DEVELOPMENT OF A LOOP-MEDIATED ISOTHERMAL AMPLIFICATION ASSAY FOR SENSITIVE AND RAPID DETECTION OF TOXIGENIC *Vibrio cholerae*

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by

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DEDICATIONS

This dissertation is dedicated to my precious husband, my late father, my beloved mum, dear siblings, lecturers and colleagues for their encouragement, support and endless patience.

They guided the basic principle of life;

There is no such thing as 'busy'.
If you truly prioritise your work and do it accordingly,
you will make time for it.
You can do it.

Hard-works will be rewarded,
the rest is to believe yourself and have faith in Allah Azza wa Jalla.

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LIST OF SYMBOLS AND ABBREVIATIONS

%	Percentage
μl	Microliter
°C	Degree celcius
BLAST	Basic Local Alignment Search Tool
cAMP	cyclic adenosine monophosphate
DNA	Deoxyribonucleic acid
Bst	*Bacillus stearothermophilus*
et al.	et alii
dNTPs	nucleotide triphosphates containing deoxyribose
mM	milimolar
pmol	picomol
CFU	colony forming unit
kb	kilobase
WHO	World Health Organization
kDa	kiloDalton
GM_1	monosialotetrahexosylganglioside
ng	nanogram
pg	picogram
fg	femtogram
ag	attogram
g	gravity
Y-1	Y receptors on mouse adrenocortical
ELISA	Enzyme-Linked Immunosorbent Assay
RPLA	Reverse passive latex agglutination
CT	cholera toxin
Taq	*Thermus aquaticus*
FIP	Forward inner primer
BIP	Backward inner primer
LB	Backward loop primer
LF	Forward loop primer

F3	Forward outer primer
B3	Backward outer primer
EDTA	Ethylenediaminetetraacetic acid
FASTA	Text-based format for representing either nucleotide sequences or peptide sequences, in which nucleotides or amino acids are represented using single-letter codes
GC-	guanine cytosine
AT-	adenosine thymine
mg	miligram
μg	microgram
HPLC	High Performance Liquid Chromatography
bp	base pairs
UV	Ultraviolet
$MgSO_4$	Magnesium sulphate
M	Molar
U	Unit

PEMBANGUNAN ASAI GELUNG-DIMEDIASI AMPLIFIKASI ISOTHERMAL BAGI PENGESANAN YANG PANTAS DAN SENSITIF TERHADAP *Vibrio cholerae* TOKSIGENIK

ASBTRAK

Taun dikenali sebagai masalah kesihatan awam yang ketara di seluruh dunia yang disebabkan oleh *Vibrio cholerae* toksigenik. Pada masa ini, rutin diagnosis bagi taun melibatkan bakteria kultur dan ujian biokimia tetapi kaedah konvensional ini memakan masa, kurang cekap, sukar dan kakitangan yang mahir diperlukan untuk melaksanakan tugas-tugas tersebut. Sehingga kini, tiada kit diagnostik molekul yang pantas di mana ianya boleh didapati secara komersial dan boleh digunakan di makmal dan kerja lapangan. Oleh itu, kajian ini telah memberi tumpuan kepada membangunkan asai multipleks LAMP untuk diagnosis taun yang mudah, cepat dan sangat sensitif. Primers pada asalnya direka untuk mengesan gen toksigenik, *ctxA* yang hanya wujud dalam strain toksigenik *V. cholerae* dan satu gen kawalan dalaman (SSP2). Spesifikasi primers telah disahkan oleh analisis carian BLAST dan diuji pada strain bakteria lain bagi memastikan bahawa gen sasaran adalah sangat spesifik. Kawalan dalaman telah digunakan untuk pengesahan pada kebolehpercayaan asai LAMP bagi mengelakkan keputusan negatif palsu. Asai multipleks LAMP stabil suhu terdiri daripada format kering dan basah. Bagi format kering, komponennya adalah 0.6 mM campuran dNTPs, 1.6 pmol/ ul setiap FIP dan BIP bagi kedua-dua sasaran dan kawalan dalaman, 0.8 pmol/ ul LB dan LF untuk kedua-dua sasaran dan kawalan dalaman, 0.2 pmol/ ul setiap F3 dan B3 untuk kedua-dua sasaran dan kawalan dalaman, 4.27 U / ul daripada *Bst* 2.0 WarmStart Polymerase DNA dan

kombinasi penstabil enzim dipilih (8% trehalose + 0.25 mg/ ml BSA + 6% FICOLL) dengan 0.05% Orange G pewarna gel. Format basah terdiri daripada 1X LAMP penguatan penampan, 6 mM $MgSO_4$, dan 0.4 M betaine dan disimpan sebagai penampan akueus. 13.3 pg / ul daripada SSP2 plasmid DNA telah disepadukan dalam setiap larian yang berfungsi sebagai kawalan dalaman. Multipleks LAMP stabil suhu telah diuji bagi ujian kestabilan dipercepatkan pada 25 dan 37 °C selama 1 bulan. Campuran multipleks LAMP stabil suhu disimpan pada 25 dan 37 °C adalah stabil sehingga 1 bulan. Walau bagaimanapun, selepas pengiraan dipercepatkan ujian kestabilan haba, ia mampu bertahan pada suhu bilik selama 3.14 bulan dalam hubungan dengan suhu simpanan 37 °C. Keputusan multipleks LAMP stabil suhu yang diperolehi menunjukkan 100% spesifik terhadap 73 strain *V. cholerae* sahaja dan bukan kepada 5 spesies *Vibrio* dan 37 strain bukan *Vibrio*. Sensitiviti asai multipleks LAMP stabil suhu yang dikesan di peringkat genomik adalah 100 fg/ ul dan 10^2 CFU/ ml pada peringkat bakteria. Pembangunan asai multipleks LAMP stabil suhu adalah pantas, sangat sensitif dan spesifik untuk mengesan *V. cholerae* yang toksigenik. Asai ini mempunyai potensi untuk mengawal wabak penyakit berjangkit dan secara tidak langsung membantu dalam mengurangkan morbiditi dan kematian yang disebabkan oleh taun.

DEVELOPMENT OF A LOOP-MEDIATED ISOTHERMAL AMPLIFICATION ASSAY FOR SENSITIVE AND RAPID DETECTION OF TOXIGENIC *Vibrio cholerae*

ABSTRACT

Cholera is known as significant public health problem worldwide which is caused by toxigenic *Vibrio cholerae*. Currently, the routine cholera diagnosis involves bacterial culture and biochemical tests but conventional methods are time consuming, inefficient, laborious and skilled personnel is required to perform the tasks. Until now, no rapid molecular diagnostic kits commercially accessible that can be used in laboratory and field settings. Consequently, this study was focused on developing a multiplex LAMP assay for detection of cholera that is simple, fast and highly sensitive. The designed primers were constructed for the detection of toxigenic gene, *ctxA* that presents only in toxigenic strains of *V. cholerae* and one internal control (SSP2). BLAST search analyses were used to confirm the primers specificity and the same primers were also tested on other non-*Vibrio* bacterial strains to ensure that the target gene is highly specific. The reliability of LAMP assay to rule out false negative result was validated with the inclusion of an internal control. The optimised thermostabilised multiplex LAMP assay consists of dry and wet format. For dry format, the components were 0.6 mM dNTPs mixture, 1.6 pmol/ μl of each FIP and BIP for both target and internal control, 0.8 pmol/ μl of LB and LF for both target and internal control, 0.2 pmol/ μl of each F3 and B3 for both target and internal control, 4.27 U/ μl of *Bst* 2.0 WarmStart DNA polymerase and enzyme stabiliser combinations selected (8% trehalose + 0.25 mg/ ml BSA + 6% FICOLL) with 0.05%

Orange G loading dye. The wet format composed of 1X LAMP amplification buffer, 6 mM $MgSO_4$, and 0.4 M betaine and kept as aqueous buffer. A 13.3 pg/ μl of SSP2 plasmid DNA was integrated in each run which functions as internal control. The thermostabilised multiplex LAMP was tested for accelerated stability test at 25 and 37 °C for 1 month. Results showed that the thermostabilised multiplex LAMP mixture stored at 25 and 37 °C was stable until 1 month. After calculation of accelerated heat stability test, it can last at room temperature for 3.14 months in correlation with 37 °C storage. The multiplex LAMP assay results obtained showed 100% specific with 73 *V. cholerae* strains only and not for 5 *Vibrio* species and 37 non-*Vibrio* strains. The analytical sensitivity of the multiplex LAMP assay at genomic level was 100 fg/ μl whereas at bacterial level, the limit of detection was found to be 10^2 CFU/ ml, respectively. The developed thermostabilised multiplex LAMP assay is fast and distinctly sensitive and specific for the recognition of toxigenic *V. cholerae*. This assay has potential to be implemented during cholera outbreak and consequentially helpful in reducing the morbidity and mortality of cholera cases.

CHAPTER 1

INTRODUCTION

1.1. *Vibrio cholerae*

Vibrio cholerae is classified under genus *Vibrio* and known to be Gram-negative, facultative anaerobic organism, about 1.4 - 2.6 μm long curved-rod bacteria, and motile caused by the presence of a single flagellum. It is oxidase positive (from Oxidase test) and reduces nitrates to nitrites (Nair, 2002). It is known as the causative agent for profuse, severe watery diarrhoea disease to human being called cholera. Even so, not all *V. cholerae* strains are toxigenic to human due to wide diversity of strains with varies of virulence gene contents (Heidelberg et al., 2000). Unlike different *Vibrio* species, *V. cholerae* are also natural free-living inhabitants at riverine, estuarine and coastal water areas.

V. cholerae can be distinguished from other *Vibrio* species through application of biochemical tests, the components of lipopolysaccharide (LPS) and the ability to produce cholera toxin (Finkelstein, 1996). The chemical structure of *V. cholerae* LPS consists of three distinct regions known as lipid A, the core oligosaccharide and the O antigen (Manning et al., 1994). Particularly, the O antigen carries the heat-stable O-serotype specificity and also antigen determinants which is widely used to distinguished between *V. cholerae* strains in serology.

These bacteria grow rapidly in adequate media within 30 minutes period with vigorous aeration with an optimum temperature of 37 ºC. Although they are able to live at pH range of 5.0 - 9.6, they can live optimally at pH 7.6 but die quickly at pH less than 6.0 (Desmarchelier, 1997). Even so, they are able to grow at pH 8.0 - 9.5

which impede most of Gram-negative bacteria. The tolerance of *V. cholerae* to alkaline conditions has been utilised in media preparation for isolation process in the laboratory (Finkelstein, 1996). Naturally, this gives the bacteria to well adapt in intestinal environment by adhering to small bowel and release their toxigenic cholera toxin protein although mostly are killed in the stomach acid barrier.

1.1.1. Taxonomy of *V. cholerae*

The hierarchy categorisation of *V. cholerae* is listed in Table 1.1.

Table 1.1: Taxonomy classification of *V. cholerae* bacterium

Classification order	Details
Kingdom	Bacteria
Phylum	Proteobacteria
Class	Gamma Proteobacteria
Order	Vibrionales
Family	*Vibrionaceae*
Genus	*Vibrio*
Species	*V. cholerae*

1.1.2. Nomenclature and strain categorisation of *V. cholerae*

V. cholerae is a clearly described organism based on the biochemical tests performed and also from DNA homologous research (Baumann et al., 1984) but not homogenous in terms of toxigenic potential. In specific, to distinguish within *Vibrio* species are based on the cholera toxin production, serogroup and possibility of causing infestation outbreak. Previously, the public health declared the *V. cholerae* O1 strains were correlated with epidemic and pandemic cholera while other serogroups were either non-toxigenic or only occasionally causing disease (Chowdhury et al., 2015; Rashed et al., 2012). However, in early 90s, a new serogroup O139 had caused epidemic outbreak in eastern India and Bangladesh. Thus, in present, major cholera outbreaks have been associated with these two major serogroups, O1 and O139 (Raychoudhuri et al., 2009) while other serogroup which caused seasonal and sporadic diarrhoea cases are mentioned as non-O1/ non-O139 serogroups. The identified *V. cholerae* strains are found to be approximately 200 serogroups which based on specific agglutination test against O antigen (Faruque et al., 2003).

In history of cholera disease, *V. cholerae* O1 strain has been correlated with infestation and widespread of cholera due to production of cholera toxin. However, there are certain isolates of *V. cholerae* O1 serogroup did not produce cholera toxin particularly the environmental strains (Kaper et al., 1981; Levine et al., 1982). This serogroup is subcategorised into serotypes mainly Ogawa and Inaba while Hikojima is seldom isolated. The Classical and El Tor biotypes are categorised under *V. cholerae* O1 serogroup and each of serotypes combinations can be formed from these two biotypes. The El Tor biotype is different from Classical biotype by being

haemolytic, positive in the Voges-Proskauer test which detects specific fermentation pathway while negative in Polymyxin B sensitivity test (Bopp et al., 1999; Feng et al., 2008).

Recently, before appearance of new serogroup O139, each of the *V. cholerae* isolates were referred as non-O1 *V. cholerae* due to the absence of agglutination with O1 antisera but with the presence of O139 serogroup, one might categorise the O2 until O138 as non-epidemic *V. cholerae*. The non-O1/ non-O139 strains were determined based on the LPS somatic antigen in which the cholera toxin is not produceable but they are able to produce enterotoxins similar to cholera toxin (Begum et al., 2006). Even so, they were not associated with epidemic and pandemic cholera cases (Morris, 1990). These strains are commonly responsible for sporadic, localised outbreaks (Dutta et al., 2013), and mainly due to ingestion of shellfish and also from variety of extra intestinal disorders inclusive of lesions, ear, sputum, urine and cerebrospinal fluid (Hughes et al., 1987; Morris, 2003).

The first case of O139 serogroup was isolated from Eastern India and Bangladesh where at first it was mentioned as non-O1 *V. cholerae* due to absence of agglutination with O1 antisera. Nonetheless, later investigation has disclosed this bacteria belonged to a new serogroup called O139 and synonym of "Bengal" for the origin of this strain (Bhattacharya et al., 1993; Faruque et al., 2003; Kaper et al., 1995). Researchers emphasised that *V. cholerae* O139 Bengal are acquired from the pandemic of O1, El Tor biotype due to lateral gene transfer between O1 and non-O1 strains (Nair, 2002). It produces similar cholera toxin of O1 strains but does not have similar characteristic of somatic O1 antigen.

1.1.3. Virulence related genes

Cholera toxin genetic elements (CTX) and pathogenicity islands (VPI) are the main virulence genes of *V. cholerae* located in two regions of the chromosome (Faruque et al., 1998; Karaolis et al., 1998; Rajanna et al., 2003). The 4.5 kb core region virulence cassette of toxigenic factors grouped in the CTX genetic elements contains cholera enterotoxin, the main protein secretion that causes severe watery diarrhoea, is encoded by *ctx*AB genes (comprising A and B subunits) which is absent in non-cholera Vibrios (Nair, 2002). Other virulence factors includes zonula occludens toxin (*zot*), core-encoded pilin (*cep*), accessory cholera enterotoxin (*ace*), and a product of unknown function (*orf*U) (Fasano et al., 1991; Trucksis et al., 1993). Whereas VPI comprises virulence genes sets, virulence gene regulators, transposase gene, specific attachment sites flanking each end of the island, and a integrase with homology to a phage integrase gene (Faruque et al., 1998). The virulence proteins available on VPI integrate with toxin-coregulated pili (TCP) and the accessory colonisation factor (ACF) during colonisation in human intestine.

The TCP acts as the receptor of CTX phage that is important for colonisation of *V. cholerae* in small intestine. The expression of cholera toxin and TCP are co-regulated by the regulator proteins for virulence. The site-specific recombination process of excision and insertion of 41.2 kb VPI into the host chromosome involves termini integrase gene and the attachment sites, whereas the transposase gene linking its left and right ends to mediate the circularisation of the excised VPI (Almagro-Moreno et al., 2010; Rajanna et al., 2003). Thus, the VPI can construct a non-replicative circular intermediate product that needs transferring mechanism like conjugative transposons,

generalised transducing phages or uptake by transformation process (Almagro-Moreno et al., 2010).

1.2. Summary of cholera disease

Cholera is an acute diarrhoea, clinical-epidemiologic illness caused by toxigenic *V. cholerae* strains. The severity of the infection is described as profuse and high voluminous passage of stools of rice water characteristic, accompanied by severe dehydration, acidosis, vomiting, hypovolemic shock and death if not treated rapidly (Ghose, 2011). The vehicle of transmission mainly through poor sanitation practice, famine incident, lack of treated and clean water supplies and also a proper sewage system which is commonly occurred in the Third World countries. Since 1961, the *V. cholerae* El Tor biotype caused the seventh pandemic of cholera until today. The new serogroup O139 has been reported to cause major outbreaks in certain countries which has increase the awareness among researchers that this might be the causative agent for eighth pandemic of cholera.

1.2.1. Epidemiology of cholera

Originally, cholera belonged to old world and turn out to be one of the main infectious illness in previous time with increment of incidence in recent epidemics in Haiti (2010 - 2012), Vietnam (2008) and Zimbabwe (2009). In 2013, WHO has recorded 129 064 cases of cholera from 47 countries in which 43% were described in Africa and 47% described in America with an approximate burden of 1.4 to 4.3

million cases, and every year from 28 000 to 142 000 of mortality cases globally (World Health Organization, 2015b).

V. cholerae has been indicated as etiologic agent for cholera in 1883 where fifth pandemic was occurred at the moment (Koch, 1884). Since 1817 until present day, the world has been stroked by seven pandemics and the sixth pandemic between 1899 and 1923 is responsible by classical biotype (Pollitzer, 1954). Nevertheless, this classical biotype is no longer exists and has been replaced (Samadi et al., 1983) with the seventh causative agent for seventh pandemic, the El Tor biotype. According to previous studies, the seventh pandemic strain was first isolated in Sulawesi, Indonesia and later outspread all over Southeast Asia including Malaysia to Asian mainland (Kaper et al., 1995). It also causes large-scale epidemics in the Middle East areas and neighbouring countries (Cohen et al., 1971; Goodgame and Greenough, 1975; Kaper et al., 1995).

However, the emergence of new serogroup O139 Bengal in India (1992), has alarmed the researchers of the possibility for eighth pandemic cholera to occur (Bhattacharya et al., 1993). Though this strain has persistently been isolated in Asia but it has the possibility to continue and outspread to other mainlands. Presently, the cholera outbreaks are responsible by *V. cholerae* O1 and O139 serogroups.

1.2.2. Outbreaks in Malaysia

Although Malaysia is categorised as one of the non-endemic countries in cholera cases worldwide (Ali et al., 2015), but there are still some cases occasionally occurred all over the country. In Malaysia, cholera outbreaks mainly occur due to El Tor biotype of O1 serogroup periodically and for O139 serogroup occurs sporadically while non-O1/ non-O139 serogroup so far has not been involved in any major outbreak (Ang et al., 2010; Chen et al., 2004; Teh et al., 2011). In 2006, there were 237 cholera cases with 2 deaths reported by Department of Public Health (DPH), Ministry of Health Malaysia (http://www.dph.gov.my/survelans/Statistik).

In three years time, another cholera outbreak has struck Terengganu, Peninsular Malaysia in November 2009 with 187 cases and one death that were caused by two *V. cholerae* El Tor biotypes that were unsusceptible to particular antibiotics (Teh et al., 2012). In August 2012, cholera outbreak has been reported in Bintulu, Sarawak (East Malaysia) with 27 *V. cholerae* samples confirmed for toxigenic *ctx* gene (Bilung et al., 2014). The latest cholera cases reported occurred in Kota Belud, Sabah (East Malaysia) by the local media with three cases due to lack supply of treated water (Rintod, 2013). This reveals that within recent years, a serious increment of cholera cases have occurred and need to be carefully observed.

1.2.3. Pathophysiology of cholera

V. cholerae is widely known to cause infection in human though it also exists in environmental. The vehicle of transmission mainly from fecal-oral route particularly from consumption of contaminated food and water with *V. cholerae*. Even though *V. cholerae* is killed in the acid barrier of stomach but some of it might pass through and colonise the small intestine epithelium via their fimbria and TCP to perforate the mucus lying over the mucosa (Sack et al., 2004).

When the pathogens settled in epithelium, the toxigenic *V. cholerae* started to secrete 84 kDa cholera toxin protein which is expressed by *ctx*AB gene (subunit A and B) (Harris et al., 2012). The secretion of cholera toxin goes into the lumen of the extracellular surroundings which disrupts the ion transportation and leads to the opening of normally gated channels in the intestinal epithelial cells. The B subunits (56 kDa) started to bind the toxin to the GM_1 ganglioside receptors, natural receptor of cholera toxin in human while the A subunit activates adenylate cyclase after transported into the cell results in increment of cyclic AMP (cAMP) together with excretion of chloride inside crypt cells and inhibits the absorption of sodium chloride in the villus cells (Banerjee, 2010; Sack et al., 2004). This results in an enormous reflux of fluid and electrolyte loss in the form of watery diarrhoea as one of cholera symptoms.

Generally cholera patients produced a high concentration of toxigenic *V. cholerae* up to 10^8 per gram which possibly contaminate the water supplies and food and also seen as environmental reservoir for transmission of cholera mainly in countries that lack of proper human sewage system and sanitation practice (Sack et al., 2004).

1.2.4. Clinical presentations of cholera

Generally, only minority of people developed cholera gravis, which is the most severe manifestation after infected with toxigenic *V. cholerae*. In report by WHO, about 75% of people infected with cholera were asymptomatic and 80% of them developed mild diarrhoea. However in 20% cases, they developed a very severe cholera manifestation that can lead to death within hours compared to a healthy adult (World Health Organization, 2014). The symptom of loss in electrolyte fluid leads to depletion of blood volume resulted in hypotension and shock while potassium and bicarbonate ions loss through diarrhoea resulted in metabolic acidosis and deficiency in potassium ions in the body, and subsequently, death in untreated cases (Harris et al., 2012; Sack et al., 2004).

After 24 to 48 hours of incubation period, usually cholera patients begin to show symptoms such as painless abdominal cleanse of immense stools that resembles rice water accompanied by intense fishy odour, where the rate of purging is quick and can reach up to 500 - 1000 ml/ h and results in acute dehydration (Fazil and Singh, 2011; Harris et al., 2012). The characteristic of vomit characteristic usually clear, watery, alkaline fluid and thus, an immediate admission of rehydration fluids must be provided to reduce the risks of death within few hours of onset of disease.

1.2.5. Cholera interventions

The typical and effective intervention for cholera patients is by admitting with rehydration fluids to recover the body fluid loss and also to maintain normal hydration status. Usually the administration of rehydration fluids are carried out based on the severity of the dehydration status as guided by WHO which based on clinical signs and symptoms showed from patients.

Patients with a very severe dehydration status should be given lactated Ringer solution or isotonic sodium chloride solution in every 2 - 5 hours intravenously based on age and body weight (World Health Organization, 1993). Administration of oral rehydration salt (ORS) can also be given to patients as it contains potassium, bicarbonate, glucose and chloride via intravenous or nasogastric tube to helps restoring the patient condition with moderate dehydration status.

Antibiotics have been implemented as an adjunct to hydration intervention, however, they alone are insufficient to hinder the mortality of cholera (Centers for Disease Control and Prevention, 2015; Das et al., 2013). Generally, moderate and severely ill patients older than two years old are administered with antibiotics concurrence with rehydration therapy to lessen the fluid lost and to reduce the secretion of *V. cholerae* (Seas et al., 1996). Usually about 50% of illness can be decreased with an effective antibiotic therapy accompanied by shorter elimination of *V. cholerae* in stool in one or two days in comparison to exceed five days without any antibiotics intervention (Nelson et al., 2011).

Doxycycline antibiotic has been endorsed by WHO as the first choice of antimicrobial agent by giving as a single dose of 300 mg for an adult and 6 mg/ kg in children between 1 and 14 years of age to cholera infected patients. Azithromycin

and erythromycin have been used as first line regimen in children and pregnant women and they were found to be effective and appropriate (Centers for Disease Control and Prevention, 2015; Nelson et al., 2011).

Apart from these therapies, zinc supplementation admission occurrence with antibiotic and rehydration therapy has shown in reduction of period and acuteness of diarrhoea and chances of following infections for 2 - 3 months in children (Roy et al., 2008).

1.2.6. Prevention controls and public sanitation

Even though the cholera intervention seems to be simple and uncomplicated by just administering rehydration fluids to replace lost body fluids and electrolytes, however, in countries which experience poverty and lack of clean water supplies, this problem might arises particularly when lacking of medical assistance and supplies. In such countries, the poorly treated cholera cases can result in increasing of death rates from 20% to 50% during the epidemic season. Thus, a proper management in environmental hygienic practices and sanitation and also vaccination can be carried out to hinder and control the outspread of cholera as well as reducing the mortality rates.

The spreading of cholera disease is due to inaccessible to treated, good quality of water supplies in a sufficient amount. Chlorination of water reservoir is effective in improving the quality of water supply by diminishing the existence of pathogens in the water. An efficient sanitation of personal hygiene and domestic hygiene such as

food handling and sewage management system can minimise the transmission of cholera disease.

1.2.7. Cholera diagnosis

Cholera diagnosis mainly implements the gold standard method by identification of causative agent by culture method, biochemical and serological test (Dick et al., 2012). The clinical specimens collected from diarrhoea patients are either stool or rectal swab. Isolation of *V. cholerae* is performed using alkaline peptone water (APW) due to its capability of reproducing effectively in alkaline pH for a duration of 6 to 8 hours (Kaper et al., 1995). Then, the isolates are cultured on thiosulphate-citrate-bile salts-sucrose (TCBS) agar which gives rise to large and smooth yellow colonies that differ from other non-sucrose fermenting bacteria colonies.

Despite having more than 200 serogroups of *V. cholerae* described so far, only serogroup O1 and O139 are routinely screened as these serogroups are the causative agents for prominent epidemic and pandemic cholera cases worldwide. The identification of *V. cholerae* O1 serogroup can be performed using polyvalent antiserum against O1 antigen while for *V. cholerae* O139 serogroup identification using monovalent antiserum against O139 antigen. Whereas, non-O1/ non-O139 strains are categorised for isolates that give non-reactive result with both antisera during agglutination test.

There are some biochemical tests done for recognition of *V. cholerae* bacterium such as Oxidase test, String test and Triple sugar iron test. In purpose for cholera intervention, identification of *V. cholera* O1 isolate's serotype and biotype is

unnecessary, however, the data collected is helpful in epidemiological study. In serotyping test, a monovalent antiserum of Inaba and Ogawa is used whereas biotyping test can be performed using several tests inclusive of Voges-Proskauer test, polymyxin B susceptibility test, hemagglutination test with chicken red blood cells and susceptibility to bacteriophages to distinguish either Classical or El Tor biotype (Bopp et al., 1999; Feng et al., 2008; Kaper et al., 1995).

During the early stages of an outbreak where the incidence of cholera is still low, an expression of CT is compulsory to perform in cholera endemic settings for surveillance and as precautions for potential of outbreaks to occur. Production of CT enterotoxin can be done using rabbit ileum loop test, cell culture-based tests, GM_1-ELISA assay and reversed passive latex agglutination test (RPLA) that is commercially available.

Nevertheless, these assay's processes exceed 12 hours of incubative period and in consequence, the CT recognition using rapid tests such as G_{M1}-ELISA or RPLA assay still needs 2 days to obtain the results. In addition, these assays are time-consuming, laborious, and limited sensitivity, particularly for improper sample collections or patient's samples that previously treated with antibiotic or with traditional home-remedies.

The diagnosis process may also be hindered in conditions of low numbers of viable organisms present or exist in a viable but non-culturable state (Senoh et al., 2010). Therefore, a molecular amplification-based technique is the best possible detection method for detecting the toxigenic genes of *V. cholerae* strains as the results can be acquired within a few hours compared with conventional methods.

1.2.8. Loop-mediated isothermal amplification (LAMP)

Molecular amplification-based techniques are one of the most precious implements in all life science fields like in diagnosis of infectious diseases, genetic disorders and even in agriculture and fisheries. Rapidity, ease of operation handling, highly sensitive and specific is criteria needed in facilitating a particular reliable and efficient diagnosis system. Loop-mediated isothermal amplification (LAMP) technique is one of DNA amplification methods which possesses these criteria compared with other molecular amplification-based techniques referred to PCR, nucleic acid sequence-based amplification (NASBA), strand displacement amplification (SDA) and other molecular techniques. It is also be claimed to be stable compared with PCR and real time PCR when using clinical samples in which some components that present in the sample may be hinder or inhibit the *Taq* polymerase unlike *Bst* polymerase.

Since it was first reported by the Japanese research groups in 2000 (Notomi et al. 2000), LAMP has been extensively applied from conventional LAMP until recently reported as real-time LAMP by using DNA probes for multiplex target. The entire process is straightforward and rapid where under isothermal conditions, the DNA amplification can be finished in less than 60 minutes by using three sets of specific primers which recognise eight individual sequences of target gene and it occurs in the same single tube.

The principle of LAMP involves the *Bst* DNA polymerase enzyme performing auto-cyclic strand displacement activity under isothermal temperatures is explained with Figure 1.1.

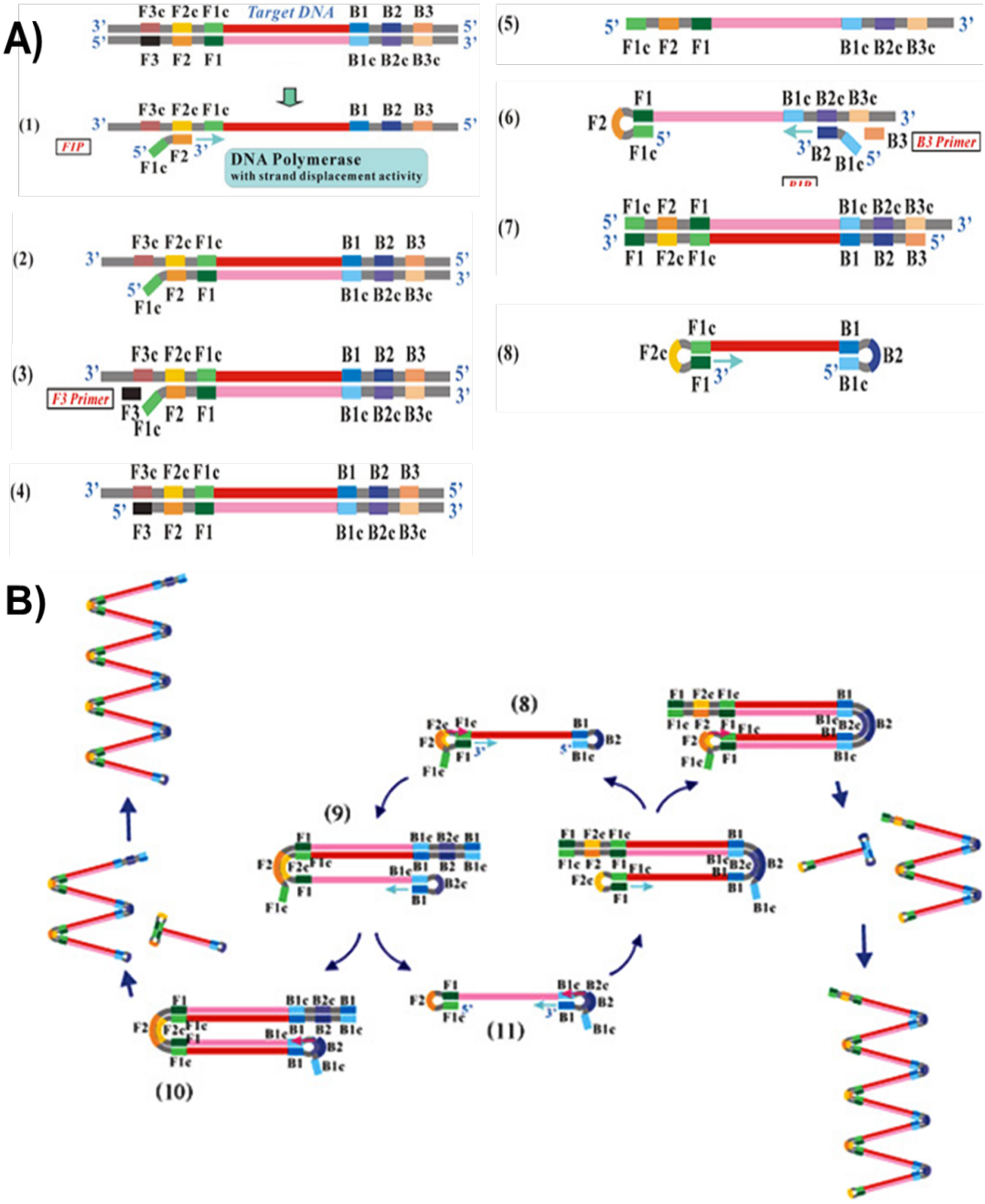


Figure 1.1 Principles of LAMP amplification. Non-Cyclic Step [1—8]: Generation of stem loop DNA with dumbbell-shaped structure at both ends that is ready to enter into cyclic amplification step. Initially, the strand displacement activity of *Bst* DNA polymerase helps in synthesis of complementary DNA strand, starting with FIP. The outer primer (F3) then displaces the FIP-linked complementary strand, which forms a stem-loop structure at the 5' end. This serves as a template for BIP-initiated DNA synthesis and subsequent B3-primed strand displacement DNA synthesis. The final product is a structure with stem-loops at each end. Cyclic Amplification Step [9—11]: Exponential amplification of original dumbbell-shaped stem-loop DNA employing internal primers. The product is the differently sized structures consisting of alternately inverted repeats of the target sequence on the same strand, giving a cauliflower-like structure. Adopted from Eiken Chemical Co. Ltd., Japan.

The LAMP amplification process comprises two main steps which are non-cyclic and cyclic steps. A stem-loops DNA was formed at every one of structure during non-cyclic process and they function as the beginning structure for LAMP cyclic steps. It started with annealing of FIP primer to complementary nucleotide sequences which later DNA amplification is initiated by *Bst* DNA polymerase and releasing a single stranded DNA due to its strand displacement activity of this enzyme.

The single stranded DNA produced is beginning from 3' end of FIP (F2 region). Then, the F3c region located outside of FIP is annealed by F3 primer and commence the activity of strand replacement, freeing the FIP-linked complementary strand. The complementary regions between F1c and F1 on this single stranded DNA leads to formation of stem-loop structure at 5' end which functions as DNA template for BIP-initiated DNA synthesis and following B3-primed strand displacement DNA synthesis. A similar process happens to BIP region and lastly when the BIP-linked complementary strand displaced; it constructs a stem-loops at every one of structure which appears as dumbbell shape. This dumbbell shape functions as DNA template for LAMP cyclic amplification process.

In next LAMP series, the FIP primer hybridises to the dumbbell shape loop then commence the strand displacement DNA combination, generating the first stem-loop DNA and also recently developed stem-loop DNA with size twice long. Briefly the FIP recombines to single stranded district in the stem-loop DNA followed by strand displacement activity, loose out the formerly synthesised strand. A single strand was released and shaped into stem-loop structure at the 3' end due to complementary B1c and B1 districts. At that time, beginning from 3' end of the B1 district, DNA construction begins utilising self-structure as DNA template and loose out FIP-linked

complementary strand. The loose out single strand then shaped into dumbbell-like structure as both ends have complementary F1-F1c and B1c-B1 districts, respectively. Moreover, BIP recombines to B2c district followed by strand displacement activity, loosing out the B1-primed DNA strand. Consequently, assorted proportions of DNA structure comprises of alternately inverted repeats of target sequence on the same strand are constructed.

The rotation series reaction persist and produce a mass of 10^9 copies of interested gene in less than 60 minutes. Eventually, the end products are stem-loop DNAs with assorted inverted repeats of target sequence and cauliflower-like shapes with numerous loops shaped by recombining between alternately inverted repeats of the target in the same strand.

1.3. Rationale of study

Despite the fact that great immense of global development in medical field, cholera still remains a major challenge at global level particularly in Sub-Saharan Africa known to be cholera hotspot. In previous years back, a chain of cholera pandemic disasters occurred which describe the pathogenicity of the disease caused by *V. cholerae* O1 serogroup.

Diagnostic method for cholera mainly focus on isolation and detection of the bacteria by culture method and biochemical tests in which are time-consuming and tedious in the case of bacterial culture (Dick et al., 2012). In addition, skilful and trained personnel are required to perform the tests. However, limited facilities exist at developing countries have delaying the diagnosis process.

As a result, some studies have shown attempts to develop a rapid and specific diagnostic test that allows *V. cholerae* diagnosis in shorter time and assists the intervention like Cholera SMART kit which provides 100% specificity and 96% sensitivity in comparison with gold standard tests (Hasan et al., 1994). However, a detection kit rarely gives 100% sensitivity in which it is unable to detect the presence of low concentration of analyte target in specimens.

Molecular amplification method like PCR known as highly adaptable and powerful molecular tool that is utilised across divergent disciplines. Nonetheless, this tool has yet to be intensively utilised particularly in the diagnosis of microbial agents while the time-consuming conventional tests still been used for detection of *V. cholerae*. Thus, the consequence barriers which hinder the full implementation of PCR as the main diagnostic tool are requirement for expensive thermocycler machine, time-

consuming (requires at least 2 - 3 hours), skilled expertise to perform the task, and detection of PCR amplicons using conventional agarose gel electrophoresis.

Whereas, LAMP method only requires simple instrument which can provide a stable temperature control like heating block or water bath, rapid (requires at least 1 - 2 hours), simple operating procedure to perform and the amplicons can be detected merely by turbidity level, or by adding fluorescence dye or even by agarose gel electrophoresis. The LAMP method also offers high sensitivity until femtogram level and high specificity (due to implementation of three sets of primers) in detection of target genes and rapid compared with PCR method. It also can be used directly from clinical samples without concerning any inhibitors which might present unlike PCR (Kaneko et al., 2007).

The present study aims to develop a LAMP assay for the detection of toxigenic *V. cholerae*. This assay is made easy to perform and also eliminates the need for cold-chain transportation and storage via thermostabilization technology. An internal control is also integrated into the proposed assay to ensure the validity of the assay results. This study describes for the first time complete in-house development LAMP assay kit that features the use of thermostabilized ready-to-use assay reagents.

1.4. Objective (s) of research study

The aim of this study is to develop a thermostabilised multiplex LAMP assay for the detection of toxigenic *V. cholerae*.

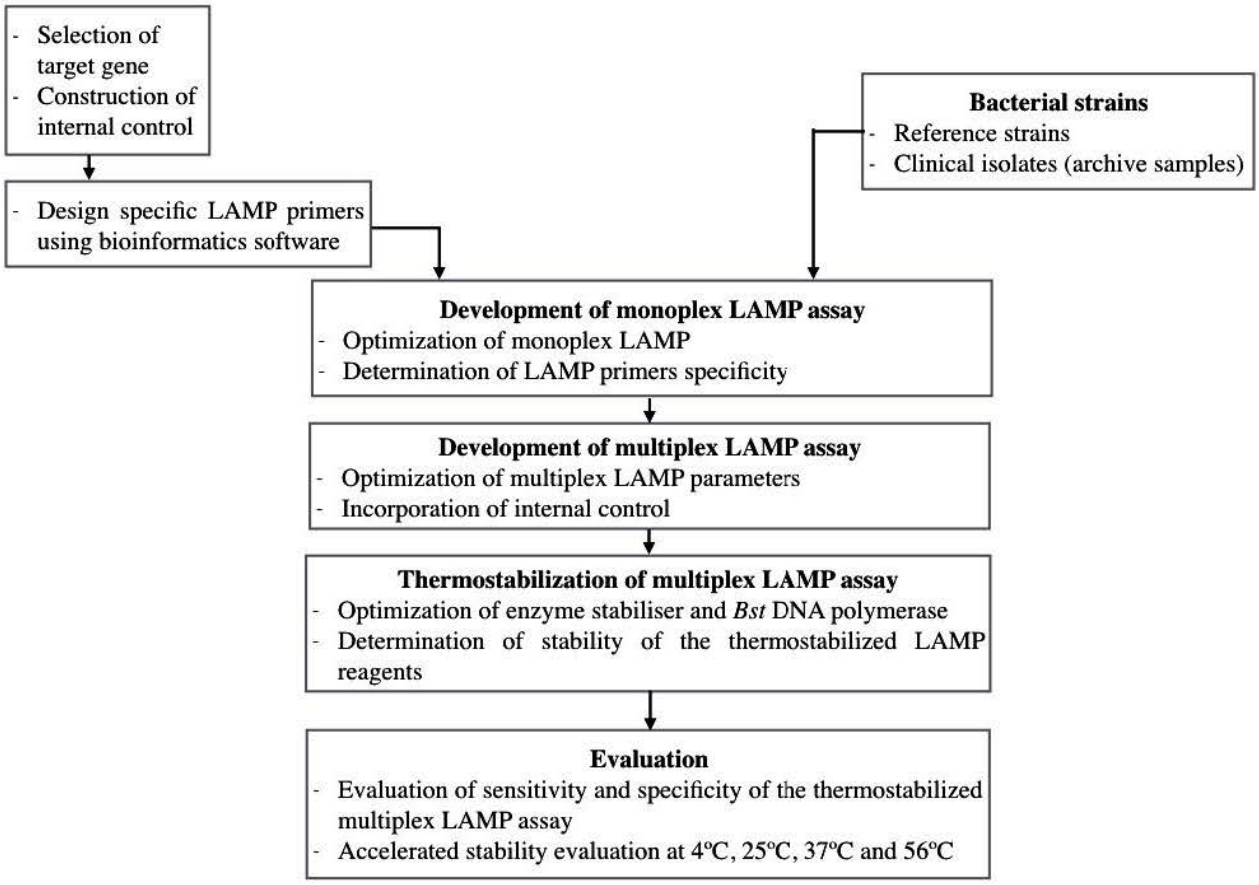
The objectives of this research project are as follows:

1. To develop multiplex LAMP assay for detection of toxigenic *V. cholerae* strains
   - To construct specific LAMP primers targeting *ctxA* gene and internal control
   - To optimise the monoplex LAMP assay parameters
   - To integrate and optimise an internal control for validation of multiplex LAMP assay

2. To develop a thermostabilised multiplex LAMP assay utilising the designed LAMP primers
   - To optimise the enzyme stabiliser combinations on the thermostabilised multiplex LAMP assay
   - To determine the accelerated stability of the thermostabilised multiplex LAMP assay

3. To evaluate the developed thermostabilised multiplex LAMP assay
   - To evaluate the analytical sensitivity of the developed thermostabilised multiplex LAMP assay
   - To evaluate the analytical specificity of the developed thermostabilised multiplex LAMP assay on Gram positive bacteria, Gram negative bacteria and *V. cholerae* clinical isolates

1.5. Experimental overview of study



CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Bacterial strains

A total of 100 *V. cholerae* strains, 5 *Vibrio* species and 91 Gram positive and Gram negative bacterial strains were acquired from the archive of Cholera Research Laboratory, Department of Medical Microbiology and Parasitology, School of Medical Sciences, Health Campus, Universiti Sains Malaysia (USM) and also Stock Culture Laboratory, Department of Medical Microbiology and Parasitology, USM. Apart from total 196 bacterial strains collected, 81 bacterial strains were used for preliminary specificity test as mentioned in section 3.2.1 while 115 bacterial strains were used in analytical specificity for thermostabilised multiplex LAMP assay as mentioned in section 3.5.3. Reference strain of *V. cholerae* J 3321, serogroup O1, El Tor biotype was obtained from archive of Cholera Research Laboratory and it was used for standardisation and optimization for LAMP assay. The list of bacterial strains utilised in present study are recorded in Table 2.1.

2.1.2. DNA Plasmid

The DNA plasmid which has been utilised in this study was available in Cholera Research Laboratory, Department of Medical Microbiology and Parasitology, School of Medical Sciences, Health Campus, Universiti Sains Malaysia (USM) and stored in archive plasmid collections at - 80 ºC until use. The DNA plasmid used is a SSP2