, Prof. M. Ravichandran, for his support throughout this study. He is a patient and dedicated teacher and friend to me and has guided me through my whole study. I am also grateful to my co-supervisors, Prof. Mustafa Musa and Dr. Habsah for their guidance and advice that have refined my study and motivate me to become a better student and researcher.

To my dear fellow seniors and colleagues in the laboratory, DR Atif Ali, DR Chan Yean Yean, DR Lee Su Yin, Ms. Melissa Chan, Mr. Kurunathan and Mr. Atif Amin, I would like to express my heartfelt appreciation for their encouragements and support that have helped me through numerous obstacles during my study. My appreciation also goes out to my other labmates and friends who have helped me during the course of this study: Elina, Balqis, Nik, Chandrika, Kak Shikin, Nas, Nisha, Syazwan, Syazman, AngLim and ZiNing. I am grateful to the lecturers, administrative staff and lab technologists from the Department of Medical Microbiology and Parasitology, Department of Pathology, Institute for Research in Molecular Medicine, Microscopy Unit of School of Biological Science and Animal Research Unit, Universiti Sains Malaysia for their help and the use of their excellent facilities.

I would also like to thank Prof. P. Lalitha, DR See Thoe, DR Few Ling Ling, DR Khoo Boon Yin, DR Lim Boon Huat, Dr Shyamoli and DR Fando (Cuba) for their valuable advices and guidances. Special thanks to the Ministry of Science, Technology and Innovations (MOSTI) for offering NSF fellowship to support my PhD and also providing USM-Cuba Initiatives grant to fund this study.

Last but not least, my deepest gratitude to my family for their support and care. They are my strongest motivation and inspiration. I dedicate this thesis to them as my appreciation to them.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS	ii
TABLE OF CONTENTS	iii
LIST OF TABLES	x
LIST OF FIGURES	xi
LIST OF SYMBOLS AND ABBREVIATIONS	xvii
ABSTRAK	xx
ABSTRACT.....	xxii
CHAPTER 1 : INTRODUCTION.....	1
1.1 Cholera	1
1.2 <i>Vibrio cholerae</i>	1
1.2.1 Taxonomy of <i>V. cholerae</i>	3
1.2.2 Subtyping	3
1.2.2.1 Serogroup.....	3
1.2.2.2 Biotypes	4
1.2.2.3 Serotypes.....	4
1.2.3 CTX phage	6
1.3 Epidemiology	8
1.3.1 Early pandemics	8
1.3.2 Seventh pandemic, 1961-present	8
1.3.3 Possibility of eighth pandemic	11
1.4 Ecology of <i>V. cholerae</i>	12
1.4.1 Environment.....	12
1.4.2 Human	12
1.5 Pathogenesis.....	13
1.5.2 Cholera toxin.....	13
1.5.3 MARTX toxin.....	14
1.5.4 Other Toxins	20
1.5.4.1 Haemolysin (Hly)	20
1.5.4.2 Zonula occludens toxin (Zot).....	20
1.5.4.3 Accessory cholera enterotoxin (Ace).....	21
1.5.4.4 Shiga-like toxin (SLT).....	21
1.6 Transmission and prevention of cholera	22
1.6.1 Transmission of cholera	22
1.6.2 Prevention of cholera	22
1.6.2.1 Disinfection and sanitation	22
1.6.2.2 Immunization	23
1.7 Cholera vaccines	23
1.7.1 Immunity to <i>V. cholerae</i>	24
1.7.2 Cytokines during infection.....	24
1.7.3 Killed oral cholera vaccine.....	26
1.7.4 Attenuated live oral cholera vaccine	27
1.7.5 Vaccine under development.....	27

1.8	Diagnosis and treatments	28
1.8.1	Diagnosis.....	28
1.8.2	Treatments.....	29
1.9	Development of VCUSM vaccine candidate	29
1.10	Rationale of the study.....	30
1.11	Objectives of the study.....	31
1.12	Overview of the study	32

CHAPTER 2 : MATERIALS AND METHODS..... 33

2.1	Materials.....	33
2.1.1	Chemical and reagent.....	33
2.1.2	Consumables and kits.....	35
2.1.3	Antibodies	38
2.1.4	Culture media	39
2.1.4.1	Alkaline peptone water (APW).....	39
2.1.4.2	AKI Medium.....	39
2.1.4.3	Columbia horse blood agar	39
2.1.4.4	Luria Bertani (LB) medium	40
2.1.4.4.1	LB broth	40
2.1.4.4.2	LB agar	40
2.1.4.4.3	LB agar for stab culture	40
2.1.4.4.4	LB agar for motility test	41
2.1.4.4.5	LB broth 2X.....	41
2.1.4.4.6	LB broth 2X (without sodium chloride).....	41
2.1.4.4.7	LB broth with 15% Sucrose	41
2.1.4.4.8	LB agar 2X (without sodium chloride)	42
2.1.4.4.9	LB agar 15% sucrose without sodium chloride.....	42
2.1.4.5	Skimmed milk agar	42
2.1.4.6	TCBS agar.....	42
2.1.4.7	Addition of antibiotics/ supplements to the agar based media	43
2.1.4.8	Cell culture medium.....	43
2.1.4.8.1	RPMI medium	43
2.1.4.8.2	PFHMII medium	43
2.1.5	General buffers, stock solutions and antibiotics	44
2.1.5.1	δ -Aminolevulinic acid (40 mg/ml)	44
2.1.5.2	Acid alcohol.....	44
2.1.5.3	Ammonia water (0.2%).....	44
2.1.5.4	Ammonium persulfate (20%)	44
2.1.5.5	Ampicillin (100 mg/ml).....	45
2.1.5.6	Calcium chloride (100 mM)	45
2.1.5.7	Carbonate buffer (60 mM), pH 9.6.....	45
2.1.5.8	Cacodylate buffer (0.2 M), pH 7.4.....	45
2.1.5.9	Cholera toxin (0.5 μ g/ml) for ELISA	46
2.1.5.10	Citrate buffer (10mM), pH 6.0.....	46
2.1.5.11	Coomassie blue solution	46
2.1.5.12	Ethanol	46
2.1.5.13	EDTA (0.5M).....	46
2.1.5.14	Ethidium bromide (10 mg/ml)	47
2.1.5.15	Evans blue (1%).....	47

2.1.5.16	Glycerol (80%)	47
2.1.5.17	Hydrochloric acid (1N).....	47
2.1.5.18	Hydrogen peroxide (3%)	47
2.1.5.19	Kanamycin sulfate (10 mg/ml)	48
2.1.5.20	Karnovsky's fixative.....	48
2.1.5.21	Krebs Ringer Tris (KRT).....	48
2.1.5.22	Lead citrate solution.....	48
2.1.5.23	Lipopolysaccharide (1 mg/ml).....	49
2.1.5.24	Magnesium chloride (100 mM)	49
2.1.5.25	Neutral buffer formalin (10%).....	49
2.1.5.26	Normal saline (0.9% NaCl)	49
2.1.5.27	Osmium tetroxide solution.....	49
2.1.5.28	Paraformaldehyde (10%)	50
2.1.5.29	Phosphate buffered saline (PBS) 1X	50
2.1.5.30	Phosphate buffered saline-Tween-20.....	50
2.1.5.31	Phosphate buffered saline-Triton-X.....	50
2.1.5.32	Polymyxin B sulfate 0.75 mg/ml (4500 units).....	51
2.1.5.33	Ponceau S solution.....	51
2.1.5.34	SDS PAGE running buffer, pH 8.3.....	51
2.1.5.35	SDS PAGE destaining solution	51
2.1.5.36	SDS PAGE resolving buffer, pH 8.8	51
2.1.5.37	SDS PAGE sample buffer, pH 6.8.....	52
2.1.5.38	SDS PAGE stacking buffer, pH 6.8.....	52
2.1.5.39	Sodium hydroxide (1 M).....	52
2.1.5.40	Skimmed milk (5%).....	52
2.1.5.41	Toluidine blue solution	52
2.1.5.42	Tris Borate EDTA (TBE) buffer, pH8.3.....	52
2.1.5.43	Type I ganglioside (1 µg/ml)	53
2.1.5.44	Uranyl acetate (2%)	53
2.1.5.45	Western blotting transfer buffer.....	53
2.1.5.46	4-chloro-1-naphtol substrate solution	53
2.1.5.46.1	Substrate A solution	54
2.1.5.46.2	Substrate B solution.....	54
2.1.6	Bacterial strains and cancer cell lines	54
2.1.6.1	<i>E.coli</i> BW 20767- λ pir.....	54
2.1.6.2	<i>E.coli</i> TOP10 XL1-Blue	54
2.1.6.3	<i>V. cholerae</i> O1 El Tor, AQ1016.....	54
2.1.6.4	<i>V. cholerae</i> O1 Classical Inaba, V53.....	54
2.1.6.5	<i>V. cholerae</i> O139 Bengal, 3189.....	55
2.1.6.6	<i>V. cholerae</i> O139 Bengal, CIR134.....	55
2.1.6.7	VCUSM2 (Δ hemA)	55
2.1.6.8	VCUSM14 (Δ hemA, Δ ctxA, del-ace and del-zot).....	55
2.1.6.9	HEp-2.....	55
2.1.6.10	HT-29.....	55
2.1.7	Plasmids	56
2.1.7.1	pCR2.1 TOPO.....	56
2.1.7.2	pTZ57R.....	56
2.1.7.3	pWM91	56
2.1.7.4	pCVD-hemA/M	56
2.1.8	Animals	56

2.2	Methods.....	57
2.2.1	Bacterial culture conditions.....	57
2.2.2	Bacterial storage and recovery	57
2.2.2.1	Glycerol stock.....	57
2.2.2.2	Lyophilized stock.....	58
2.2.3	Cell culture conditions	59
2.2.4	Cell lines storage and recovery	59
2.2.5	Enumeration of cell lines	60
2.2.6	Molecular cloning	60
2.2.6.1	Polymerase chain reaction (PCR).....	60
2.2.6.1.1	Primers design	60
2.2.6.1.2	Lysate preparation	60
2.2.6.1.3	PCR amplification	62
2.2.6.1.4	Agarose gel electrophoresis.....	62
2.2.6.1.5	Purification of DNA from agarose gel	62
2.2.6.2	Cloning of PCR amplified product	63
2.2.6.2.1	Cloning of PCR amplified product into pTZ57R vector	63
2.2.6.2.2	Preparation of <i>E. coli</i> competent cells.....	64
2.2.6.2.3	Transformation	64
2.2.6.2.4	Clone screening	64
2.2.6.2.5	Plasmid purification	65
2.2.6.3	Restriction enzyme digestion.....	66
2.2.6.3.1	<i>Bst</i> XI.....	66
2.2.6.3.2	<i>Psy</i> I.....	66
2.2.6.3.3	<i>Sma</i> I.....	66
2.2.6.3.3	<i>Sac</i> I and <i>Sal</i> I.....	66
2.2.6.4	Polishing using T4 DNA polymerase	67
2.2.6.5	Dephosphorylation using shrimp alkaline phosphatase (SAP)..	67
2.2.6.6	DNA Ligation	67
2.2.6.7	Conjugation transfer of plasmid vector	68
2.2.6.8	Sucrose selection.....	68
2.2.7	<i>In vitro</i> analysis	69
2.2.7.1	Cholera toxin (CT) ELISA	69
2.2.7.2	Biofilm formation analysis	69
2.2.7.3	Haemolysis activity.....	70
2.2.7.3.1	Preparation of chicken red blood cells	70
2.2.7.3.2	Haemolysis assay	70
2.2.7.4	Protease assay	71
2.2.7.5	Swarming assay	71
2.2.7.6	Cytotoxicity assay.....	71
2.2.7.7	Actin cross linking assay	72
2.2.7.7.1	SDS PAGE	72
2.2.7.7.2	Western blot transfer	72
2.2.7.7.3	Western blot analysis.....	73
2.2.7.8	<i>In vitro</i> colonization assay	73
2.2.7.9	<i>In vitro</i> cytokines analysis	74
2.2.7.9.1	Interleukin-6 ELISA.....	74
2.2.7.9.2	Interleukin-8 ELISA.....	75
2.2.7.9.3	TNF- α ELISA.....	75
2.2.8	<i>In vivo</i> studies.....	76

2.2.8.1	Infant mice colonization assay.....	76
2.2.8.2	Rabbit ileal loop reactogenicity assay	77
2.2.8.3	Removable intestinal tie-adult rabbit diarrhea (RITARD)	77
2.2.8.4	Orogastric immunization of rabbit with <i>V. cholerae</i>	78
2.2.8.5	Rectal swab	78
2.2.8.6	Blood collection and serum preparation	79
2.2.8.7	Euthanization of rabbit.....	79
2.2.9	Pathological studies.....	79
2.2.9.1	Light microscopy analysis	80
2.2.9.1.1	Tissue processing	80
2.2.9.1.2	Tissue sectioning	80
2.2.9.1.3	Hematoxylin and eosin staining	80
2.2.9.1.4	Immunohistochemistry	81
2.2.9.2	Electron microscopy analysis	82
2.2.9.2.1	Transmission electron microscopy	82
2.2.9.2.2	Scanning electron microscopy.....	82
2.2.10	Immunological studies	83
2.2.10.1	Anti-CT ELISA.....	83
2.2.10.2	Anti-LPS ELISA.....	84
2.2.10.3	Vibriocidal assay.....	84

CHAPTER 3 : CONSTRUCTION OF MARTX AND CTXA MUTANTS..... 85

3.1	Introduction.....	85
3.2	Construction of VCUSM9 (<i>rtxC::aphA</i>).....	87
3.2.1	PCR amplification of <i>rtxC</i> gene from <i>V. cholerae</i> O139.....	88
3.2.2	Cloning of <i>rtxC</i> PCR amplicon into pTZ57R cloning vector	88
3.2.3	Amplification of <i>aphA</i> gene from pCR2.1 TOPO vector	91
3.2.4	Insertion mutation of <i>rtxC</i> gene with <i>aphA</i>	91
3.2.5	<i>SacI</i> and <i>SalI</i> digestion of pTZ57R- <i>rtxC::aphA</i>	94
3.2.6	Sub-cloning of <i>rtxC::aphA</i> into suicide plasmid pWM91	94
3.2.7	Conjugation transfer of pWM91- <i>rtxC::aphA</i> into VCUSM2	97
3.2.8	Removing the plasmid backbone and selection of <i>rtxC::aphA</i> mutant of ALA auxotroph of <i>V. cholerae</i> O139.....	97
3.3	Construction of VCUSM10 (Δ <i>rtxA/C</i>)	102
3.3.1	<i>PsyI</i> digestion of pTZ57R- <i>rtxC::aphA</i>	103
3.3.2	Blunt end ligation of 3.5kb fragments of <i>PsyI</i> digested pTZ57R- <i>rtxC::aphA</i>	103
3.3.3	<i>SacI</i> and <i>SalI</i> digestion of pTZ57R- Δ <i>rtxA/C</i>	106
3.3.4	Sub-cloning of Δ <i>rtxA/C</i> into suicide plasmid pWM91	106
3.3.5	Conjugation transfer of pWM91- Δ <i>rtxC</i> into VCUSM9.....	109
3.3.6	Removing the plasmid backbone and selection of Δ <i>rtxA/C</i> mutant of ALA auxotroph of <i>V. cholerae</i> O139.....	109
3.4	Construction of ALA prototroph strains	111
3.4.1	Construction of VCUSM9P	111
3.4.1.1	Conjugation transfer of pCVD- <i>hemA/M</i> into VCUSM9.....	111
3.4.1.2	Removing the plasmid backbone and selection of prototroph <i>rtxC::aphA</i> mutant of <i>V. cholerae</i> O139.....	114
3.4.2	Construction of VCUSM10P	114
3.4.2.1	Conjugation transfer of pCVD- <i>hemA/M</i> into VCUSM10.....	114

3.4.2.2	Removing the plasmid backbone and selection of $\Delta rtxA/C$ mutant of <i>V. cholerae</i> O139 with ALA prototrophic property	116
3.4.3	Construction of VCUSM14P	118
3.4.3.1	Conjugation transfer of pCVD- <i>hemA/M</i> into VCUSM14.....	118
3.4.3.2	Removing the plasmid backbone and selection of prototrophic <i>ctxA</i> mutant of <i>V. cholerae</i> O139	118
3.5	Summary	119

CHAPTER 4 : CHARACTERIZATION OF VCUSM MUTANT STRAINS..... 121

4.1	Introduction	121
4.2	<i>In vitro</i> characterization of <i>V. cholerae</i> mutant strains	121
4.2.1	Cholera toxin production in VCUSM9P, 10P and 14P.....	121
4.2.2	Biofilm formation by VCUSM9P, 10P and 14P.....	124
4.2.3	Haemolysis by VCUSM9P, 10P and 14P	127
4.2.4	Proteolytic activity of VCUSM9P, 10P and 14P	127
4.2.5	Motility of VCUSM9P, 10P and 14P.....	131
4.2.6	Cytotoxicity assay	131
4.2.7	Actin cross linking assay.....	134
4.2.8	<i>In vitro</i> colonization assay	135
4.2.9	Cytokines analysis.....	135
4.2.9.1	Interleukin 6 (IL-6)	135
4.2.9.2	Interleukin 8 (IL-8)	138
4.2.9.3	Tumor necrosis factor-alpha (TNF- α)	138
4.3	<i>In vivo</i> characterization of VCUSM strains	142
4.3.1	Intestinal colonization property of WT O139 <i>V. cholerae</i> , VCUSM9P, VCUSM10P and VCUSM14P	142
4.3.2	Reactogenicity study of WT O139 <i>V. cholerae</i> , VCUSM9P, 10P and 14P in rabbit ileal loop	144
4.3.3	Pathological studies of wild type O139 <i>V. cholerae</i> , VCUSM9P, VCUSM10P and VCUSM14P in ileal loop model.....	148
4.3.3.1	Hematoxylin and eosin staining.....	148
4.3.3.2	Immunohistochemistry staining.....	153
4.3.3.3	Scanning electron microscopy (SEM) analysis	153
4.3.3.4	Transmission electron microscopy (TEM) analysis	156
4.3.4	Reactogenicity study of VCUSM14P in RITARD model	156
4.4	Summary	160

CHAPTER 5 : IMMUNOLOGICAL ANALYSIS OF VCUSM14P VACCINE CANDIDATE 162

5.1	Introduction	162
5.2	Protective efficacy of VCUSM14P vaccinated in rabbit ileal loop reactogenicity assay	162
5.3	Protective efficacy of VCUSM14P vaccinated rabbit in WT O1 and O139 RITARD challenge.....	164
5.4	Antibodies responses.....	164
5.4.1	Determination of anti-CT IgA and IgG antibodies titer.....	166
5.4.2	Determination of Anti-LPS IgA and IgG antibodies titer	166
5.4.3	Determination of vibriocidal antibodies titer	169

5.5	Summary	171
CHAPTER 6 : GENERAL DISCUSSION.....		172
SUMMARY AND CONCLUSION.....		186
LIMITATION OF THE RESEARCH		188
LIMITATION AND RECOMMENDATIONS FOR FUTURE RESEARCH.....		1888
REFERENCES.....		1899
APPENDICES		202
	Appendix A	202
	Appendix B	2077
	Appendix C	2099
LIST OF PUBLICATIONS & PRESENTATIONS		2111

LIST OF TABLES

	Page
Table 1.1 Biochemical tests for differentiation of <i>V. cholerae</i> from related species (Adapted from table 2, Kaper et al., 1995)	2
Table 1.2 Differentiation of classical and El Tor biotypes of <i>V. cholerae</i> O1 (Adapted from table 4, Kaper et al., 1995)	5
Table 1.3 Cholera pandemics 1817-1923/5 (Adapted from table 1; Chapter 1, Barua, 1992)	10
Table 2.1 List of antibodies used in this study	38
Table 2.2 List of primers and their sequences used in the study	61
Table 4.1 Fluid accumulation ration from ileal loop assay of WT O139 <i>V. cholerae</i> , WT O1 <i>V. cholerae</i> , VCUSM9P, VCUSM10P and VCUSM14P in rabbit small intestine (n=2)	147
Table 5.1 Wild type challenge of immunized rabbit with <i>V. cholerae</i> O139 Bengal and O1 El Tor for protective efficacy of VCUSM14P vaccination	163

LIST OF FIGURES

		Page
Figure 1.1	Panel A, schematic representation of the genetic organization of CTX ϕ comprising an RS2 region and a core region. Panel B, schematic representation of the genetic organization of RS1 element. Solid arrows represent ORFs, whereas bend arrows indicate the promoters for <i>rstR</i> , <i>rstA</i> and <i>ctxAB</i> . (Adapted from figure 1, Faruque et al., 2002)	7
Figure 1.2	Cholera, countries reporting outbreaks and imported cases, 2006-2008 (WHO, 2009b)	9
Figure 1.3	Schematic diagram showing the binding of cholera toxin molecule through its pentameric CT-B to the GM1 pentasaccharides of the epithelial cell membrane. (Adapted from figure 7.7 Cholera Toxins, Chaudhuri and Chatterjee 2009c)	15
Figure 1.4	Schematic representation of the genetic organization of <i>V. cholerae</i> MARTX gene clusters. (Adapted from figure 1a, Boardman et al., 2004)	17
Figure 1.5	Mechanism of disruption of actin cytoskeleton by MARTX toxin by two different pathways : action crosslinking and Rho GTPase inactivation. (Adapted from figure 13.8, Cholera Toxins, Chaudhuri and Chatterjee, 2009a)	19
Figure 1.6	Study overview	32
Figure 3.1	Conjugation transfer of suicide plasmid and homologous recombination process in <i>V. cholerae</i>	86
Figure 3.2	Construction of VCUSM9 (<i>rtxC::aphA</i>) of ALA auxotrophic O139 <i>V. cholerae</i> strain	87
Figure 3.3	Panel A, diagrammatic representation of <i>rtxC</i> gene and binding sites of <i>rtxC</i> -R1 and <i>rtxC</i> -F1 primers. Panel B, agarose gel electrophoresis result of 996 bp <i>rtxC</i> PCR amplification using <i>rtxC</i> -R1 and <i>rtxC</i> -F1 primers	89
Figure 3.4	Panel A, diagrammatic representation of pTZ57R- <i>rtxC</i> . Panel B, agarose gel electrophoresis result of 996 bp <i>rtxC</i> PCR amplification using <i>rtxC</i> -R1 and <i>rtxC</i> -F1 primers	90
Figure 3.5	Panel A, diagrammatic representation of pCR2.1 TOPO. Panel B, agarose gel electrophoresis result of 1.0 kb <i>aphA</i> PCR amplification using Kan <i>PsyI</i> -F1 and Kan <i>PsyI</i> -R1 primers	92

	Page
Figure 3.6 Panel A, diagrammatic representation of pTZ57R- <i>rtxC::aphA</i> . Panel B, agarose gel electrophoresis result of 2.0 kb <i>rtxC::aphA</i> PCR amplification using <i>rtxC-R1</i> and <i>rtxC-F1</i> primers	93
Figure 3.7 Panel A, diagrammatic representation of pTZ57R- <i>rtxC::aphA</i> and restriction enzyme sites for <i>SacI</i> and <i>SalI</i> . Panel B, agarose gel electrophoresis result of <i>SacI</i> and <i>SalI</i> digested pTZ57R- <i>rtxC::aphA</i>	95
Figure 3.8 Panel A, diagrammatic representation of pWM91- <i>rtxC::aphA</i> . Panel B, agarose gel electrophoresis result of 2.0 kb <i>rtxC::aphA</i> PCR amplification using <i>rtxC-F1</i> and <i>rtxC-R1</i> primers	96
Figure 3.9 Agarose gel electrophoresis of PCR screening of <i>rtxC-rtxC::aphA</i> merodiploids for 996 bp WT <i>rtxC</i> and 2.0 kb <i>rtxC::aphA</i> PCR amplicons using <i>rtxC-F1</i> and <i>rtxC-R1</i> primers	98
Figure 3.10 Agarose gel electrophoresis result of PCR screening of sucrose selected <i>rtxC::aphA</i> merodiploids for 996 bp WT <i>rtxC</i> and 2.0 kb <i>rtxC::aphA</i> PCR amplicons using <i>rtxC-F1</i> and <i>rtxC-R1</i> primers	100
Figure 3.11 Agarose gel electrophoresis result of PCR screening of sucrose selected <i>rtxC-rtxC::aphA</i> merodiploids for 1.4 kb <i>SacB</i> PCR amplicons using <i>SacB-F2</i> and <i>SacB-R</i> primers	101
Figure 3.12 Construction of VCUSM10 (Δ <i>rtxC/A</i>) of ALA auxotrophic O139 <i>V. cholerae</i> mutant	102
Figure 3.13 Panel A, diagrammatic representation of pTZ57R- <i>rtxC::aphA</i> and restriction enzyme sites for <i>PstI</i> . Panel B, agarose gel electrophoresis result of <i>PstI</i> digested pTZ57R- <i>rtxC::aphA</i>	104
Figure 3.14 Panel A, diagrammatic representation of pTZ57R- Δ <i>rtxA/C</i> . Panel B, agarose gel electrophoresis result of PCR screening of transformed colonies for 656 bp Δ <i>rtxA/C</i> PCR amplicons using <i>rtxC-F1</i> and <i>rtxC-R1</i> primers	105
Figure 3.15 Panel A diagrammatic representation of pTZ57R- Δ <i>rtxA/C</i> . Panel B, agarose gel electrophoresis result of <i>SacI</i> and <i>SalI</i> digested pTZ57R- Δ <i>rtxA/C</i>	107
Figure 3.16 Panel A, diagrammatic representation of pWM91- Δ <i>rtxA/C</i> . Panel B, agarose gel electrophoresis result of PCR screening of transformed colonies for 656 bp of Δ <i>rtxA/C</i> PCR amplicons using <i>rtxC-F1</i> and <i>rtxC-R1</i> primers	108

	Page
Figure 3.17 Agarose gel electrophoresis result of PCR screening of <i>rtxC::aphA-ΔrtxA/C</i> merodiploids for 2.0 kb <i>rtxC::aphA</i> and 656 bp <i>ΔrtxA/C</i> PCR amplicons using <i>rtxC-F1</i> and <i>rtxC-R1</i> primers	110
Figure 3.18 Agarose gel electrophoresis result of PCR screening of sucrose selected <i>rtxC::aphA-ΔrtxA/C</i> merodiploids for 2.0 kb <i>rtxC::aphA</i> and 656 bp <i>ΔrtxA/C</i> PCR amplicons using <i>rtxC-F1</i> and <i>rtxC-R1</i> primers	112
Figure 3.19 Agarose gel electrophoresis result of PCR screening of sucrose selected <i>rtxC::aphA-ΔrtxA/C</i> merodiploids for 1.4 kb <i>SacB</i> PCR amplicons using <i>SacB-F2</i> and <i>SacB-R</i> primers	113
Figure 3.20 Agarose gel electrophoresis result of PCR screening of sucrose selected <i>ΔhemA-hemA/M</i> merodiploids for 1.4 kb <i>SacB</i> PCR amplicons using <i>SacB-F2</i> and <i>SacB-R</i> primers for VCUSM9	115
Figure 3.21 Agarose gel electrophoresis result of PCR screening of sucrose selected <i>ΔhemA-hemA/M</i> merodiploids for 1.4 kb <i>SacB</i> PCR amplicons using <i>SacB-F2</i> and <i>SacB-R</i> primers for VCUSM10	117
Figure 3.22 Agarose gel electrophoresis result of PCR screening of sucrose selected <i>ΔhemA-hemA/M</i> merodiploids for 1.4 kb <i>SacB</i> PCR amplicons using <i>SacB-F2</i> and <i>SacB-R</i> primers for VCUSM14	120
Figure 4.1 Phenotypic characterization of constructed <i>V. cholerae</i> mutant strains	122
Figure 4.2 Production of cholera toxin by VCUSM9P, VCUSM10P, VCUSM14P and WT O139 <i>V. cholerae</i> in LB broth at 30°C with shaking at 200 rpm for 12 hrs (n=4)	123
Figure 4.3 Biofilm formation by WT O139 <i>V. cholerae</i> , VCUSM9P, VCUSM10P and VCUSM14P in LB broth at 30°C after (A)12 hrs, (B)24 hrs and (C)48 hrs. Red circles indicate the formation of biofilm	125
Figure 4.4 Biofilm formation by WT O139 <i>V. cholerae</i> , VCUSM9P, VCUSM10P and VCUSM14P in LB broth at 37°C after (A)12 hrs, (B)24 hrs and (C)48 hrs. Red circles indicate the formation of biofilm	126
Figure 4.5 Haemolysis of chicken RBC by WT O139 <i>V. cholerae</i> , VCUSM9P, VCUSM10P and VCUSM14P at room temperature after 12 hrs of incubation with bacterial cell concentration of A) 10 ⁸ CFU/ml and B) 10 ⁶ CFU/ml. O1 Classical Inaba <i>V. cholerae</i> strain was used as negative control	128

	Page
Figure 4.6 Haemolysis of RBC in commercial horse blood agar by WT O139 <i>V. cholerae</i> , VCUSM9P, VCUSM10P and VCUSM14P at 37°C after 16 hrs of incubation. Halo regions indicate the lysis of horse RBC by the vibrios	129
Figure 4.7 Protease activity by WT O139 <i>V. cholerae</i> , VCUSM9P, VCUSM10P and VCUSM14P in modified LB agar with 5% skim milk at 37°C for 16 hrs. Halo areas surrounding the bacterial cells indicate utilization of casein in the agar medium as a result of protease activity	130
Figure 4.8 Swarming activity of WT O139 <i>V. cholerae</i> , VCUSM9P, VCUSM10P and VCUSM14P in motility agar plate incubated at room temperature for 24 hrs. The motility was measured by the size of the motility zone	132
Figure 4.9 The Cytotoxicity effect of <i>V. cholerae</i> strains on HEp-2 cells in culture. Subconfluent HEp-2 monolayer were exposed for 1hr at m.o.i. of 100 with PBS as negative control (blank)	133
Figure 4.10 The action cross-linking activities of <i>V. cholerae</i> strains on HEp-2 cells in culture. At 1 hr after infection, cells were resuspended in sample buffer, boiled and subjected to SDS-PAGE and Western blotting with an anti-actin antibody	136
Figure 4.11 <i>In vitro</i> colonization of WT O139 <i>V. cholerae</i> , VCUSM9P, VCUSM10P and VCUSM14P in differentiated HT-29 cell lines (n=4)	137
Figure 4.12 IL-6 secretion by HT-29 infected with WT O139 <i>V. cholerae</i> , VCUSM9P, VCUSM10P and VCUSM14P at 2 hrs, 4 hrs and 8 hrs intervals, (n=2)	139
Figure 4.13 IL-8 secretion by HT-29 cells infected with WT O139 <i>V. cholerae</i> , VCUSM9P, VCUSM10P and VCUSM14P at 2 hrs, 4 hrs and 8 hrs intervals, (n=2)	140
Figure 4.14 TNF- α secretion by HT-29 cells infecteds with WT O139 <i>V. cholerae</i> , VCUSM9P, VCUSM10P and VCUSM14P at 2 hrs, 4 hrs and 8 hrs intervals, (n=2)	141
Figure 4.15 Intestinal colonization	143
Figure 4.16 Immunohistochemical detection of WT O139 <i>V. cholerae</i> , VCUSM9P, VCUSM10P and VCUSM14P intestinal colonization in 3-5 days old suckling mice. The vibrios were identified as brown staining (red arrows). Original magnification X200	145
Figure 4.17 Ileal loop assay of WT O139 <i>V.cholerae</i> , WT O1 <i>V. cholerae</i> , VCUSM9P, VCUSM10P and VCUSM14P in rabbit small intestine	146

	Page
Figure 4.18 Histopathological features of rabbit ileum section infected with WT O139 <i>V. cholerae</i> . The muscular layer (thick arrow) and villi of the mucosal layer (thin arrows) were severely damaged. Original magnification X200	149
Figure 4.19 Histopathological features of rabbit ileum section infected with VCUSM9P. The villi (thin arrow) of the mucosal layer were severely blunted and RBCs (thick arrow) were detected in between muscle fibers. Original magnification X200	150
Figure 4.20 Histopathological features of rabbit ileum section infected with VCUSM10P . The villi (thin arrow) of the mucosal layer suffer from mild blunting, haemorrhage (thick arrow) and congested blood vessels (arrow head) were also observed within the tissue. Original magnification X200	151
Figure 4.21 Histopathological features of rabbit ileum section infected with VCUSM14P. The mucosal and muscular layer were intact in the tissue. Original magnification X100	152
Figure 4.22 Immuno histochemical staining of rabbit ileum sections infected with (A) WT O139 <i>V. cholerae</i> , (B) VCUSM9P, (C) VCUSM10P and (D) VCUSM14P*. The vibrios were identified as brown staining (red arrows). Original magnification X20 *Original magnification is X400	154
Figure 4.23 Scanning electron microscopy of rabbit ileum infected with (A) WT O139 <i>V. cholerae</i> , (B) VCUSM9P, (C) VCUSM10P and (D) VCUSM14P. The vibrios were observed colonizing the mucosal surface of the intestine. Original magnification X5000	155
Figure 4.24 Transmission electron microscopy of rabbit ileum section infected with WT O139 <i>V. cholerae</i> . The vibrios (thick arrows) appear to be endocytosed by APC (arrow heads) in between muscle fiber (thin arrow). N represents nucleus of the APC. Original magnification X4500	157
Figure 4.25 Transmission electron microscopy of rabbit ileum section infected with VCUSM9P. The vibrios (thick arrows) appear to be endocytosed by APC (arrow heads) in between muscle fiber (thin arrow).N represents nucleus of the APC. Original magnification X4500	158
Figure 4.26 Transmission electron microscopy of rabbit ileum section infected with VCUSM10P. The vibrios (thick arrows) appear to be endocytosed by APC (arrow heads) in between muscle fiber (thin arrow).N represents the nucleus of APC. Original magnification X4500	159

	Page
Figure 5.1 Scanning electron microscopy of VCUSM14P immunized rabbit's small intestine after RITARD challenge with wild type O139 <i>V. cholerae</i> . Original magnification X250 and X9210	165
Figure 5.2 Geometric mean titer (GMT) of anti-cholera toxin IgA and IgG antibodies in VCUSM14P vaccinated rabbits (n=3)	167
Figure 5.3 Geometric mean titer (GMT) of anti-lipopolysaccharide IgA and IgG antibodies in VCUSM14P vaccinated rabbits (n=3)	168
Figure 5.4 Geometric mean titer of vibriocidal antibodies in VCUSM14P vaccinated rabbits (n=3)	170

LIST OF SYMBOLS AND ABBREVIATIONS

+	positive
-	negative or minus
>	more than
<	less than
10X	ten times
2X	two times
A ₂₆₀	absorbance at 260nm
ALA	Aminolevulinic acid
AP	Alkaline Phosphatase
APCs	Antigen Presenting Cells
APW	Alkaline Peptone Water
ATP	Adenosine Tri-phosphate
BLAST	Basic Local Alignment Search Tool
bp	base pair
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
cm	centimeter
CFU	colony forming unit
CT/CTX	Cholera toxin
CT-A	Cholera toxin A subunit
CT-B	Cholera toxin B subunit
CTXΦ	Cholera toxin phage
dH ₂ O	distilled water
ddH ₂ O	double distilled water
DMSO	dimethyl sulfoxide
DNA	Deoxy Ribonucleic Acid
DNase	deoxyribonuclease
dNTP	deoxynucleoside triphosphate
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
<i>et al.</i>	et alii
EtOH	ethanol
FAR	fluid accumulation ratio
FCS	feotal calf serum
FDA	Food and Drug Administration

g	gram
GM1	Ganglioside type I
H ₂ O ₂	Hydrogen peroxide
HCl	Hydrochloric acid
Hg	Mercury
hr	hour
HRP	Horse Radish Peroxidase
Ig	immunoglobulin
IG	intra gastric
IM	intramuscular
IP	intraperitoneal
IPTG	Isopropyl-beta-D-thiogalactopyranoside
IV	intravenous
kb	kilo base
kDa	kiloDalton
kg	kilogram
L	liter
LB	Luria Bertani
LPS	lipopolysaccharide
mA	milliamperes
MARTX	Multifunctional Repeat-in-Toxin
M cells	Modified epithelial cells
MCS	Multiple cloning site
mg	miligram
MHC	Major Histocompatibility
min	minute
ml	milliliter
mm	millimeter
mmol	millimole
m.o.i.	multiplicity of infection
MSHA	Mannose Sensitive Hemagglutinin
MW	molecular weight
n	sample size
nm	nanometer
°C	degree Celsius
OD ₆₀₀	optical density 600nm
OG	orogastric

PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline-Tween 20
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
pg	picogram
pmol	picomole
pH	Potential hydrogeni
RBC	Red Blood Cell
RE	restriction enzyme
RT	room temperature
rpm	revolution per minute
SD	standard deviation
SDS	Sodium dodecyl sulphate
sec	second
spp.	Species
T _a	annealing temperature
TAE	Tris/acetate/EDTA (buffer)
Taq	Thermus aquaticus
TBE	Tris/borate/EDTA (buffer)
TCBS	Thiosulfate Citrate Bile salt Sucrose
T _m	Melting temperature
Tris	Tris(hydroxymethyl)aminomethane
U	unit
UV	Ultraviolet
V	Volt
v/v	volume/volume
w/v	weight/volume
WHO	World Health Organization
WT	wild type
X	Times/multiplication
ZOT	Zonnula occludens toxin
α	alpha
β	beta
μm	Micrometer
μg	Microgram
μM	Micromolar

**PEMBANGUNAN VAKSIN KOLERA MELALUI PEMAHAMAN MEKANISMA
MOLEKUL ENTEROPATOGENESIS BAGI MUTASI GEN-GEN TOKSIN MARTX
DAN GEN TOKSIN KOLERA *VIBRIO CHOLERAE* KUMPULAN SERO O139**

ABSTRAK

Kolera merupakan penyakit cirit-birit yang boleh membawa maut. Ia disebabkan oleh mikroorganisma *V. cholerae* kumpulan sero O1 dan O139 yang bertoksik. Pengesanan awal kolera dan rawatan segera ke atas pesakit adalah amat diperlukan bagi mencegah berlakunya kematian. Walaupun penyebaran penyakit ini dapat dikawal dengan penyediaan sistem kumbahan yang teratur, namun cara terbaik untuk mengatasinya adalah melalui program vaksinasi. Terdapat beberapa vaksin kolera bagi *V. cholerae* kumpulan sero O1 namun, vaksin-vaksin ini tidak dapat mencegah jangkitan daripada *V. cholerae* kumpulan sero O139. Walaupun terdapat vaksin kolera bivalen O1/O139, ujian klinikal menunjukkan gerakbalas imun perlindungan terhadap kumpulan sero O139 adalah kurang memuaskan. Maka, objektif dalam kajian ini adalah untuk menghasilkan vaksin hidup teratenuat kolera terhadap *V. cholerae* kumpulan sero O139. Kajian ini berfokus kepada analisis terhadap kesan mutasi bagi toksin MARTX dan toksin kolera dalam *V. cholerae* kumpulan sero O139. Ciri-ciri sitotoksik mutan *rtxC* (VCUSM9P) dan mutan *rtxA/C* (VCUSM10P) *V. cholerae* kumpulan sero O139 didapati hilang. Analisis “Western blot” menunjukkan mutasi gen *rtxC* sahaja adalah tidak memadai untuk menghapuskan kesan toksik toksin MARTX. Kesan reaktogenik pada model gelung ileum arnab bertambah walaupun aktiviti toksin MARTX mutan *rtxC* dan *rtxA/C* berkurang. Walaubagaimanapun, mutan *ctxA* (VCUSM14P) dengan CT-A yang telah dinyahaktif tidak menunjukkan sebarang kesan reaktogenik pada model gelung ileum arnab dan masih mengekalkan aktiviti sitotoksik yang sama sepertimana yang ditunjukkan genotip liar O139 *V. cholerae* pada sel-sel HEp-2. Selain daripada itu, mutan-mutan MARTX dan CT juga berupaya untuk mencetuskan rembesan sitokin-sitokin proinflamasi dalam sel-sel

HT-29 yang telah dijangkiti. Maka, strain mutan yang tidak reaktogenik VCUSM14P telah dipilih untuk kajian pemvaksinan seterusnya. Setelah pemvaksinan dua dos VCUSM14P melalui laluan oral, didapati antibodi-antibodi anti-CT, anti-LPS dan “vibriocidal” dihasilkan pada titer yang tinggi. Kajian cabaran juga menunjukkan perlindungan terhadap jangkitan *V. cholerae* kumpulan sero O139. Kesimpulannya, dengan kajian eksperimen kami telah membuktikan mutasi pada toksin MARTX tidak mengurangkan kesan reaktogenik dalam *V. cholerae* O139 kumpulan sero O139 dan juga kesan mutasi tunggal terhadap gen *rtxC* adalah tidak memadai untuk merencatkan fungsi paut silang aktin toksin MARTX. Selain itu, penilaian ke atas VCUSM14P menunjukkan potensi mutan *ctxA* sebagai calon vaksin kolera yang tidak reaktogenik dan selamat terhadap *V. cholerae* kumpulan sero O139.

**UNDERSTANDING THE MOLECULAR ENTEROPATHOGENESIS OF MARTX
TOXIN GENES AND CHOLERA TOXIN GENE MUTATION OF *VIBRIO
CHOLERAE* O139 SEROGROUP TOWARDS CHOLERA VACCINE
DEVELOPMENT**

ABSTRACT

Cholera is a lethal diarrheal disease caused by toxigenic *V. cholerae* of O1 and O139 serogroups. Early diagnosis and prompt treatments of the disease are needed to prevent death in these patients. Even though the spreading of the disease could be controlled with proper sanitation, the best way to prevent cholera is through vaccination. There are several cholera vaccines available for O1 serogroup *V. cholerae* but they do not cross-protect against O139 serogroup. The bivalent O1/O139 cholera vaccine also did not show satisfactory protective immune response against O139 serogroup in clinical trials. Hence, the objective of this study is to construct an attenuated live cholera vaccine against *V. cholerae* O139 serogroup. In the present study, we focused on mutational analysis of MARTX toxin and cholera toxin of *V. cholerae* O139 serogroup. Constructed *rtxC* (VCUSM9P) and *rtxA/C* (VCUSM10P) mutants of *V. cholerae* O139 serogroup showed depleted in cytotoxic properties. Western blot analysis indicated mutation of *rtxC* gene alone is not sufficient to abolish MARTX toxin toxicity. Despite disrupted MARTX toxin activity in *rtxC* and *rtxA/C* mutants, increase reactivity were observed in ileal loop model. However, *ctxA* mutant (VCUSM14P) with mutated CT-A did not show any reactivity in ileal loop model, but retain MARTX cytotoxicity activity similar to WT O139 strain in HEp-2 cells. These MARTX and CT mutants were also found to induce proinflammatory cytokines in infected HT-29. Non-reactogenic mutant strain, VCUSM14P, was selected for vaccination study. High titers of anti-CT, anti-LPS and vibriocidal antibodies were induced after two regimens vaccination through oral route of VCUSM14P. Challenge study with VCUSM14P also showed

protection against WT O139 *V. cholerae*. In conclusion, we have experimentally proven that the MARTX toxin mutation does not reduce reactogenicity in *V. cholerae* O139 serogroup and mutation in *rtxC* alone do not completely remove the actin crosslinking property of MARTX toxin. The evaluation on VCUSM14P vaccine indicated that it is a non-reactogenic and protective vaccine candidate against *V. cholerae* O139 serogroup.

CHAPTER 1

INTRODUCTION

1.1 Cholera

Cholera is a diarrheal disease caused by certain types of *Vibrio cholerae*. It begins with sudden onset of massive diarrhea, with the loss of enormous amount of protein-free fluid. The loss of electrolytes, bicarbonates and ions within short period of time will results in hypovolemic shock and acidosis in the patients (Chaudhuri and Chatterjee, 2009a, Tauxe, 1998). If left untreated without prompt attention, the resulting dehydration will lead to tachycardia, hypotension, and also vascular collapse that will lead to deaths (Chaudhuri and Chatterjee, 2009a, Ryan, 2004).

1.2 *Vibrio cholerae*

V. cholerae, a member of the family *Vibrionaceae*, is a facultative anaerobic, Gram-negative, non-spore-forming and highly motile organism with the length of 1.4-2.6 μm (Kay *et al.*, 1994). The genus *Vibrio* also includes many different species, of which *V. parahaemolyticus*, *V. alginolyticus*, *V. mimicus*, and *V. vulnificus* are associated with human diseases (Sakazaki, 1992). Among the vibrio species, *V. cholerae* is a well-defined species based on biochemical tests. The *Vibrio* species can be differentiated by a variety of tests as shown in Table 1.1. Most of the environmental *V. cholerae* are non-pathogenic and the organism became pathogenic mainly through acquiring virulence genes by lateral gene transfer from other species (Gonzalez-Fraga *et al.*, 2008).

Table 1.1 Biochemical tests for differentiation of *V. cholerae* from related species (Adapted from table 2, Kaper *et al.*, 1995).

Test	<i>V. cholerae</i>	Other <i>Vibrio</i> species
Oxidase	+	+ ^a
String test	+	+/-
Acid from mannitol	+	+/-
Acid from sucrose	+	+/-
Lysine decarboxylase	+	+/-
Arginine dihydrolase	-	+/-
Ornithine decarboxylase	+	+/-
Growth in 0% NaCl	+	- ^b

^aExcept for *V. metchnikovii*

^bExcept for *V. mimicus*

1.2.1 Taxonomy of *V. cholerae*

The taxonomy of *V. cholerae* is given below:

Kingdom	: Bacteria
Phylum	: Proteobacteria
Class	: Gamma Proteobacteria
Order	: Vibrionales
Family	: <i>Vibrionaceae</i>
Genus	: <i>Vibrio</i>
Species	: <i>Vibrio cholerae</i>

1.2.2 Subtyping

V. cholerae is not homogeneous in many aspects and subtyping of the species are based on important distinctions within the species such as serogroup, cholera enterotoxin production and potential for epidemic spread.

1.2.2.1 Serogroup

Serogrouping are based on highly diverse heat-stable lipopolysaccharide O-antigen produced by *V. cholerae*. The heat-stable lipopolysaccharide O-antigen can be differentiated by its differences in the sugar composition of the terminal sugar group (Shimada *et al.*, 1994, Tauxe, 1998). Currently, there are around 200 serogroups (O1 to O200) of *V. cholerae* had been identified, and more may surface in the future (Shimada *et al.*, 1994). Before 1992, all major epidemics of cholera were exclusively caused by the O1 serogroup of *V. cholerae* strains and the simple distinction of *V. cholerae* O1 and *V. cholerae* non-O1 was used for classification. But in 1993, the emergence *V. cholerae* O139 as new serogroup associate with cholera (Faruque *et al.*, 2003, Morris, 1994), the designation of *V. cholerae* non-O1 non-O139 has been used to categorize the epidemic causing serogroups of *V. cholerae* O1 and

O139 (Kaper *et al.*, 1995). To date, only strains from *V. cholerae* O1 and O139 serogroups, which produce cholera toxin (Angelichio *et al.*, 1999) are associated with epidemic cholera (Trucksis *et al.*, 1993). Strains of these serogroups which do not produce CT are unable to cause cholera and are not involved in epidemics. *V. cholerae* of serogroups other than O1 and O139 do occasionally produce cholera outbreaks, but yet to cause large epidemic or worldwide pandemic (Kaper *et al.*, 1995). The emergence of *V. cholerae* O139 as new serogroup associated with cholera, and its possible evolution from horizontal gene transfer between O1 and non-O1 strains (Bik *et al.*, 1995), has led to heightened interest in the study of *V. cholerae* non-O1 non-O139 serogroups.

1.2.2.2 Biotypes

V. cholerae O1 serogroup consists of two different biotypes, classical and El Tor (Kaper *et al.*, 1995). These subdivisions are based on several characteristics and are as described in Table 1.2. It is believed that *V. cholerae* O1 serogroup classical biotype was the causative agent for the first six cholera pandemic and the El Tor biotype caused the seventh cholera pandemic (Blake, 1994).

1.2.2.3 Serotypes

Classical and El Tor biotypes are subdivided into two major serotypes, Ogawa and Inaba, through their antigenic factors. Ogawa serotype is said to express A and B antigens and small amount of C antigen, whereas Inaba serotype express only the A and C antigens. A third serotype, Hikojima, which is rare and unstable is said to express all three antigens - A, B and C (Kaper *et al.*, 1995, Stroehrer *et al.*, 1992).

Table 1.2 Differentiation of classical and El Tor biotypes of *V. cholerae* O1 (Adapted from table 4, Kaper *et al.*, 1995).

Test Used	Result	
	Classical	El Tor
Haemolysis	–	+/-
Agglutination of chicken erythrocytes	–	+
Voges-Proskauer reaction	–	+
Inhibition by polymyxin B (50-U disc)	+	–
Lysis by Group IV cholera phage	+	–
Lysis by FK cholera phage	+	–

1.2.3 CTX phage

Cholera toxin is encoded by *ctxAB* genes which are part of a gene cluster normally referred to as CTX genetic element (Davis and Waldor, 2003, Pearson *et al.*, 1993). Initial studies had revealed that *ctxAB* genes were only present in toxigenic *V. cholerae* (Trucksis *et al.*, 1993). It was also discovered that the *ctxAB* genes were not integral components of the *V. cholerae* genome, but instead correspond to the genome of an integrated filamentous bacteriophage designated CTX ϕ (Waldor and Mekalanos, 1996). The CTX ϕ genome has two regions, the “core” and RS2. The core region contains the genes encoding CT (*ctxA/B*), zonula occludens toxin (*zot*), accessory toxin (*ace*), core-encoded pilin (*cep*) and functions related to phage morphogenesis (Waldor and Mekalanos, 1996). The RS2 region contains three open reading frame (ORF), designated *rstR*, *rstA*, and *rstB*, and two apparently untranslated regions called intergenic regions Ig-1 and Ig-2 (Boyd *et al.*, 2000). These genes code for products that control phage replication and site-specific integration. The CTX ϕ prophage is often flanked by a related genetic element known as the RS1. The RS1 element is very similar to the RS2 region of CTX ϕ in genetic and functional aspects (Waldor *et al.*, 1997). Different from RS2, RS1 carries an additional ORF termed *rstC*, which serve as antirepressor that controls CTX ϕ lysogeny, production of CTX ϕ particles, and expression of cholera toxin (Davis *et al.*, 2002). The CTX genetic elements are as shown in Figure 1.1. The CTX ϕ utilizes the toxin-coregulated pili (TCP) as its functional receptor for infection of *V. cholerae* cells (Heilpern and Waldor, 2000, Levin and Tauxe, 1996). Understanding the mode of integration of phage DNA into the host chromosome has been a problem and two different views have been separately proposed by McLeod and Waldor (2004) and Val *et al.* (2005). Two chromosome-encoded tyrosine recombinases, XerC and XerD, were found to assist in the recombination process that leads to integration of phage DNA into host chromosome of *V. cholerae* (Huber and Waldor, 2002, McLeod and Waldor, 2004, Val *et al.*, 2005). The CTX ϕ phage enters into a lysogenic state following its integration into the

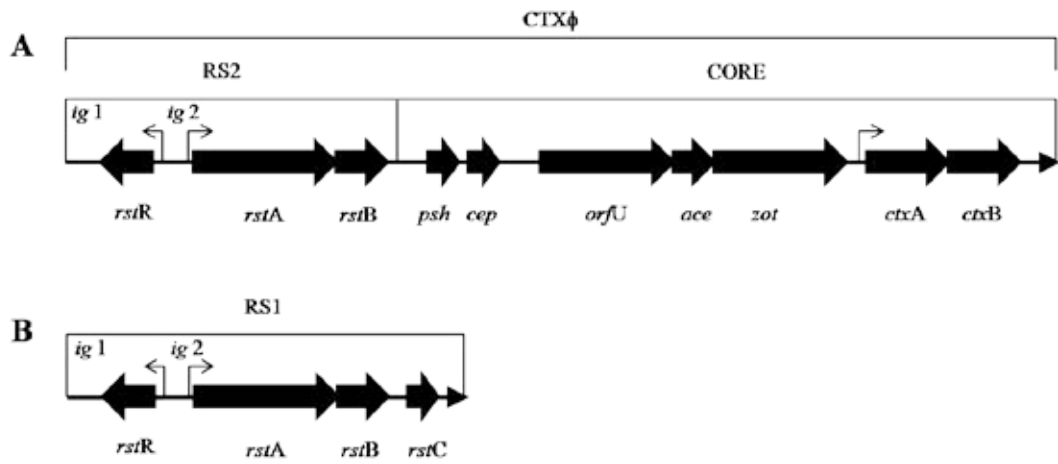


Figure 1.1 Panel A, schematic representation of the genetic organization of CTXφ comprising an RS2 region and a core region. Panel B, schematic representation of the genetic organization of RS1 element. Solid arrows represent ORFs, whereas bend arrows indicate the promoters for *rstR*, *rstA* and *ctxAB*. (Adapted from figure 1, Faruque *et al.*, 2002)

host chromosome. Like other filamentous phages, CTX ϕ is secreted from *V. cholerae* without cell lysis.

1.3 Epidemiology

There were 190,130 cholera cases reported from 53 countries worldwide to WHO in 2008, which involved 5,143 cases of death cause by cholera (WHO, 2009a). The figures represent 7.6% increase in the number of cases and 27% increase in the number of deaths reported as compared with 2007. Prabably, the true number of cholera cases and deaths were far more than those reported. Due to the limitation in facilities and surveillance system in developing and under-develop countries, the under-reported cases were expected to be high. Besides, cases of reported cholera to WHO also excluded numerous cholera cases which were labeled as acute watery diarrhea (AWD) with estimated 500,000-700,000 cases. Figure 1.2 shows cholera cases reported by countries in year 2006-2008.

1.3.1 Early pandemics

There were six cholera pandemics recorded in the period of 1817-1923, and was described as early history of cholera (Kaper *et al.*, 1995). Though, the dates of these pandemics differ among different writers as shown in Table 1.3. But, the most commonly used dates of the first six pandemics are of those proposed by Politzer (1959), the fifth and sixth pandemics were caused by *V. cholerae* of classical biotype, and it is believed that the earlier pandemics were also caused by classical biotype (Blake, 1994, Tauxe, 1998).

1.3.2 Seventh pandemic, 1961-present

The seventh cholera pandemic began in 1961, distinct from previous six pandemics; the causative agent was *V. cholerae* O1 El Tor biotype. The pandemic was originated from th

Table 1.3 Cholera pandemics 1817-1923/5 (Adapted from table 1: Chapter 1, Barua, 1992)

Pandemic	According to					
	Haesser (1882)	Hirsch (1883)	Sticker (1912)	Kolle and Priggs (1928)	Pollitzer (1959)	Wilson and Miles (1975)
1	(a)1816-1823 (b)1826-1837	1817-1823	1817-1838	–	1817-1823	1817-1823
2	1840-1850	1826-1837	1840-1864	–	1829-1851	1826-1837
3	1852-1860	–	1863-1975	–	1852-1859	1846-1862
4	1863-1873	–	1881-1896	–	1863-1879	1864-1875
5	–	–	1899	1883-1896	1881-1896	1883-1896
6	–	–	–	1902-1923	1899-1923/5	1899-1923

island of Sulawesi in Indonesia and spread to the surrounding islands (Faruque *et al.*, 1998). The entire Southeast Asia and Asian mainland were invaded with *V. cholerae* O1 El Tor biotype in between 1962-1969. By 1970s, the pandemic spread to the African continent and reached South America in 1990s with an explosive epidemic in Peru. The pandemic spread to surrounding countries in South America and further north to Central America (PAHO, 1991). The pandemic is still ongoing, and it continues to cause seasonal outbreaks in many developing countries (Faruque *et al.*, 1998). The emergence of *V. cholerae* O1 El Tor biotype has completely displaced classical biotype worldwide, except in Bangladesh where reappearance of the classical biotype at epidemic proportions in 1982 and persist for several years. But, the classical biotype seems to have become extinct again (Shimada *et al.*, 1994, Siddique *et al.*, 1991).

1.3.3 Possibility of eighth pandemic

In late 1992, a new cholera epidemic erupted in Madras, India and in Southern Bangladesh (Faruque *et al.*, 1998). The causative organism isolated from this outbreak was *V. cholerae* of non-O1, which produced cholera toxin and is now known as *V. cholerae* O139 serogroup (Shimada *et al.*, 1994). Rapid spreading of *V. cholerae* O139 serogroup to surrounding countries and Southeast Asia raise concern of the possibility of the eighth pandemic. Outbreaks or cases due to *V. cholerae* O139 have since been reported in countries such as Pakistan, Nepal, China, Thailand, Kazakhstan, Afganistan and Malaysia (Kaper *et al.*, 1995). Imported cases were also reported in the United Kingdom and United States of America. Surveillance during 1996 and 1997 has shown that *V. cholerae* O139 serogroup continues to cause cholera outbreaks in India and Bangladesh and coexists with the El Tor vibrios (Faruque *et al.*, 1997).

1.4 Ecology of *V. cholerae*

1.4.1 Environment

V. cholerae is part of the normal, free-living bacterial flora in riverine and estuarine areas. It has been regarded as a member of organisms whose major habitats are aquatic ecosystems. But, toxigenic strains of *V. cholerae* O1 and O139 serogroups are rarely isolated from the environment, even in epidemic settings which fecal contamination of the environment might be expected (Faruque *et al.*, 1998). However, CT-producing *V. cholerae* O1 and O139 can persist in environment with the absence of known human disease. The persistence of *V. cholerae* within the environment may be facilitated by its ability to assume survival forms, including viable but nonculturable state (VBNC) and the “rugose” survival form (Baker *et al.*, 1983, Colwell and Huq, 2001). Microbiology studies also shown that zooplankton and phytoplankton plays a significant role as a reservoir for *V. cholerae* in the environment (Colwell and Huq, 2001, Tamplin *et al.*, 1990), hence planktonic blooms had been related to seasonal outbreak of cholera (Alam *et al.*, 2006). The *V. cholerae* was also found to be able to survive through attaching to the crustacean and algae from the aquatic environments (Castro-Rosas and Escartin, 2005, Colwell *et al.*, 1992, Estrada-Garcia and Mintz, 1996).

1.4.2 Human

Human can serve as reservoir and source of toxigenic *V. cholerae*. Person with acute cholera can excrete 10^7 - 10^8 CFU of *V. cholerae* organisms per gram of stool and with the output of *V. cholerae* ranging from 10^{11} - 10^{13} CFU (Kaper *et al.*, 1995). Even after cessation of symptoms, patients whom did not receive antibiotics treatment may continue to excrete vibrios for weeks (Dizon *et al.*, 1967). In some cases, a very small minority of patients may continue to excrete the organism for even longer periods of time, for up to 10 years (Kaper *et al.*, 1995). So it is suggested that the asymptomatic human carrier of the toxigenic *V. cholerae* is the primary reservoir for cholera. Yet, opposing views claim that humans do not

constitute a true reservoir for disease causing strains, but there is clearly a dynamic relationship between human and environmental sources of the organism.

1.5 Pathogenesis

1.5.1 *V. cholerae* infection

Infection due to *V. cholerae* begins with the ingestion of food or water contaminated with the organism. Most of the vibrios will be killed by the low pH stomach condition. After surviving passage through the gastric acid barrier in the stomach, the vibrios will penetrate the mucous coat of the intestinal epithelium with the help of its motility properties and enzymes secretion (Faruque *et al.*, 1998, Kaper *et al.*, 1995). Upon reaching the endothelial surface, colonization of the organisms onto microvilli will be achieved with the help of toxin-coregulated pili (TCP) (Kaper *et al.*, 1995, Tacket *et al.*, 1998, Voss *et al.*, 1996). The adhering vibrios through TCP activate expression and secretion of CT by *V. cholerae* that will disrupt ion transport in the intestinal epithelial cells and lead to subsequent loss of water and electrolytes (Field *et al.*, 1972). The severity of symptoms varies with the *V. cholerae* strain ingested, the infection dose, the effect of gastric acid and also patient's blood type (Tauxe, 1998).

1.5.2 Cholera toxin

Cholera toxin is the most significant pathogenic factor secreted by *V. cholerae* subsequent to colonization at the small bowel. The holotoxin CT (comprising the subunits A and B) are encoded by *ctxAB* genes which are part of a cluster of genes normally referred to as the CTX genetic element (Faruque *et al.*, 1998). The CTX genetic element also carries the *zot* gene coding for zonula occludens toxin (Zot), the *ace* gene encoding accessory cholera toxin (Ace), a core-encoded pilin (*cep*) responsible for enhancing colonization and an open reading

frame (ORF) of unknown function (*orfU*) (Waldor and Mekalanos, 1996) as shown in Figure 1.1.

The enterotoxin is an oligomeric complex made up of six protein subunits: one A subunit (CT-A) and five B subunits (CT-B). The CT-B forms a symmetrical pentamer from five identical CT-B subunits with a cylindrical central pore (Chaudhuri and Chatterjee, 2009b). While the A1 fragment of the CT-A subunit rest on the B pentamer, with the elongated A2 fragment alpha helix tail that extends through the pore of CT-B holotoxin. The A2 fragment alpha helix tail is mainly responsible for the interaction between the CT-A and CT-B subunits. In nature, the CT binds to ganglioside GM1 receptor in the intestinal epithelial cell by its CT-B subunits (Holmgren *et al.*, 1975, Kaper *et al.*, 1995). Figure 1.3 shows binding of CT to GM1 on the surface of epithelial cell membrane. Upon successful binding of pentameric CT-B to GM1, the entire CT were which are linked by an exposed loop containing a site for proteolytic cleavage (after arginine 192) and a disulfide bond that bridges the cleavage site are then proteolytically cleaved and reduced. This will generate enzymatically active A1 peptide, ADP-ribosyl transferase (22 kDa) and A2 peptide (5 kDa) (Chaudhuri and Chatterjee, 2009b). The A1 peptide migrates into the cytosol of the cell and activates adenylate cyclase by catalyzing the ADP-ribosylation of the subunit of the heterotrimeric GTPase (Lencer *et al.*, 1993). This will cause a sustained increase of intestinal cAMP levels (Bennett *et al.*, 1975), which results in excess secretion of isotonic fluid into the intestinal lumen. The A2 peptide forms the scaffolding that tethers the A and B subunits together (Chaudhuri and Chatterjee, 2009b).

1.5.3 MARTX toxin

Multifunctional autoprocessing repeat-in-toxin (MARTX) was discovered by Lin *et al.*, 1999 in a search for undiscovered potential virulence determinants in *V. cholerae* based on similarities to virulence genes of other pathogenic microorganisms (Lin *et al.*, 1999). The MARTX gene cluster was uncovered during analysis for contigs located in the vicinity of the

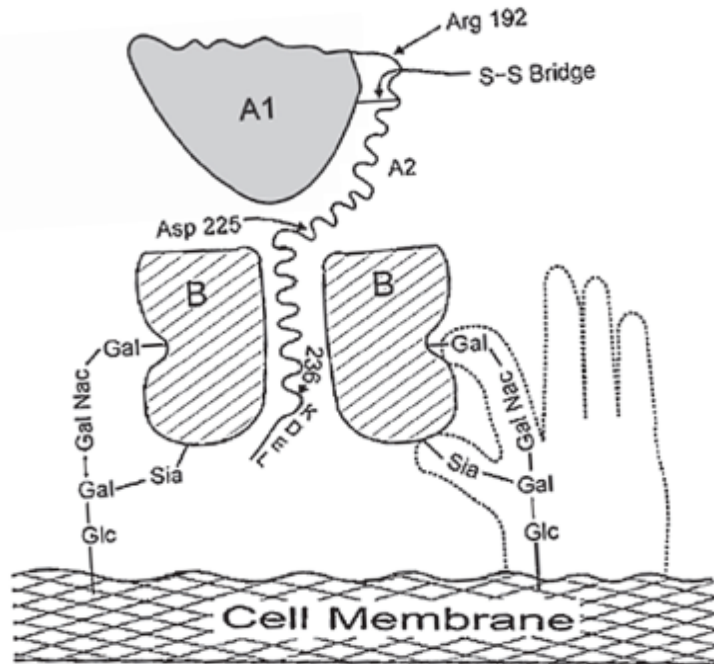


Figure 1.3 Schematic diagram showing the binding of cholera toxin molecule through its pentameric CT-B to the GM1 pentasaccharides of the epithelial cell membrane. (Adapted from figure 7.7 Cholera Toxins, Chaudhuri and Chatterjee 2009c)

CTX genetic element. Four genes were found to display high degrees of similarity to the biogenesis of RTX toxins in several Gram-negative organisms. Hence, it is categorized in RTX toxin family of secreted bacterial cytotoxins. The RTX toxin nomenclature is derived from the presence of a C-terminal calcium-binding domain of acidic glycine-rich nonapeptide repeats in the members of the RTX toxin family. Most of *V. cholerae* isolates belonging to O1 El Tor, O139, and non-O1 non-O139 serogroups, exhibited intact MARTX gene clusters (Chow *et al.*, 2001, Cordero *et al.*, 2007). However, MARTX gene clusters in classical *V. cholerae* strains was found to have deletion that removes 5' end of *rtxA*, whole *rtxC* and 5' end *rtxB* (Lin *et al.*, 1999). Hence, the classical *V. cholerae* strains do not cause cell rounding in eukaryotic cell lines.

MARTX toxin of *V. cholerae* is encoded by *rtxA* gene which resides within the MARTX gene cluster together with *rtxB*, *rtxC*, *rtxD*, *rtxE* and VC1449 (Boardman and Satchell, 2004, Sheahan *et al.*, 2004) as shown in Figure 1.4. These *rtxB*, *rtxC*, *rtxD* and *rtxE* genes play an important role in production and transportation of the MARTX toxin. The *rtxC* encodes an acyltransferase, which acts as an activator of MARTX toxin (Cheong *et al.*, 2010), whereas *rtxB*, *rtxD* and *rtxE* encode transportation proteins that are involved in the transport of the toxin into the environment. The VC1449 codes for a conserved hypothetical protein (Boardman and Satchell, 2004, Sheahan *et al.*, 2004).

Unlike members of RTX family of toxins, *V. cholerae* MARTX toxin does not disrupt membrane integrity and do not cause necrosis or leakage of cytoplasmic products (Lin *et al.*, 1999, Saha *et al.*, 1996). Functional analysis revealed that the toxin is responsible for the rounding and detachment of HEp-2 cells *in vitro* and mediate actin crosslinking (Cordero *et al.*, 2007, Fullner and Mekalanos, 2000) or inactivation of Rho GTPases (Sheahan *et al.*, 2007). Since then, the toxin was renamed as multifunctional autoprocessing repeat-in-toxin (MARTX).

Despite the difference between MARTX of *V. cholerae* and other RTX toxins of Gram negative organisms, the MARTX of *V. cholerae* is also secreted by type 1 secreting system (T1SS) (Boardman and Satchell, 2004). Study by Boardman and Satchell (2004)

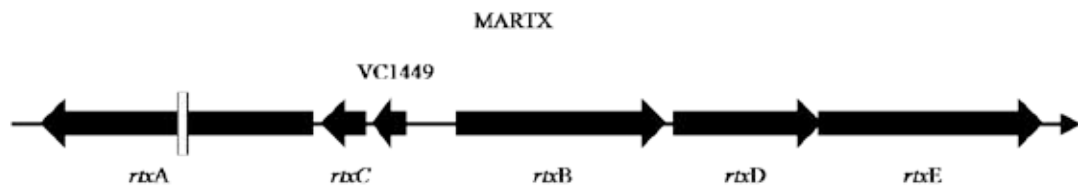


Figure 1.4 Schematic representation of the genetic organization of *V. cholerae* MARTX gene clusters. (Adapted from figure 1a, Boardman *et al.*, 2004)

shows that *V. cholerae* MARTX toxin is secreted by a four component T1SS encoded by *rtxB*, *rtxD* *rtxE* and *tolC*. Comparatively, the *rtxB* operon is a putative ATPase gene and *rtxD* operon is a putative periplasmic linker protein gene. Both genes were overlapped by 46 nucleotides and encodes proteins that are 63% and 50% similar to *hlyB* and *hlyD* genes exemplified by *E. coli* haemolysin T1SS (Lin *et al.*, 1999). The *rtxE* operon which located downstream to *rtxD*, encodes a transport ATPase that is 63% similar to *hlyB* and 60% similar to *rtxB* of *V. cholerae* (Boardman and Satchell, 2004). The involvement of *tolC* which is unlinked to MARTX gene cluster in toxin secretion was proposed from evidence that the absence of cell rounding by *V. cholerae* in *tolC* mutant (Bina and Mekalanos, 2001).

V. cholerae MARTX did not disrupt membrane integrity and did not cause leakage of cytoplasmic components in eukaryotic cells, which distinct it from pore forming RTX family of toxins. The toxin causes cell rounding of eukaryotic cell types such as Hep-2 and T84 cell lines without direct effect on cell viability by means of cytoskeleton alteration (Steele-Mortimer *et al.*, 2000). It was proposed that cytoskeleton alteration by *V. cholerae* MARTX toxin occurs through two different pathways: either by actin crosslinking (Fullner and Mekalanos, 2000, Lin *et al.*, 2000) or by Rho GTPases inactivation (Sheahan *et al.*, 2007), as illustrated in Figure 1.5. MARTX toxin targets globular monomer (G-actin), resulting in the crosslinking of actin into dimers, trimers, and higher-order crosslinked products, disrupting the equilibrium between filamentous polymer (F-actin) and G-actin, ultimately resulting in F-actin disassembly and an increase in paracellular permeability (Fullner and Mekalanos, 2000). Cell rounding and actin depolymerization associated with MARTX was also prevented in Rho GTPases activation, suggesting involvement of Rho GTPase signaling pathways in MARTX activity. However, the extent to which these activities of RTX influence pathogenesis *in vivo* is largely unknown.

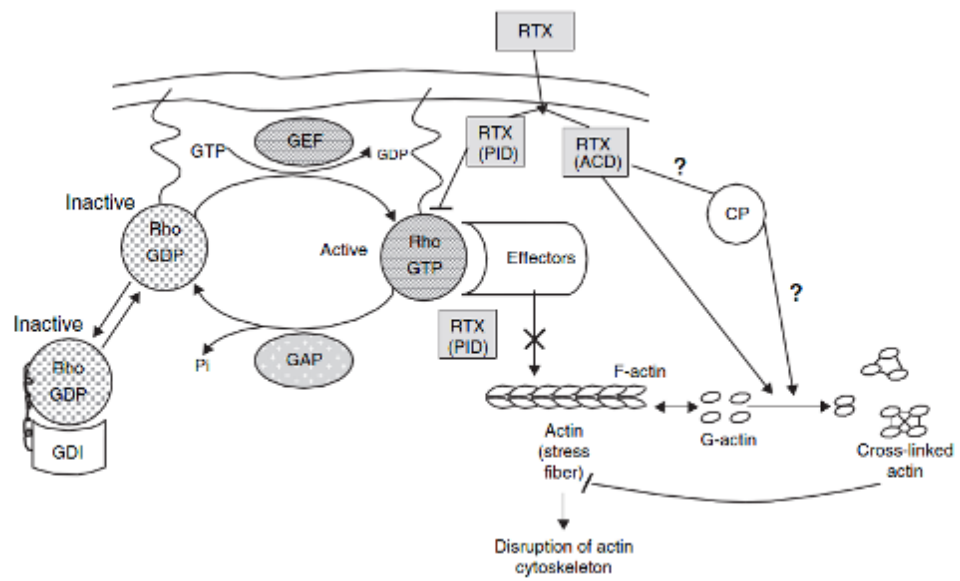


Figure 1.5 Mechanism of disruption of actin cytoskeleton by MARTX toxin by two different pathways: action crosslinking and Rho GTPase inactivation. (Adapted from figure 13.8, Cholera Toxins, Chaudhuri and Chatterjee, 2009a)

1.5.4 Other Toxins

1.5.4.1 Haemolysin (Hly)

Haemolysin of *V. cholerae*, often called *V. cholerae* cytolysin (VCC), is a 65 kDa exotoxin encoded by *hlyA* gene. It is produced by most *V. cholerae*, including many non-CT producing strains (Goldberg and Murphy, 1984, Honda and Finkelstein, 1979, Yamamoto *et al.*, 1984). Mature VCC forms heptameric oligomers, insert into lipid bilayers to generate noncylindrical anionselective membrane pores about 1–2 nm in internal diameter (Galdiero and Gouaux, 2004). The VCC attacks red blood cell membranes and causes cell rupture with the liberation of haemoglobin (Honda and Finkelstein, 1979). It has been proposed that VCC contributes to the pathogenesis of gastroenteritis caused by *V. cholerae*, especially with non-CT producing strains (Pichel *et al.*, 2003), although the significance of VCC in the disease in humans has been questioned.

1.5.4.2 Zonula occludens toxin (Zot)

Zot is a single polypeptide chain of 44.8 kDa in size and 399 amino acids in length. It has a bacteriophageal origin, and is present in toxigenic strains of *V. cholerae* (Baudry *et al.*, 1992, Fasano *et al.*, 1991). Studies also showed that the activity of Zot is reversible, heat-labile and sensitive to protease digestion. The toxin reversibly alters the permeability of intestinal epithelial tight junctions, allowing the passage of macromolecules through mucosal barriers (Salama *et al.*, 2006). This alteration was found only confined to the small intestine, since Zot does not affect the colon permeability. The selective effect of the toxin on the small intestine seems related to the regional distribution of the Zot receptors that are present in the jejunum and ileum, but not in the colon (Chaudhuri and Chatterjee, 2009c).

1.5.4.3 Accessory cholera enterotoxin (Ace)

Ace is another toxin produced by *V. cholerae*, its open reading frame is located upstream of *zot* and *ctx* gene in the filamentous phage, CTX ϕ . The termination codon of *ace* overlaps with the initiation codon of *zot*, an arrangement involving translational coupling between *ace* and *zot*. The toxin comprises a 289 bp gene coding for 96 amino acids with a predicted molecular mass of about 11.3 kDa (Trucksis *et al.*, 1993). The mechanism of action of Ace has been delineated in a human colonic tumor cell line, T84 cells (Trucksis *et al.*, 2000). It has been suggested that Ace may contribute to an early phase of intestinal secretion in *V. cholerae* infection before the onset of the secretion stimulated by the more slowly acting cholera toxin (Trucksis *et al.*, 2000).

1.5.4.4 Shiga-like toxin (SLT)

Shiga-like toxins (or verotoxins) in *V. cholerae* was reported by O'Brien *et al.*, 1984 on the basis of cytotoxic activity of cell lysates towards HeLa cells, and this activity was found to be neutralized by antibody against purified Shiga toxin produced by *Shigella dysenteriae* type 1 (O'Brien and Holmes, 1987). SLT is moderate to high levels of heat-stable (56 °C, 15 min) and cause cytotoxicity to certain cell lines, enterotoxic in rabbit ileal loops, and neurotoxic (causing limb paralysis and death) in rabbits and mice (Chaudhuri and Chatterjee, 2009c). Genes encoding SLT have not been cloned from *V. cholerae* despite repeated efforts as there is no homology could be found in genome sequences of *V. cholerae* with SLT *Shigella dysenteriae* type 1 and *slt-1* of *E. coli* (Chaudhuri and Chatterjee, 2009c).

1.6 Transmission and prevention of cholera

1.6.1 Transmission of cholera

Cholera can be transmitted through ingestion of contaminated food or water. In epidemics and endemics, the primary source of the contaminant may be the faeces of the infected humans, and food serve an important role as vehicle for the transmission (Estrada-Garcia and Mintz, 1996, Tauxe, 1998). Most of the vibrios will not survive the passage through stomach gastric acid, but the organisms hidden within the food particles are spared (Rabbani and Greenough, 1999). Besides, foods also serve as culture medium for *V. cholerae* to multiply and achieve sufficient dose for causing disease (Estrada-Garcia and Mintz, 1996). As mentioned earlier, *V. cholerae* could also survive within crustacean and many sea creatures, and the general preference to eat raw or partially cooked seafood makes seafood the most commonly implicated vehicle in foodborne cholera.

1.6.2 Prevention of cholera

The existence of environmental reservoirs of toxigenic *V. cholerae* means the organism is likely to persist indefinitely. As long as the organism persists, the potential for epidemic cholera exist, wherever and whenever conditions are ripe for sustained transmission. Therefore proactive steps should be taken to prevent the transmission of the disease.

1.6.2.1 Disinfection and sanitation

The success of control efforts depends on knowing the actual mechanisms or vehicles of transmission and having the tools available to block them. *V. cholerae* disseminate through contaminated food and water, hence the best way to prevent the disease is to provide clean water and food. The organisms can be killed through boiling of drinking water or chlorinating the water supply. Eating hot serving food also helps to prevent the spreading of

the disease. Generally, the practice of proper hygiene will help to prevent the spreading of the organism (Estrada-Garcia and Mintz, 1996). In the long term, improvements in water supply, sanitation, food safety and community awareness of preventive measures are the best means of preventing cholera and other diarrheal diseases.

1.6.2.2 Immunization

Immunization is the ideal prophylactic intervention for cholera prevention. There are two oral cholera vaccine currently available, WC/rBS - killed whole cells vaccine with a recombinant B subunit of CT and CVD103-HgR - live attenuated cholera vaccine (WHO, 2001). However, these vaccines are for *V. cholerae* O1 serogroup and do not confer cross-protection against toxigenic *V. cholerae* O139 serogroup (Tacket and Sack, 2008, WHO, 2001). Hence, *V. cholerae* O139 serogroup may pose as a threat to the world as it is capable of causing cholera pandemic.

1.7 Cholera vaccines

The early attempt of cholera vaccine was by Ferran (1884) shortly after the discovery of *V. cholerae* by Koch. After Ferran, Haffkine also began to work on cholera vaccine and he was convinced of the efficacy of his vaccine as none of the immunized person developed cholera (Tacket and Sack, 2008). The vaccine, which was made of killed *V. cholerae* strains from both Inaba and Ogawa serotypes, was never recommended by WHO, but still may be available in some countries. Two types of cholera vaccines have been developed since then, a killed oral vaccine and a live attenuated oral vaccine; both have been shown to be safe, immunogenic and efficacious.

1.7.1 Immunity to *V. cholerae*

It is believed that cholera is a non-inflammatory infection. There are often no gross changes to the intestinal mucosa or the architectural integrity of the small bowel. However, we discover that our *V. cholerae* strain of O139 serogroup causes pathological changes to the small intestine at high dose of 10^8 CFU in 1cm intestinal loop (Amin *et al.*, 2009). There are also upregulation of pro-inflammatory cytokines production in the intestinal cells (Nandakumar *et al.*, 2009, Stokes *et al.*, 2004, Zhou *et al.*, 2004) and migration of neutrophils to the lamina propria during acute cholera (Qadri *et al.*, 2004).

Study had demonstrated that *V. cholerae* infection induces protective immunity against subsequent infection (Kaper *et al.*, 1995). It was suggested that secretory immunoglobulin A (sIgA) protects against colonization of the mucosa. Increase of antigen-specific lymphocytes in circulation (Bhuiyan *et al.*, 2009) and recruitment to intestinal mucosa leads to elevated sIgA secretion in intestinal mucosa. Serum vibriocidal antibody and anti-toxins antibodies were also elicited besides sIgA, which correlate with protective immune response. But these antibodies decrease to baseline levels one year after infection. Similarly, mucosal sIgA levels decrease to baseline levels in months after infection (Nelson *et al.*, 2009). However, when challenged with *V. cholerae* antigens, anamnestic immune response was demonstrated with rapid increase in antibody secretion in the intestine. Hence, it is the anamnestic immune response that mediates protection against cholera, which also supported by presence of cholera induced memory B cell even after one year of cholera infection (Longini *et al.*, 2002).

1.7.2 Cytokines during infection

Cytokines are central to the development of effective immunity against microbial pathogens. It is well documented that cytokines involvement in immune reaction during active infection. Different cytokines were secreted by the infected cells in response to the damage caused by the pathogen. The understanding of secreted cytokines by these cells during infection will