

**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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List of abbreviations

Δ Kan	mutated <i>aphA</i> 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
CTX Φ	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 *hemA*, 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- λ pir 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 *hemA* 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 *hemA* 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- λ pir *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 *hemA* gene.

Chapter 1: Introduction

1.1 Cholera

Cholera is an acute watery diarrheal disease caused by toxigenic strains of *Vibrio 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 *ctxA* 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 *ctxAB* 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 *ctxB* 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 *thyA* and *hap* mutants from SG-25-1a, a spontaneous Δ CTX Φ 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 *ctxB*.

The strain in which this study is involved and interested in is VCUSM2, a *hemA* 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 CTX Φ Phage

Cholera toxin or CT is encoded by the *ctx* operon carried by the CTX Φ 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 CTX Φ related element RS1. RS1 is a filamentous phage that is not autonomously transmissible but is instead a 'satellite' phage of CTX Φ . It is inserted adjacent to their CTX prophages and depends on the CTX Φ coat and secretion proteins for packaging. Both CTX Φ and RS1 have identical sequences at the 5' ends. They encode proteins used for replication, integration and regulation of gene expression. The remaining genes in CTX Φ 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)

CTX Φ is more dependent on host-encoded proteins compared to the prototypical F Φ 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 CTX Φ 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). CTX Φ 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 CTX Φ . Thus, CTX Φ 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 *orfU*), *ace*, *zot*, *ctxA* and *ctxB*. 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 CTX Φ infection as well as played a role in CTX Φ 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 Ca²⁺ as a second messenger and it stimulates a novel Ca²⁺-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 CTX Φ 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 *ctxB*) and 1 A subunit (coded by *ctxA*).

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 *hemaA* 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 *ctxAB* 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 *ctxA* 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, reactivity and colonization abilities.

Chapter 2: Materials and Methods

2.1 Experimental overview

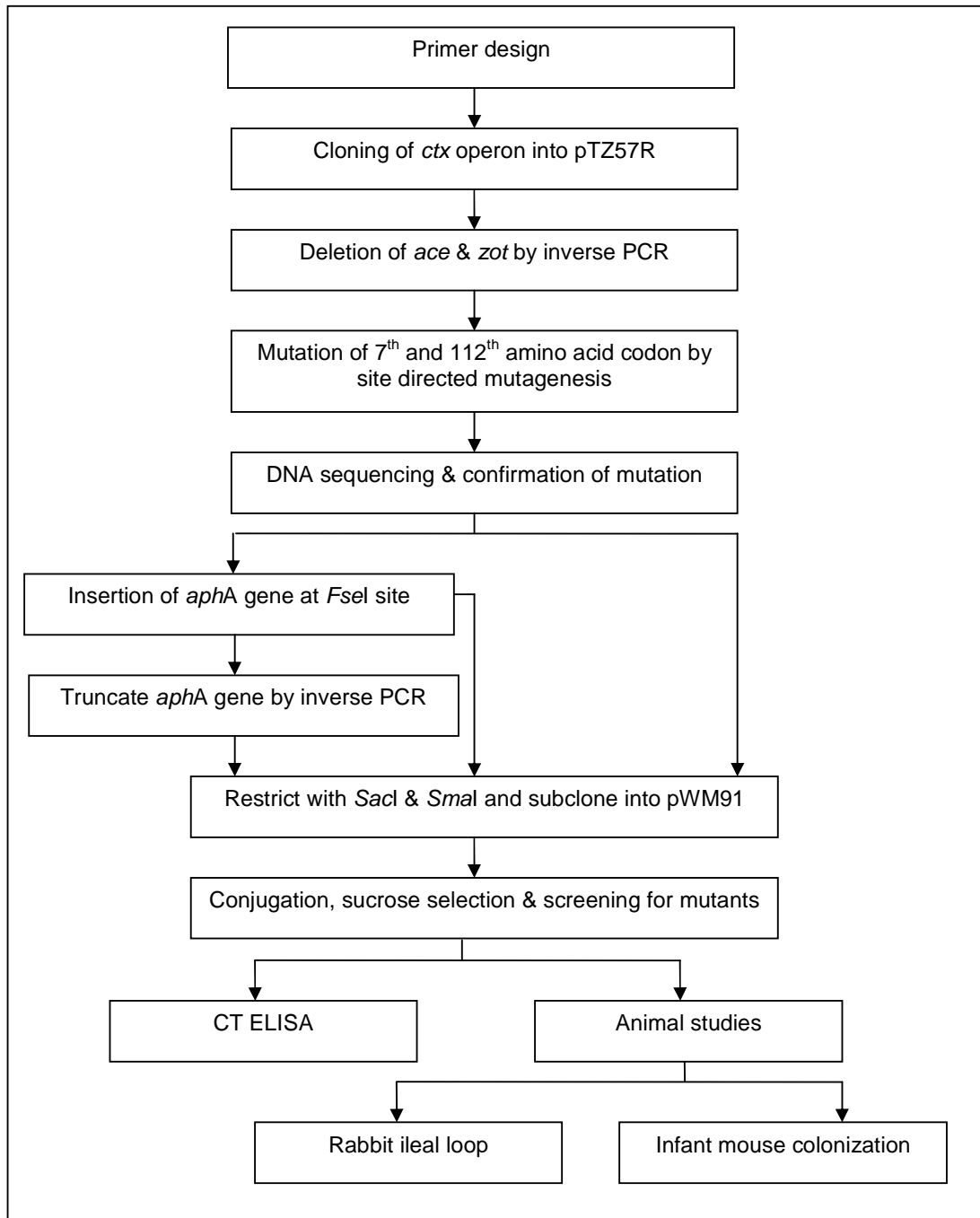


Figure 1: Experimental overview

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 μ m 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 μ m 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 μ m 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 μ m nitrocellulose membrane, and stored at room temperature (21-23°C) for not more than 30 days.

2.2.2.5 Cholera toxin (0.5 μ g /ml)

Cholera toxin (1 mg /ml)	5 μ l
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 μ m 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 μ m 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 μ m 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

(*RP4 2tet: mu-1kan::Tn7integrant leu 63:: rec A1 cre(510 hsdR17 end A1 Zbf-5 uid)(Δ MluI)*:

pir thi was gifted from Dr. Copass of Harvard Medical School, Boston, USA.

2.2.3.2 *E. coli* TOP10

(*FmerA Δ (mrv hsdRMS mcrBC) ϕ 80lacZ Δ M15 Δ lac X74 deOR recA1araD139 Δ (ara-leu)7697 galU galKrpsL(*str*^R) endA1 nupG*) 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 *hemA* mutant, derived from VCUSM1 by removing Kanamycin gene cassette from *hemA* 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} *lacZ* α of pBluescript II (SK⁺) unique polylinker sites:*ApaI*,*BamHI*,*NotI*,*SacI*,*SmaI*,*SpeI* and *XhoI* *SacB* *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.