SITE DIRECTED MUTATION OF *ctx* OPERON IN VCUSM2, *Vibrio cholerae* VACCINE CANDIDATE STRAIN: TOWARDS THE DEVELOPMENT OF A VACCINE FOR CHOLERA

by

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TABLE OF CONTENTS

Acknowledgements ii

Table of Contents iii

List of Tables vi

List of Figures vii

List of abbreviations ix

Abstrak x

Abstract xi

Chapter 1: Introduction 1
- 1.1 Cholera 1
- 1.2 Epidemiological data and statistics 1
- 1.3 Pathophysiology 2
- 1.4 Prevention & vaccines & cost 3
- 1.5 Strains 5
  - 1.5.1 Serogroup 5
  - 1.5.2 Serotype 6
  - 1.5.3 Biotype 6
- 1.6 CTXΦ Phage 7
- 1.7 Toxins & Proteins 8
- 1.8 VCUSM2 10
- 1.9 Objectives 11

Chapter 2: Materials and Methods 12
- 2.1 Experimental overview 12
- 2.2 Reagents, chemicals, kits 13
  - 2.2.1 Culture media 13
    - 2.2.1.1 Luria Bertani (LB) agar 13
    - 2.2.1.2 Luria Bertani (LB) broth 13
    - 2.2.1.3 Modified LB agar without NaCl and with 10% sucrose 14
    - 2.2.1.4 Modified LB broth without NaCl and with 10% sucrose 14
    - 2.2.1.5 TCBS Agar 15
    - 2.2.1.6 Addition of antibiotics/ supplements to the agar based media 15
  - 2.2.2 General buffers, stock solutions, antibiotics 16
    - 2.2.2.1 δ-Aminolevulinic Acid 40 mg /ml 16
    - 2.2.2.2 Ampicillin 100 mg/ml 16
    - 2.2.2.3 Calcium chloride 100 mM 16
    - 2.2.2.4 Carbonate buffer 60 mM pH 9.6 17
    - 2.2.2.5 Cholera toxin (0.5 µg /ml) 17
    - 2.2.2.6 Ethanol 70% 17
    - 2.2.2.7 EDTA 0.5 M 17
    - 2.2.2.8 Ethidium bromide 10 mg /ml 18
    - 2.2.2.9 Gel loading dye 6X 18
    - 2.2.2.10 Glycerol 80% 18

2.2.2.11 Hydrochloric Acid 1N 19
2.2.2.12 Kanamycin sulfate 50 mg /ml 19
2.2.2.13 Magnesium chloride 100 mM 19
2.2.2.14 Normal saline (0.9% NaCl) 19
2.2.2.15 Phosphate Buffered Saline (PBS) 10 X 20
2.2.2.16 Phosphate Buffered Saline-Tween 20 20
2.2.2.17 Polymyxin B sulfate 0.75 mg /ml (4500 units) 20
2.2.2.18 Sodium bicarbonate 60 mM 21
2.2.2.19 Sodium carbonate 60 mM 21
2.2.2.20 Sodium hydroxide 10M 21
2.2.2.21 Sodium hydroxide 1M 21
2.2.2.22 Skimmed milk 5% 22
2.2.2.23 Tris Acetic Acid EDTA (TAE) 50X 22
2.2.2.24 Tris Acetic Acid EDTA (TAE) 1X 22
2.2.2.25 TypeIII Ganglioside (10 µg /ml) 22
2.2.3 Bacterial species and strains 23
2.2.3.1 *E. coli* BW 20767- λ pir 23
2.2.3.2 *E .coli* TOP10 23
2.2.3.3 *V. cholerae* O139 Bengal 23
2.2.3.4 VCUSM2 23
2.2.4 Plasmids 24
2.2.4.1 pTZ57R 24
2.2.4.2 pWM91 24
2.2.5 Animals 24
2.3 Methods 25
2.3.1 Bacterial strains and culture conditions 25
2.3.1.1 Medium term storage of bacterial strains as glycerol stock 25
2.3.1.2 Recovery of bacteria from glycerol stock 26
2.3.1.3 Long term storage of bacterial strains as lyophilized stock 26
2.3.1.4 Recovery of bacteria from lyophilized stock 26
2.3.2 Cloning and mutagenesis 28
2.3.2.1 PCR 28
2.3.2.1.1 Primer designing 28
2.3.2.1.2 Preparation for bacterial lysate for PCR 31
2.3.2.1.3 PCR amplification 31
2.3.2.1.4 Inverse PCR 32
2.3.2.2 Site-directed mutagenesis 32
2.3.2.2.1 Designing mutagenic primers 32
2.3.2.2.2 Site directed mutagenesis master mix 33
2.3.2.2.3 Temperature cycling 34
2.3.2.2.4 Plasmid Digestion 34
2.3.2.3 Ligation 35
2.3.2.4 Transformation 36
2.3.2.4.1 Competent cell preparation 36
2.3.2.4.2 Transformation by Calcium Chloride method 36
2.3.2.5 Restriction enzyme 37
2.3.2.5.1 *Fse*I 37
2.3.2.5.2 *Sma*I 37
2.3.2.5.3 *Sac*I & *Sal*I 37
2.3.2.5.4 *Sac*I & *Sma*I 37
2.3.2.5.5 *Sac*I, *Sma*I & *Bgl*I 38
2.3.2.6 Blunting DNA strands 38
2.3.2.7 Kinasing 38
2.3.2.9 Electrophoresis 39
2.3.3 Conjugation 40

2.3.3.1 Preparation of donor cells ... 40
2.3.3.2 Preparation of recipient cells ... 40
2.3.3.3 Conjugative mating ... 41
2.3.3.4 Phenotypic screening ... 41
2.3.3.5 Genotypic screening ... 42
2.3.4 Sucrose selection ... 42
2.3.4.1 Phenotypic screening ... 42
2.3.4.2 Genotypic screening ... 43
2.3.4.3 Serotyping ... 44
2.3.5 Cholera Toxin ELISA ... 44
2.3.6 Ileal Loop Ligation ... 45
2.3.7 Infant Mouse colonization ... 45

Chapter 3: Results ... 46
3.1 Plasmid construction ... 48
3.1.1 pTZctxOp ... 49
3.1.2 pTZctxOp-MD ... 53
3.1.3 pcODmA ... 57
3.1.3a pWM-coDmA ... 64
3.1.4 pcoDmAKan ... 68
3.1.4a pWM-coDmAKan ... 72
3.1.5 pcoDmA ΔKan ... 75
3.1.5a pWM-cODmA ΔKan ... 79
3.2 Sequencing ... 83
3.3 Conjugation ... 84
3.4 Serotyping ... 95
3.5 Cholera Toxin ELISA ... 95
3.6 Rabbit ileal loop ... 96
3.7 Infant mouse colonization ... 97

Chapter 4: Discussion ... 98

References ... 103

Appendices ... 107

List of Tables

Table 2.1: Bacterial strains and culture conditions 25

Table 2.2: Primers used in this study, their sequences and functions 29

Table 2.3: Primers used for mutagenesis and screening of mutations, their sequences and function in this study 30

Table 2.4: Composition of a standard PCR master mix for a 20 µl reaction 31

Table 2.5: Typical PCR cycling conditions used in this study 32

Table 2.6: Mutagenic primer sequences and %GC 33

Table 2.7: PCR program for site directed mutagenesis 34

Table 2.8: Culture plates used in phenotypic screening of merodiploids and *V. cholerae* mutants 43

Table 3.1: Constructed plasmids, inserts and description of the inserted genes 48

Table 3.2: Fluid accumulation results from rabbit ileal loop ligation experiment 96

Table 3.3: Inoculum size and recovery of *V. cholerae* strains in infant mouse colonization experiments 97

List of Figures

Figure 1: Experimental overview 12

Figure 2: Schematic representation of conjugation and allele replacement towards the construction of VCUSM14 47

Figure 3: Amplification of *ctx* operon 49

Figure 4: PCR screening of *ctx*AB in pTZ-ctxOp 51

Figure 5: Diagram of pTZ-ctxOp 52

Figure 6: Inverse PCR for the deletion of *ace* and *zot* 54

Figure 7: PCR screening for deletion of *ace* and *zot* in mutant plasmids 55

Figure 8: Restriction enzyme analysis of pTZctxOp-MD 56

Figure 9: Site directed mutagenesis of 7th amino acid codon 58

Figure 10: PCR screening for mutated 7th amino acid codon using mutant specific primer, MctxA7MS-F 59

Figure 11: PCR screening of mutated 7th amino acid with wild type primer, MctxA7WS-F 60

Figure 12: Site directed mutagenesis of 112th amino acid codon 61

Figure 13: PCR screening of 112th amino acid codon mutation with mutant specific (MctxA112MS-F) and wild type specific (MctxA112WS-F) primers 62

Figure 14: Diagram of pcODmA 63

Figure 15: Restriction of pcODmA with *Sac*I and *Sal*I 65

Figure 16: PCR screening for pWM-cODmA by amplifying *ctx*A 66

Figure 17: Diagram of pWM-cODmA 67

Figure 18: Restriction enzyme analysis of pcODmAKan and pcODmA to compare insert size 69

Figure 19: Restriction of pcODmAKan to remove insert from plasmid for subcloning purposes 70

Figure 20: Diagram of pcODmAKan 71

Figure 21: PCR screening of pwm-coDmAKan 73

Figure 22: Diagram of pWM-cODmAKan 74

Figure 23: Inverse PCR of pcODmAKan to truncate *aph*A gene 76

Figure 24: PCR screening of pcODmA ΔKan by PCR 77

Figure 25: Diagram of pcODmA ΔKan 78

Figure 26: Restriction of pcODmA ΔKan using *Sac*I, *Sma*I and *Bgl*I 80

Figure 27: PCR screening for pWMcODmA ΔKan 81

Figure 28: Diagram of pWM--cODmA ΔKan 82

Figure 29: PCR screening for merodiploid for *ctx* operon 85

Figure 30: PCR screening of *ace* and *zot* genes in VCUSM11 86

Figure 31: PCR screening merodiploid (potential VCUSM12) for *sac*B gene 87

Figure 32: PCR screening for the loss of *ace* gene in VCUSM12 88

Figure 33: PCR screening of merodiplod (potential VCUSM13) for *sac*B gene 89

Figure 34: PCR screening of possible VCUSM13 for deletion of *aphA* gene 90

Figure 35: PCR screening of merodiploids (potential VCUSM14) for *sac*B gene 91

Figure 36: PCR screening for *aph*A and truncated *aph*A in VCUSM14 92

Figure 37: PCR screening for mutated 7th amino acid in VCUMS14 93

Figure 38: PCR screening for mutated 112th amino acid codon in VCUSM14 94

List of abbreviations

ΔKan	mutated *aph*A gene
10X	10 times
ALA	δ-aminolevulinic acid
bp	base pair
ChrI	chromosome 1
CNIC	Centro Nacional de Investigaciones Científicas, Cuba
CT	cholera toxin
CTA	cholera toxin subunit A
CTB	cholera toxin subunit B
CTXΦ	cholera toxin phi phage

Mutasi tertumpu tapak operon *ctx* dalam VCUSM2, calon vaksin *Vibrio cholerae*: ke arah pembangunan vaksin kolera

ABSTRAK

Kolera ialah penyakit cirit-birit akut dan dahsyat yang disebabkan oleh jangkitan bakteria *Vibrio cholerae* O1 dan O139. Ia merupakan penyakit endemik di negara-negara yang tidak mempunyai sumber air yang bersih mahupun sistem kumbahan yang baik. Jumlah kes akibat kolera dilaporkan semakin meningkat. Vaksin yang memberi perlindungan terhadap jangkitan *V. cholerae* O139 belum wujud lagi. Objektif kajian ini adalah untuk membangunkan vaksin O139 yang telah dilemahkan melalui mutasi operon *ctx* dalam VCUSM2. Mutasi ini akan menjadikan VCUSM2 tidak toksik tetapi mengekalkan keupayaan perangsangan sistem imun badan. VCUSM2, adalah mutan *hem*A, strain O139, yang memerlukan bekalan ALA luaran dan mempunyai dua salinan operon *ctx*. Operon ini diklonkan dalam pTZ57R, *ace* dan *zot* dilupuskan melalui PCR songsang, dan asid amino ke-7 dan ke-112 subunit A toksin kolera dimutasikan. VCUSM14 dihasilkan melalui siri eksperimen konjugasi VCUSM2 dengan BW20767-λpir yang membawa pWM91 yang mengandungi gen-gen tertentu, diikuti pemilihan sukrosa. VCUSM14 tidak mengakibatkan cecair berkumpul dalam kajian model usus arnab (‘rabbit ileal loop model’) tetapi mengkolonikan usus anak mencit (‘infant mouse’) dengan lemah. Molekul toksin kolera yang dihasilkan berjaya dikenalpasti melalui ujian CT-ELISA dengan menggunakan antibodi poliklonal. VCUSM14 mempunyai potensi dibangunkan sebagai calon vaksin sekiranya keupayaan mengkolonisasikan usus berjaya ditingkatkan melalui pengembalian gen *hem*A yang sempurna ke dalamnya.

Site Directed Mutation of *ctx* operon in VCUSM2, *Vibrio cholerae* vaccine candidate strain:
Towards the development of a vaccine for cholera

ABSTRACT

Cholera is an acute diarrheal disease caused by *Vibrio cholerae* O1 and O139. It affects countries with scarce clean water supplies and poor sanitation systems. Reported cases and mortality rates are increasing. No vaccines are available that confers protection against the O139 serotype. This study aims to create an attenuated O139 vaccine strain by mutating the *ctx* operon in VCUSM2 through site-directed mutagenesis, rendering it non-toxigenic but retaining its immunogenicity. VCUSM2 is a *hem*A mutated, ALA auxotrophic O139 strain with two copies of *ctx* operon in tandem. The operon was cloned into pTZ57R, *ace* and *zot* genes were deleted by inverse PCR and the cholera toxin A subunit was mutated at the 7th (arginine to lysine, R7K) and 112th (glutamate to glutamine, E112Q) amino acid positions. VCUSM14 was obtained through a series of conjugation with BW20767-λpir *E. coli* harboring pWM91 carrying respective inserts, and sucrose selection. VCUSM14 does not cause fluid accumulation in rabbit ileal loop model and is a poor colonizer in the infant mouse model. CT-ELISA showed that it produces toxin molecules recognizable by polyclonal anti-CT antibodies. This strain shows potential for vaccine development once colonization abilities are improved through reinsertion of the *hem*A gene.

Chapter 1: Introduction

1.1 Cholera

Cholera is an acute watery diarrheal disease caused by toxigenic strains of *Vibiro cholerae*, a Gram negative bacillus. The disease is characterized by rice watery stool, vomiting, dehydration and in its extreme form cholera gravis, hypovolemic shock and death. It is a disease that mainly affects the poor, in countries where clean water and sanitation systems are not readily available.

Historically, there have been 7 recorded pandemics of the disease. The first 6 pandemics were caused by the classical biotype of O1 *V. cholerae.* Although strains of the El Tor biotype were known to cause sporadic infections, it was only in 1961 that this strain emerged to cause the 7th pandemic.

In 1992, a new strain of *V. cholerae* was discovered to be the etiologic agent for epidemic outbreaks of cholera in India and Bangladesh. As it did not agglutinate in *V. cholerae* O1 antiserum or in any of the 137 non-O1 serogroup antisera, this strain was designated the name O139, a new serogroup of *V. cholerae*. *V. cholerae* O139 is also known as *V. cholerae* Bengal. The concern with regards to the Bengal strain is that it seems highly capable of being the etiologic agent for an 8th cholera pandemic.

1.2 Epidemiological data and statistics

The number of cholera cases reported in 2005 has increased 30% compared to the number of cases reported in 2004. In 2005, there were 131,943 cases, with 2272 deaths reported, representing a 30% increase compared to the numbers in 2004 (WHO 2006).

Interestingly, despite major natural disasters such as Hurricane Katrina and the tsunami, almost no cholera cases were reported from these affected areas with only 4 cases being reported in the United States being linked to Hurricane Katrina (WHO 2006).

Officially notified cases in Asia showed an 18% increase in 2005, with a total of 6824 cases and 37 deaths in 9 Asian countries. The Indian continent reported 46% of all cases reported in Asia. Indonesia reported 20% of the total cases in Asia and 19 deaths. However, none of these cases reported were from the region affected by the tsunami in 2004 (WHO 2006).

Cholera cases are much under reported because of the affect it may have on the tourism industry of a country. Although many cases meet the WHO case definition of cholera, they are reported as acute watery diarrhea and thus does not describe the actual incidence of this disease. In Afghanistan alone, 150,000 cases of acute watery diarrhea were reported in 26 provinces that, according to WHO case definition, are cholera (WHO 2006).

Information with regards to infection with *Vibrio cholerae* O139 was only available from China where 35% of the laboratory confirmed cases of cholera were attributed to be by the O139 strain (WHO 2006).

1.3 Pathophysiology

Cholera results from the ingestion of pathogenic *Vibrio cholerae*, usually from a contaminated food or water source. The incubation period for the organism varies from 12 to 72 hours, depending on the size of the inoculum and also the susceptibility of the individual (Cash *et al.*, 1974).

V. cholerae is highly acid labile and most of the organism would be killed in the acidic environment of the gastric pouch. Thus, a relatively large inoculum size (10^6-10^8) compared to other enteric pathogens is required to establish infection.

Individual susceptibility revolves around the pH of the stomach, where the infectious dose is lower and the risk of illness is higher in persons who are hypochlorhydric (Wachsmuth IK, 1994). In one study, volunteers who were administered sodium bicarbonate to neutralize the gastric acid showed that the required infectious dose was much lower than in a normal individual. A study with heavy cannabis users reported increased risk of developing diarrhea following ingestion of *V. cholerae* O1 compared with persons who did not smoke cannabis or who smoked cannabis less frequently. This finding may explain the patterns of infection in the Ganges delta, where cannabis use is common in parts of the population (Wachsmuth IK, 1994).

Vibrio cholerae colonizes the epithelial cells of the intestines but does not invade nor alter the structure of these cells (Wachsmuth IK, 1994). Once the organism successfully colonizes the intestines, the production of cholera toxin leads to the increase of chloride and bicarbonate secretion into the intestinal lumen. At the same time, there is a decrease in villous absorption of sodium chloride. This causes intracellular fluid to be secreted into the intestinal lumen and subsequently leads to the acute diarrhea symptoms characteristic of cholera.

1.4 Prevention & vaccines & cost

Extensive and in depth studies of *V. cholerae*, its genes, their functions and the proteins they code for have been carried out and are still on-going. Yet many people still die of cholera yearly (WHO 2006). The long term solution for the problem is to provide safe and clean

drinking water and to establish good sanitation systems in communities where cholera is endemic. The short term solution would be, and is, to develop a safe, immunogenic, affordable vaccine to be administered to the masses in countries where clean water and sanitation are not readily available.

However, these countries often do not have the resources to fund mass health campaigns, what more to purchase readily available cholera vaccines for a large scale vaccination program. Hence, there is a need for a vaccine to be made available to these nations at the lowest possible cost. Currently, apart from being too expensive for mass vaccination, the available cholera vaccines do not confer long term immunity (Trach *et al.*, 1997, WHO 2004) and do not produce convincing protection in differing communities (Richie *et al.*, 2000, WHO 2004).

Studies have shown that patients who experience cholera exhibit long term immunity towards reinfection (Wachsmuth IK, 1994). This would mean that to produce a long term immune affect, the ideal vaccine would need to mimic a true infection. Thus, an attenuated live oral vaccine would perhaps offer better protection as compared to whole killed vaccines.

Currently, available vaccines and candidate strains which are mostly of the O1 serogroup (Cryz *et al.*, 1995, Fontana *et al.*, 2000, Garcia *et al.*, 2005, Liang *et al.*, 2003, Qadri *et al.*, 2006, Thungapathra *et al.*, 1999, Yu *et al.*, 2005) do not protect against O139 infection (Albert *et al.*, 1994a, Qadri *et al.*, 1997). CVD 103-HgR is an O1 classical Inaba strain that has 94% of the *ctx*A gene absent (Ketley *et al.*, 1993) and is now a commercially available vaccine that does not claim to protect against O139 infection (Albert *et al.*, 1994a, Albert *et al.*, 1994b).

IEM101 is an attenuated strain that was found to be naturally deficient of the *ctx*AB gene (Liu *et al.*, 1995). Without this gene, the bacteria lacks toxigenicity and does not cause fluid

accumulation. However, often efforts to develop a cholera vaccine would seek to make available the B subunit in the formulation (Liang *et al.*, 2003, Svennerholm *et al.*, 1984, Sack *et al.*, 1997, Ketley *et al.*, 1993, Waldor and Mekalanos, 1994). Hence, IEM108 was developed from IEM101 with the insertion of the *ctx*B gene (Liang *et al.*, 2003). Other O1 serogroup vaccine strains include the Cuban 638 strain, Peru-15, and Texas Star-SR

Upon discovery that current O1 based vaccines do not help in protecting against O139 infections, research groups have increased efforts to develop live attenuated O139 strains that may be used as vaccines. One such example is Ledon *et. al*. with the construction of *thy*A and *hap* mutants from SG-25-1a, a spontaneous ΔCTXΦ mutant O139 strain (Ledon *et al.*, 2003).

Coster *et. al.* describe the construction and evaluation of Bengal-15 (Coster *et al.*, 1995). The strain is a stable spontaneous non-motile derivative of Bengal-3, which is absent for the CTX element but has been inserted with a construct that encodes for *ctx*B.

The strain in which this study is involved and interested in is VCUSM2, a *hem*A mutant O139 strain constructed by Ravichandran *et. al.* (Ravichandran *et al.*, 2006). Further description of this strain can be found in 1.8 of this chapter.

1.5 Strains

1.5.1 Serogroup

Different O antigens differentiate the serogroups of Vibrios. Currently, more than 139 different O antigens of *V. cholerae* have been identified. These O antigens are thermostable polysaccharides that are part of the cell wall lipopolysaccharide (LPS) (Wachsmuth IK,

1994). The serogroups of *V. cholerae* can not be chemically identified but are determined by respective antisera.

However, only the *V. cholerae* carrying O1 and O139 antigens are associated with epidemics. The remaining serogroups are collectively known as non-O1, non-O139 strains. These strains may not be significant as etiologic agents of epidemics but might cause sporadic diarrhea, gastroenteritis and occasionally extraintestinal infection such as wound infections and acute sepsis. This is observed in people with liver disease or immunosuppression (Ramamurthy *et al.*, 1993).

1.5.2 Serotype

On the basis of variation in antigenic form and certain traits, epidemic O1 strains can be further differentiated into three serotypes, namely Inaba, Ogawa and Hikojima. The 3 serotypes have been designated by the antigenic formulas AB (Inaba), AC (Ogawa) and ABC (Hikojima) respectively; with the A antigen common to all serotypes.

1.5.3 Biotype

Vibrio cholerae of the O1 serotype can be further classified into El Tor and Classical biotypes. They are distinguished by their hemolytic activity, agglutination reaction with erythrocytes, and Polymyxin B resistance.

The Classical biotype was responsible for the pandemics from 1817 till 1923, spread from the Indian subcontinent to most other parts of the world. The seventh and ongoing pandemic which began in 1961 however, is predominated by the El Tor biotype. This biotype largely replaced the Classical biotype in India and other parts of the world.

1.6 CTXΦ Phage

Cholera toxin or CT is encoded by the *ctx* operon carried by the CTXΦ bacteriophage. Not all *Vibrio cholerae* strains are toxigenic and can produce this toxin. A subset of *V. cholerae* acquired these toxin genes by means of bacteriophage infection or horizontal gene transfer (Davis and Waldor, 2003).

Toxigenic *V. cholerae* O1 El Tor and O139 typically demonstrate possession of multiple CTX prophages that are inserted site-specifically near the terminus of ChrI, the larger of the two *Vibrio* chromosomes (Heidelberg *et al.*, 2000, Das *et al.*, 1993, Ehara *et al.*, 1997). Hence, in these strains, it is possible to observe multiple *ctx* operons and reinfection by the bacteriophage. The O1 Classical biotype shows immunity to reinfection of the phage when an existing phage is present. Therefore, O1 Classical strains have only one copy of the *ctx* operon (Kimsey and Waldor, 1998).

Contemporary toxigenic *V. cholerae* also contain the CTXΦ related element RS1. RS1 is a filamentous phage that is not autonomously transmissible but is instead a 'satellite' phage of CTXΦ. It is inserted adjacent to their CTX prophages and depends on the CTXΦ coat and secretion proteins for packaging. Both CTXΦ and RS1 have identical sequences at the 5' ends. They encode proteins used for replication, intergration and regulation of gene expression. The remaining genes in CTXΦ encode for proteins that are needed for packaging and secretion ($pIII^{CTX}$, *ace* and *zot*) and CT, which is not involved in virion formation (Davis *et al.*, 2002)

CTXΦ is more dependent on host-encoded proteins compared to the prototypical Ff filamentous coliphages it is similar to. Studies have shown that chromosomal gene products facilitate the integration and secretion of this phage. The genome of the CTXΦ does not contain a sequence similar to any known integrase. Instead, chromosome-encoded

recombinases XerC and XerD are required for its integration (Huber and Waldor, 2002). CTXΦ relies also upon a chromosome-encoded secretin, EpsD for its secretion. Interestingly, the Eps system also mediates release of cholera toxin (Sandkvist *et al.*, 1997, Sandkvist *et al.*, 1993). The secretion of the phage by the host cell allows for the simultaneous vertical and horizontal transmission of CTXΦ. Thus, CTXΦ can simultaneously be transferred to a new host cell and retained within the genome of the old host.

1.7 Toxins & Proteins

In the *ctx* operon, there are five identified genes, $pIII^{CTX}$ (formerly called *orf*U), *ace*, *zot*, *ctx*A and *ctx*B. All these genes, except $pIII^{CTX}$, are involved in or assist in fluid secretion.

The gene $pIII^{CTX}$ was formerly referred to as an open reading frame with unknown function. Heilpern *et. al.* hypothesized and found that the protein functioned in a similar manner to that of the coliphage fd protein pIII (Heilpern and Waldor, 2003). The protein was found to mediate CTXΦ infection as well as played a role in CTXΦ assembly and release.

Another potential enterotoxin produced by *V. cholerae* that has been identified is the accessory cholera enterotoxin (Trucksis *et al.*, 1993). It has been demonstrated that the 11.3-kDa product can cause fluid accumulation in adult rabbit ileal loops and in a sealed infant mouse model (Trucksis *et al.*, 1993). Culture supernatants from constructs devoid of CT and Zot but containing the *ace* gene demonstrated a significant increase in short circuit current secondary to an increase in potential difference in Ussing preparations. This toxin is suspected to be the cause behind the mild to moderate diarrhea experienced by volunteers of vaccine candidates, JBK70 and CVD101. It has also been found that the mechanism of secretion by this toxin involves Ca^{2+} as a second messenger and it stimulates a novel Ca^{2+}-dependent synergy (Trucksis *et al.*, 1993).

The zonula occludens toxin coded by *zot*, is a 44.8kDa protein that is cleaved and gives rise to a 33kDa N-terminal fragment and a 12 kDa or smaller C-terminal fragment. This toxin activates a complex intracellular sequence of events that regulate tight junction permeability of the small intestinal mucosa (Fasano *et al.*, 1995). The increase in the permeability of the mucosa enables the bacteria to colonize more easily and also enhances the influx of cholera toxin into intestinal cells. It has been observed that purified Zot primarily binds to a cell receptor, rearranges the cell cytoskeleton(Fasano *et al.*, 1995), and induces secretion in the small intestines in rabbit models (Fasano *et al.*, 1997). It is also suggested that *zot* is involved in the CTXΦ morphogenesis together with $pIII^{CTX}$, to assemble the filamentous phage at the bacterial envelope (Uzzau *et al.*, 1999).

The cholera toxin (CT) itself however is the main cause of the characteristic rice watery stool diarrhea of cholera. CT is a hexameric protein that consists of 5 B subunits (coded by *ctx*B) and 1 A subsunit (coded by *ctx*A).

The pentamer B subunit contains the GM1 ganglioside receptor binding site and is responsible for the binding properties of the protein. When the toxin is secreted in the small intestines, the B subunit will bind to the GM1 ganglioside, a glycolipid which is practically ubiquitous in eukaryotic cell membranes. The binding of the toxin will then lead to the release of the A subunit into the intestinal cells.

The A subunit is an ADP-ribosyltransferase, an enzyme responsible for the toxicity of the molecule and the diarrheal effect characteristic of cholera. The A subunit will then cleave into two peptides, A1 and A2. The two proteolytically cleaved proteins are still linked by disulphide bonds before internalization (Gill and Rappaport, 1979). The A1 peptide will enzymatically transfer ADP-ribose from nicotinamide adenine dinucleotide (NAD) to a target protein. The target protein is the guanosine 5'-triphosphate (GTP)-binding regulatory protein associated with membrane bound adenylate cyclase. It 'locks' adenylate cyclase in its

‘on mode’ and this leads to excessive production of cyclic adenosine 51-monophosphate (cAMP). The accumulation of cAMP in the intestinal cells will cause hypersecretion of chloride, bicarbonate and finally, water into the intestinal lumen.

As mentioned, the A subunit is an enzyme. Thus, it has an active site with a specific configuration (Fontana *et al.*, 1995, Jobling and Holmes, 2001). This configuration is produced through the interaction of certain amino acids through hydrogen bonds and disulphide bonds. If these bonds are changed, certain configurations may be altered. These alterations are particularly important in the inactivation of enzymes. This is because enzymes have specific sites, known as active sites, that are used to bind to specific sites of particular substrates. Once the active sites have been altered, the enzymes cannot bind to their substrates and therefore cannot perform their intended functions. By altering the active site of the A subunit, the molecule is unable to function enzymatically and its toxigenicity is addressed (Fontana *et al.*, 1995).

1.8 VCUSM2

VCUSM2 is an ALA (δ-aminolevulinic acid) auxotrophic O139 strain in which a frameshift mutation has been carried out on the *hem*A gene. The gene encodes for glutamyl-tRNA reductase, an important enzyme in the C5 pathway of ALA biosynthesis. Studies have shown that VCUSM2 is able to colonize and induces good immune response in animal models. Rabbit ileal loop ligation experiments show that VCUSM2 is less reactogenic than its parental wildtype O139 strain. However, slight fluid accumulation was still observed at higher doses of CFU (Ravichandran *et al.*, 2006).

Although studies have shown that VCUSM2 is less toxigenic than the parental wildtype O139 strain, it is still essentially toxigenic as it possesses the complete *ctx*AB gene. VCUSM2 has been identified to possess double copies of *ctx* operon, arranged in tandem

(unpublished data). This data was acquired by digesting the purified genomic DNA with specific enzymes and performing a Southern Blot with specially designed probes. The experiments were performed and analyzed by researchers in Cuba. The double copies of *ctx* operon in VCUSM2 are fully functional and cause significant amounts of fluid accumulation as well as hemorrhaging in rabbit ileal loop model experiments (Ravichandran *et al.*, 2006).

1.9 Objectives

It is the objective of this study to genetically manipulate VCUSM2 that its toxigenicity may be reduced. The study aims to delete the *ace* and *zot* genes in the operon by means of inverse PCR because these genes can contribute to mild diarrhea. Also, the study aims to mutate the *ctx*A gene at two specific sites so that two amino acids involved in the formation of the active site of the enzyme will be changed, rendering it non-functional. The target amino acids are the 7th and 112th amino acid of the A subunit. The 7th is arginine and has been substituted for lysine (R7K). The 112th is glutamate and was substituted with glutamine (E112Q).

The obtained *ctx* operon mutant *Vibrio cholerae* was then subjected to evaluation in areas of CT production, reactogenicity and colonization abilities.

Chapter 2: Materials and Methods

2.1 Experimental overview

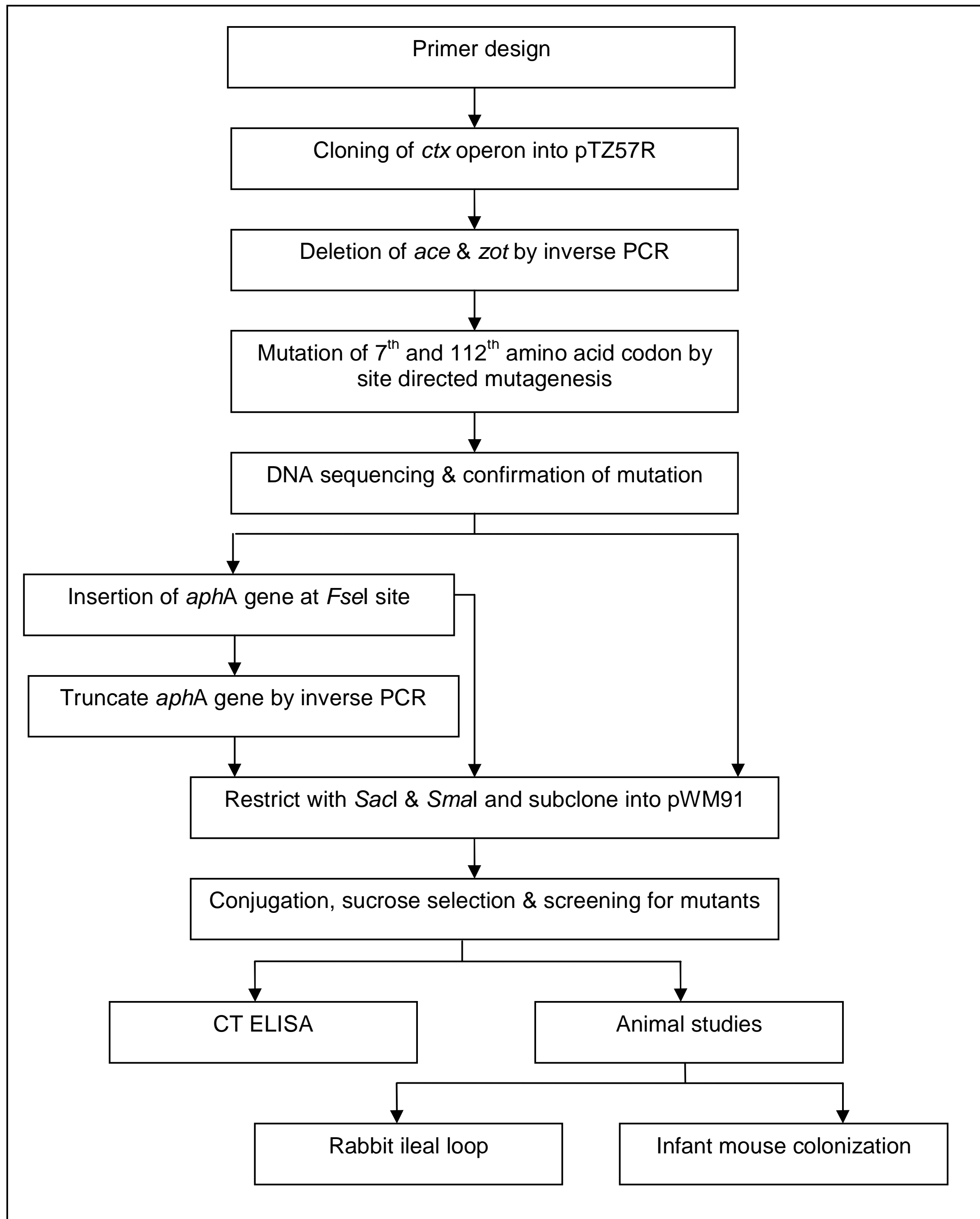

Figure 1: Experimental overview

2.2 Reagents, chemicals, kits

2.2.1 Culture media

2.2.1.1 Luria Bertani (LB) agar

Tryptone ………………………………… 15 g

Yeast extract …………………………….. 5 g

Sodium Chloride ………………………… 10 g

Agar ……………………………………… 15 g

Distilled water …………………………… to 1L

The ingredients were dissolved in 750 ml of distilled water. The pH was adjusted to 7.2 using 1M NaOH. The volume was made up to 1 L with distilled water and the medium was autoclaved at 121°C for 15 minute. The medium was allowed to cool at around 45°C and poured aseptically into the plastic Petri plates. The Petri plates were incubated at 37°C for 12 h and visually inspected for the presence of any bacterial/ fungal contamination. The plates were stored at 4°C for not more than 30 days.

2.2.1.2 Luria Bertani (LB) broth

Tryptone ………………………………… 15 g

Yeast extract …………………………….. 5 g

Sodium Chloride ………………………… 10 g

Distilled water …………………………… to 1L

The ingredients were dissolved in 750 ml of distilled water and the pH was adjusted to 7.2 using 1M NaOH. The volume was made up to 1 litre with distilled water. The medium was distributed in 10 ml aliquots in universal bottles and autoclaved at 121°C for 15 minutes. The

medium was incubated at 37°C for 12 h and visually inspected for the presence of any bacterial/ fungal contamination. The medium was stored at room temperature for not more than 30 days.

2.2.1.3 Modified LB agar without NaCl and with 10% sucrose

Tryptone ………………………………… 15 g

Yeast extract …………………………….. 5 g

Agar ……………………………………… 15 g

Distilled water …………………………… to 500 ml

The ingredients were dissolved in 200 ml of distilled water and the pH was adjusted to 7.2 using 1M NaOH. The volume was made up to 250 ml with distilled water and the medium was autoclaved at 121°C for 15 minutes. The medium was allowed to cool to around 55°C and added with 250 ml of sterile, warm (about 50°C) 20% sucrose solution.

2.2.1.4 Modified LB broth without NaCl and with 10% sucrose

Tryptone ………………………………… 15 g

Yeast extract …………………………….. 5 g

Distilled water …………………………... to 500 ml

The ingredients were dissolved in 200 ml of distilled water and the pH was adjusted to 7.2 using 1M NaOH and the volume was made up to 250 ml with distilled water. The medium was distributed in 5 ml aliquots in universal bottles and autoclaved at 121°C for 15 minutes. Each bottle was then added with 5 ml of 20% sterile sucrose solution. The medium was incubated at 37°C for 12 h and visually inspected for the presence of any bacterial/ fungal contamination.

2.2.1.5 TCBS Agar

Dehydrated TCBS Powder ………………. 80 g

Distilled water …………………………… to 1L

TCBS agar was dissolved in 1L of autoclaved distilled water and the medium was brought to a boil on a Bunsen burner. The medium was allowed to cool to 50°C and poured in Petri plates. The plates were incubated at 37°C for 12 h and visually inspected for the presence of any bacterial or fungal growth. The plates were stored at 4°C for not more than 30 days.

2.2.1.6 Addition of antibiotics/ supplements to the agar based media

The medium was allowed to cool to about 45°C and sterile stock solutions of antibiotics or other supplements were added aseptically to the culture medium. The final concentration of various antibiotics and other supplements used in the present study were Ampicillin (100 µg /ml); Polymyxin (0.75 µg /ml or 450 unit/ ml); Kanamycin (50 µg /ml); ALA (80 µg /ml). For medium used in selection of merodiploids, ampicillin concentration was quadrupled. The medium was swirled to mix the contents well and poured aseptically into the plastic Petri plates. The plates were incubated at 37°C for 12 h and visually inspected for the presence of any bacterial or fungal growth. The plates were stored at 4°C for not more than 30 days.

2.2.2 General buffers, stock solutions, antibiotics

Following are the general buffers, stock solutions and antibiotics used throughout this study.

2.2.2.1 δ-Aminolevulinic Acid 40 mg /ml

δ-Aminolevulinic acid …………………… 400 mg

Distilled water …………………………… up to 10 ml

δ-Aminolevulinic acid salt was dissolved in autoclaved distilled water and filter sterilized by passing through 0.22 µm nitrocellulose membrane. The solution was stored in 1 ml aliquots in sterile 1.5 ml micro-centrifuge tubes at -20°C.

2.2.2.2 Ampicillin 100 mg/ml

Ampicillin ………….. …………………… 500 mg

Distilled water …………………………… up to 5 ml

Salt was dissolved in autoclaved distilled water and filter sterilized by passing through 0.22 µm nitrocellulose membrane. The ampicillin solution was stored in 1 ml aliquots in sterile 1.5 ml micro-centrifuge tubes at -20°C.

2.2.2.3 Calcium chloride 100 mM

Calcium chloride dihydrate ……………… 14.7 g

Distilled water …………………………… up to 1 L

Calcium chloride was dissolved in autoclaved distilled water and filter sterilized by passing through a 0.22 µm nitrocellulose membrane. The solution was stored at 4°C.

2.2.2.4 Carbonate buffer 60 mM pH 9.6

Sodium bicarbonate 60 mM ……………… 100 ml

Sodium carbonate 60 mM ………………… 20 ml

Sodium carbonate and sodium bicarbonate solutions were mixed, filter sterilized by passing through a 0.22 µm nitrocellulose membrane, and stored at room temperature (21-23°C) for not more than 30 days.

2.2.2.5 Cholera toxin (0.5 µg /ml)

Cholera toxin (1 mg /ml) …………………. 5 µl

Carbonate buffer 60 mM (pH 9.6) ………… to 10 ml

This solution was used immediately as a control in ELISA.

2.2.2.6 Ethanol 70%

Absolute ethanol ………………………….. 700 ml

Distilled water ……………………………. 300 ml

The solution was stored at room temperature.

2.2.2.7 EDTA 0.5 M

EDTA disodium dihydrate ……………….. 186.1 g

Distilled water …………………. ………... up to 1L

EDTA was added into distilled water and the pH was adjusted to 8.0 with NaOH pellets. The volume was made up to 1L and the solution was stored at room temperature.

2.2.2.8 Ethidium bromide 10 mg /ml

Ethidium bromide tablet………………… 100 mg

Distilled water ………………….............. 10 ml

The tablet was dissolved and kept in an opaque bottle. The solution was stored at room temperature.

2.2.2.9 Gel loading dye 6X

Orange G ………………………………… 200 mg

Glycerol .………………………………… 60 ml

EDTA 0.5M.…………………………….. 12 ml

Distilled water ………………………….. up to 100 ml

EDTA solution, glycerol and Orange G powder were added together and mixed thoroughly. Distilled water was added to the solution to make it 100ml. The solution was filter sterilized by passing through 0.22 µm nitrocellulose membrane and stored at -20°C.

2.2.2.10 Glycerol 80%

Glycerol ……………………………….. 80 ml

Distilled water ………………………… 20 ml

Glycerol was mixed with autoclaved distilled water and sterilized by autoclaving at 121°C for 15 minutes. The solution was stored at room temperature.

2.2.2.11 Hydrochloric Acid 1N

Hydrochloric acid (11.6 N) ……………… 8.62 ml

Distilled water …………………………… 91.3 ml

The solution was stored at room temperature.

2.2.2.12 Kanamycin sulfate 50 mg /ml

Kanamycin sulfate ……………………….. 500 mg

Distilled water …………………………… 10 ml

The solution was filter sterilized by passing through a 0.22 µm nitrocellulose membrane. The solution was stored in 1 ml aliquots at -20°C.

2.2.2.13 Magnesium chloride 100 mM

Magnesium chloride hexahydrate ……….……..20.33 g

Distilled water…………………………………..up to 100 ml

Magnesium chloride was dissolved in autoclaved distilled water and filter sterilized by passing through 0.22 µm nitrocellulose membrane. The solution was stored at 4°C.

2.2.2.14 Normal saline (0.9% NaCl)

Sodium chloride …………………………. 9 g

Distilled water …………………………… up to 1 L

Sodium chloride was dissolved in distilled water and sterilized by autoclaving at 121°C for 15 minutes. The solution was stored at room temperature.

2.2.2.15 Phosphate Buffered Saline (PBS) 10 X

Sodium chloride …………………………. 80 g

Potassium chloride ………………………. 2 g

Sodium phosphate dibasic heptahydrate … 14.4 g

Potassium phosphate monobasic anhydrous 2.4 g

Distilled water ………………………….up to 1L

The salts were dissolved in distilled water and the pH was adjusted to 7.4 using 1N HCl/ 1M NaOH. The solution was sterilized by autoclaving at 121°C for 15 minutes and stored at room temperature.

2.2.2.16 Phosphate Buffered Saline-Tween 20

Phosphate buffered saline 10X …………... 100 ml

Tween 20 ………………………………… 0.5 ml

Distilled water …………………………… 900 ml

The solution was stored at room temperature.

2.2.2.17 Polymyxin B sulfate 0.75 mg /ml (4500 units)

Polymyxin B sulfate (6000 units/ mg) …..... 7.5 mg

Distilled water …………………………..... 10 ml

Polymyxin B was dissolved in autoclaved distilled water and filter sterilized by passing through 0.22 µm nitrocellulose membrane. The solution was stored in 1 ml aliquots at -20°C.

2.2.2.18 Sodium bicarbonate 60 mM

Sodium bicarbonate ……………………..….. 5.04 g

Distilled water …………………………… up to 1 L

Sodium bicarbonate was dissolved in autoclaved distilled water and filter sterilized. The solution was stored at room temperature.

2.2.2.19 Sodium carbonate 60 mM

Sodium carbonate ………………………..…….. 6.36 g

Distilled water …………………………… up to 1 L

Sodium carbonate was dissolved in autoclaved distilled water and filter sterilized. The solution was stored at room temperature.

2.2.2.20 Sodium hydroxide 10M

Sodium hydroxide ………………………..……. 40 g

Distilled water …………………………… up to 100ml

Sodium hydroxide was dissolved in distilled water and stored at room temperature.

2.2.2.21 Sodium hydroxide 1M

Sodium hydroxide 10 M …..……….……. 10 ml

Distilled water …………………………… 90 ml

The solution was stored at room temperature.

2.2.2.22 Skimmed milk 5%

Skimmed milk …………………………… 5 g

Distilled water …………………………… up to 100 ml

When used in freeze drying of bacterial stocks, the solution was steam autoclaved and stored at -20°C. When used as blocking agent in ELISA, the solution was prepared fresh and used immediately.

2.2.2.23 Tris Acetic Acid EDTA (TAE) 50X

Tris base …………………………………. 242 g

Acetic acid glacial ……………………….. 57.1 ml

EDTA (0.5 M) …………………………… 100 ml

Distilled water …………………………… up to 1L

The solution was sterilized by autoclaving at 121°C for 15 minutes and stored at room temperature.

2.2.2.24 Tris Acetic Acid EDTA (TAE) 1X

TAE (50X) ……………………………….. 20 ml

Distilled water …………………………… up to 1L

The solution was store at room temperature.

2.2.2.25 TypeIII Ganglioside (10 µg /ml)

TypeIII ganglioside (10 mg /ml) ………… 10 µl

Carbonate buffer 60 mM (pH 9.6) ..……… up to 10 ml

This solution was used immediately to coat the ELISA plates.

2.2.3 Bacterial species and strains

Following is a list of bacterial strains that have been used throughout this study.

2.2.3.1 *E. coli* BW 20767- λ pir

(*RP*4 *2tet: mu-1kan::Tn7integrant leu* 63:: *rec A*1 *cre(*510 *hsdR*17 *end A1 Zbf*-5 *uid)(ΔMluI): pir thi* was gifted from Dr. Copass of Harvard Medical School, Boston, USA.

2.2.3.2 *E .coli* TOP10

(*F⁻merA Δ(mrv hsdRMS mcrBC)ϕ*80*lacZΔM*15 *Δlac X*74 *deOR rec*A1*ara*D139 Δ*(ara-leu)*7697 *gal*U *gal*K*rps*L*(str^R) end*A1 *nup*G) was obtained from Invitrogen Corp. USA

2.2.3.3 *V. cholerae* O139 Bengal

Isolated from a patient in Hospital Universiti Sains Malaysia, Kubang Kerian, 16150, Kelantan, Malaysia.

2.2.3.4 VCUSM2

This strain is a *hem*A mutant, derived from VCUSM1 by removing Kanamycin gene cassette from *hem*A gene. (Ravichandran *et al.*, 2006)

2.2.4 Plasmids

Following is a list of plasmid that have been purchased or procured from other research labs to be used in the present study.

2.2.4.1 pTZ57R

(CAP protein binding site, mRNA (LacZ), *lac* repressor (LacI) binding site, f1 packaging signal) was obtained from Fermentas.(Mead *et al.*, 1986). The plasmid map can be found in Appendix A.

2.2.4.2 pWM91

(f1(+) ori lacZα of pBluescript II (SK^+) unique polylinker sites:*Apa*I,*Bam*HI,*Not*I,*Sac*I, *Sma*I,*Spe*I and *Xho*I *Sac*B *Suc*^{R} Amp^R) was gifted by Dr. Copass of Harvard Medical School, Boston, USA. (Metcalf *et al.*, 1996)

2.2.5 Animals

BALB/c albino mice and New Zealand white rabbits have been used throughout this study. All animals were obtained from Rumah Haiwan, Kampus Kesihatan, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan.