CONSTRUCTION OF RECOMBINANT *rtxH* HYPOTHETICAL GENE OF *Vibrio cholerae* O139

By

WONG TZE HANN

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ABSTRACT

CONSTRUCTION OF RECOMBINANT *rtxH* HYPOTHETICAL GENE OF *Vibrio cholerae* O139

Wong Tze Hann

Vibrio cholerae O1 and O139 are the pathogenic strains in *V. cholerae* which could lead to cholera outbreak, a disease that demonstrated as severe watery diarrhea and vomiting. Cholera is a major public health problem in developing country and causes an estimated of 100 000–120 000 deaths annually. *V. cholerae* O1 and O139 contain several virulence factors that contribute to their pathogenicity. In this research, *rtxH* gene of MARTX gene cluster was selected for cloning. The gene is a hypothetical gene where its function and structure still remain unknown. This study aimed to construct recombinant *rtxH* in expression vector pHIS. Firstly the *rtxH* gene was amplified through polymerase chain reaction (PCR), then cloned directionally into expression vector pHIS using *Bam*HI and *Not*I restriction endonuclease (RE) site. To achieve this, linkers containing the RE sequences were added to both forward and reverse primers of *rtxH*. The amplified *rtxH* gene (~380 bp) together with pHIS vector were subjected to RE digestion. The *rtxH* gene was then cloned into the expression vector by sticky end ligation, to construct the recombinant plasmid. Initial PCR screening was performed to confirm the successful insert of *rtxH* into pHIS vector. Successive RE analysis further confirmed the correct insert in the

constructed plasmind. DNA sequencing was done to check for correct insert and to confirm the absence of mutation within the *rtxH* gene in the recombinant plasmid. PCR screening and RE analysis verified that *rtxH* gene was successfully cloned into pHIS vector. Sequencing result verified that no mutation in the *rtxH* gene and the *rtxH* gene was found to be in-frame with the vector's protein expression. The recombinant plasmid construct of *rtxH* is ready for protein expression upon transformation into expression host. Future study on the purified recombinant *rtxH* will allow us to understand the structure and function of this hypothetical protein.

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Thank you very much.

DECLARATION

I hereby declare that the project report is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

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WONG TZE HANN

APPROVAL SHEET

This project report entitled **"CONSTRUCTION OF RECOMBINANT *rtxH* HYPOTHETICAL GENE OF *Vibrio cholerae* O139"** was prepared by WONG TZE HANN and submitted as partial fulfillment of the requirements for the degree of Bachelor of Science (Hons) Biomedical Science at Universiti Tunku Abdul Rahman.

Approved by:

_____

(Dr. TAN GIM CHEONG) Date: …….

Supervisor

Department of Biomedical Science

Faculty of Science

Universiti Tunku Abdul Rahman

FACULTY OF SCIENCE

UNIVERSITI TUNKU ABDUL RAHMAN

Date: _____

PERMISSION SHEET

It is hereby certified that **WONG TZE HANN** (ID No: **10ADB04284**) has completed this final year project entitled **"CONSTRUCTION OF RECOMBINANT *rtxH* HYPOTHETICAL GENE OF *Vibrio cholerae* O139"** under supervision of Dr. Tan Gim Cheong from the Department of Biomedical Science, Faculty of Science.

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(WONG TZE HANN)

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LIST OF ABBREVIATIONS

%	Percent
~	Approximately
$\times g$	Gravity
3D	3 Dimensional
Ace	Accessory cholera enterotoxin
ATP	Adenosine triphosphate
bp	Base pair
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
CT	Cholera toxin
CTXϕ	CTX bacteriophage
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
E-value	Expected-value
HCl	Hydrochloric acid
HlyA	Hemolysin
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kb	Kilo base pair
kDa	Kilo Dalton

LB	Luria Bertani
LPS	Lipopolysaccharide
MARTX	Multifunctional autoprocessing repeat-in-toxin
$MgCl_2$	Magnesium chloride
NaOH	Sodium hydroxide
OD	Optical density
ORF	Open reading frame
PCR	Polymerase chain reaction
pHIS P2	pHIS Parallel 2
RE	Restriction endonuclease
RNA	Ribonucleic acid
rpm	Revolutions per minute
RS	Repetitive sequence
RTX	Repeat-in-toxin
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SLT	Shiga-like toxin
ST	NAG-specific heat-stable toxin
SXT	Trimethoprim-sulfamethaxazole
TBE	Tris-Borate-EDTA
TCBS	Thiosulphate-citrate-bile salts-sucrose
TCP	Toxin coregulated pilus
TISS	Type I secretion system

U	Unit
UV	Ultraviolet light
V	Volt
v/v	Volume/volume
VPI	*V. cholerae* Pathogenicity Island
w/v	Weight/volume
Zot	Zonula occludens toxin
ΔG	Delta G

CHAPTER 1

INTRODUCTION

Diarrheal diseases are the leading cause of morbidity in the developing world and the second-most common cause of death among children under the age of 5 worldwide (Kosek et al., 2003). Cholera is one of the acute diarrheal diseases that usually presents as abrupt massive watery diarrhea and vomiting (Wong et al., 2010). It is caused by the bacterium *Vibrio cholerae* (Albert 1994). In the last decade, the life-threatening diarrheal disease cholera has reached a more extensive distribution than at any other time in the 20th century. This acute intestinal infection (Lowden et al., 2010) is still a major problem in developing countries with poor socio-economic conditions where access to safe water cannot be assured (Kumar et al., 2009). It causes an estimated case of 100,000 deaths annually and resulting in major microeconomic and macroeconomic losses (Clemens 2011).

V. cholerae can be classified based on their somatic O antigens, with more than 200 serogroups identified to date (Chatterjee and Chaudhuri 2003). Among the various serogroups of *V. cholerae*, strains belong to the O1 and O139 serogroups are responsible for the severe epidemic and pandemic potential (Kumar et al., 2009). *V. cholerae* O1 is the only strain that known to cause cholera. However, *V. cholerae* O139 emerged in late 1992 in Southern India and Bangladesh as

pandemic strain (Waldor et al., 1996). The initial *V. cholerae* O139 isolates, recovered during 1992 to 1993, were sensitive to tetracycline and resistant to trimethoprim-sulfamethoxazole (SXT) and streptomycin, because they are protected by an integrating conjugative element, termed the SXT element, on the chromosome (Basu et al., 2000; Faruque et al., 2003).

V. cholerae, as a non-invasive bacterium (Chun et al., 1999), expresses several factors to establish and cause disease in the host (Boardman and Satchell 2004). Many researchers have been focused on understanding the basis of *V. cholerae* pathogenicity and its pandemic potential. Numerous studies have emphasized the necessity of three essential virulent factors in pathogenic *V. cholerae* strains: the filamentous CTX bacteriophage (CTXϕ) that encodes cholera toxin (CT), the toxin coregulated pilus (TCP) pathogenicity island encoding the TCP pili, which is a colonization factor and receptor for CTXϕ, and *toxR*, an essential virulence regulatory gene (Faruque et al., 1998). These are the most important virulence genes in the toxigenic strains and are known to contribute to the pathogenicity of *V. cholerae*. CT causes outflow of fluids from the intestinal cells, leading to profuse watery diarrhea, which is the hallmark of cholera, and TCP helps in the colonization of the organism to the endothelial lining of intestine (Kirn and Taylor 2005). This indicates that other toxins or factors produced by *V. cholerae* might also play a role in the pathogenesis of the disease. Other than CT, *V. cholerae* also produces other toxins such as the zonula occludens toxin (Zot), accessory cholera enterotoxin (Ace), hemolysin (HlyA), NAG-specific heat-stable toxin (ST), and

multifunctional autoprocessong repeat-in-toxin (MARTX) (Lin et al., 1999; Mohapatra et al., 2007; Satchell 2007).

RTX (repeat-in-toxin) is a novel toxin in *V. cholerae* which is produced by several pathogenic Gram-negative bacteria (Lin et al., 1999). Due to the variability in fuction of *V. cholerae* RTX, hence it is called MARTX. The MARTX toxin gene cluster in *V. cholerae* encodes cytotoxin (*rtxA*), an acyltransferase (*rtxC*), and an associated ATP-binding cassette transporter system (RtxB and RtxD, two proteins for toxin transportation). It also contains an ATPase RtxE and a conserved hypothetical protein with unknown function (Boardman and Satchel 2004). It is physically linked to the core element in the *V. cholerae* genome but the activity of this cluster is independent of the core element (Lin et al., 1999).

In this study, hypothetical gene *rtxH* was chosen due to its unknown function and structure. This gene was not previously studied and the product of this gene still remains uncharacterized. Therefore, in this research, *rtxH* gene was cloned to further characterize the function of this protein. VC1449 is the open reading frame (ORF) for *rtxH*. In the research did by Safa (2010), this ORF is believed to be present only in the El Tor biotype; and PCR analysis demonstrated that these ORFs are present in the genomes of all atypical El Tor *V. cholerae* O1 and typical El Tor strains examined in his study. Other than that, Lin et al. (1999) reported

that MARTX gene cluster sequence was absent in the classical strains but present in the E1 Tor strains. The intact sequence could also be found in O139 strain.

Objectives:

- To amplify the *rtxH* gene of *V. cholerae* serogroup O139
- To clone the *rtxH* gene into pHIS P2 expression vector
- To verify and identify any frameshift mutation occurred in the cloned gene

CHAPTER 2

LITERATURE REVIEW

2.1 *Vibrio cholerae*

2.1.1 Taxonomy

V. cholerae is a member of the family Vibrionaceae. The genus *Vibrio* consists of 63 species and 11 of them are known to be pathogenic to humans including *V. cholerae* (O1 and O139), *V. parahaemolyticus*, *V. vulnificus*, *V. mimicus*, *V. hollisae*, *V. fluvialis*, *V. alginolyticus*, *V. damsela*, *V. furnissii*, *V. metschnikovii* and *V. cincinnatiensis*. Among these, the first three species can cause serious human infections. *V. cholerae* is categorized based on its somatic antigens (O antigens) into different serovars or serogroups (Shimada et al., 1994). Currently, there are over 200 known serogroups and of which two major serogroups, O1 and O139 are the causative agent of cholera (Bhunia 2008). The O1 serogroup presents as two biotypes, classical and El Tor; antigenic factors allow further categorization into two major serotypes — Ogawa and Inaba. Strains of the Ogawa serotype are able to express the A and B antigens and a small amount of C antigen, while Inaba strains express only the A and C antigens. A third serotype, Hikojima expresses all three antigens but is rare and unstable (WHO, 2002). Figure 2.1 shows the classification of *V. cholerae* into different serogroups and biotypes.

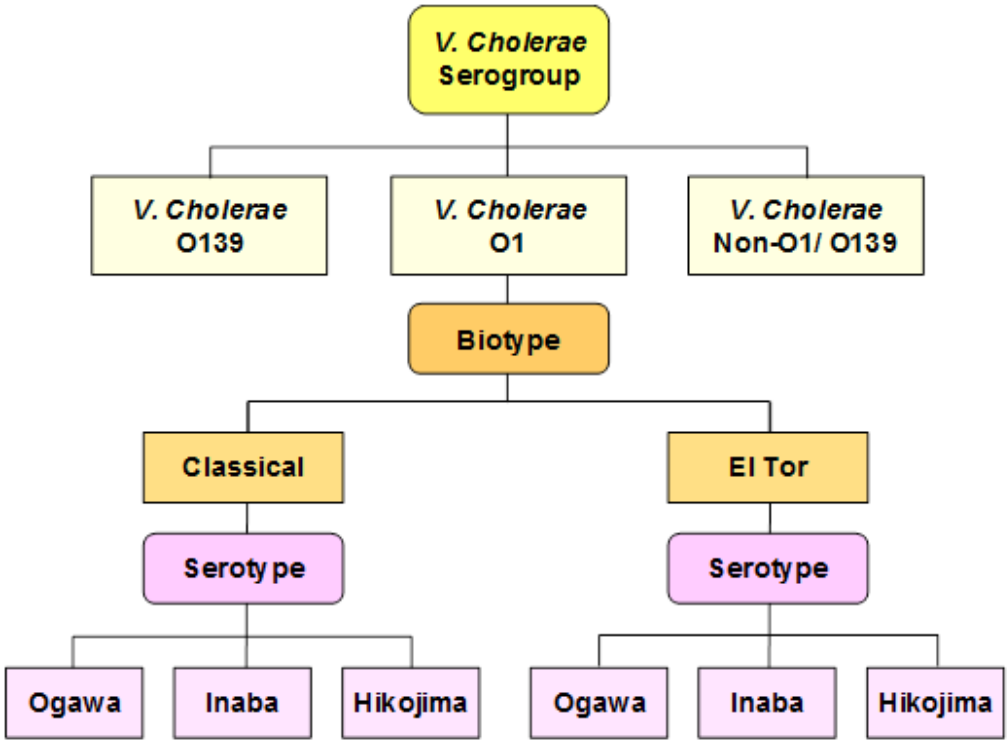


Figure 2.1: Serogroups and biotypes of *V. cholerae* (Ali 2004).

2.1.2 Morphological and Biochemical Characteristics

V. cholerae is a Gram-negative rod or curved shaped bacterium (0.7-1.0 × 1.5-3.0 μm) as shown in Figure 2.2. It is a facultative anaerobe and non-spore forming microorganism (Maneshwari et al., 2011). It produces pale-yellow, translucent colonies that are about 2-3 mm in diameter on thiosulfate citrate bile salts sucrose (TCBS) agar. *V. cholerae* can grow at temperature range of 15-45 ℃, pH range of 6-10 and salt (NaCl) concentration of 6% (Bhunia 2008). However, an important distinction from other *Vibrio* spp. is the ability of *V. cholerae* to grow in nutrient broth without added NaCl. The bacterium is gelatinase-positive (Albert 1994),

oxidase-positive, reduces nitrate, and is motile because it possesses single, sheathed, polar flagellum (WHO 2002).

Studies of the aquatic environment have shown that *V. cholerae*, including strains of O1 and O139 are normal inhabitants of surface water. They live particularly in brackish waters, survive and multiply in association with zooplankton and phytoplankton (Huq et al., 1983). However, scientists discovered that this bacterium is a natural inhabitant of riverine, estuarine, and coastal waters throughout both temperate and tropical regions of the world (Colwell 1996). *V. cholerae* O139 has a mannose-sensitive haemagglutinin fimbria (Chiavelli et al., 2001), a polyaccsharide capsule (Johnson et al., 1993) and a lipopolysaccharide (LPS) (Knirel et al., 1997). Both the capsule and LPS O side chain of *V. cholerae* O139 are functioned as virulence factors (Waldor et al., 1994) and were found to be important factors for colonization in the mammalian intestine (Nesper et al., 2002). The LPS may function as an adhesion factor (Alam et al., 1997), and the capsule enhances intestinal colonization (Waldor et al., 1994). In addition, it was confirmed that the capsule of *V. cholerae* O139 has a tendency to contribute to effective adherence to the human intestinal mucosa (Nesper et al., 2002) and resistance to phagocytosis by leukocytes (Albert et al., 1999). Besides, it is also known that the mannose-sensitive haemagglutinin fimbriae of *V. cholerae* O139 aids in the attachment to plankton in aquatic habitats (Chiavelli et al., 2001). *V. cholerae* O139 has ligands and receptors for mannose (Dearborn and Korn 1974; Tarsi and Pruzzo 1999) and it requires neither capsule nor LPS O side chain to

grow and survive inside the free-living amoeba *Acanthamoeban castellanii* (Abd et al., 2009).

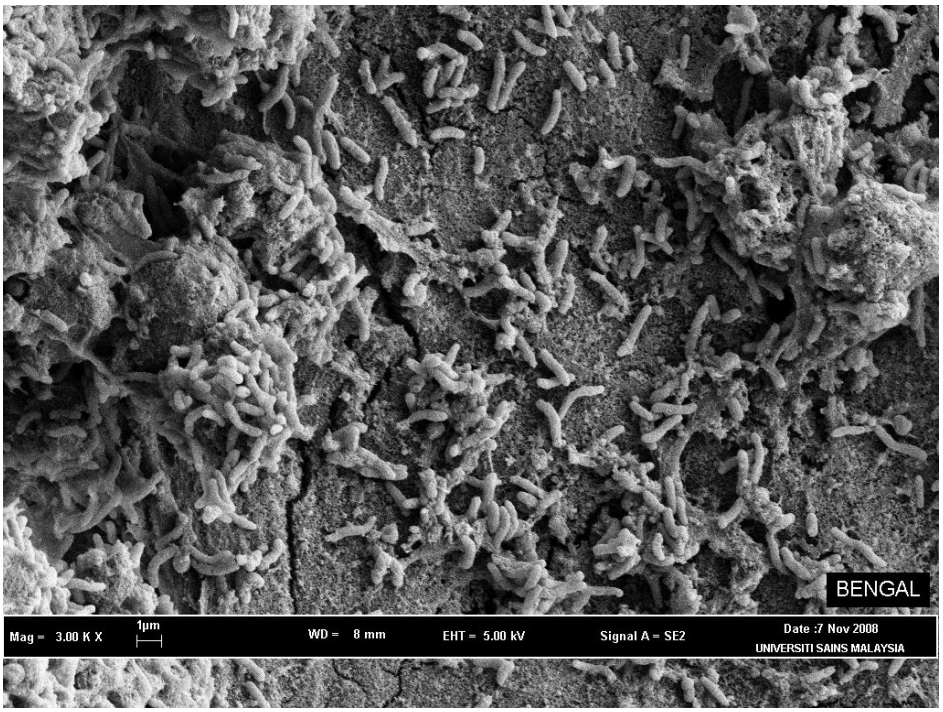


Figure 2.2: *V. cholerae* under scanning electron microscope (SEM) with 3000X magnification (Courtesy of Tan 2012).

2.1.3 Epidemiology of Cholera

Cholera is a major public health problem in many developing countries of Asia, Africa and Latin America (Faruque et al., 1998). Historically, epidemics and pandemics were strongly linked to poor sanitation, unprotected water source, and crowded living conditions (Kindhauser 2003). Since 1817, seven cholera pandemics have occurred around the world (Safa et al., 2008). Firm evidence suggests that the fifth (1881-1896) and sixth (1899-1923) cholera pandemics were

caused by the *V. cholerae* O1 classical biotype while the most extensive seventh and current pandemic (1961-present) is caused by the El Tor biotype. The sixth cholera pandemic strain was apparently persisted only in Southern Bangladesh. The seventh pandemic of cholera began in Sulawesi, Indonesia in 1961 and spread through Asia and Middle East in the 1960s. This infection did not reach Africa until 1970 and Latin America until 1991. There are an estimated 3-5 million cholera cases and 100,000-120,000 deaths due to cholera every year (WHO 2012a). Of the 33 countries that reported deaths from cholera, 23 were from the African continent accounting for 53% of the global total (WHO 2012b). In endemic areas, children were more susceptible to this disease due to the lacking of acquired immunity (WHO 1995). Since the onset of El Tor dominance in 1961, the classical strains were gradually replaced by the El Tor strains and are now believed to have extinct (Safa et al., 2008).

In 1992, a new cholera strain, *V. cholerae* O139 emerged in India and Bangladesh. It spread west to Pakistan and east to Western China. The infection causes an estimated 100,000 cases and 1000 deaths in Southern Bangladesh in the early months of 1993 (WHO 1995). Surveillance during 1996 and 1997 reported that *V. cholerae* O139 continues to cause cholera outbreaks in India and Bangladesh and coexists with the El Tor vibrios (Faruque et al., 1997; Mukhopadhyay et al., 1998).

In 2011, cholera cases caused by *V. cholerae* have been reported from all regions of the world. A total of 58 countries reported a cumulative total of 589 854 cases including 7816 deaths with a case fatality rate of 1.3% (WHO 2012b). Until now, cholera remains a global treat and has the potential to cause pandemic.

2.2 Virulence Factor of *V. cholerae*

V. cholerae comprise both pathogenic and nonpathogenic strains that vary in their virulence gene content (Faruque et al., 1998). This bacterium contains a wide variety of strains and biotypes, receiving and transferring genes horizontally as well as vertically for toxins (Waldor and Mekalanos 1996), colonization factors (Karaolis et al., 1999), antibiotic resistance (Hochhut and Waldor 1999), capsular polysaccharides that provide resistance to chlorine (Yildiz and Schoolnik 1999) and new surface antigens, such as the O139 lipopolysaccharide and O antigen capsule (Bik et al., 1995; Waldor et al., 1996). The lateral or horizontal transfer of these virulence genes by filamentous bacteriophage (Waldor and Mekalanos 1996), pathogenicity islands (Karaolis et al., 1998; Kovach et al., 1996) and other accessory genetic elements (Mekalanos et al., 1997) provides insights into how bacterial pathogens emerge and contribute to microbial evolution.

2.2.1 CTX Genetic Element

The major virulence-associated factors are present in cluster (Hacker et al., 1997) with at least three regions in *V. cholerae* chromosome. The first is the CTX genetic element (Pearson et al., 1993) which has been reported that it is resided in the genome of a filamentous bacteriophage, CTXϕ (Waldor and Mekalanos 1996). Studies have previously shown that the genome of CTXϕ contains a 4.5 kb central core region comprising the *ctxAB, zot, ace, orfU* and *cep* genes. The CTX genetic element comprises a central core with a repetitive sequence (RS) of 2.4-kb, designated as RS2 (Pearson et al., 1993). This RS2 region is located at the upstream of CTX central core and consists of three ORFs that are involved in the regulation (*rstR*), replication (*rstA*) and integration (*rstB*) of CTXϕ, together with two intergenic sequences, *ig1* and *ig2*, which can be found at the left and right ends of *rstR*, respectively (Chun et al., 1999; Sharma et al., 1997). The CTX genetic element can also be flanked by one or more copies of a 2.7 kb RS, designated RS1. The RS1 element, a satellite phage, is very similar to the RS2 region of CTXϕ, but with an additional ORF named *rstC* (Faruque et al., 2002; Nusrin et al., 2004). Figure 2.3 shows the schematic diagram of CTX genetic element.

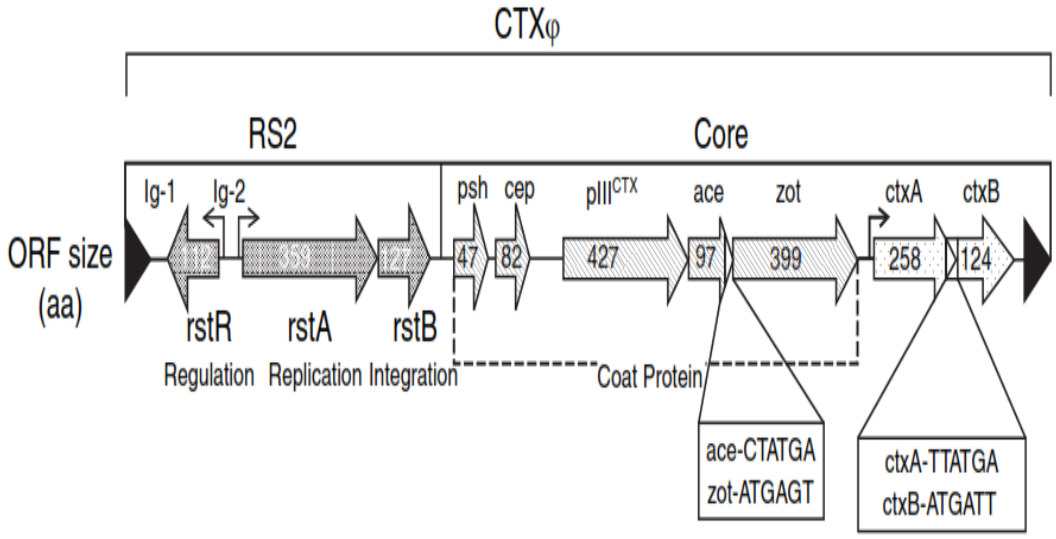


Figure 2.3: Genetic organization of CTXϕ showing the direction of transcription of each gene. Numbers in the ORF indicate the amount of amino acid present in the protein (Chaudhuri and Chatterjee 2009).

2.2.1.1 Cholera Toxin (CT)

CT is composed subunits encoded by of two *ctxA* and *ctxB*, which are located on a transmissible filamentous phage (Waldor and Mekalanos 1996). *ctxA* and *ctxB* have coding sequences of 777 bp and 375 bp respectively. The primary translational product for *ctxA* is 258 amino acid peptides whereas *ctxB* encodes a gene product with 124 amino acid polypeptides (Chaudhuri and Chatterjee 2009). The pathogenic strain of *V. cholerae* is mainly associated with the secretion of the CT, which is a protein complex that responsible for the diarrhea effects in cholera infection. CT belongs to the AB toxins superfamily and is an oligomeric protein consists of a heterodimeric A-subunit (CT-A, ~27.4 kDa) and a homopentameric B-subunit (CT-B, ~58 kDa), with each of the subunit has a specific function (Chinnapen et al., 2007).

The CT A-subunit contains two domains: an 11 kDa N-terminal chain (A1), which plays a role in the catalytic activity of the toxin, and an 18 kDa C-terminal chain (A2), which anchors the A-subunit to the central pore in the pentameric B-subunit. The A1 and A2 chains are connected sequentially via a serine protease-containing flexible linker, which is bridged by a disulfide bond. Enzymatic cleavage at this site is needed for toxicity and it is often occurs after release from the bacterium in the intestinal lumen (Fujinaga et al., 2003; Lencer et al., 1995). The B subunit has five identical B monomers (~11.6 kDa) that are arranged in a ring-like configuration. Each monomer possesses a binding site for the plasma membrane receptor of the jejunal intestinal epithelial cells, the monosialoganglioside GM_1 (Chinnapen et al., 2007). CT is the causal mediator of severe diarrhea by acting on the cystic fibrosis transmembrane conductance regulator (CFTR). This results in the net increase of chloride ions (Cl^-) secretion, accompanied by the osmotic movement of a large quantity of water into the intestinal lumen (Sánchez and Holmgren 2005).

2.2.1.2 Zonula Occludens Toxin (Zot)

Zot gene is located immediately upstream to the *ctx* locus (Baudry et al., 1992). Along with *ace* and CT gene, *zot* is located in the CTX genetic element (Waldor and Mekalanos 1996). The *zot* gene sequences are present in both *V. cholerae* O1 and non-O1 strains, and strains that contain *ctx* sequences almost always contain *zot* sequences and vice versa. The *zot* gene contains a 1.3 kb ORF which encodes

a single polypeptide chain of 44.8 kDa with 399 amino acids in length (Baudry et al., 1992). Zot possesses multiple domains that contribute to its dual functionality: it can act as a morphogenetic phage protein and as an enterotoxin. Subsequent to cleavage at amino acid residue 287, a carboxy-terminal fragment of 12 kDa that responsible for the biological effect of the toxin is excreted. The ~33 kDa N-terminal left after cleavage is probably involved in CTXϕ assembly and remains correlated with the bacterial cell membrane (Chaudhuri and Chatterjee 2009).

The protein, Zot may contribute to residual diarrhea in human (Fasano et al., 1991). When tested on rabbit ileal mucosa, *Zot* is able to affect the structure of epithelial tight junctions and thereby increases the permeability of the small intestine. This permits the passage of macromolecules through the paracellular route (Fasano et al., 1991; Fasano et al., 1995). Due to protein kinase C alpha-dependent F-actin polymerization, the mechanism of action of *Zot* on tight junctions involves rearrangement of the epithelial cell cytoskeleton (Fasano et al., 1995). It is notable that *Zot* does not lead to tissue damage and its effect on intestinal permeability is time- and dose-dependent and fully reversible (Fasano et al., 1991; Fasano et al., 1995). The selective effect of the toxin on the small intestine is most likely related to the regional distribution of the Zot receptors which are present in the jejunum and ileum, but not the colon (Chaudhuri and Chatterjee 2009).

2.2.1.3 Accessory Cholera Enterotoxin (Ace)

The *ace* ORF is located upstream of *zot* and *ctx* (Heidelberg et al., 2000). The Ace protein comprises a 294 bp gene coding for a 97 amino acid residue peptide with a predicted molecular mass of about 11.3 kDa. The analysis of the predicted protein sequence showed the presence of an alpha helical C-terminal region. This 20-residue region has an amphipathic nature in which nearly all of the hydrophobic residues are on one side of the helix. Hence, researchers suggested that multimers of Ace possibly insert into the eukaryotic membrane, with the hydrophobic parts facing the lipid bilayer while the hydrophilic sides facing the interior of the transmembrane pore (Trucksis et al., 1993).

The termination codon of *ace*, TGA, overlaps with the initiation codon of *zot*, which is in the different reading reading frame (Trucksis et al., 1993). This unique arrangement suggests the involvement of translational coupling between *ace* and *zot*, like the one found between the *ctxA* and *ctxB* gene (Lockman & Kaper 1983). Ace, similar to CT, is secreted by *V. cholerae* independent of phage production, and exhibits physiological activity (Trucksis et al., 1997). However, its role in causing cholera has not been characterized with the use of isogenic mutants in human volunteers. It has been proposed that Ace may contribute to an early stage of intestinal secretion in *V. cholerae* infection before the onset of the secretion stimulated by cholera toxin (Trucksis et al., 1997).

2.2.2 *V. cholerae* Pathogenicity Island (VPI)

The second region is a large pathogenicity island for *V. cholerae* (VPI) (Karaolis et al., 1998). The chromosomal VPI is 41.2 kb in size in both classical and El Tor biotype strains and encodes 29 potential proteins (Karaolis et al., 1998; Karaolis et al., 2001). This pathogenicity island comprises the *tcp-acf* gene cluster that encode a TCP (Kovach et al., 1996; Waldor and Mekalanos 1996), a type IV pilus that function as an essential colonization factor (Taylor et al., 1987); *toxT*, *tcpP*, and *tcpH* which involved in the regulation of the cholera toxin (*ctx*) and other virulence genes (Kovach et al., 1996; Karaolis et al., 1998); transposase-like gene (*vpiT*) and integrase-like gene (*int*) which belongs to the family of site-specific recombinases (Kovach et al., 1996; Karaolis et al., 1998); and DNA of uncharacterized but potentially important function (Karaolis et al., 1998). Besides, this pathogenicity island also acts as a CTXϕ receptor (Waldor and Mekalanos 1996). The VPI is flanked by *att* sites that most likely function as specific attachment sites between this VPI element and the host bacterial chromosome. It shows that the possession of the VPI has allowed specific strains of *V. cholerae* which is normally an aquatic or estuarine water bacterium to become adapted to the human intestinal environment and successfully colonize it. During diarrheal disease, numerous *V. cholerae* cells could be produced due to their capability to colonize intestine and secrete CT. These factors would thereby allow for the organism's continual survival in nature and has selective advantage over nonpathogenic strains. The recognition of potential integrase and transposase genes at each end of the VPI propose that these genes may have played a role in

the transfer and integration of the VPI into epidemic and pandemic strains (Karaolis et al., 1998). Figure 2.4 shows the schematic diagram of VPI which contains *tcp-acf* gene cluster.

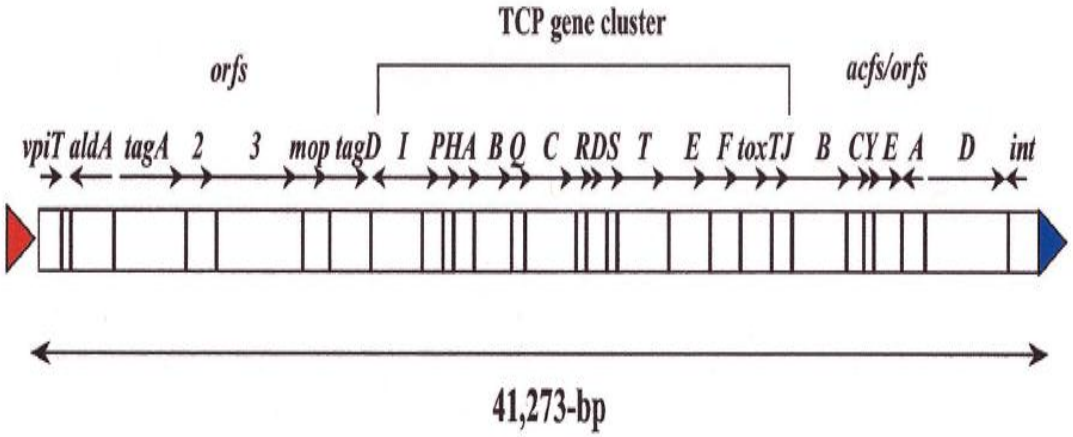


Figure 2.4: Schematic representation of VPI in *V. cholerae* chromosome (Rajanna et al., 2003).

2.2.3 Hemolysin (HlyA)

Hemolysin, also known as cytolysin, is an exotoxin that targets on red blood cell membrane that eventually lead to cell rupture with the release of hemoglobin. Hemolysin produced by *V. cholerae* non-O1 strain is both structurally and immunologically indistinguishable from El Tor hemolysin (Yamamoto et al., 1984; Yamamota et al., 1986). The nucleotide sequence of *hlyA* shows an ORF of 2,223 bp and is predicted to encode a precursor protein with molecular weight of 82 kDa. After the cleavage of 25 amino acids at the N-terminal, the mature form of protein is predicted to have a molecular weight of 79.4 kDa with 691 amino

acids in length (Chaudhuri and Chatterjee 2009). Two other gene products, HlyB and HlyC, are also crucial for the production of El Tor hemolysin. The genes *hlyA*, *hlyB* and *hlyC* form an operon. *hlyB* encodes a product which that is highly homologous to methyl-accepting chemotactic proteins (Mcps). This protein is linked to the motility and chemotaxis of bacteria. *hlyC* encodes a protein homologous to lipases of other bacteria (Chaudhuri and Chatterjee 2009).

This exotoxin is a pore-forming toxin (PFT) that can lyse rabbit erythrocytes and human intestinal cells in culture (Honda and Finkelstein 1979; Zitzer et al., 1997). It increases vascular permeability of rabbit skin and is lethal to mice (Yamamoto et al., 1984). HlyA also involved in cell-vacuolating activity in Vero cells (Figueroa-Arredondo et al., 2001). Recently, an apoptosis-inducing property of HlyA has also been established (Chaudhuri and Chatterjee 2009).

Accoding to Ichinose et al. (1987), some *V. cholerae* strains that lack of CT gene may contribute to the pathogenesis of gastroenteritis by secreting hemolysin. Research by Menzl et al. (1996) suggested that the toxin was able to form oligomer in the culture supernatants. The toxin was only active in lipid bilayer membranes and in erythrocytes when in the oligomeric form. They also illustrated that the toxin's oligomer can form asymmetric and voltage-dependent channels in lipid bilayer membranes and are hemolytically active. However, the activity El Tor hemolysin was difficult to preserve for longer time (Menzl et al., 1996).

2.2.4 Shiga-like Toxins (SLTs)

SLTs or verotoxins were named after the prototype toxin produced by *Shigella dysenteriae* type 1. Other than Vibrio species, certain strains of *Escherichia coli* (*E. coli*) also produced SLTs. They are well-documented toxin that cause bloody and nonbloody diarrhea. SLTs are cytotoxic to certain cell lines, enterotoxic in rabbit ileal loops and caused limb paralysis and death in rabbits and mice (O'Brien and Holmes 1987). It was suggested that SLT also responsible for diarrhea. Consistent with the hypothesis, supporting evidence revealed that the vaccine strain *V. cholerae* CVD103-HgR (*Δctx*), which does not produce detectable Shiga-like activity, caused little or no reactogenicity (Levine et al. 1988).

2.2.5 MARTX Gene Cluster

The third gene cluster, MARTX gene cluster can be found in the *rtx* locus. The *rtx* locus of the *V. cholerae* genome consists of two divergent operons (*rtxHCA* and *rtxBDE*) and containing a total of six ORFs (Heidelberg et al., 2000; Boardman and Satchell 2004). According to Lin et al. (1999), the first ORF in the left-oriented operon is a conserved hypothetical gene, VC1449. The second ORF is the *rtxC* gene that encodes a putative MARTX toxin-activating acyltransferase followed by the toxin structural gene *rtxA*. The contiguous operon encodes a putative type I secretion system (TISS) and is transcribed separately. The first ORF in the right-oriented operon is *rtxB*, a putative transport ATPase gene, and

subsequently the second ORF is a putative periplasmic linker protein gene, *rtxD* (Lin et al., 1999). Downstream to *rtxD* is another ORF, known as *rtxE*, which encodes a protein that is 60% similar to RtxB. Hence, there are two TISS transport ATPases located within this operon (Boardman and Satchell 2004).

Although MARTX activity is independent of the core element, it is physically linked to the CTX prophage in the *V. cholerae* genome (Lin et al., 1999). MARTX gene cluster has cytotoxic activity and is associated with the CTX prophage in the *V. cholerae* genome. The MARTX toxin of *V. cholerae* might be associated with residual adverse properties displayed by certain live, attenuated cholera vaccines (Faruque and Mekalanos 2003). Figure 2.5 shows the schematic diagram of MARTX gene cluster.

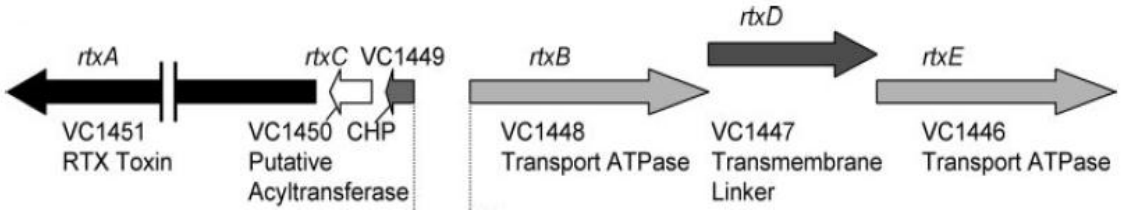


Figure 2.5: Schematic illustration of the *rtx* locus of *V. cholerae* which contains six genes in two divergently transcribed operons (Boardman et al., 2007).

2.2.5.1 *rtxA* (VC1451) – MARTX Toxin

rtxA is the largest ORF of the *V. cholerae* genome with the size of 13,635 bp encoding the MARTX toxin (Heidelberg et al., 2000; Lin et al., 1999). The gene *rtxA* is the third gene in an operon with two other genes, *rtxH* (VC1449) and *rtxC*

(VC1450) (Proft 2009). This gene is closely linked to the *ctx* genes which encode cholera toxin (Lin et al., 1999; Chow et al., 2001). *rtxA* gene is carried by both clinical and environmental isolates of *V. cholerae*, but not in O1 classical biotypes (Chow et al., 2001; Faruque et al., 2004; Lin et al., 1999).

V. cholerae MARTX toxin is a member of the RTX family of toxins (Lin et al., 1999) that includes *Escherichia coli* hemolysin and *Bordetella pertussis* adenylate cyclase-hemolysin toxin (Rose et al., 1995). Characterization of the MARTX toxin revealed that it is distinct from other members of the RTX family of toxins (Sheahan and Satchell 2007). The full-length 4545-amino acid MARTX toxin is predicted to be 484 kDa in size and is secreted from the bacterium through TISS (Boardman and Satchell 2004). The secretion system generally has three components: an inner membrane transport ATPase, a transmembrane linker protein, and an outer membrane porin (Andersen et al., 2001). In addition, approximately one-quarter of this large toxin is comprised of 18- to 20-amino-acid glycine-rich repeats that are localized at the extreme N- and C-terminus of MARTX as opposed to the C-terminal nonapeptide repeats of most RTX toxins (Lin et al., 1999). However, it contains a nonapeptide motif G*G*X*G*X*D*XXX* which is common to all RTX exoproteins (Cordero et al., 2006).

The gene necessary for MARTX toxin production is found in *rtx* locus. This locus contains repeat regions, which are thought to be crucial for toxin binding and translocation into cells, while the inner part of the toxin is hypothesized to hold

cytopathic activities (Sheahan et al., 2004). MARTX toxin of *V. cholerae* is not a pore-forming toxin, but it changes the structure of cells. Due to the depolymerization of actin stress fibres and an increase in paracellular permeability, the toxin causes rounding of a wide range of cells grown in culture (Fullner et al., 2001; Lin et al., 1999; Fullner and Mekalanos 2000). This depolymerization of actin takes place through a mechanism unique to this toxin, resulting in the formation of covalently cross-linked actin dimers, trimers and higher-order multimers that can be visualized using Western blotting (Fullner and Mekalanos 2000). A region of the toxin situated between residues 1963 and 2419 is denoted as the actin cross-linking domain (ACD). In the research of Sheahan et al. (2004), this domain has been identified as the portion of the toxin which contains 412 amino acids and it is present within the 4,545-amino-acid holotoxin. It is involved in cross-linking activity and directly catalyzes the cross-linking reaction (Cordero et al., 2006) when it is expressed as a transgene in eukaryotic cells (Sheahan et al., 2004). Besides, this toxin has been implicated in the pathogenesis of cholera disease (Proft 2009).

2.2.5.2 *rtxBDE* (VC1448, VC1447 & VC1446) – ABC Transporter

The *rtxBDE* operon encodes three components of the T1SS. *rtxB* is transcribed as the ATP-binding transporter protein, RtxD is the membrane fusion protein (MFP) and RtxE has been characterized as a transport ATPase, a membrane-associated protein (Boardman and Satchell 2004; Lee et al., 2008). Recently, research on the

regulatory mechanism of *rtxE* was carried out. Researchers demonstrated that the *rtxBDE* genes are arranged as a single transcriptional unit and transcribed from a single promoter, P*rtxBDE*, and that the activity of P*rtxBDE* is induced by the exposure to mammalian cells. The *rtxE* gene has also been identified as essential to the secretion of the RtxA toxin (Lee et al., 2008).

The *V. cholerae* Rtx exporter atypically carries two ATP-binding cassettes (ABCs), *rtxB* and *rtxE* that may function as a heterodimer (Boardman and Satchell 2004). RtxB and the ATP-binding protein RtxE appeared to be necessary for MARTX protein secretion (Boardman and Satchell, 2004; Lee et al., 2008). Recent study also found that the growth phase regulation of *rtxBDE* expression is not linked to any of the three known quarum-sensing pathways of *V. cholerae* (Boardman et al., 2007).

2.2.5.3 *rtxC* (VC1450) – Acyltransferase

rtxC encodes an acyltransferase, which acts as an activator of MARTX toxin (Boardman and Satchell 2004). According to Tan et al. (2010), the research indicated that in *V. cholerae rtxC* is not an essential for the MARTX toxin function, but the MARTX toxin without *rtxC* activation showed a reduction in actin cross-linking activity, while a deletion of *rtxC* had no effect on the virulence of *V. vulnificus* (Lee et al., 2007; Liu et al., 2007). These results suggest that not all MARTX toxins required acylation (Satchell 2007). Recent studies have

reported that *rtxC* is absent from strains of classical biotype and present in the El Tor biotype only (Dziejman et al., 2002; Lin et al., 1999; O'Shea et al., 2004). This gene encodes a protein similar to the *hlyC* acyltransferase that is required for maturation of *E. coli* α-hemolysin and it is located in the same operon as the *rtxA* toxin gene (Boardman and Satchell 2004, Lin et al., 1999).

2.2.5.4 *rtxH* (VC1449) – Hypothetical Protein

rtxH is a conserved hypothetical gene of unknown function (Proft 2009). This gene is 360 bp in length and encodes a 120-amino-acid hypothetical protein (Lin et al., 1999). It can only be found in the MARTX gene clusters (Boardman and Satchell 2004). RtxH is an uncharacterized protein hence the 3D structure of this protein remains unknown. More research should be carried out to investigate this gene because it might play a role in the pathogenesis of cholera.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Bacteria Strains

The bacteria used in this study are *Vibrio cholerae* O139, *E. coli* TOP10 and *E. coli* BL21 DE3. These strains are obtained from Cholera Research Group Universiti Sains Malaysia.

3.1.2 Plasmid

The plasmid that used in this study is the expression plasmid, pHIS Parallel 2 (pHIS P2) as shown in Figure 3.1.

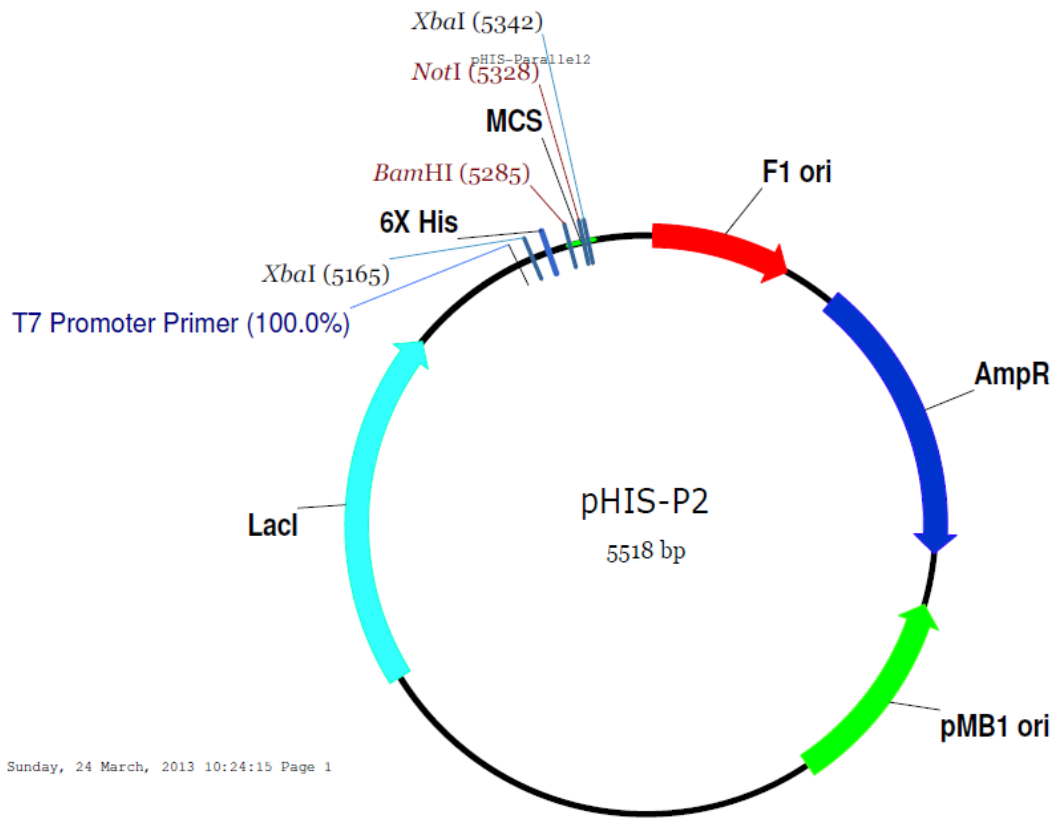


Figure 3.1: pHIS Parallel 2 plasmid map.

3.1.3 Chemicals and Reagents

Table 3.1 shows details of the chemicals and reagents used.

Table 3.1: List of chemicals and reagents used and their manufacturers.

Chemicals/Reagents	Manufacturers
100 bp DNA ladder	Philekorea Technology Inc.
95% ethanol	Copens Scientific (M) Sdn. Bhd.
Agarose powder	Choice-Care Sdn. Bhd.

Table 3.1: Continued.

Chemicals/Reagents	**Manufacturers**
Alkaline phosphatase	Thermo Scientific
Ampicillin	Fisher Scientific
*Bam*HI	BIORON
Boric acid	QR ëc
Bovine serum albumin (BSA)	BIORON
Calcium chloride ($CaCl_2$) anhydrous	GENE Chem
dNTP mix	Philekorea Technology Inc.
EDTA disodium dihydrate	SYSTERM
Ethidium bromide	Bio Basic Inc.
Gel/PCR DNA fragments extraction kit	Geneaid
Glycerol	QR ëc
High speed plasmid mini kit	Geneaid
Hydrochloric acid (HCl)	R&M Chemicals
Luria Bertani (LB) agar	MERCK
Luria Bertani (LB) broth	MERCK
Magnesium chloride ($MgCl_2$)	R&M Chemicals
NEB3 buffer	New England Biolabs
*Not*I	BIORON
Pfu DNA polymerase	Philekorea Technology Inc.
Sodium hydroxide (NaOH)	Bio Basic Inc.

Table 3.1: Continued.

Chemicals/Reagents	Manufacturers
Thiosulfate-citrate-bile-sucrose (TCBS) agar	MERCK
T4 DNA ligase	Enzymatics
Tango Buffer	Thermo Scientific Inc.
Taq DNA polymerase	Philekorea Technology Inc.
Tris	Bio Basic Inc.
*Xba*I	BIORON

3.1.4 Instruments and Labwares

Table 3.2 shows details of the instruments and labwares used in this study.

Table 3.2: List of instruments and labwares used and their manufacturers.

Instruments/Labwares	Manufacturers
-20 ℃ freezer	Haier
-80 ℃ Ultra-low temperature freezer	Thermo Scientific Inc.
15 mL conical tube centrifuge machine	Dynaminca
Autoclave sterilizer	HIRAYAMA HVE-50
Drying incubator	Memmert
Electrophoresis system	Major Science
Hotplate stirrer	Copens Scientific (M) Sdn. Bhd.

Table 3.2: Continued.

Instruments/Labwares	**Manufacturers**
Incubator	Memmert
Laminar airflow	ESCO Airstream
Microcentrifuge	Sartorius
Micropipette	ViPRΩ
Microwave oven	Sharp
Nanospectrophotometer	IMPLEN
PCR machine	Kyratec
pH meter	Sartorius
Power unit	Medigene Sdn. Bhd.
Refrigerator 4 ℃	Copens Scientific (M) Sdn. Bhd.
Shaking incubator	Daihan Labtech
Spectrophotometer	Biorad
UV Transilluminator	V-BioScience Sdn. Bhd.
Vortex machine	VELP Scientifica
Water bath	Memmert
Weighing balance	OHAUS
White light board	Vilber Lourmat

3.2 Preparation of Solutions

3.2.1 EDTA, 0.5 M

To prepare 0.5 M EDTA, 186.1 g of EDTA disodium dehydrate was added into 750 mL of deionized distilled water and the pH was adjusted to pH 8.0 using 1 M NaOH. The volume was made up to 1 L. Then, the solution was autoclaved at 121 ℃ for 15 minutes and stored at room temperature.

3.2.2 Tris Borate EDTA (TBE) Buffer, 10X

To prepare 10X TBE buffer, 108 g of Tris base, 55 g of boric acid and 40 mL of 0.5 M EDTA were added into 800 mL of deionized distilled water and the pH was adjusted to pH 8.3 using 1 M NaOH. The volume was made up to 1 L and the buffer was stored at room temperature after autoclaved at 121 ℃ for 15 minutes. To prepare 1X TBE buffer, 100 mL of 10X TBE buffer was mixed with 900 mL of distilled water and is ready for use.

3.2.3 Hydrochloric Acid (HCl), 1 M

To prepare 1 M of HCl, 8.3 mL of 37% (v/v) HCl (12 M) was measured and mixed evenly with 91.7 mL of deionized distilled water. The solution was stored at room temperature.

3.2.4 Sodium Hydroxide (NaOH), 1 M

To prepare 1 M NaOH, 4 g of sodium hydroxide pellet was measured and dissolved in 100 mL of deionzed distilled water. The solution was stored at room temperature.

3.2.5 Calcium Chloride ($CaCl_2$), 100 mM

To prepare 100 mM of $CaCl_2$, 1.1 g of calcium chloride anhydrous was measured and dissolved in 100 mL of deionized distilled water. The solution was filter sterilized using 0.22 μm syringe filter before used and stored at 4 ℃.

3.2.6 Magnesium Chloride ($MgCl_2$), 100 mM

To prepare 100 mM of $MgCl_2$, 0.95 g of magnesium chloride powder was measured and dissolved in 100 mL of deionized distilled water. The solution was filter sterilized using 0.22 μm syringe filter before used and stored at 4 ℃.

3.2.7 Ethanol, 70% (v/v)

To prepare 70% (v/v) ethanol, 736.8 mL of 95% (v/v) ethanol was measured using measuring cylinder and distilled water was added to 1 L to dilute it. The ethanol solution was stored at room temperature.

3.2.8 Ampicillin Stock Solution, 100 mg/mL

To prepare 100 mg/mL ampicillin stock solution, 1 g of sodium ampicillin was measured and dissolved in 10 mL of deionized distilled water. The solution was mixed evenly and filter sterilized using 0.22 μm syringe filter. Ampicillin stock solution was then aliquoted into microcentrifuge tubes and stored at -20 ℃.

3.2.9 Agarose Gel, 1.5% (w/v)

To prepare 1.5% (w/v) of agarose gel, 0.75 g of agarose powder was measured and dissolved in 50 mL of 1X TBE buffer using the microwave oven. The bottle was swirled to mix the solution evenly and ensure the powder was fully dissolved. Next, the agarose solution was poured onto a clean gel cast and the comb with desired size was inserted. The solution was left in room temperature for it to solidify.

3.2.10 Glycerol, 80% (v/v)

To prepare 80% (v/v) glycerol solution, 80.4 mL of 99.5% glycerol stock was measured and dissolved in 19.6 mL of deionized distilled water. The solution was autoclaved at 121 ℃ for 15 minutes before used and stored at room temperature.

3.3 Preparation of Culture Media

3.3.1 Luria Bertani (LB) Agar

A total of 37 g of LB agar powder was measured and dissolved in 1 L of deionized distilled water. Mixture was then autoclaved at 121℃ for 15 minutes. Lastly, the medium was poured into sterile petri dish and left to solidify at room temperature. The LB agar will be stored at 4℃ until use.

3.3.2 Luria Bertani (LB) Broth

A total of 25 g of LB broth powder was measured and dissolved in 1 L of deionized distilled water. Mixture was then mixed evenly and poured into sample vials. Then, all sample vials with 5 mL of LB broth was autoclaved at 121℃ for 15 min and stored at room temperature.

3.3.3 Thiosulfate-Citrate-Bile-Sucrose (TCBS) Agar

A total of 88.0 g of TCBS agar powder was measured and added into 1 L of deionized distilled water. The mixture was boiled until the powder was fully dissolved. At the same time, the mixture was stirred so that the powder can dissolve faster and the solution was mixed evenly. Lastly, the medium was poured into sterile petri dish and left to solidify at room temperature. The TCBS agar will be stored at 4℃ until use.

3.4 Methods

3.4.1 Overview

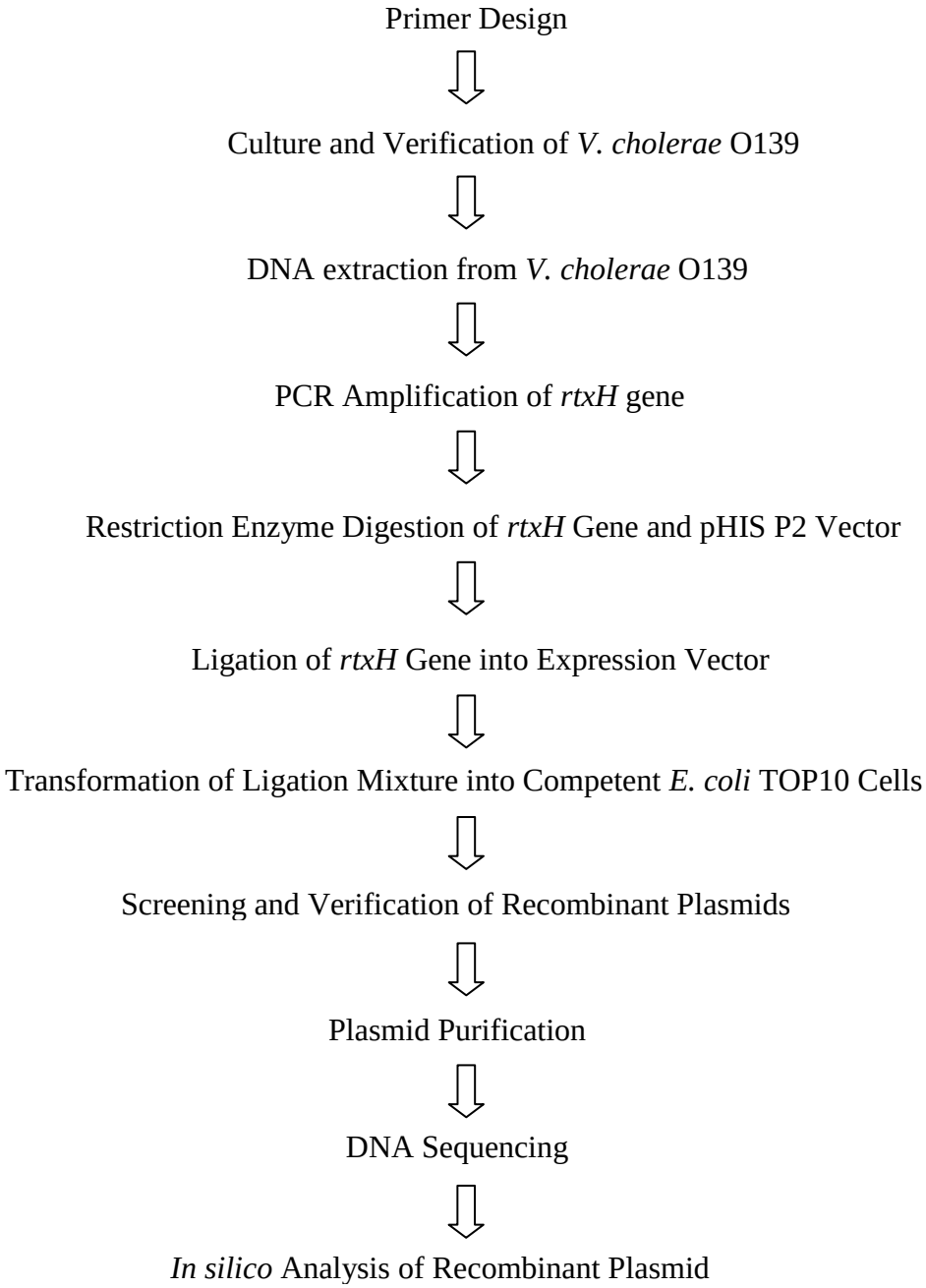


Figure 3.2: Flow chart of research methodology in this study.

3.4.2 Culture and Verification of *V. cholerae* O139

Glycerol stock of *V. cholerae* O139 was taken out from -80 ℃ freezer and thawed in the ice box. Then, 500 μL of bacteria suspension was pipette immediately into the sample vial containing 5 mL LB broth. The bacteria was incubated in shaking incubator at 37 ℃ for few hours to revive it. After that, the turbidity of the broth was observed. Higher turbidity indicated higher concentration of bacteria presence in the broth. Next, revived bacteria culture was inoculated and streaked on TCBS agar. The agar plate was incubated overnight at 37 ℃. The confirmed *V. cholerae* strain was maintained in LB agar and subcultured every two weeks.

3.4.3 DNA Extraction from *V. cholerae* O139

A single colony of *V. cholerae* was inoculated into a 1.5 mL microcentrifuge tube containing 50 μL of deionized distilled water. The bacteria were boiled for 10 minutes to lyse the cells so that DNA genome could be released. After that, the microcentrifuge tube was spun for 1 minute at 11,000 ×*g* centrifugation. All the cell debris was pellet down and the supernatant that containing DNA template was used to carry out PCR.

3.4.4 PCR Amplification of *rtxH* Gene

3.4.4.1 Primer Design

A set of primers flanking the *rtxH* gene was specifically designed. The primer sequences were compared with the sequence in GenBank (accession number: AE003852.1). Then, primer dimer analysis was performed for both forward and reverse primer using OligoAnalyzer 3.1. The annealing temperatures also calculated to analyze the efficacy of PCR amplification. A formula was used to calculate the annealing temperature: [(A+T) × 2 + (C+G) × 4]-2. Based on the formula, the calculated annealing temperature for forward and reverse primers were 52 ℃. Besides, restriction enzyme analysis was carried out using NEBcutter V2.0 to determine the suitable enzymes that can be used in this study. The forward primer (VCrtxH-F) was designed with a *Bam*HI restriction enzyme flanking at 5'end and the reverse primer (VCrtxH-R) consists of a *Not*I restriction site at 5' end (Table 3.3). Primers were synthesized by 1st BASE Laboratories Sdn. Bhd. (Selangor).

Table 3.3: List of primers in this study. The underlined nucleotides, ATG, represent the start codon. Nucleotides in bold represent the RE sites added into the primers.

Primer	**Primer Sequence**
VCrtxH-F	5'**AAGGATCC**ATGGGGAAATTTATGATGGA3'
VCrtxH-R	5'**AAGCGGCCGC**GGTTACTTTTTCACC3'

3.4.4.2 PCR Optimization and Amplification

Gradient PCR was done using *Taq* DNA polymerase to optimize the annealing temperature of the *rtxH* gene. It was performed at six different temperatures, which were 50 ℃, 52 ℃, 54 ℃, 56 ℃, 58 ℃ and 60 ℃. Annealing temperature that gave the brightest and clearest band was chosen to carry out the subsequent PCR amplification step. After the optimization of annealing temperature, a one-step PCR was performed in a 20 µL mixture containing 1X *Pfu* enzyme buffer, 75 µM deoxyribonucleotide triphosphate (dNTPs), 1.25 mM of $MgCl_2$, 1 µM of each primer (VCrtxH-F and VCrtxH-R), 2 U of *Pfu* enzyme, deionized distilled water and 1 µL of the extracted template DNA. The mixture was then subjected to 35 amplification cycles using Kyratech PCR thermocycler according to the reaction condition below (Table 3.4).

Table 3.4: Gradient PCR and standard PCR conditions for *rtxH* gene.

Step	**Temperature (℃)**	**Duration (s)**	**Number of cycle (s)**
Pre-denaturation	95	600	1
Amplification			
i. Denaturation	95	30	
ii. Annealing	50-60	30	35
iii. Extension	72	45	
Final extension	72	300	1
Hold on completion	8		1

3.4.4.3 Agarose Gel Electrophoresis for PCR Products

After PCR, 5 μL of PCR product was mixed with 1 μL of 6X loading dye and resolved into the gel electrophoresis system. The gel was run at 100 V for the first two minutes and subsequently changed to 80 V for 45 minutes. Then, the gel was stained with ethidium bromide for 10 minutes and destained in water for 1 minute. Finally, the agarose gel can be viewed under UV transilluminator and image was captured.

3.4.5 Purification of PCR Products

Purification process was performed to obtain the desired gene fragment with a DNA size of 380 bp after amplification. The *rtxH* gene was purified using Gel/PCR DNA Extraction Kit (Geneaid). Before the kit was used, absolute ethanol was added into the wash buffer. Up to 100 μL of PCR products was transferred to a 1.5 mL microcentrifuge tube and the DF buffer was added in a ratio of 5:1 to denature enzymes in the PCR mixture. The DF buffer was mixed with the PCR sample by vortex and transferred to DF column with a 2 mL collection tube. Then, the PCR mixture was subjected to spin at 16,000 ×*g* for 30 seconds. The flow-through was discarded and the DF column was placed back to the collection tube. Next, 600 μL of wash buffer was added to the centre of the column and stood for 1 minute. This step is to remove the contaminants that present in the mixture. The column was then placed in a microcentrifuge machine for 30 seconds at a speed of 16,000 ×*g*. The flow-through was discarded and the

DF column was put back to the microcentrifuge to dry the column matrix at 16,000 ×*g* for 2 minutes. After that, the column was transferred to a new 1.5 mL microcentrifuge tube and 50 µL of deionized distilled water was added to the center of the column to elute the DNA. The tube was stood at room temperature for at least 2 minutes so that the DNA was completely dissolved. The purified DNA could be obtained by centrifugation at 16,000 ×*g* for 2 minutes. Finally, the DF column was removed and the purified DNA was stored in -20 ℃.

3.4.6 Cloning of *rtxH* Gene into pHIS P2 Vector

3.4.6.1 Restriction Enzyme (RE) Digestion of *rtxH* Gene and Vector

Prior to ligation, both the purified 380 bp *rtxH* gene and 5518 bp pHIS P2 vector underwent double digestion using *Bam*HI and *Not*I to facilitate the directional cloning at the multiple cloning site. One µg of both *rtxH* and pHIS P2 were treated with 5 U of *Bam*H1 and 5 U of *Not*I at the same time. Both tubes had a total volume of 20 µL containing 1X NEB 3 Buffer and 0.1 mg/mL bovine serum albumin (BSA). The mixture was incubated at 37 ℃ in a water bath for 1.5 hours and the reaction was stopped by inactivation of enzyme at 80 ℃ for 20 minutes.

3.4.6.2 Dephosphorylation of Vector

After RE digestion, the vector was undergone dephosphorylation before ligation is performed. This process is needed to prevent the religation of vector that results

in reduction of cloning efficacy. The plasmid was treated with 1 U of alkaline phosphatase in NEB3 buffer and incubated at room temperature (25 ℃) for 10 minutes. Then, the enzyme was inactivated in an 80 ℃ water bath for 15 minutes. After that, 5 μL of the mixture was run through gel electrophoresis to observe the bands using 1.5% (w/v) TBE agarose gel at 80 V for 45 minutes.

3.4.6.3 Ligation of *rtxH* Gene into Vector

After restriction enzyme digestion *rtxH* gene and dephosphorylation of plasmid, purification process was carried out using Gel/PCR DNA fragments extraction kit (Geneaid). Then, the purified digested product was ligated together by T4 DNA ligase. A total of 20 μL of reaction mixture consists of 1X T4 DNA ligase reaction buffer, digested plasmid, digested *rtxH* gene and 5 U of T4 DNA ligase. The ratio of the vector and insert is 1:3. The mixture was incubated at room temperature for 5 hours and transformed into *E. coli* TOP10 competent cells.

3.4.7 Plasmid Transformation

3.4.7.1 Preparation of Competent Cells Using Calcium Chloride Method

A single colony of *E. coli* TOP10 that grew on LB agar plate was inoculated into a 5 mL LB broth and grown for 2 hours at 37 ℃ in a shaking incubator with speed 200 rpm. Subsequently, the optical density (OD) of the bacterial suspension was measured. To obtain high efficacy of *E. coli* competent cells, the cells should

reach 0.5-0.6 absorbance unit at OD_{600}. Next, the bacterial suspension was transferred to a 15 mL conical tube and stood on ice for at least 15 minutes. The chilled *E. coli* cells were then pelleted by centrifugation at 3,000 rpm for 10 minutes at 4℃. The supernatant was removed and 600 μL of chilled 100 mM $MgCl_2$ was added to resuspend the cells. Then, the mixture was incubated on ice for 45 minutes. The cells were subjected to centrifugation again at 3,000 rpm for 10 minutes after the incubation. The supernatant was discarded and the pellet was resuspended using 300 μL of chilled 100 mM $CaCl_2$. The competent *E. coli* TOP10 cells were stored on ice and ready to use.

3.4.7.2 Transformation Using Heat Shock Method

Firstly, 100 μL of competent *E. coli* cells was aliquoted into three 1.5 mL microcentrifuge tubes labeling positive control, negative control and ligation mixture. Negative control was used to test the sterility of the competent cells and there were no plasmid or ligation mixture added. One μL of extracted pHIS P2 plasmid was added to the positive control tube to determine the efficiency of competent cells. Ten μL of ligation mixture was added to another tube and transformation was carried out subsequently. Three tubes were stood on ice for 1-2 minutes and then placed on a water bath at 42℃ for 45 seconds. Then, the tubes were put on ice again for 1-2 minutes after the heat shock method. Four hundred μL of LB broth was added to each tube and incubated in a shaking incubator with 200 rpm at 37℃ for 30 minutes. This step is to regenerate the weaken *E. coli*

cells. Later, the microcentrifuge tubes were subjected to centrifugation at 3000 rpm for 10 minutes. Four hundred μL of supernatant was discarded and the pellets were resuspended with the remaining supernatant. Approximately 100 μL of each sample was spread on the LB agar medium with ampicillin and incubated overnight at 37 ℃.

3.4.8 Screening and Verification of Recombinant Plasmids

3.4.8.1 Screening of Positive Clones by Colony PCR

Bacterial colonies that grown on LB agar added with ampicillin were subjected to colony PCR. The purpose of colony PCR is to screen the positive clones that carry the *rtxH* gene fragment. Ten bacterial colonies were picked and each was suspended in separate 1.5 mL microcentrifuge tube containing 50 μL of sterile deionized distilled water. The colonies were subcultured from the master plate using the bacterial suspension. Then, the tubes were heated at 100 ℃ for 10 minutes to lyse the *E. coli* cells to release the plasmid into water. Then, tubes were centrifuged at 11,000 ×*g* for 1 minute. One μL of the supernatant that contained plasmid was used as the DNA template and would be added to the PCR mixture. After that, the PCR mixture was set up in a total volume of 20μL for each sample containing 1X *Taq* Buffer, 75 μM dNTPs, 2 U *Taq* enzyme, 1 μM of primers (VCrtxH-F and VCrtxH-R), DNA template and deionized distilled water. The PCR mixture was subsequently subjected to 35 amplification cycles in Kyratech PCR Thermocycler according to the parameters listed as in Table 3.5.

Finally, a gel electrophoresis of amplified PCR products was performed using 1.5% (w/v) TBE agarose gel.

3.4.8.2 Plasmid Purification

After determined the correct band size of targeted recombinant plasmid by colony PCR screening, the positive clones was subcultured into a 5 mL sterile LB broth that supplemented with 100 μg/mL ampicillin and incubated overnight at 37 ℃ in shaking incubator. The plasmid extraction was done by using High-speed Plasmid Mini Kit (Geneaid). Prior to initial use, the provided RNase A was added to the PD1 buffer and stored at 4 ℃. Besides, absolute ethanol was added to the wash buffer before use. After overnight incubation, 1.5 mL of cultured bacteria cells was transferred to a 1.5 mL microcentrifuge tube. The cells were harvested by centrifugation at 16,000 ×*g* for 1 minute and the supernatant was discarded. This step was repeated twice to get a higher plasmid concentration. Then, 200 μL of PD1 buffer was added to the tube and the cells were resuspended by vortex or pipetting. Two hundred μL of PD2 buffer was added and mixed gently by inverting the tubes 10 times. The tube was stood at room temperature for 2 minutes or until the lysate is homogenous. Next, 300 μL of PD3 buffer was added and mixed immediately by inverting the tube 10 times. The tube was subjected to centrifugation at 16,000 ×*g* for 3 minutes in this neutralization step. The supernatant was then transferred to a PD column with 2 mL collection tube and centrifuged at 16,000 ×*g* for 30 seconds. The flow-through was discarded and the

400 µL of W1 buffer was added into the PD Column. The column was subjected to centrifuge at 16,000 ×*g* for 30 seconds. The flow-through was discarded again and 600 µL of wash buffer was added. After that, the centrifugation step was repeated at 16,000 ×*g* for 30 seconds. The flow-through was discarded and the column was centrifuged again at the same speed for 3 minutes to dry the column matrix. The dried PD column was transferred to a new 1.5 mL microcentrifuge tube. Fifty µL of autoclaved deionized distilled water was added to the center of the column matrix and stood for 2 minutes or until the water was completely absorbed. Finally, the tube was centrifuged at 16,000 ×*g* for 2 minutes to elute the plasmid DNA. The plasmid DNA was then be used directly or stored at -20 ℃. The concentration and the purity of extracted plasmid were determined using nanospectrophotometer. Five µL of the extracted plasmid was also visualized qualitatively through a 1.5% (w/v) TBE agarose gel at 80 V for 45 minutes.

3.4.8.3 RE Analysis on Recombinant Plasmid

Purified recombinant plasmid was subjected to RE digestion to confirm the presence of gene insert in the pHIS P2 plasmid. About 1 ng of the recombinant plasmid was added to the RE digestion mixture containing 1X NEB3 Buffer, 0.1 mg/mL BSA, 5 U of *Bam*HI and 5 U of *Not*I. The mixture was incubated at 37 ℃ water bath for 1.5 hours. At the same time, the recombinant plasmid was also being digested using *Xba*I to confirm the ligation vector was pHIS P2 plasmid. 10 U of *Xba*I and 1X Tango Buffer was used to perform the RE digestion. The

mixture was incubated at 37 ℃ water bath for 4 hours. Five µL of both mixtures were then electrophorezed through a 1.5% (w/v) TBE agarose gel at 80 V for 45 minutes.

3.4.9 DNA Sequencing

After the verification step and RE digestion, the purified recombinant plasmid was sent for sequencing at 1st BASE Laboratories Sdn. Bhd. Malaysia. The plasmid was sequenced using T7 promoter-F primer. Alignment was done by comparing the sequenced results against known full-length *rtxH* sequence from GenBank. Similarities and frameshift mutation were identified using BLAST software.

3.4.10 *In silico* Analysis of Recombinant Plasmid

This analysis was done using Vector NTI software. The peptide sequence of the recombinant *rtxH* was compared with the original peptide sequence of *rtxH*. Frameshift mutation and point mutation could be determined in this analysis from the differences of amino acids in the peptide sequence.

CHAPTER 4

RESULTS

4.1 Culture and Verification of *V. cholerae* O139

V. cholerae O139 was streaked on the TCBS agar to verify the *Vibrio* spp. Yellow colonies were observed after overnight incubation.

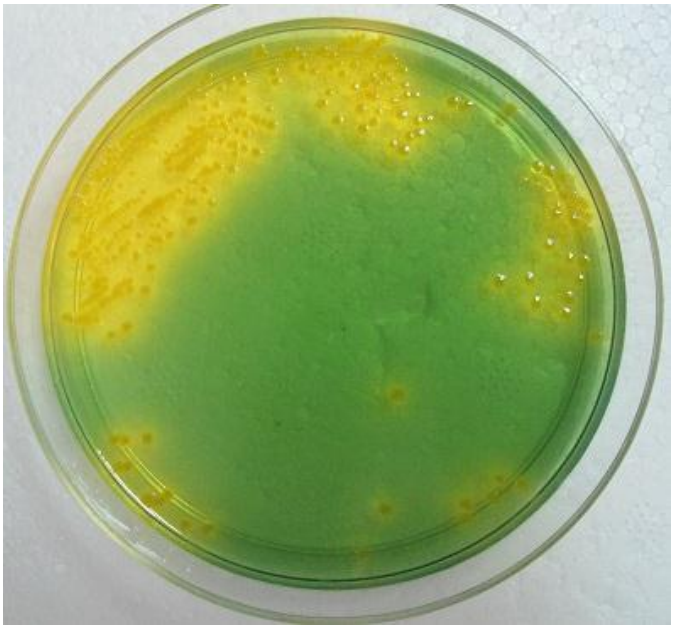


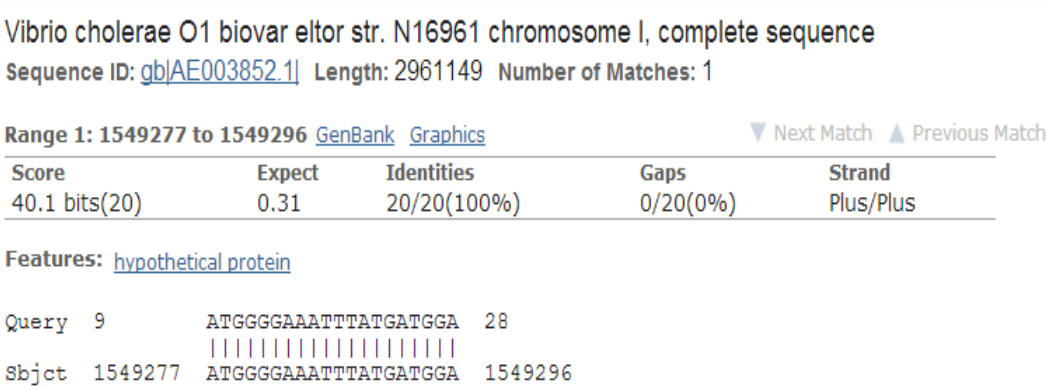
Figure 4.1: *V. cholerae* O139 cultured in TCBS agar.

4.2 PCR Amplification of *rtxH* Gene

4.2.1 Primer Design

The primers was compared with the nucleotide sequence in GenBank and results show that both forward and reverse primer has 100% similarity to the

chromosome of *V. cholerae* O1 biovar El Tor strain N16961 (Figure 4.2 and Figure 4.3). The hetero-dimer analysis revealed that the maximum Delta G value and Delta G value are -55.91 and -5.46 kcal/mole, respectively (Appendix A). In homo-dimer analysis, the highest Delta G values for forward and reverse primer are -11.52 and -22.78 kcal/mole (Appendix B and Appendix C). Gradient PCR result (Figure 4.4) shows clear and thick band in the temperature range of 50 ℃ - 60 ℃. *rtxH* gene was amplified and formed a band with band size of ~380 bp.


Vibrio cholerae O1 biovar eltor str. N16961 chromosome I, complete sequence
Sequence ID: gb|AE003852.1| Length: 2961149 Number of Matches: 1

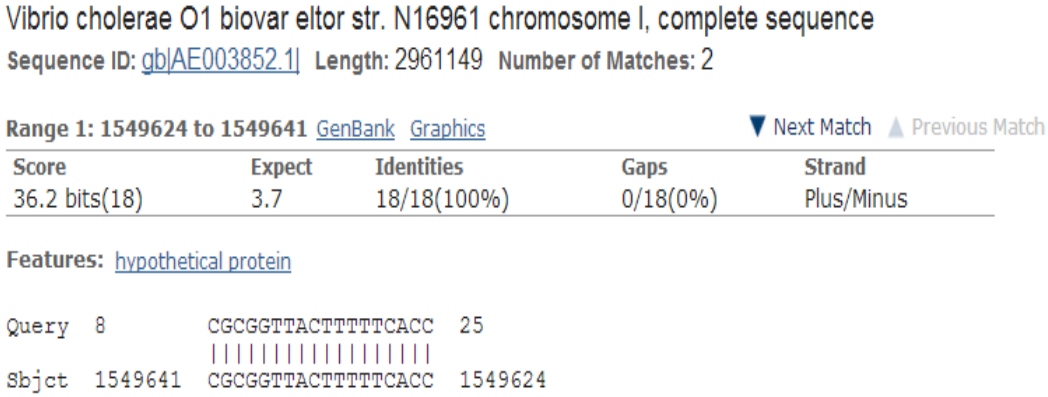
Range 1: 1549277 to 1549296 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
40.1 bits(20)	0.31	20/20(100%)	0/20(0%)	Plus/Plus

Features: hypothetical protein

```
Query  9        ATGGGGAAATTTATGATGGA  28
                ||||||||||||||||||||
Sbjct  1549277  ATGGGGAAATTTATGATGGA  1549296
```

Figure 4.2: Analysis of forward primer (VCrtxH-F) using nucleotide BLAST.


Vibrio cholerae O1 biovar eltor str. N16961 chromosome I, complete sequence
Sequence ID: gb|AE003852.1| Length: 2961149 Number of Matches: 2

Range 1: 1549624 to 1549641 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
36.2 bits(18)	3.7	18/18(100%)	0/18(0%)	Plus/Minus

Features: hypothetical protein

```
Query  8        CGCGGTTACTTTTTCACC  25
                ||||||||||||||||||
Sbjct  1549641  CGCGGTTACTTTTTCACC  1549624
```

Figure 4.3: Analysis of reverse primer (VCrtxH-R) using nucleotide BLAST.

4.2.2 Optimization of Annealing Temperature

The annealing temperatures used in this gradient PCR were 50 ℃, 52 ℃, 54 ℃, 56 ℃, 58 ℃ and 60 ℃. As shown in Figure 4.4, each annealing temperature gave a thick band with band size 380 bp. In this study, the annealing of 58 ℃ was selected.

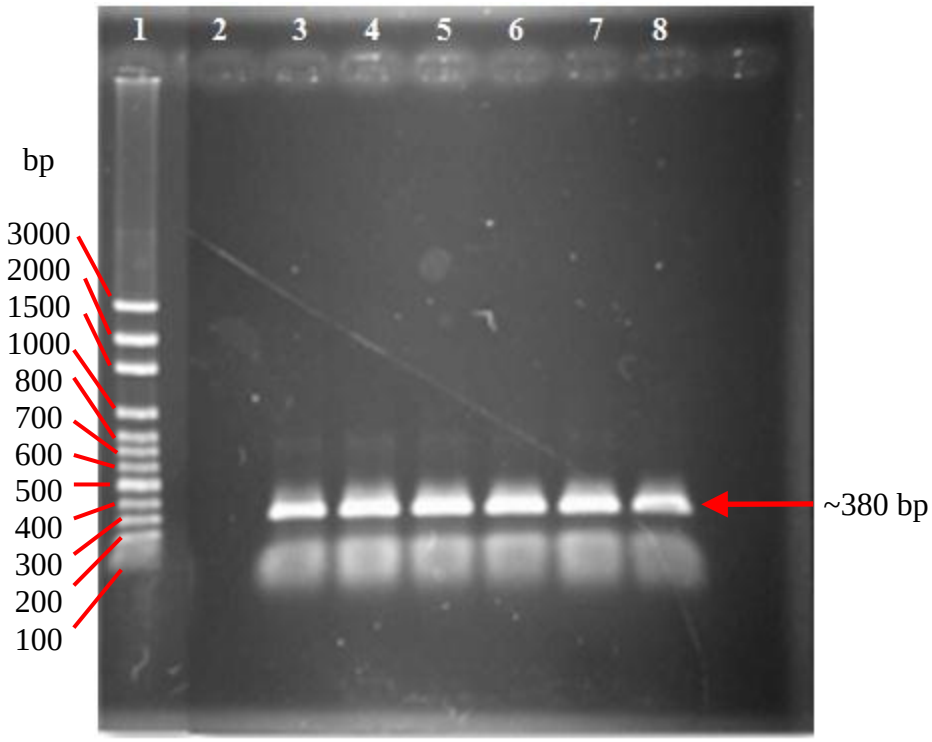


Figure 4.4: Gradient PCR amplification of *rtxH* gene. Lane 1: 100 bp DNA ladder; Lane 2: Negative control; Lane 3-8: *rtxH* gene, with annealing temperature of 50 ℃, 52 ℃, 54 ℃, 56 ℃, 58 ℃ and 60 ℃ for each sample, respectively.

4.2.3 PCR Amplification

The *rtxH* gene was successfully amplified using VCrtxH-F primer and VCrtxH-R primer. The gene was amplified using DNA genome of *V. cholerae* O139. A single band with band size of ~380 bp can be seen clearly on the agarose gel (Figure 4.5). The absence of band in lane 1 indicated that the PCR reagents were not been contaminated by other DNA templates.

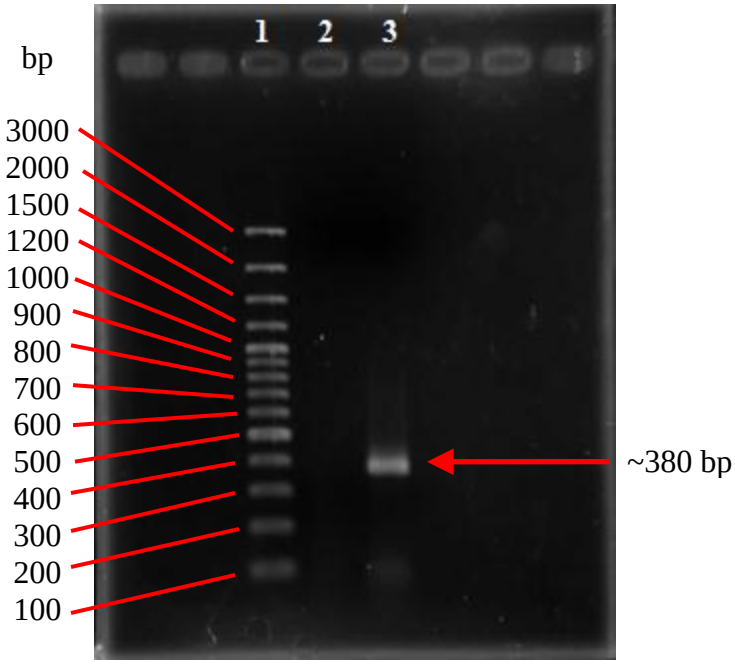


Figure 4.5: PCR amplification of *rtxH* gene. Lane 1: 100 bp DNA ladder; Lane 2: Negative control; Lane 3: PCR product.

4.3 Cloning of *rtxH* Gene into *E. coli* TOP10 Competent Cells

4.3.1 RE Digestion of *rtxH* Gene and pHIS P2 Plasmid

The PCR product and pHIS P2 plasmid was subjected to RE digestion using two different enzymes, namely *Not*I and *Bam*HI. The undigested pHIS P2 plasmid

showed 2 bands which had the band size of 5518 bp (Figure 4.6). After RE digestion, the plasmid was linearized and only one band can be observed. The linearized plasmid had an estimated band size of 5479 bp. The PCR product showed a band that was ~380 bp before digestion. The band remained at the same position after RE digestion.

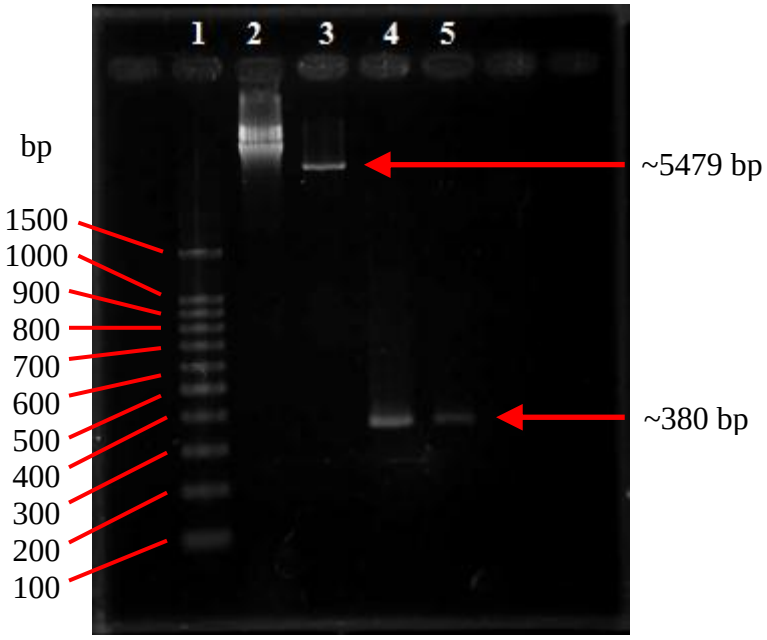


Figure 4.6: RE digestion of pHIS P2 plasmid and purified PCR product, *rtxH*. Lane 1: 100 bp DNA ladder; Lane 2: Undigested pHIS P2 plasmid; Lane 3: pHIS P2 plasmid digested with *Not*I and *Bam*HI; Lane 4: Undigested *rtxH* gene; Lane 5: *rtxH* gene digested with *Not*I and *Bam*HI.

4.3.2 Transformation of Ligation Mixture into *E. coli* TOP10 Competent Cells

There was no colonies observed in negative control plate (Appendix D) indicated that the competent cells were not contaminated. *E. coli* cells that possessed

ampicillin-resistant gene only can grow on the LB-ampicillin agar. The positive control plate (Appendix E) contained undigested pHIS P2 plasmids which were successfully transformed into the competent cells. The ligation mixture and vector were ligated in a ratio of 3:1. Twenty-four colonies can be observed on the LB-ampicillin plate (Appendix F). The recombinant plasmid as shown in Figure 4.7 indicates the position of *rtxH* gene in pHIS P2 plasmid.

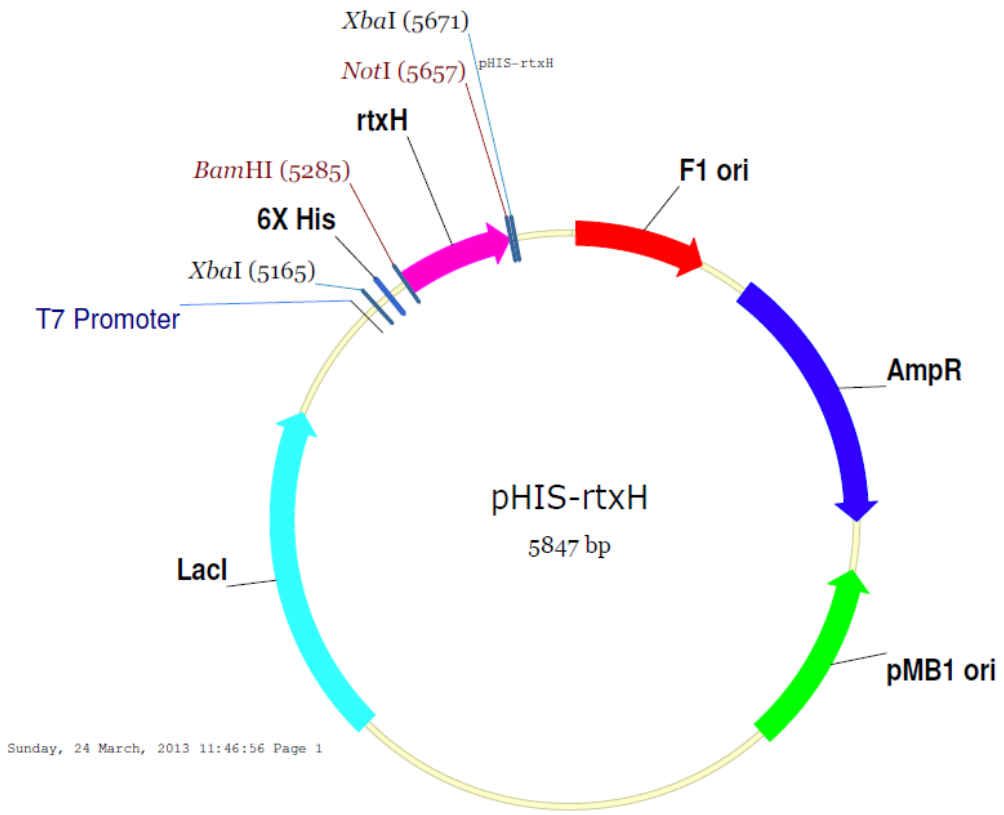


Figure 4.7: Recombinant plasmid map shows the location of *rtxH* gene after ligated into pHIS P2 vector.

4.4 Screening and Verification of Recombinant Plasmids

4.4.1 Colony PCR

PCR screening was performed to check whether the insert was successfully cloned into the vector. Ten colonies were picked to screen the presence of insert. It was found that there were three ampicillin-resistant transformants containing the gene insert of ~380 bp (Figure 4.8).

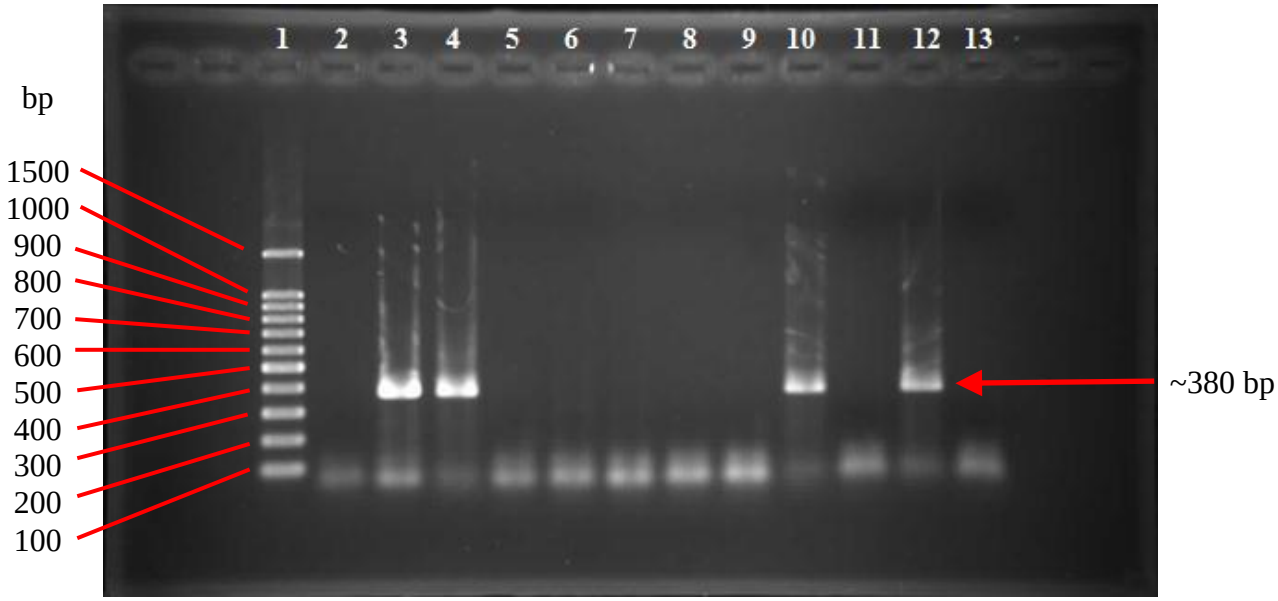


Figure 4.8: PCR screening on 10 ampicillin-resistant plasmids. Lane 1: 100 bp DNA ladder; Lane 2: Negative control; Lane 3: Positive control; Lane 4-13: Colony 1-10.

4.4.2 Plasmid Purification

One of the three colonies with insert was chosen and it was incubated in a shaking incubator for overnight to get a high concentration of plasmid. The extracted plasmid showed two bands with different band size in the agarose gel (Figure

4.9). Because the insert is only 380 bp, there is no significance difference on the band size of the plasmid. The concentration and purity of the recombinant plasmid are shown in Table 4.1.

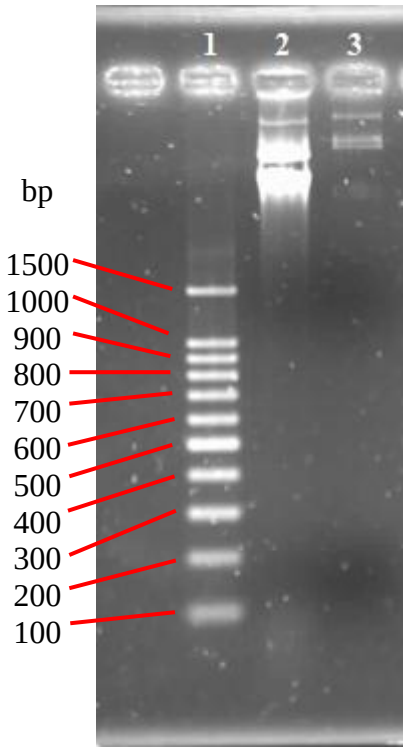


Figure 4.9: Plasmid extraction of recombinant *rtxH*. Lane 1: 100 bp DNA ladder; Lane 2: pHIS P2 plasmid; Lane 3: Recombinant *rtxH*.

Table 4.1: Concentration and purity of recombinant plasmid.

Concentration, ng/µL	195
Purity A260/A280	1.902

4.4.3 PCR Verification of Recombinant Plasmid

The recombinant plasmid was subjected to PCR after plasmid extraction. The PCR product showed a clear band at ~380 bp. The position of PCR product is same as the positive control (Figure 4.10).

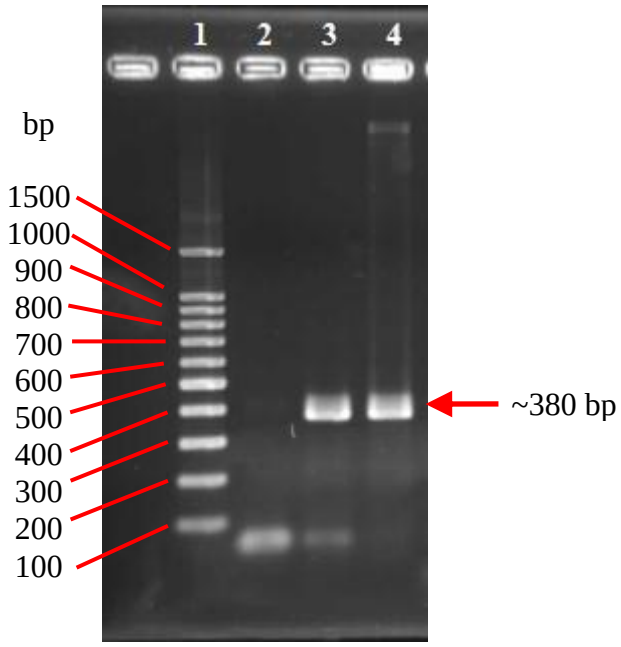


Figure 4.10: PCR verification of recombinant plasmid using VCrtxH-F and VCrtxH-R primers. Lane 1: 100bp DNA ladder; Lane 2: Negative control; Lane 3: Positive control; Lane 4: PCR product of recombinant plasmid.

4.4.4 RE Analysis of Recombinant Plasmid

The recombinant plasmid was digested by *Xba*I to confirm the vector is pHIS P2 plasmid. A single linearized band with ~5335 bp and a single band with 512 bp could be observed after the digestion of *Xba*I (Figure 4.11). At the same time, the recombinant clone was also digested with *Not*I and *Bam*HI. After the digestion, a band with ~5479 bp and another band with ~380 bp were shown.

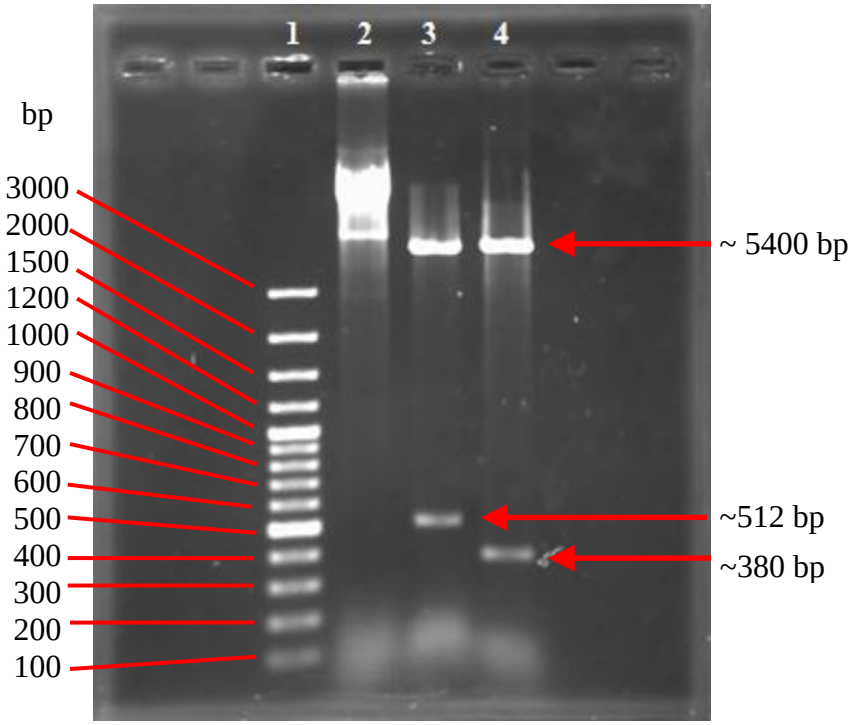


Figure 4.11: RE analysis of recombinant plasmid. Lane 1: 100 bp DNA ladder; Lane 2: Undigested recombinant plasmid; Lane 3: Recombinant plasmid digested with *Xba*I; Lane 4: Recombinant plasmid digested with *Not*I and *Bam*HI.

4.4.5 Sequence Analysis

4.4.5.1 DNA Sequencing

The recombinant *rtxH* was sent for DNA sequencing using T7 promoter-F primer to confirm the identity of recombinant plasmid. The *rtxH* gene sequence showed 100% similarity with the chromosome of *V. cholerae* O1 biovar strain N16961 and had E-value of 0.0 using nucleotide BLAST (Figure 4.12). DNA sequencing result also shows that there were no mutations in this gene.

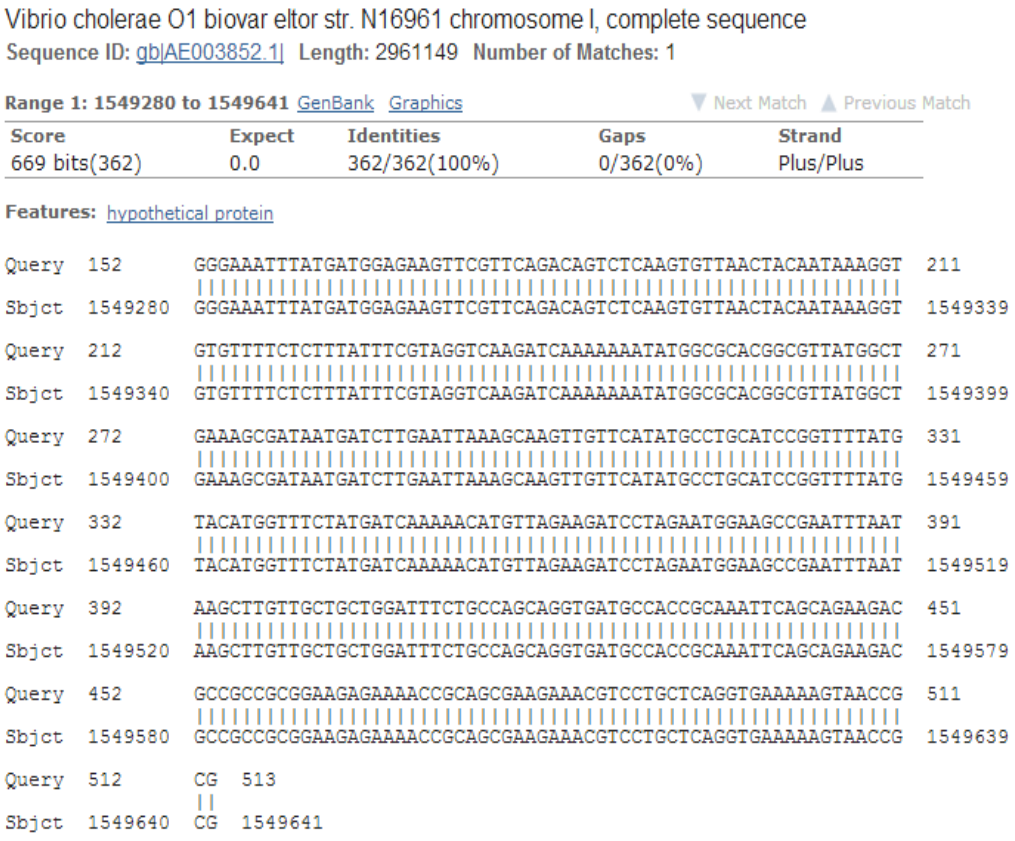


Figure 4.12: BLAST analysis of nucleotide sequence *rtxH* gene obtained from DNA sequencing.

4.4.5.2 *In silico* Analysis of Recombinant Plasmid

In silico analysis shows that the recombinant *rtxH* has a similar peptide sequence to the original *rtxH* sequence. rTEV protease cleavage site and 6X Histidine Tag were also being transcript as shown in Figure 4.13.

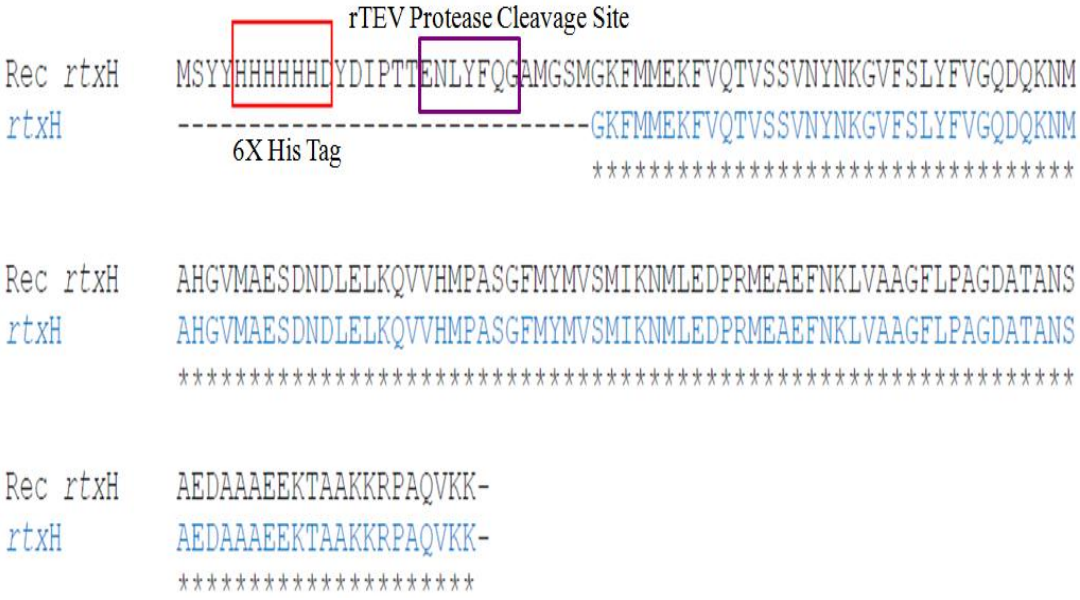


Figure 4.13: Comparison of peptide sequence of recombinant *rtxH* and the original *rtxH* peptide sequence using Vector NTI software.

4.5 Transformation of Recombinant Plasmid into *E. coli* BL21 DE3 Competent Cells

The purified recombinant clone was transformed into *E. coli* BL21 DE3 competent cells. Due to high concentration of the plasmid, 0.5 µL was used for transformation to obtain single colony. Colonies that possess circular plasmid with ampicillin-resistant gene can grow on the LB-ampicillin agar. No colony could be observed in the negative control plate (Figure 4.14A). There are about 180 colonies could be found on the agar plate (Figure 4.14B).

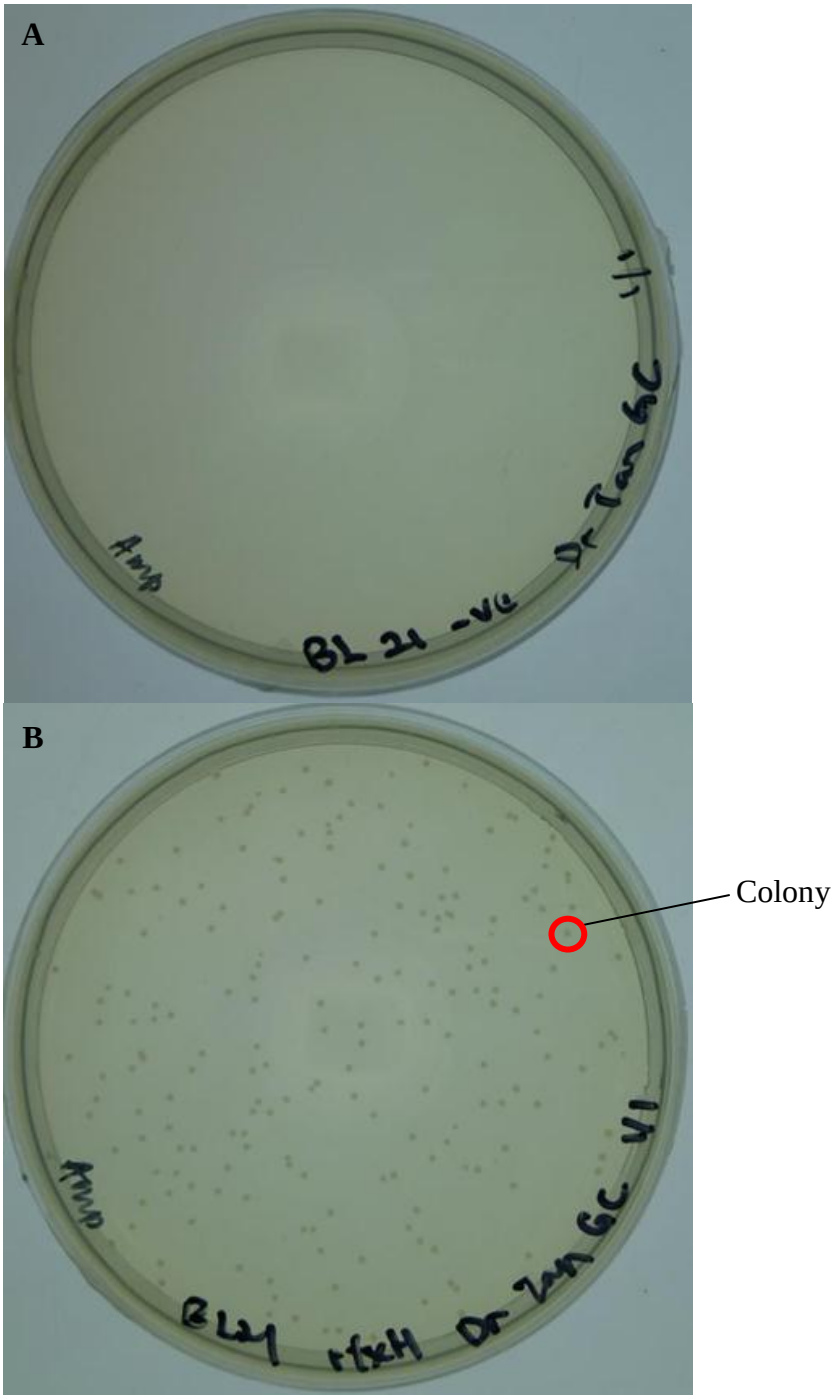


Figure 4.14: (A) Negative control for transformation (B) Formation of colonies after overnight incubation at 37 ℃ using recombinant plasmid.

4.6 Screening of Recombinant Plasmid (*E. coli* BL21 DE3)

Ten colonies were picked to perform colony PCR. Each colony showed a band with ~380 bp in the PCR gel image (Figure 4.15).

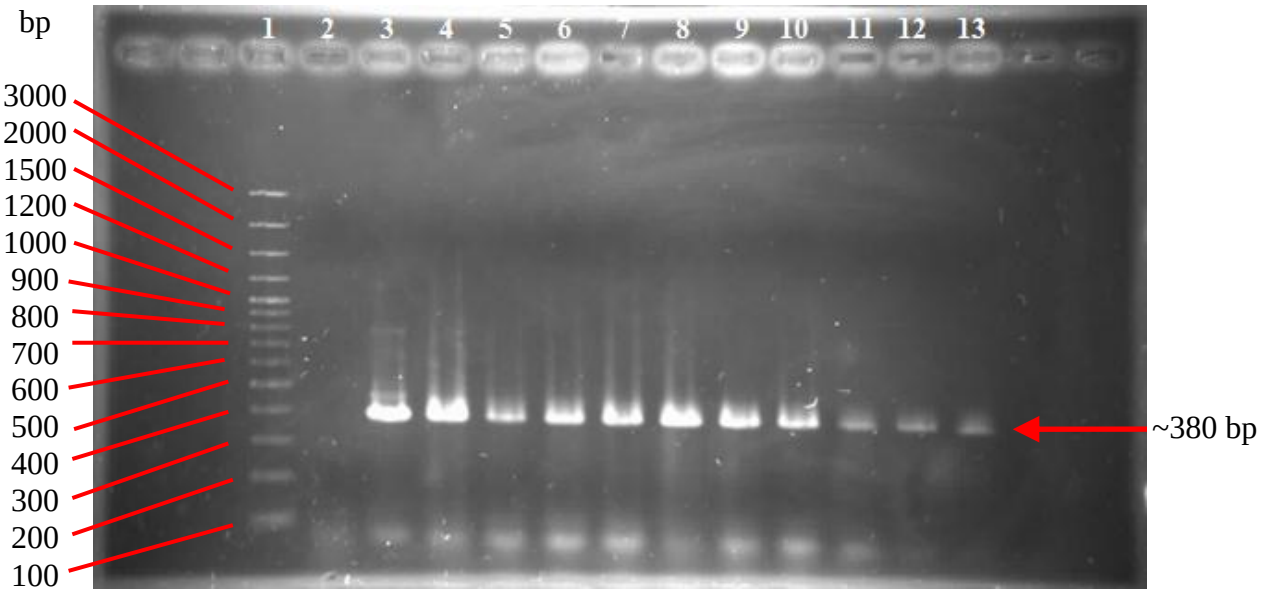


Figure 4.15: PCR screening for transformants of *E. coli* BL21 DE3. Lane 1: 100 bp DNA ladder; Lane 2: Negative control; Lane 3: Positive control; Lane 4 – 13: Colony 1-10.

CHAPTER 5

DISCUSSION

5.1 Culture and Verification of *V. cholerae* O139

Vibrios are usually isolated and cultured using TCBS agar, which is the most commonly used medium for the selection and differentiation of *Vibrio* species (Harwood et al., 2004). Bacteria colonies appeared as yellow or green are depends on their ability to ferment sucrose. Sucrose positive bacteria such as *V. cholerae* are able to ferment sucrose and thus produce yellow colonies on the TCBS agar (Kobayashi et al., 1963). *V. cholerae* glycerol stock was streaked on TCBS agar. Based on Figure 4.1, the yellow colonies formed on the agar plate confirmed the strain *V. cholerae*. The most important component in TCBS agar is the selective agents which include sodium citrate, sodium thiosulfate and ox bile. These agents provide an alkaline pH to suppress the Gram-positive organisms and inhibit coliforms. Alkaline pH also enhances the growth of vibrios, because these organisms are sensitive to acid environments. Bromothymol blue and thymol blue are pH indicators in the agar (Rivera-Posada et al., 2011).

5.2 PCR Amplification of *rtxH* Gene

Taq DNA polymerase is the most widely used enzyme in PCR (Hamilton et al., 2001). However, the amplicons produced tend to have unexpected mutations due

to its high error rate (12.13×10^{-6}) (Lee et al., 2009). It also lacks the ability to synthesize DNA fragments longer than 4 kb (Hamilton et al., 2001). *Pfu* DNA polymerase was used in this PCR amplification step rather than *Taq* because it possesses high proofreading activity and able to reduce the misincorporations produced in PCR (Lee et al., 2009). *Pfu* enzyme has a lower error rate compared to *Taq* DNA polymerase (2.18×10^{-6}) (Lee et al., 2009). *Pfu* polymerases are known to have associated 3' → 5' exonuclease activity. They can remove misincorporated nucleotides up to five nucleotides after the misincorporation (Hamilton et al., 2001; Jacewicz et al., 2007). The 3' → 5' exonuclease activity confers proofreading capability and enhances PCR fidelity (Kahler and Antranikian 2000), which is an intrinsic property of DNA polymerase and represents the frequency of correct nucleotide insertion per incorrect insertion (Pavlov et al., 2004). The proofreading activity of *Pfu* is important in the cloning part to produce a gene which has 100% similarity with the original template. In Figure 4.7, the agarose gel image shows a band size of approximately 380 bp. This DNA size is matched with the expected *rtxH* gene size and the extra 20 bp of linkers which are the additional nucleotides added to the 5'end of the forward and reverse primers.

Thick band could be observed in the gel image indicates that the PCR reaction is highly effective. According to Figure 4.2 and Figure 4.3, the designed primers can bind specifically to the gene interest because they have 100% similarity to the *V. choleare* O1 biovar El Tor strain N16961 chromosome. Besides, the results

revealed that both primer sequences are encoded for hypothetical protein, which is referred to *rtxH* gene in this study. Other than that, these BLAST results also indicate the primers are specifically bind to the *rtxH* gene sequence but not to other region in the *V. cholerae* genome. In Appendix A, B and C show the homo-dimer and hetero-dimer analyses of both forward and reverse primers. The maximum ΔG in the analysis denotes the energy needed to break apart a fully complementary set of oligos. Ideally the ΔG values for both primers should be more positive than -9 kcal/mole (Integrated DNA Technologies, Inc. 2013a). In hetero-dimer analysis, the forward and reverse primers have more positive value, which is -5.46 kcal/mole. On the other hand, in homo-dimer analysis both primers show a more negative value, which are -11.52 and -22.78 kcal/mole respectively. However it will not necessary cause problems if the ΔG values are more negative than this (Integrated DNA Technologies, Inc. 2013a). Based on the agarose gel image (Figure 4.5), formation of primer dimers does not affect the result. Gradient PCR was performed prior to the amplification step to optimize the annealing temperature of *rtxH* gene. Agarose gel image in Figure 4.4 shows that each annealing temperature has a clear band. This indicates that the annealing temperature range is optimum for the PCR amplification step. Hence, any temperature in this range could be used. In this study, the annealing temperature of 58℃ is chosen for all PCR. According to Qu et al. (2012), the annealing temperature of a successful PCR is always more than 55℃. Higher annealing temperature will improve the specificity of the primers and to prevent possible non-specific binding (Qu et al., 2012).

To facilitate the ligation of *rtxH* gene to the vector, the primer was slightly modified as shown in Table 3.3 to carry out directional cloning. The forward primer was added with *Bam*HI recognition site at 5'end (G↓GATCC) while *Not*I restriction enzyme recognition site (GC↓GGCCGC) was added at the 5'end of reverse primer. Hence the amplified PCR products would have these extended enzyme cutting sites with *Bam*HI at upstream and *Not*I at downstream of *rtxH* gene. Two additional nucleotides, AA were inserted adjacent to the *Bam*HI and *Not*I recognition sites to facilitate the binding of REs onto respective recognition site and this will also increase the efficiencies of cleavage by the REs (Elghanam et al., 2012).

5.3 RE Digestion of *rtxH* Gene and pHIS P2 Plasmid

In Figure 4.6, the gel image showed that the undigested plasmid contained multiple bands. The migration of DNA molecules can be affected by the conformation of DNA molecules, molecular size of DNA molecule and applied voltage (Vennison 2009). There were two major conformation of plasmid DNA, which are supercoiled and relaxed circular. Supercoiled molecule is a compact structure of coiled DNA helix that wound around histone-like proteins. Supercoiled plasmid DNA moves most easily through the agarose matrix due to its compact structure (Micklos et al., 2003). Therefore, the fastest-moving band is assumed to be supercoiled. On the other hand, relaxed circle is the slowest-migrating form of plasmid DNA. This open circular structure is formed when the

enzyme topoisomerase I introduces a nick into one strand of the DNA helix during DNA replication. Nicking results in the release the torsional strand that holds the molecule in a supercoiled (Micklos et al., 2003). After the double digestion, plasmid DNA shows only one band and the molecular weight is even smaller than the two conformations of plasmid. This is because there are 39 base pairs of nucleotides in between *Bam*HI and *Not*I cut site. When RE digestion was performed, these nucleotides were being removed and produced a linearized plasmid with smaller band size. At the same time, *rtxH* gene also underwent RE digestion using *Bam*HI and *Not*I. This is because the PCR products generated by *Pfu* enzyme are blunt-ended (Cáceres 2011). RE digestion was required to produce cohesive ends in PCR products to facilitate the directional cloning process. The gel image shows that the band size of the digested *rtxH* gene remains in the same position as the undigested *rtxH* gene. This is due to the minor difference occurred in the gene which is too little to be visualized. Around 10 bp of nucleotides were removed by the restriction enzymes to produce a 5' overhang at both ends of the gene.

5.4 Dephosphorylation of pHIS P2 Plasmid

Dephosphorylation of vector was achieved prior to ligation by using shrimp alkaline phosphatase. Dephosphorylation step is important to remove the additional phosphate group at the 5' overhang of the linearized vector. Besides, it can also improve the cloning efficiency (Shih et al., 2005). Vector has to be

dephosphorylated to prevent a high non-recombinant background (Aslanidis and de Jong 1990) and to prevent self-ligation of the plasmid (Pal et al., 2011). A buffer that is compatible with the restriction endonuclease digestion and the alkaline phosphatase dephosphorylation was utilized to optimize enzymatic processing of vector DNA (Costa and Weiner 1994) and to prevent additional steps of clean up that might be lead to losses of precious DNA.

5.5 Transformation of Ligation Mixture into *E. coli* TOP10 Competent Cells

Recombinant vectors as shown in Figure 4.7 were transformed into *E. coli* TOP10 competent cells. The entired surface of positive control plate is covered with bacteria but single colonies still can be discerned (Appendix E). This indicates a high transformation efficacy. However, it gives a poor result when come to ligated DNA. Only 24 colonies could be observed in the LB-ampicillin plate (Appendix F). Ligated DNA is composed of relaxed circular plasmid and linear plasmid, and these forms produce 5-100 times fewer transformants than an equivalent mass of intact supercoiled plasmid (Micklos et al., 2003). Successive ligation step also depends on the nature of the termini created on fragmented genomic DNA (Zane et al., 2002). The RE site of *rtxH* gene and vector may not be fully digested and this could result in low production of positive clones. The incomplete digestion is probably due to the short incubation time during the RE digestion step. After that, both insert and vector should undergo gel electrophoresis and purification by using gel extraction method. Through this

method, left over fragments and enzymes can be removed thoroughly thus improving ligation efficacy. Optimum experimental conditions should be established in order to maximize efficiency and to minimize unwanted concatamerization in linearized plasmid and amplified PCR products (Sambrook and Russell 2001). The factors that might affect transformation efficiency include the competency of *E. coli* cells, how well the cells are suspended in calcium chloride, the temperature and duration of heat shock method, amount of plasmid DNA used, spreading technique and the length of recovery period (Micklos et al., 2003).

The competence of a microorganism is dependent on its ability to uptake recombinant DNA and survives the introduction of foreign DNA into the cell (Life Technologies Corporation 2013). For *E. coli* the optimal stage of the growth cycle occurs at a cell concentration corresponding to an optical density of 0.6 at a wavelength of 600 nm. When an OD_{600} of a value 0.6 or 0.7 was reached, *E. coli* cells were stored in ice to slow down the growth. It was found that the stage of the growth cycle at which cells are harvested is critical to success. There is a narrow window in the cell concentration at harvest. Above or below this window, the transformation efficacy drops rapidly (Brambach et al., 2013).

Positive control and negative controls play a significant role in transformation step. *E. coli* cells without plasmid were used as negative control there is no colony formed in the LB-ampicillin plate. Negative control is important in determining

the contamination in competent cells. Positive control contains intact circular pHIS P2 plasmid which the plasmid holds ampicillin-resistance gene. Hence, the colonies are able to grow on the LB-ampicillin plate. Positive control is used to verify the efficacy of the competent cells.

Ampicillin is a bacteriostatic agent which blocks the synthesis of peptidoglycan layer in *E. coli* cells. Ampicillin is chosen but not other antibiotics because the plasmid used in this study, pHIS P2, contains ampicillin resistant gene (*AmpR*) as selective marker. This gene act as a selective marker where *E. coli* cells do not possess *AmpR* gene could not survive in the LB-ampicillin plate. *AmpR* gene codes for a protein known as beta-lactamase which confers resistance to ampicillin (Fuat 2006). Beta-lactamase is an enzyme that degrades beta-lactam antibiotics (Fernández et al., 2012) by cleaving a specific bond in the beta-lactam ring.

Ligation mixture was transformed into *E. coli* TOP10 competent cells at first because these cells allow stable replication of high-copy number plasmids. This give rise to a higher chance of successful transformation as the recombinant plasmids produced are increase. However, TOP10 cells do not contain the gene encoding for T7 RNA polymerase and thus are not sensitive to isopropyl β-D-1-thiogalactopyranoside (IPTG) induction. Eventually it generates an "induction free" system (Palacios et al., 2011).

5.6 Screening and Verification of Recombinant Plasmids

Colony PCR was used to select positive clones with the correct insert size into pHIS P2 plasmid. As shown in Figure 4.8, 10 out of 24 colonies were picked for screening. There are three positive clones in the 10 colonies. It is probably due to the partially digested *rtxH* gene and vector and also incomplete dephosphorylation of pHIS P2 plasmid.

At the bottom of the gel, there is the presence of primer dimers. Primer dimers are formed by the left over primers when the thermal cycler ramping to the initial denaturation temperature during PCR reaction. Under these conditions, the primers, which are in a large molar excess over target DNA, can bind nonspecifically to other primer molecules. Primer dimers may undergo amplified oligomerization during PCR to create a complex mixture of primer artifacts (Chou et al., 1992). This primer dimer formation may be due to the linker sequence added to the primers (*Bam*HI and *Not*I recognition sites). However, the primer dimer formation did not affect the results as the primer dimer analysis and optimization of PCR condition was done in the previous step. Annealing temperature had been optimized using gradient PCR to maximize amplification efficiency without non-specific products and primer dimer formation (Kalender et al., 2011).

5.7 Plasmid Purification

Purpose of plasmid purification is to remove all the enzymes and unwanted genomic fragments (Integrated DNA Technologies, Inc. 2013b). High molecular weight of plasmid DNA was trapped at the front edge of the gel. Lane 2 in Figure 4.9 shows the pHIS P2 plasmid with two different conformations. The recombinant plasmid in Lane 3 also shows two bands. The supercoiled form of the recombinant plasmid has a slightly higher molecular weight compared to the pHIS vector alone. Through the visual inspection of band size in agarose gel, preliminary deduction can be made where the insert is successfully cloned into the pHIS vector. At the same time, the concentration and purity of recombinant plasmid was measured using nanospectrophotometer. A260/A280 ratio must range in between 1.7–2.0. If A260/A280 ratio is lower than 1.7, it indicates the presence of proteins and organic solvents. If A260/A280 ratio is greater than 2.0, it indicates the presence of RNA content in the sample (Praveen et al., 2013). In Table 4.1, the absorbance ratio of A260/A280 is 1.902, which is under the acceptable range.

5.8 PCR Verification and RE Analysis of Recombinant Plasmid

The agarose gel image in Figure 4.10 shows a band size which is similar to positive control. Hence it is assumed that the gene insert is successfully cloned into the vector. In the RE analysis, double digestion were performed using *Bam*HI and *Not*I. The recombinant plasmid also cut with *Xba*I at the same time.

Restriction enzyme digestion was carried out to determine the presence of insert in the recombinant plasmid and prior estimation has made to predict restriction fragment sizes (Costa et al., 1994). The recombinant plasmid produced two fragments after cut with *Xba*I, which are the linearized plasmid backbone and another smaller DNA fragment (Figure 4.13). According to the plasmid map, pHIS vector has two *Xba*I recognition site (T↓CTAGA). The gene insert of approximately 380 bp is laid between the restriction sites. Therefore, the 512 bp DNA that shown in the gel image is the correct band size and it matched the prediction. In Figure 4.11, the double digestion of recombinant plasmid using *Bam*HI (G↓GATCC) and *Not*I (GC↓GGCCGC) also yielded two fragments, the linearized plasmid and the gene insert. This indicates that the gene is successfully cloned into the vector. Both results support each other with the band size of 380-bp and 512 bp respectively.

5.9 DNA Sequencing

DNA sequencing is important to confirm the identity of the gene insert. DNA sequencing was run using T7 promoter-F primer. The gene insert sequence was BLAST in GenBank. Sequencing result in Figure 4.12 shows 100% similarity with the bits-score 669 and expected value 0.0. The bit score gives an indication of how good the alignment is for both query and subject sequences; the higher the score, the better the alignment. This score is calculated from a formula that takes into account the alignment of similar or identical residues, as well as any gaps

introduced to align the sequences. The E-value denotes the statistical significance of a given pairwise alignment and reflects the size of the database and the scoring system used. The lower the E-value, the more significant the hit. In this study, the high value of bit score and E-value of 0.0 indicate that the alignment is very good and the query sequence is totally matched with the subject sequence (Madden 2002). The perfect match of the *rtxH* gene in the recombinant plasmid is relied on the utilization of *Pfu* DNA polymerase in the PCR amplification step. High fidelity rate of this enzyme contributes to the precise amplification hence no mutation can be detected in the recombinant plasmid (Kahler and Antranikian 2000). Other than that, based on the sequencing result, the subject sequence is *V. cholerae* O1 biovar strain N16961 chromosome and the query sequence is *rtxH* gene in the *V. cholerae* O139. 100% similarity of recombinant *rtxH* gene to the subject sequence suggesting that the *V. cholerae* O1 and O139 strains have the identical *rtxH* gene sequence. However, this gene is absent in the *V. cholerae* classical strain because they have a deletion in DNA sequences that overlap the *rtxA*, *rtxC*, and *rtxB* genes (Lin et al., 1999). According to Dziejman et al. (2002), they further confirmed the absence of the *rtxC* gene and the adjoining gene VC1449 in classical strain through array analysis.

5. 10 *In silico* Analysis of Recombinant Plasmid

In silico analysis of recombinant transcription was performed to analyse the recombinant *rtxH* with original sequence of *rtxH*. Analysis shows that the *rtxH*

gene cloned into pHIS P2 expression vector was in-frame (Figure 4.13). It is important to perform such analysis to ensure the expression of correct recombinant protein. Deletion or insertion of nucleotides with a number other than three often leads to a frameshift in translation (Zhao et al., 2011). Frameshift mutation may result in a truncated protein product with abnormal function. In addition, single nucleotide change of the mutation can also lead to the replacement of an amino acid or create a stop codon which will terminate the translation (Béroud and Soussi 1996). Therefore, different structure of proteins could be obtained and this will affect the following step in the research.

5.11 Transformation of Recombinant Plasmid into *E. coli* BL21 DE3 Competent Cells

Once the DNA sequence is correct, the recombinant plasmid was incubated overnight and purified using plasmid extraction kit. Purified recombinant plasmids were transformed into *E. coli* BL21 DE3 competent cells. This transformation step is repeated but using different strain of *E. coli* because BL21 DE3 cells contain the gene encoding for T7 RNA polymerase. This gene is under the control of lacUV5 promoter and allows the expression of T7 RNA polymerase to be induced by IPTG. IPTG binds to the *lac* repressor in *E. coli* and thereby induce protein expression of the gene. The BL21 strains yield an "on-demand" system as these strains need induction to develop a potential message (Palacios et al., 2011). In the transformation process, 0.5 µL of purified recombinant plasmid was used due to the high concentration obtained during the extraction process.

Such low amount of recombinant plasmid was able to produce about 150 colonies as shown in Figure 4.14B. This is because circular plasmid is easier to be transformed as compared to ligation mixture in the previous step. Increase the volume of recombinant plasmids in this step will lead to the formation of bacteria 'lawn' and single colony could not be discerned (Micklos et al., 2003). The circular recombinant plasmids act as a positive control in the transformation. No bacteria colony can be observed in negative control (Figure 4.14A) indicates that there is no contamination happened during the preparation of competent cells.

5.12 Future Research Work

After the construction of recombinant plasmid, the recombinant plasmid can be induced by IPTG to undergo protein expression. The protein can be analyzed and detected using SDS-PAGE. Subsequent to SDS-PAGE, the protein can be purified using His-tag purification kit. This purified protein is very useful for further research as the 3D structure and activity of this hypothetical protein can be investigated. Structural study of protein is important because it is a preliminary step to predict the function of the protein. Structure of protein can be determined via various techniques such as X-ray crystallography, nuclear magnetic resonance (NMR) spectroscopy and electron microscopy.

Other than that, antibody which specifically binds to the recombinant *rtxH* protein can be derived. The antibody will be used during Western blotting to detect the

protein. Western blot is more accurate than SDS-PAGE in identification of a particular protein. It helps investigators to determine the molecular weight and to measure relative amounts of the protein present in the samples.

CHAPTER 6

CONCLUSION

In this study, the *rtxH* gene was successfully PCR amplified from *V. cholerae* O139. The purified PCR amplified, *rtxH* gene was successfully cloned into the expression vector. Recombinant plasmids containing *rtxH* gene was sequenced and analyzed. DNA sequencing gave a satisfied result where the cloned *rtxH* gene shows 100% similarity to the nucleotide sequence in GenBank. There are absence of frameshift mutation and point mutation in the cloned gene. Hence, recombinant constructs containing *rtxH* gene were successfully created. The plasmid was transformed into *E. coli* BL21 DE3 and ready for the expression of recombinant *rtxH* protein.

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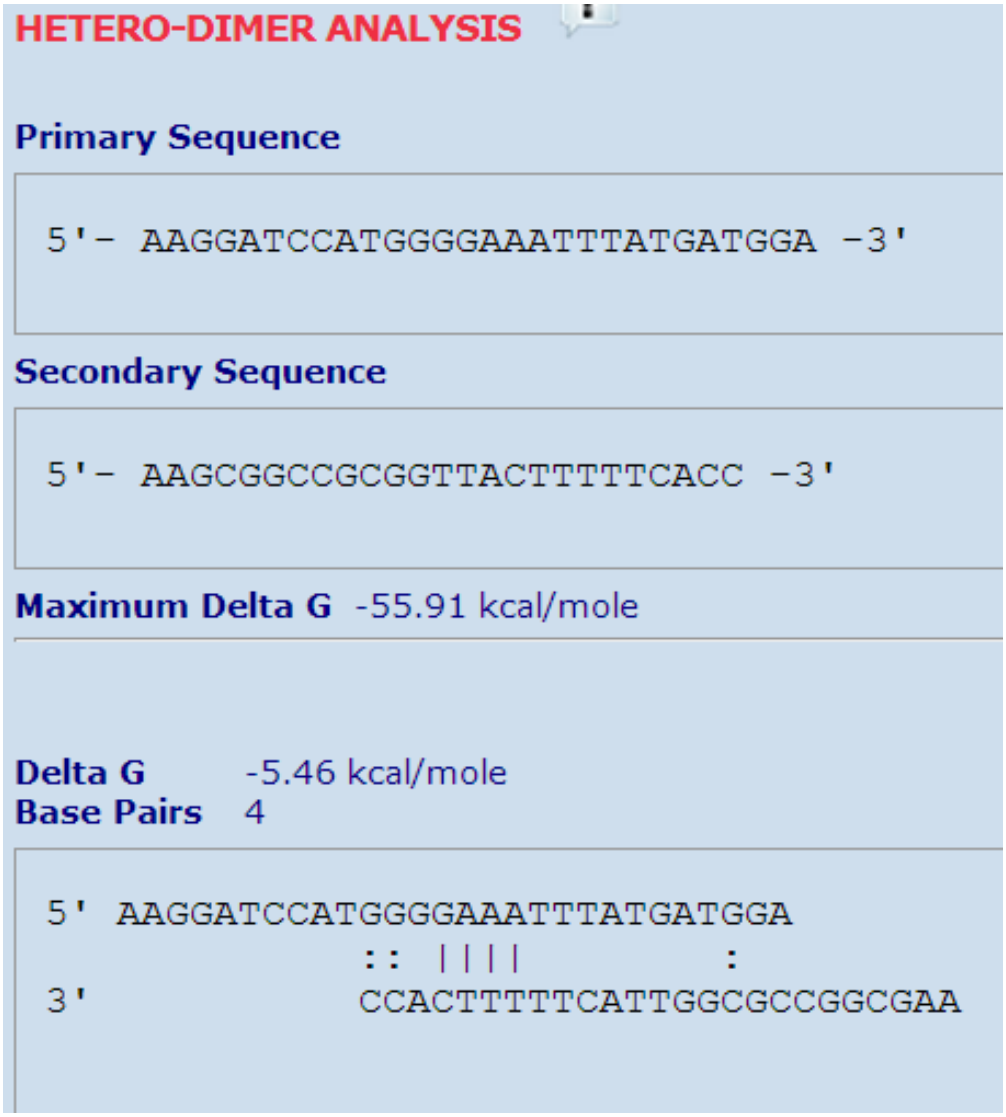
APPENDICES

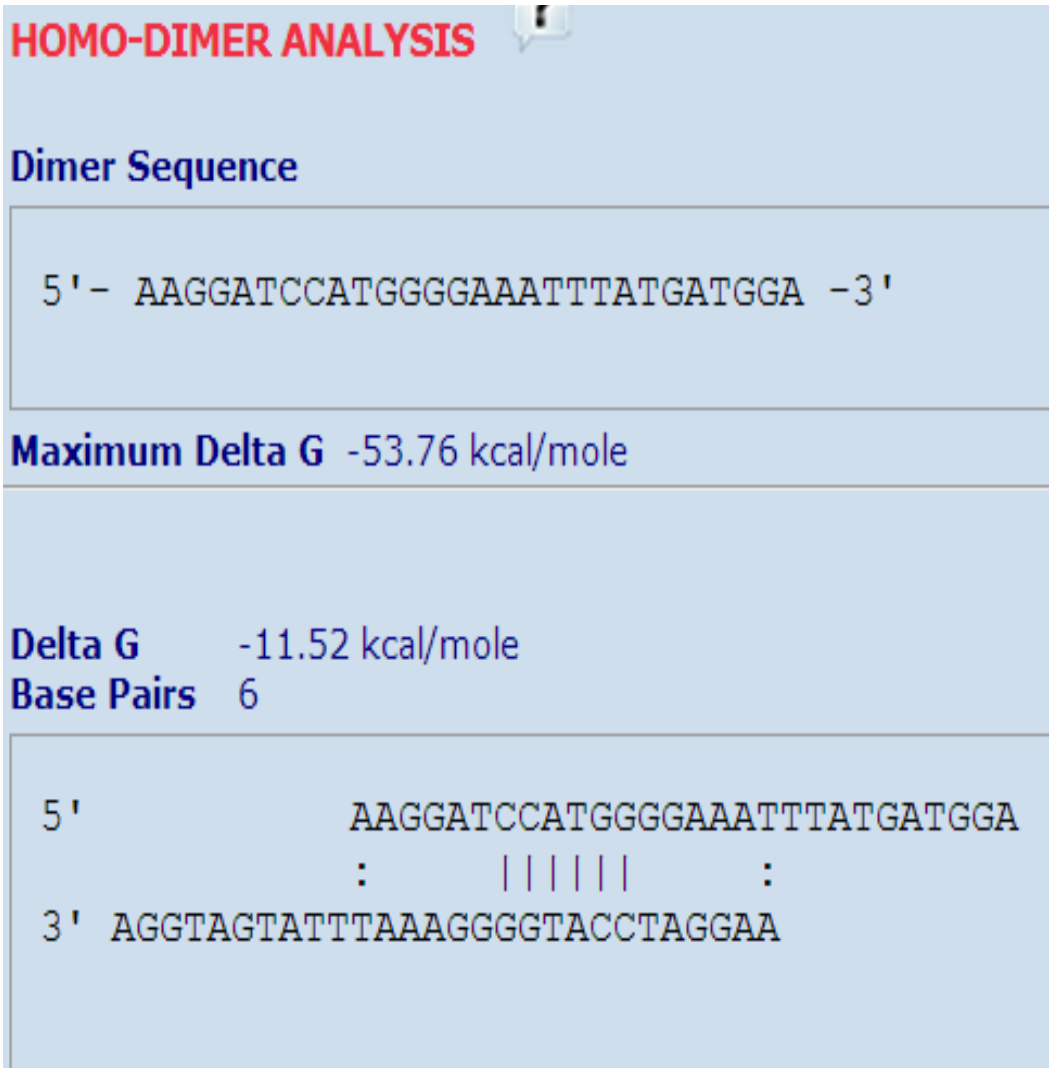
Appendix A

Hetero-dimer analysis of VCrtxH-F and VCrtxH-R



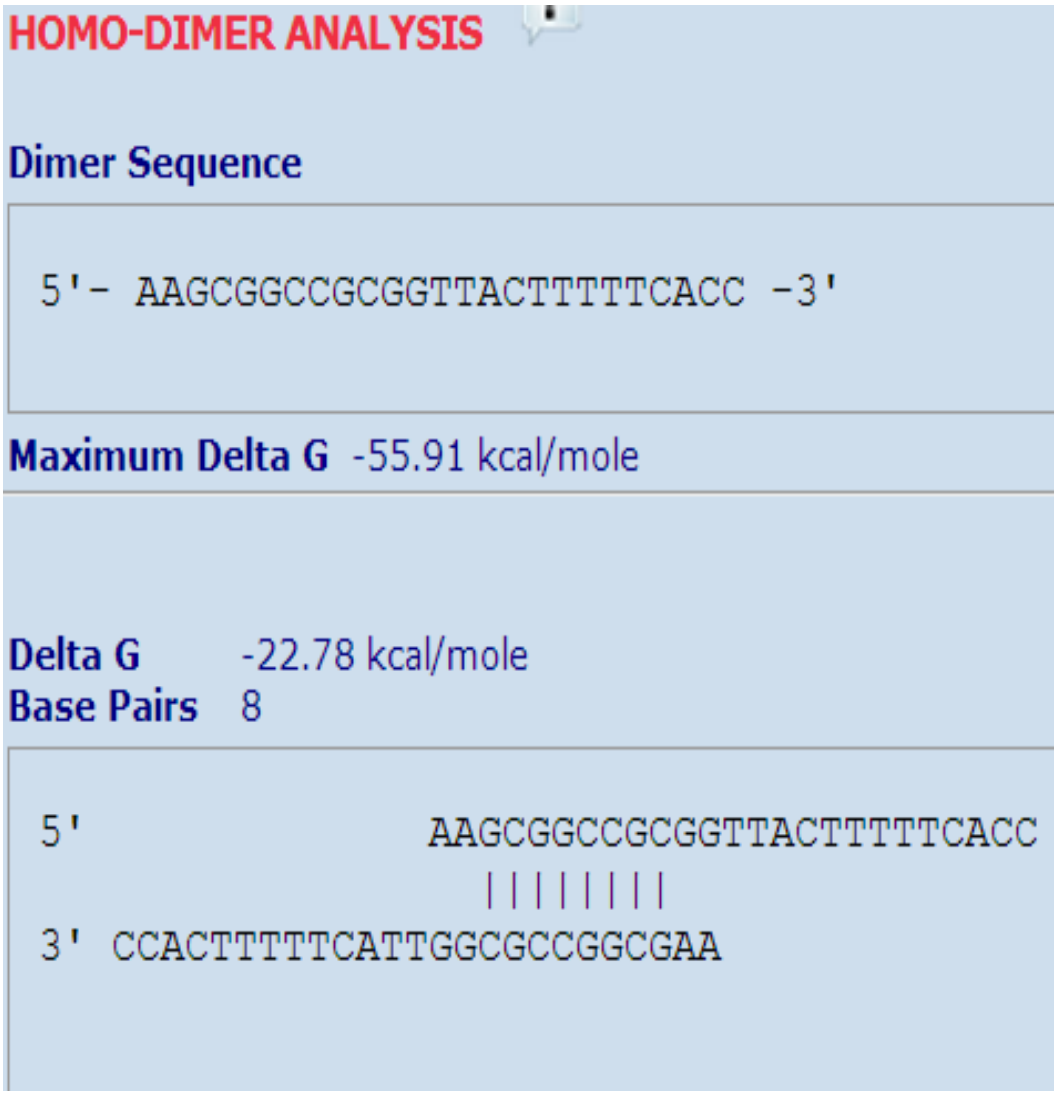
Appendix B

Homo-dimer analysis of VCrtxH-F



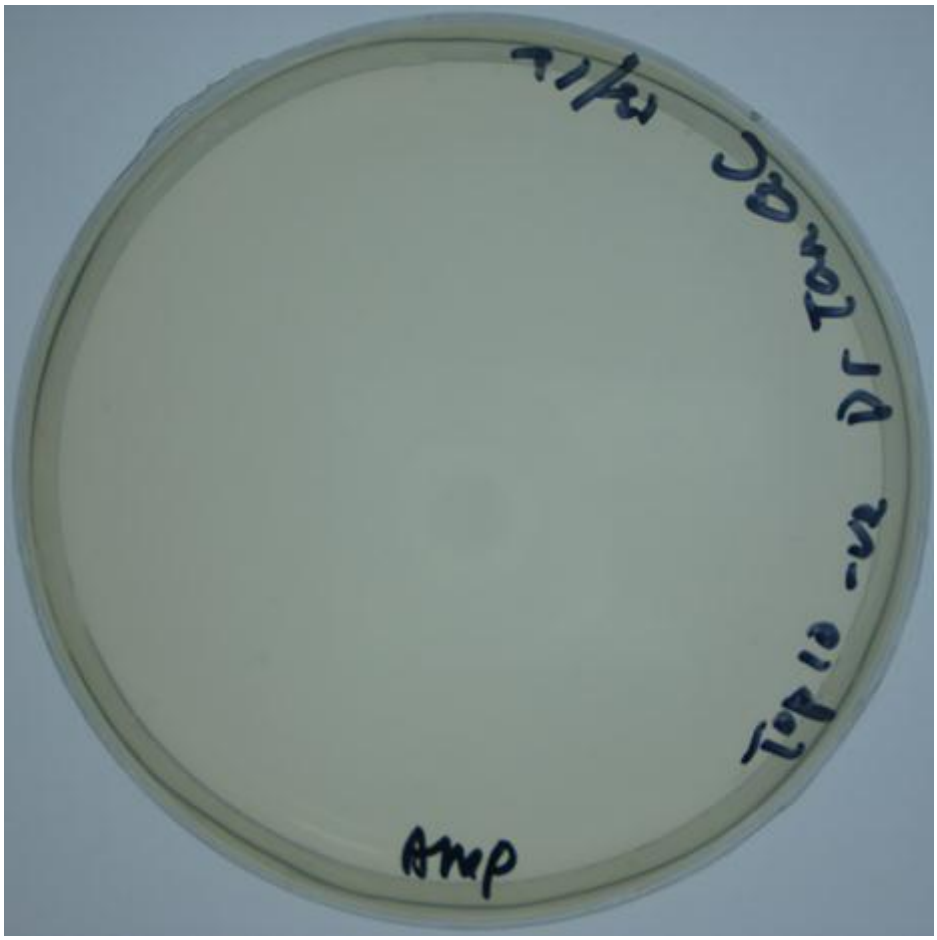
Appendix C

Homo-dimer analysis of VCrtxH-R



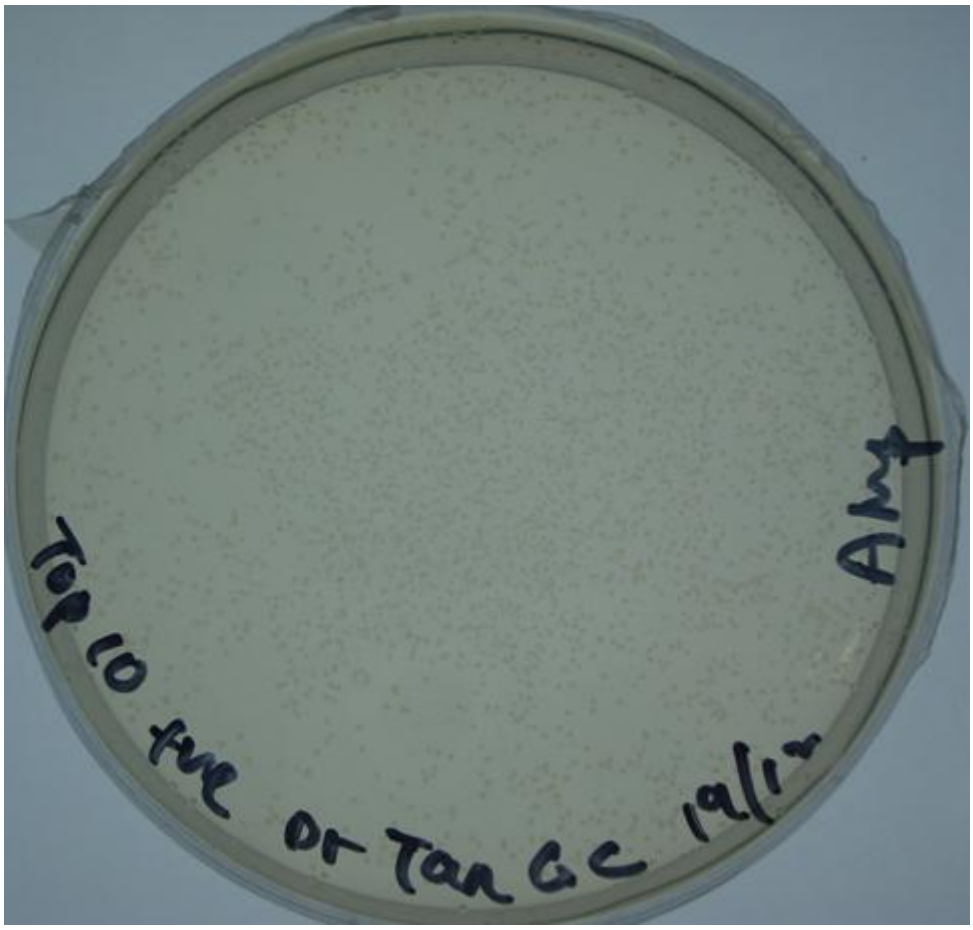
Appendix D

Negative control for transformation (*E. coli* TOP10 competent cells)



Appendix E

Positive control for transformation (*E. coli* TOP10 competent cells)



Appendix F

Formation of colonies after overnight incubation at 37 ℃ using ligation mixture (*E. coli* TOP10 competent cells)