DEVELOPMENT AND EVALUATION

OF A NASBA SYSTEM

FOR THE DIAGNOSIS OF CHOLERA

USING ELISA AND BIOSENSOR METHODS

LEE SU YIN

UNIVERSITI SAINS MALAYSIA

2008

DEVELOPMENT AND EVALUATION OF A NASBA SYSTEM FOR THE DIAGNOSIS OF CHOLERA USING ELISA AND BIOSENSOR METHODS

by

LEE SU YIN

Thesis submitted in fulfillment of the

requirements for the degree of

Doctor of Philosophy

April 2008

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my supervisor, Dr P. Lalitha, who has been helpful and supportive throughout this study. Her critical comments and valuable insights have guided me in the preparation of this thesis. I am grateful to my co-supervisor, Prof. Zainul F. Zainuddin and Assoc. Prof. M. Ravichandran for their guidance and advice that have motivated me to become a better student and researcher.

To my dear friends and comrades, Melissa Chan, Yean Yean and Kurunathan, I express my heartfelt appreciation for their encouragements and support that have helped me through numerous failed experiments and mental roadblocks. My appreciation also goes out to my labmates and friends who have helped me in one way or another during the course of this study: Elina, Balqis, Donn, Chua, Siew Keah, Nik, Nurul, Kak Shikin, Nas, Nisha, Chandrika, Syazwan, Kun Lee, Narjit and Kavitha. In addition, I am grateful to the lecturers, administrative staff and lab technologists from the Department of Medical Microbiology and Parasitology, and Institute for Research in Molecular Medicine, Universiti Sains Malaysia for their help and the use of their excellent facilities.

I would like to thank Prof. Mitsuaki Nishibuchi from the Center for Southeast Asia Studies, Kyoto University, Japan; Communicable Disease Hospital, Thandiarpettai, Chennai, India; National Public Health Laboratory, Ministry of Health, Malaysia; Institute for Medical Research (IMR), Malaysia and Kak Liza from the Medical Microbiology and Parasitology laboratory, Universiti Sains Malaysia for providing the various bacterial strains used in this study. I am also grateful to the Ministry of Science, Technology and Innovations (MOSTI) for the IRPA and NBD grants that have funded part of this study.

On a more personal note, my family has been my strongest motivation and inspiration. No words could ever express my gratitude to them. As a sign of appreciation, I dedicate this thesis to them and hope that I have made them proud.

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Symbols/ Abbrev-iations/ Acronyms	Definition	Symbols/ Abbrev-iations / Acronyms	Definition
--	nil	cDNA	complimentary deoxyribonucleic acid
%	percent	CFU	colony forming unit
~	approximately	Cl^-	chlorine ion
+	positive	cm	centimeter
−	negative/ minus	CT	cholera toxin
<	less than	CTP	cytidine 5'-triphosphate
>	more than	CV	coefficient of variation
±	plus / minus	dATP	deoxyadenosine triphosphate
→	to	dCTP	deoxycytidine triphosphate
°C	degree Celsius	ddt	dideoxythymidine
A	adenine / adenosine; absorbance	DEPC	diethylpyrocarbonate
A_{260}	absorbance at 260 nm	dG	Gibbs free energy
A_{280}	absorbance at 280 nm	dGTP	deoxyguanosine triphosphate
A_{450}	absorbance at 450 nm	dH_2O	distilled water
ADP	adenosine diphosphate	DMSO	dimethyl sulfoxide
Ag/AgCl	silver/silver chloride	DNA	deoxyribonucleic acid
AMV-RT	avian myeloblastosis virus-reverse transcriptase	DNase	deoxyribonuclease
APW	alkaline peptone water	dNTP	deoxynucleoside triphosphate
ATP	adenosine 5'-triphosphate	DTT	dithiothreitol
BLAST	Basic Local Alignment Search Tool	dTTP	deoxythymidine triphosphate
bp	basepair	ECL	electrochemiluminescence
BSA	bovine serum albumin	EDTA	ethylenediaminetetraacetic acid
C	cytosine; current	ELGA	enzyme-linked gel assay
cAMP	adenosine 3',5'-cyclic monophosphate	ELISA	enzyme-linked immunosorbent assay

et al.	et alii / and other	IU	infectious unit; international unit
FAM	carboxyfluorescein	J	Joule
FDA	Food and Drug Administration	*k*	kappa value
Flu	fluorescein	K^+	potassium ion
G	guanine / guanosine	kcal/mol	kilocalories per mol
g	gram	KCl	potassium chloride
GHKB	General Hospital Kota Bharu	kDa	kilodalton
GITC	guanidine thiocyanate	KIA	Kligler iron agar
GM1	monosialotetrahexosylganglioside	L/hour	liter per hour
GMO	genetically modified organism	LB	Luria Bertani
GTP	guanosine 5'-triphosphate	LCD	liquid crystal display
H_2	hydrogen	LCR	ligase chain reaction
H_2O	water	LoD	limit of detection
H_2O_2	hydrogen peroxidase	M	molar / molarity
H_2S	hydrogen sulphate	mbar	millibar
HCl	hydrogen chloride	MCS	multiple cloning site
HCO_3^-	hydrogen carbonate ion	mg	milligram
HIV-1	human immunodeficiency virus 1	$MgCl_2$	magnesium chloride
HPLC	high performance liquid chromatography	min	minute
hr	hour	MKAK	National Public Health Laboratory
HRP	horseradish peroxidase	ml	milliliter
HUSM	Hospital Universiti Sains Malaysia	mm	millimeter
i.e.	*id est* / that is	mM	millimolar
IFU	inclusion-forming unit	M-MLV RT	Moloney murine leukemia virus reverse transcriptase
IMR	Institute for Medical Research	MOPS	3-(*N*-morpholino)propanesulfonic
IPA	intermittent pulse amperometry	mRNA	messenger ribonucleic acid

N	normal	psi	pounds per square inch
n	sample size	p_{sn}	specificity
N/A	not available / not applicable	p_{sp}	sensitivity
Na^+	sodium ion	R^2	correlation coefficient
NAD	nicotinamide	red	reduced / reduction
NaOH	sodium hydroxide	RNA	ribonucleic acid
NASBA	nucleic acid sequence-based amplification	RNase	ribonuclease
NCBI	National Center for Biotechnology Information	RNase H	Ribonuclease H
nm	nanometer	RNasin	RNasin Ribonuclease Inhibitor
nmol	nanomol	rpm	revolutions per minute
NPV	negative predictive value	rRNA	ribosomal ribonucleic acid
nt	nucleotide	RSD	relative standard deviation
NTP	nucleoside triphosphate	RT	room temperature
O_2	oxygen	RT-PCR	reverse transcription-polymerase chain reaction
OD	optical density	S	Svedberg unit
OD_{450}	optical density at 450 nm	SAM	salf-assembled monolayer
OD_{600}	optical density at 600 nm	SD	standard deviation
OH	hydroxyl group	SDA	strand displacement amplification
ORS	oral rehydration solution	SDS	sodium dodecyl sulphate
ox	oxidized / oxidation	s	second
PBS	phosphate buffered saline	SPCE	screen-printed carbon electrode
PBST	phosphate buffered saline-Tween 20	SPE	screen-printed electrode
PCR	polymerase chain reaction	spp.	species
PFU	PCR-forming unit	SPR	surface plasmon resonance
pmol	picomole	SSC	sodium chloride/sodium citrate
PPV	positive predictive value	SSCT	sodium chloride/sodium citrate/Tween 20

T	thymine / thymidine	UTP	uridine 5'-triphosphate
Ta	annealing temperature	UV	ultraviolet
TAE	Tris-acetate-EDTA	V	volts
TBE	Tris-borate-EDTA	v/v	volume/volume
TCBS	thiosulphate citrate bilesalt sucrose	VBNC	viable but non-culturable
tcp	toxin coregulated pili	vs	versus
TdT	terminal deoxynucleotidyl transferase	w/v	weight/volume
TE	Tris-EDTA	www	World Wide Web
Tm	melting temperature	x	precision
TMA	transcription-mediated amplification	x *g*	relative centrifugal force
TMB	3,3',5,5'-tetramethylbenzidine	µA	microamperes
Tris	tris(hydroxymethyl)aminomethane	µg	microgram
tRNA	transfer ribonucleic acid	µl	microliter
TSI	triple sugar indole	µm	micrometer
U	units	µM	micromolar

PEMBANGUNAN DAN PENILAIAN SISTEM NASBA UNTUK DIAGNOSIS PENYAKIT TAUN MENGGUNAKAN KAEDAH ELISA DAN BIOSENSOR

ABSTRAK

Taun (kolera) ialah penyakit ciri-birit yang disebabkan *Vibrio cholerae*. Taun boleh membawa maut jika tidak dikesan dengan serta-merta. Justeru, pengesanan awal adalah penting untuk rawatan pesakit dan kawalan wabak taun. Ujian kultur dan biokimia konvensional adalah rumit, lambat dan kurang sensitif. Walaupun ujian berasaskan teknik molekul adalah lebih pantas, sensitif dan spesifik, kaedah ini memerlukan peralatan mahal dan penyimpanan sejuk bahan-bahan reagen. Ujian DNA seperti PCR tidak dapat membezakan sel hidup dengan sel mati. Kaedah amplifikasi berasaskan asid nukleik (NASBA) yang menggandakan RNA secara isoterma dapat mengesan sel hidup sahaja. Maka, objektif kajian ini adalah untuk membangunkan dan menilai suatu ujian NASBA-ELISA yang stabil haba dengan *biosensor* untuk mengesan *V. cholerae* berasaskan gen *lol*B. Pada peringkat permulaan, transkrip RNA sebagai kawalan positif dikonstruk dan primer serta prob khusus direka. Pengoptimuman ujian NASBA dan ELISA dilakukan dan spesifisiti analitikal diuji dengan 41 bakteria rujukan yang terdiri daripada *V. cholerae*, *Vibrio* spesis dan patogen enterik. Sensitiviti analitikal diuji dengan cairan bersiri transkrip RNA dan sel-sel *V. cholerae*. Penilaian klinikal dijalankan dengan menggunakan najis yang ditokok sampel (n=200). Seterusnya, kaedah *biosensor* dioptimumkan dan keputusannya dibandingkan dengan kaedah spektrofotometri. Campuran NASBA dijadikan stabil haba melalui pengeringbekuan dan kestabilannya dinilai pada suhu berlainan untuk jangkamasa tertentu. Selain itu, kesesuaian *lol*B mRNA sebagai penunjuk kebolehhidupan diselidiki dengan membunuh sel-sel *V. cholerae* melalui pelbagai kaedah dan mengesan isyarat NASBA daripadanya. Ujian kolera NASBA-ELISA yang telah dioptimumkan berdasarkan pengesanan produk amplifikasi melalui prob berlabel *fluorescein* dan isyarat TMB/HRP. Spesifisiti analitikal ujian adalah 100% manakala sensitiviti analitikal ujian adalah 10^2

molekul/µl transkrip RNA dan 10 CFU/ml sel. Penilaian klinikal memberikan keputusan 100% sensitif, 84.52% spesifik, 89.92% nilai ramalan positif dan 100% nilai ramalan negatif. Kaedah *biosensor* didapati setanding kaedah spektrofotometri dengan mencatatkan kepekaan analitikal yang sama, korelasi yang rapat (R^2<0.964) dan persetujuan kappa yang hampir sempurna (95.1%, k = 0.828). Penstabilan campuran NASBA mampu mengekalkan kestabilannya pada 8°C dan -20°C selama dua bulan. Dalam ujian kebolehhidupan sel, gen *lol*B masih dapat dikesan daripada kultur sel *V. cholerae* yang telah dibunuh selepas 48 jam, justeru gen ini tidak sesuai dijadikan penunjuk sel bernyawa. Kesimpulannya, buat pertama kalinya, kami telah membangunkan suatu ujian kolera-NASBA-ELISA dengan pengesanan *biosensor* yang sensitif dan dapat dilakukan dengan peralatan mudah dalam empat jam. Campuran NASBA kering mengurangkan langkah-langkah mempipet serta memudahkan penghantaran dan penyimpanan ujian. Ujian ini sesuai sebagai ujian diagnostik pantas dan ujian saringan di lapangan.

DEVELOPMENT AND EVALUATION OF A NASBA SYSTEM FOR THE DIAGNOSIS OF CHOLERA USING ELISA AND BIOSENSOR METHODS

ABSTRACT

Cholera is a diarrheal disease caused by *Vibrio cholerae*. Cholera is potentially lethal if not diagnosed on time. Hence, early detection is crucial for patient treatment and containment of outbreak. Conventional culture and biochemical tests are laborious, time-consuming and less sensitive. Although molecular-based methods are rapid, more sensitive and specific; they require expensive equipments and cold storage of reagents. Furthermore, DNA-based tests such as PCR, do not distinguish between viable and non-viable cells. Nucleic acid sequence-based amplification (NASBA) is an isothermal RNA amplification technique that specifically detects viable cells. Hence, the objective of this study was to develop and evaluate a thermostabilized cholera-NASBA-ELISA assay with biosensor detection for *V. cholerae* based on the *lol*B gene. RNA transcripts as positive control were first constructed and specific primers and probes were designed. NASBA and ELISA conditions were optimized and the analytical specificity was tested with 41 reference strains comprising of *V. cholerae, Vibrio* species and enteric pathogens. The analytical sensitivity was tested with serial dilutions of RNA transcripts and *V. cholerae* cells. Clinical evaluation of the assay was performed using spiked stool samples (n=200). Subsequently, biosensor detection for the NASBA-ELISA assay was optimized and the results compared to spectrophotometry. The NASBA mix was thermostabilized by freeze-drying and its stability at different temperatures was determined periodically. In addition, suitability of *lol*B mRNA as a viability indicator was investigated by subjecting cultures to lethal treatments and detecting the NASBA signal. The optimized cholera-NASBA-ELISA assay detected amplicons using fluorescein-labeled probes and TMB/HRP signal. The analytical specificity of the assay was 100%, while the analytical sensitivity was 10^2 molecules/µl RNA transcripts and 10 CFU/ml cells. Clinical evaluation gave 100%

sensitivity, 84.52% specificity, 89.92% PPV and 100% NPV. Biosensor detection was comparable to spectrophotometry, yielding similar analytical sensitivity level, excellent correlation (R^2 <0.964) and near perfect kappa agreement (95.1%, k= 0.828). Thermostabilization of the NASBA mix was able to preserve its stability at 8°C and -20°C for two months. In the viability assay, *lol*B mRNA was detected even after 48 hours post-treatment, therefore precluding its use as a viability indicator. In conclusion, we have for the first time, developed a sensitive cholera-NASBA-ELISA assay with biosensor detection that can be performed using simple equipments within four hours. The dry NASBA mix reduces multiple pipetting steps and facilitates transportation and storage. The test is suitable for use as a rapid diagnostic test or screening test in the field.

CHAPTER 1

INTRODUCTION

1.1 History of cholera

Cholera is a potentially life-threatening secretory diarrheal disease caused by the bacteria *Vibrio cholerae*. Cholera is thought to originate from the Ganges Delta of the Indian subcontinent. In the nineteenth century, seven distinct pandemic waves of cholera spread from Asia to many parts of the world. Six pandemics arose from the Indian subcontinent, while the seventh pandemic originated from the island of Sulawesi, Indonesia in 1961 (Parsi, 2001). In the late 1992, a possible eighth pandemic was reported when a large epidemic cholera spread throughout Bangladesh, India, Pakistan, Nepal, China, Thailand and Malaysia (Blake, 1994).

There are more than 150 serogroups of *V. cholerae,* however only the serogroups O1 and O139 cause epidemic and pandemic diseases (Sack *et al.*, 2004). The Classical biotype of *V. cholerae* serogroup O1 was responsible for the fifth and sixth pandemics and is believed to have been associated with the earlier pandemics as well. The seventh cholera pandemic is caused by the El Tor biotype of *V. cholerae* serogroup O1 and this biotype has completely displaced the Classical biotype worldwide, except in Bangladesh where it appeared in epidemic proportions in 1982 and now seems to have become extinct again (WHO, 2002). The newly emerged serogroup O139 or 'Bengal' in the late 1992 has been attributed to the eighth cholera pandemic and this serogroup is closely related to the Asian *V. cholerae* O1 El Tor strains associated with the seventh pandemic (Blake, 1994).

1.2 Prevalence of cholera

Cholera is one of the oldest and best-known diseases in the world, yet it continues to cause considerable suffering and needless deaths. In 2006 alone 131,943 cases of cholera were reported worldwide with 2272 fatalities (WHO, 2007). However, this number is just the tip of the iceberg because poor surveillance and fear of travel and economic sanctions lead to

under-reporting of actual cholera cases in certain countries (WHO, 2007). It is estimated that in reality, the number of deaths from cholera is probably 100 times more than was officially reported (WHO, 2007). In Malaysia, the Ministry of Health reported the incidence rate of cholera in 2005 (per 100,000 population) was 1.48 with a 0.01 mortality rate (Ministry of Health Malaysia, July 2007).

1.3 Characteristics of *V. cholerae*

Vibrio cholerae is a member of the family *Vibrionaceae*. It is a facultative anaerobe, short, curved rod about 1.4 – 2.6 µm, capable of fermentative and respiratory metabolism. It is a non-halophilic vibrio, oxidase-positive, reduces nitrate and is highly motile by means of a single sheathed polar flagellum. Even though growth of *V. cholerae* is stimulated by addition of 1% sodium chloride, it can grow in nutrient broth without added sodium chloride, which is an important distinction from other *Vibrio* spp. (Kay *et al.*, 1994). It can grow at 16 – 40°C, with an optimum temperature of 37°C and at pH 7.4 – 9.6 (WHO, 2002).

The species *V. cholerae* is divided into more than 150 serogroups based on the differences in their somatic (O) antigens. These variations are secondary to differences in the oligosaccharide side chains in parts of the cell wall (Parsi, 2001). The serogroup O1 is divided into two biotypes, Classical and El Tor, which can be differentiated by tests such as hemolysis, hemagglutination, phage typing, polymyxin B sensitivity and Voges-Proskauer reaction (table 1.1). Each of the O1 biotype can be further subdivided into two major serotypes, Ogawa and Inaba, based on variations in the polysaccharide component of the O antigen. Ogawa strains possesses only the A and B antigens while Inaba strains possesses only the A and C antigens. A third and rare serotype, Hikojima possesses all three A, B and C antigens (Kay *et al.*, 1994).

The new serogroup O139 Bengal was thought to have evolved as a result of horizontal gene transfer between O1 and non-O1 strains. The O139 strain does not produce O1 lipopolysaccharide and lacks some of the genetic material necessary for production of the O1 antigen. It has potential for capsule expression and an increased capacity for spread and proliferation within the environment (Morris and Cholera Laboratory Task Force, 1994).

Strains of *V. cholerae* identified by biochemical tests but do not agglutinate with O1 and O139 antisera are referred to as non-O1, non-O139 *V. cholerae*. The non-O1, non-O139 strains can produce enterotoxins that are similar to cholera toxin (Morris, 1994). Although this strain is not involved in cholera epidemics, it can be pathogenic and is frequently associated with small sporadic outbreaks of diarrheal disease caused by the consumption of contaminated shellfish and from a variety of extraintestinal infections (Morris, 1994).

Besides the somatic (O) antigen, all *V. cholerae* possess common heat-labile flagella (H) antigen, and this property may be used to distinguish *V. cholerae* from all other vibrios (Kay *et al.*, 1994). Table 1.2 summarizes the different *V. cholerae* serogroups and their antigenic determinants.

Table 1.1: Tests for differentiation of Classical and El Tor biotypes of *V. cholerae* serogroup O1

Test / Reaction	Biotype	
	Classical	El Tor
Hemolysis of sheep erythrocytes	−	+
Hemagglutination with fowl erythrocytes	−	+
Susceptibility to bacteriophage: i) classical phage (group IV) ii) El Tor phage (group V)	 + −	 − +
Sensitivity to Polymyxin B (50 U)	+	−
Produces acetoin in Voges Proskauer test (modified with 1% NaCl)	−	+

Table 1.2: *V. cholerae* serogroups and their antigenic determinants

Typing systems	Epidemic associated		Non-epidemic associated
Serogroup	O1	O139	Non-O1
Biotypes	Classical El Tor	None	–
Serotypes	Ogawa Inaba Hikojima	None	None
O1 Antigens	Ogawa – A and B Inaba – A and C Hikojima – A, B and C	Does not have O1 somatic antigen	Does not have O1 somatic antigen
Toxin production	Produces cholera toxin	Produces cholera toxin	Usually does not produce cholera toxin, sometimes produce other toxins
Polysaccharide capsule	None	Yes	None

Adapted from Kay, B., Bopp, C. & Wells, J. (1994) In *Vibrio cholerae* and Cholera: Molecular to global perspectives (Eds, Wachsmuth, I., Blake, P. and Olsvik, O.) American Society for Microbiology, Washington DC (table 1, pp. 4)

1.4 Ecology of *V. cholerae*

Until the late 1970s and early 1980s, it was believed that *V. cholerae* was highly host-dependant and incapable of surviving longer than a few hours or days outside the human intestine. However, studies have shown that *V. cholerae* is a normal inhabitant of brackish water and estuarine systems (Colwell and Huq, 1994a). *V. cholerae* non-O1, non-O139 strains are frequently isolated from aquatic ecosystems while O1 and O139 strains are less frequently isolated. Outside of the host and in the aquatic environment, *V. cholerae* can be found as free swimming cells, attached to aquatic plants, filamentous green algae, copepods, crustaceans, insects and egg masses of chronomids. Environmental persistence of *V. cholerae* within natural aquatic habitats during periods between epidemics is facilitated by biofilm formation and entry into a viable but non-culturable (VBNC) state (Colwell and Huq, 1994b).

1.5 Viable but non-culturable (VBNC) state

V. cholerae O1 and non-O1, non-O139 strains can enter into a VBNC state in response to nutrient deprivation, elevated salinity and reduced temperatures. In this dormant state, the *V. cholerae* cells are reduced in size, become ovoid and do not grow at all on standard laboratory media used to isolate *V. cholerae*. However, Colwell *et al.* (1994b) found that when the VBNC cells of *V. cholerae* were inoculated into rabbit ileal loops or ingested by human volunteers, it caused large accumulation of fluid in the rabbit ileal loops or diarrhea in the human volunteers. This may explain the reason attempts to culture and isolate *V. cholerae* from water sources between epidemics often fail. Since *V. cholerae* in the VBNC state in environmental samples may not grow on conventional bacteriologic media, it may go undetected unless other detection methods such as immunologic, molecular or direct microscopy are employed. Huq *et al.* (1990) showed that *V. cholerae* O1 could be detected by direct immunofluorescence microscopy throughout the year in their water reservoirs even when culturing could not isolate the organism itself. Rapid detection kits for *V. cholerae* that do not require culture called CholeraScreen (a coagglutination test), Cholera DFA (direct

fluorescent monoclonal antibody staining kit) and Cholera SMART (a colorimetric immunoassay) have been developed and these were also able to detect VBNC cells of *V. cholerae* (Colwell and Huq, 1994b).

1.6 Epidemiology of cholera

Cholera is the classic water-borne disease and an epidemiological study by John Snow in 1849 showed the association of the disease with contaminated drinking water (Parsi, 2001). Poor sewerage and sanitation practices that resulted in contamination of water supplies used for drinking and food preparation are often the causes of cholera epidemics (Mintz *et al.*, 1994). This mode of transmission is more common in less developed countries under conditions of famine, war and natural disaster. Cholera transmission has also been linked to drinking water from shallow wells, rivers, bottled water and ice (Parsi, 2001).

Food is another vehicle for transmission. Seafood, particularly raw or undercooked shellfish harvested from sewage-contaminated seabeds, meat, cooked grains, fruit and vegetables irrigated with raw sewerage have been implicated in cholera outbreaks (Parsi, 2001, Mintz *et al.*, 1994).

1.7 Pathophysiology of cholera

Upon ingestion, *V. cholerae* cells colonize the small intestines, particularly the duodenum and jejunum because the alkaline pH, nutrients and bile salts provide a suitable environment. They adhere to the microvilli with the help of a long filamentous fimbriae called toxin-coregulated pili (Tcp pili), hemagglutinins, mucinase and neuraminidase. Their motility also enables them to penetrate the mucus layer. They penetrate the host cells by elaborating a potent enterotoxin, called cholera toxin (CT), a 84 kDa protein consisting of 2 regions, which are the A region and B region (Sack *et al.*, 2004).

The B region (56 kDa) binds the glycolipid, GM1 ganglioside on the host intestinal epithelial cell membrane, enabling the A region (28 kDa) to penetrate the cell. The A region will enzymatically transfer ADP-ribose from NAD to a GTP-binding regulatory protein associated with membrane-bound adenylate cyclase enzyme. The ADP-ribosylation reaction activates adenylate cyclase, which leads to an increase of intracellular cAMP levels. This is followed by the secretion of H_2O, Na^+, K^+, Cl^- and HCO_3^- into the lumen (Kaper *et al.*, 1994). The patient may lose several litres of protein-free fluid and associated electrolytes, bicarbonates and ions within hours. The watery diarrhea (rice-water stool) is speckled with flakes of mucus, epithelial cells and contains large number of vibrios. Excessive loss of fluid may lead to dehydration, uremia, metabolic acidosis, hypovolemic shock and subsequently, death in untreated cases (Sack *et al.*, 2004, Parsi, 2001). The severity of a cholera infection depends on many factors, and it is increased with the absence of a local intestinal immunity, a high infectious dose ($>10^8$ bacteria), low gastric acid production and possession of blood group O (Sack *et al.*, 2004, Parsi, 2001).

1.8 Clinical manifestations of cholera

Most individuals infected with *V. cholerae* have no symptoms or only mild diarrhea, indistinguishable from other mild diarrheal diseases. Only a minority will develop cholera, a disease characterized by the sudden onset of vomiting and profuse watery diarrhea, following an incubation period of 6 to 48 hours. The most distinctive feature of cholera is the painless purging of voluminous stools resembling rice-water and having a fishy odour. In adults with severe cholera, the volume of watery stool may exceed 1 L/hour, leading to severe dehydration, hypovolemic shock, metabolic acidosis and renal failure. This condition may lead to death within a few hours of onset if rapid treatment is not given (Bennish, 1994, Parsi, 2001).

1.9 Treatment of cholera

Without proper treatment the case-fatality for severe cholera is about 50% (Sack *et al.*, 2004). Treatment is very simple and effective based on fluid replacement. Oral rehydration solution (ORS) should be given for mild cases of diarrhea, while intravenous fluid replacement using lactated Ringer's solution should be given for more severe cases (Parsi, 2001). The use of antibiotics shortens the duration and severity of the illness. In most cases, tetracycline and doxycycline is the antibiotic of choice. In cases of tetracycline resistance, erythromycin, cotrimoxazole, ciprofloxacine, azithromycin and trimethoprim-sulfamethoxazole may be used (Parsi, 2001).

1.10 Prevention of cholera

Transmission of *V. cholerae* through contaminated food and water can be prevented by minimizing contamination and reducing survival and growth of the organism (Sack *et al.*, 2004). The level of contamination can be minimized by practicing personal and domestic hygiene such as hand washing with soap before food handling and safe sewage disposal. Survival of the organism may be diminished by boiling or disinfecting drinking water and proper cooking and refrigeration of food. Prophylactic prevention of cholera can be achieved through the use of vaccines. Three oral vaccines (two inactivated and one live attenuated) are currently available (Sack *et al.*, 2004). These vaccines have been licensed in some countries and are mainly used by travellers. However, these vaccines are for *V. cholerae* serogroup O1 and do not give cross-protection against other serogroups such as O139 or non-O1, non-O139. Although a number of vaccine candidates for *V. cholerae* serogroup O139 are under development (Ravichandran *et al.*, 2006), they are not commercially available. Prevention of cholera using vaccines may be beneficial as a public health intervention if they are cost-effective, give long lasting protection and easy to administer.

1.11 Diagnosis of cholera

1.11.1 Conventional laboratory methods

Due to the clinical and epidemiological severity of cholera, rapid diagnosis of *V. cholerae* in clinical samples, water and food is very critical so that appropriate monitoring and effective preventive measures can be undertaken to prevent a cholera outbreak. The conventional laboratory methods employed are culturing, microscopy and biochemical testing.

Faecal specimens should be collected as early as possible in the illness, as the number of *V. cholerae* begins to decline soon after onset of symptoms, and before initiation of antibiotic therapy (Kay *et al.*, 1994). Faecal specimens or rectal swabs should be collected from patients into a sterile bottle containing transport medium such as alkaline peptone water (APW). APW is also the standard medium for enrichment of *V. cholerae* as its high pH condition preferentially supports the growth of vibrios while inhibiting other organisms and it can be used to recover low levels of vibrios in faecal specimens (Kay *et al.*, 1994).

If specimen is collected on the first day of illness, the vibrios are likely to be present in large numbers (10^7-10^9 CFU/ml), hence it is possible to make a provisional diagnosis by direct microscopy examination of a film of faeces using dark-field or phase-contrast microscopy (Kay *et al.*, 1994). Vibrios are highly motile and can be seen darting about rapidly. When specific antiserum is added to the film, the vibrios are immobilized.

For culture, fresh faecal specimens or APW-enriched specimens are plated onto thiosulfate-citrate-bile salts-sucrose (TCBS) agar, a highly differential and selective medium for *V. cholerae*. Its selective ingredients suppress the growth of most of the interfering organisms such as coliforms, psedomonads, aeromonads and other Gram-positive bacteria (WHO, 2002). The sucrose-fermenting *V. cholerae* grow on TCBS as large, slightly flattened, yellow, smooth colonies with a diameter of 2-4 mm.

Identification of O1 or O139 serogroups is performed using slide agglutination test with antiserum raised against *V. cholerae* O1 or O139. Agglutination in polyvalent O1 antiserum provides presumptive identification of serogroup O1, which must then be confirmed by agglutination with monovalent Ogawa and Inaba antisera. Identification of serogroup O139 should be performed with specific O139 antiserum (Kay *et al.*, 1994).

A series of biochemical tests may be used for further identification in addition to serological identification. A crucial biochemical test for distinguishing *V. cholerae* from other members of *Enterobacteriaceae* is the oxidase test. *V. cholerae* is oxidase-positive while all *Enterobacteriaceae* are oxidase-negative. Other important traits that may be used to distinguish *V. cholerae* include fermentation of glucose with acid production (without gas), maltose, mannitol, sucrose and trehalose. In a string test, a loopful of *V. cholerae* suspended in a drop of 0.5% aqueous deoxycholate gives a mucoid "string" when drawn. Inoculation of *V. cholerae* into Kligler iron agar (KIA) or triple sugar iron agar (TSI) produces an alkaline slant (K) over acid (A) butt, with no gas or H_2S (Kay *et al.*, 1994).

Conventional culture methods are time-consuming and labour-intensive. It takes at least 2 days to obtain results and may lack sensitivity, especially for poorly handled samples or clinical samples of patients previously treated with antibiotics as the number of viable and culturable organisms may be diminished. Detection may also be difficult if the number of viable organism is low, as in some environmental samples where *V. cholerae* can exist in a metabolic state that is non-culturable, but still remain viable and capable of causing disease (Colwell and Huq, 1994b).

1.11.2 Alternative methods of cholera diagnosis

1.11.2.1 Immunological-based tests for cholera

As an alternative to conventional culture methods, several rapid immunological-based tests have been developed. The earlier studies employed antibodies against *V. cholerae* O1 antigen in enzyme-linked immunosorbent assay (ELISA) format (Martinez-Govea *et al.*, 2001, Osek *et al.*, 1992, Bhadra *et al.*, 1991, Gustafsson, 1984, Cooper *et al.*, 1983), bead-ELISA format (Ramamurthy *et al.*, 1996, Ramamurthy *et al.*, 1992, Uesaka *et al.*, 1992, Cooper *et al.*, 1983), immunofluorescence microscopy (Goel *et al.*, 2005, Hasan *et al.*, 1994a, Huq *et al.*, 1990), coagglutination test (Goel *et al.*, 2005, Hasan *et al.*, 1995, Colwell *et al.*, 1992, Rahman *et al.*, 1989, Rahman *et al.*, 1987) and colloidal gold-based colorimetric immunoassay (Hasan *et al.*, 1994b). Immunoassays for *V. cholerae* O139 have also been developed using coagglutination test (Qadri *et al.*, 1994), immunofluorescence microscopy (Hasan *et al.*, 1995), colloidal gold-based colorimetric test (Qadri *et al.*, 1995) and dot-blot ELISA (Chaicumpa *et al.*, 1998).

More recently, antibody-based biosensors, called immunosensors, have been described. Jyoung *et al.* (2006) immobilized monoclonal antibodies against *V. cholerae* O1 on a self-assembled monolayer (SAM), whereby binding of O1 antigen to the antibody can be detected using surface plasmon resonance (SPR) spectroscopy. Rao *et al.* (2006) utilized an indirect ELISA method in developing an amperometric immunosensor for detection of *V. cholerae* O1 on disposable screen-printed electrodes.

Most of the immunological assays offer the advantages of speed, simpler reagents and equipments, better sensitivity and specificity over culture methods. Several tests such as CholeraScreen, Cholera DFA and Cholera SMART have been reported to be able to detect VBNC cells from faecal specimens (Colwell and Huq, 1994b). However, only a few tests are commercially available, namely SMART™ and Medicos™ Cholera Dipstick to detect the O1 serogroup but these tests are quite expensive and well beyond the reach of medical providers in areas at risk of cholera (Kalluri *et al.*, 2006).

1.11.2.2 Molecular-based tests for cholera

Recently, there has been a move to adopt molecular-based methods as a gold standard to replace the traditional culture methods. The use of molecular techniques that rely on nucleic acid amplification, such as polymerase chain reaction (PCR), ligase chain reaction (LCR), transcription-mediated amplification (TMA), strand displacement amplification (SDA) and nucleic acid sequence-based amplification (NASBA) have greatly improved the speed, sensitivity and specificity of diagnostic tests. This, in turn, would facilitate early and informed decision-making related to patient management, infection control, treatment and prevention.

PCR assays have been developed to detect the cholera enterotoxin operon, since most of the cholera pandemic and epidemics were associated with enterotoxin producing *V. cholerae* (Koch *et al.*, 1993, Fields *et al.*, 1992, Shirai *et al.*, 1991). Subsequently, a multiplex PCR assay was developed to detect toxigenic strains of *V. cholerae* based on the cholera toxin gene and to identify its biotype based on the *hyl* gene (Shangkuan *et al.*, 1995).

As the new cholera pandemic involving the serogroup O139 began to emerge, various PCR tests were developed to detect this new serogroup O139 alone (Albert *et al.*, 1997), or both the serogroups O139 and O1 (Gubala, 2006, Lipp *et al.*, 2003, Lyon, 2001, Singh *et al.*, 2001, Albert *et al.*, 1997, Rivera *et al.*, 1995). Besides detecting the serogroups O1 and O139, Nandi *et al.* (2000) and Di Pinto *et al.* (2005) have developed PCR assays based on non-virulence genes such as the *ompW* and collagenase gene, respectively, which were able to detect certain non-toxigenic *V. cholerae* strains of the serogroups non-O1, non-O139. This serogroup is often found in environmental samples and causes sporadic and localized diarrheal outbreaks (Vital-Brazil *et al.*, 2002, Kaper *et al.*, 1994).

1.11.2.2.1 Characteristics and advantages of molecular-based tests over conventional methods

The advantage of PCR over conventional culturing in detecting viable but non-culturable (VBNC) cells of *V. cholerae* was proven in a study by Chaiyanan *et al.* (2001). Conservation of cholera toxin-associated genes like *ctxA*, *tcpA*, *toxR* and *zot* in chromosomal DNA of VBNC cells was demonstrated using PCR. It was shown that VBNC cells retained their viability, along with their genes and chromosomal integrity up to one year.

PCR assays are rapid, highly sensitive, specific and can detect more than one gene at the same time. Although the advantages of PCR-based test over conventional culture method are numerous, there are certain limitations to the test. PCR requires the use of a thermal cycler and even with recent advances in this field, the cost and size of a thermal cycler limit its use in laboratories with limited resources (Lai *et al.*, 2006). In addition, PCR reagents must be transported and stored at –20°C and maintaining a constant cold chain may not be possible when transporting to far or remote places.

These are important considerations in developing a diagnostic test for cholera, as cholera is a disease that affects most of the poor under-developed countries, or countries ravaged by war or natural disasters. Hence, there are not many PCR-based tests for cholera in the market, while those that are available commercially are immunochromatographic-based assays, which can be quite expensive and still require refrigeration for long term storage (Kalluri *et al.*, 2006).

PCR can amplify DNA from both live (viable) and dead (non-viable) cells, since DNA can remain intact and undegraded long after cell death. Thus, PCR-based tests cannot differentiate viable from non-viable cells. Since cholera is transmitted mainly by contaminated food and water, detection of viable cells from these samples is desirable during an epidemic to identify the source of infection and implement control measures. It is important that positive test results are associated with live cells and not the remains of dead cell. Identification of live cells would also help clinicians in monitoring the effectiveness of

antimicrobial treatments in patients (Bej *et al.*, 1996). Detection of viable microorganisms after sterilization procedures is also important in the medical and food industry to ensure effectiveness of the process.

RNA has been suggested as a suitable indicator of cell viability as it rapidly degrades after cell death (Sheridan *et al.*, 1999, Sheridan *et al.*, 1998). Detection of RNA is possible through reverse-transcription PCR (RT-PCR), a modification of the PCR process, which first converts the RNA to cDNA, and subsequently the cDNA is amplified by PCR. RT-PCR is a multiple step process and even small amounts of DNA in the RNA sample might get amplified, thus giving a false-positive result. Therefore, all RNA samples need to be treated with DNase enzyme prior to RT-PCR.

1.11.2.3 Nucleic acid sequence-based amplification (NASBA)

Besides RT-PCR, another RNA-based amplification technique is nucleic acid sequence-based amplification (NASBA). NASBA is an isothermal amplification assay that specifically amplifies RNA. The reaction occurs at a relatively low temperature of 41°C that does not promote the denaturation of double-stranded DNA, hence DNA is not amplified (Deiman *et al.*, 2002). Furthermore, it can be performed using a simple water bath or heating block. NASBA produces more amplicons in a shorter time than PCR, requiring only 60 – 90 minutes of assay time, compared to 2 – 3 hours for RT-PCR. Amplification of approximately 10^6 to 10^9-fold is obtained within 90 minutes (Compton, 1991).

The single-stranded RNA amplicons can be detected using various methods, such as agarose gel electrophoresis (Jean *et al.*, 2004, Jean *et al.*, 2002b), molecular beacon-based real-time amplification technique (Loens *et al.*, 2008, Churruca *et al.*, 2007, Deiman *et al.*, 2007, Fykse *et al.*, 2007, Nadal *et al.*, 2007) and probe hybridisation techniques, such as Northern blotting (Jean *et al.*, 2004, Jean *et al.*, 2002b), ELISA (Lau *et al.*, 2008, Jean *et al.*, 2002a, Jean *et al.*, 2002b), electrochemiluminescence (ECL) (Lau *et al.*, 2008, Collins *et al.*, 2003, Hibbitts *et al.*, 2003), enzyme-linked gel assay (ELGA) (Lau *et al.*, 2008, Hibbitts *et*

al., 2003) and biosensors (Baeumner *et al.*, 2004a, Baeumner *et al.*, 2004b, Zaytseva *et al.*, 2004). These features make NASBA a suitable molecular-based test for cholera. Figure 1.1 shows an overview of the NASBA reaction.

NASBA was first reviewed in 1991 by Compton (1991), who described the salient features of the technology and its applications. Kievits *et al.* (1991) was among the earliest to describe the use of NASBA for the diagnosis of HIV-1 infection. Since NASBA is an RNA amplification technology, it is suitable for detection of RNA viruses, such as norovirus (Kou *et al.*, 2006, Patterson *et al.*, 2006, Rutjes *et al.*, 2006), enterovirus (Casper *et al.*, 2005, Landry *et al.*, 2005), hepatitis A virus (Abd el-Galil *et al.*, 2005) and cytomegalovirus (Bergallo *et al.*, 2006, Revello *et al.*, 2003).

NASBA provided researchers with a way of detecting viable microorganisms in clinical samples, food and environmental samples, as presence of intact RNA is a valuable indicator of viability. Detection of viable microorganisms is important for screening of contaminated food (Cook, 2003, Gore *et al.*, 2003) and environmental samples (Fykse *et al.*, 2007, Rutjes *et al.*, 2006, Baeumner *et al.*, 2003); monitoring the clinical course of a disease (Yoo *et al.*, 2005); and assessing the efficacy of antimicrobials and other treatments (de Vries *et al.*, 2006).

A real-time NASBA assay for *V. cholerae* was developed by Fykse *et al.* (2007) targeting genes encoding cholera (*ctxA*), toxin-corregulated pilus (*tcpA*), ctxA toxin regulator (*toxR*), hemolysin (*hlyA*) and the 60 kDa chaperonin product (*groEL*). The assay was able to detect *V. cholerae* from various environmental water samples and has a strong potential for detecting toxigenic strains using the *tcpA* and *ctxA* markers.

Besides detection of medically important microorganisms, other clinical applications of NASBA include detection of biomarkers and important human genes. NASBA has also been used to detect disease-causing microorganisms in the marine, agriculture and livestock industries (Lau *et al.*, 2008, Lau *et al.*, 2006, Olmos *et al.*, 2007, Teng *et al.*, 2006).

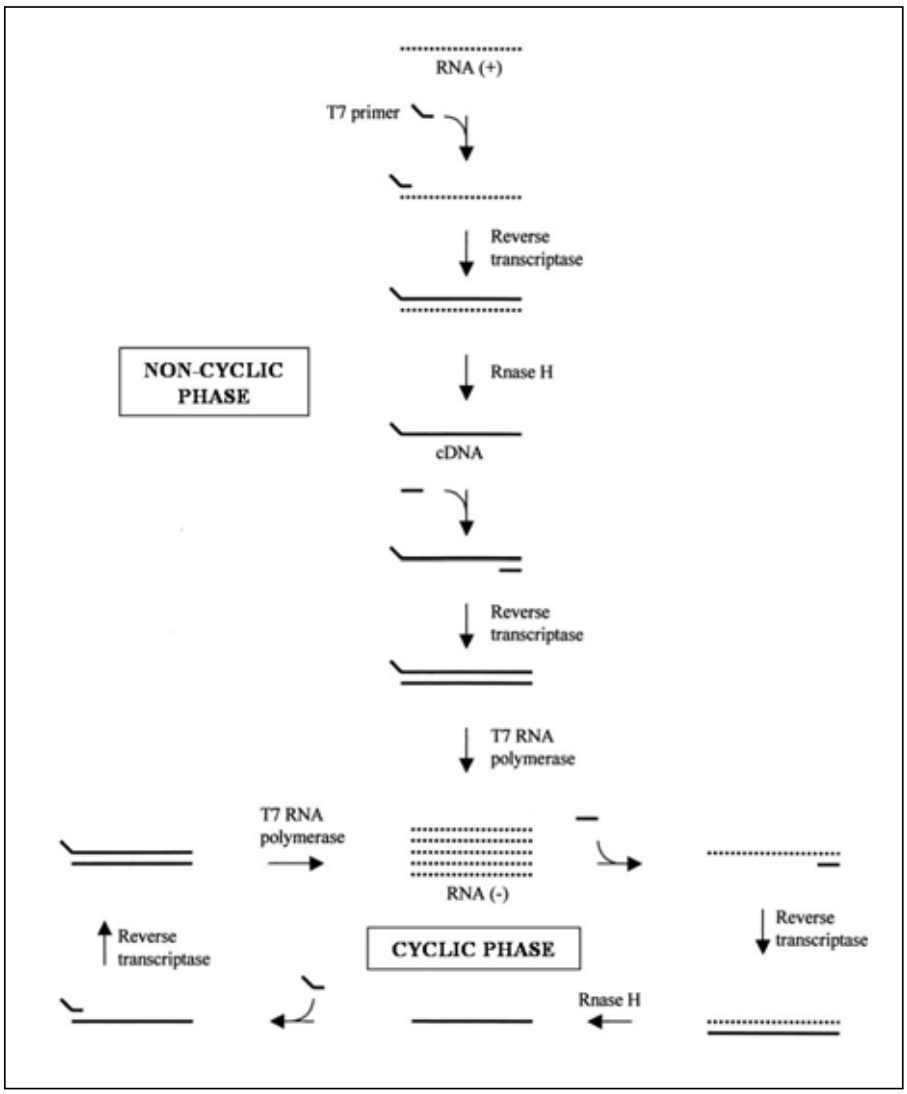


Figure 1.1: Overview of the NASBA reaction

1.12 Rationale of the study

Most of the molecular-based diagnostic tests that have been developed for *V. cholerae* are either PCR or real-time based PCR assays. These assays require constant cold chain for transport and storage of the PCR reagents, skilled laboratory technicians and sophisticated equipments to perform the tests. Moreover PCR assays detect DNA, which may still be present in dead cells; therefore it cannot be used to differentiate between viable and non-viable cells. Identification of viable *V. cholerae* is important especially during a cholera epidemic, where detection of viable cells from food and water samples is desirable to identify the source of infection and implement control measures. It is also useful for testing environmental samples for reservoirs of viable *V. cholerae*.

We have identified a non-virulence gene called *lol*B (*hem*M), which codes for an outer membrane protein in *V. cholerae*. The *lol*B gene sequence has been submitted to the Genbank under the accession number AF227752. The *lol*B gene sequence was found to be highly conserved among the different serogroups of *V. cholerae.* A PCR assay was developed that was able to detect O1, O139 and non-O1, non-O139 serogroups of *V. cholerae* from clinical and environmental samples with 98.5% sensitivity and 100% specificity (Lalitha *et al.*, 2008).

The present study used the *lol*B gene as a candidate in the development of a cholera NASBA-ELISA assay for detection of *V. cholerae*. The cholera NASBA-ELISA assay with biosensor detection would be a suitable detection method for viable *V. cholerae* as it does not require expensive and specialized equipments. The present study also aims to make NASBA easy to perform and to eliminate the need for cold storage of NASBA reagents through the use of our patent-pending thermostabilization technology (Ravichandran *et al.*, 2005), which has been successfully applied in thermostabilization PCR assays for typhoid and cholera (Aziah *et al.*, 2007, Ravichandran *et al*., 2003). To the best of our knowledge, this is the first time a thermostabilized cholera NASBA-ELISA assay featuring the use of a portable biosensor is described.

1.13 Objectives of the study

Phase 1: Development and optimization of a cholera NASBA assay

1. To design specific primers and probes for cholera NASBA assay
2. To construct *in vitro* RNA transcripts as positive control
3. To develop and optimize a cholera NASBA assay targeting the *lol*B gene of *V. cholerae*
4. To analyze the NASBA reaction and its amplicons
5. To test the suitability of *lol*B mRNA as an indicator of cell viability

Phase 2: Development and optimization of ELISA for detection of cholera NASBA amplicons (cholera NASBA-ELISA)

6. To develop and to optimize an ELISA test for detection of NASBA amplicons by comparison of two ELISA formats
7. To evaluate the cholera NASBA-ELISA assay with spiked stool samples
8. To calculate the diagnostic sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the clinical evaluation

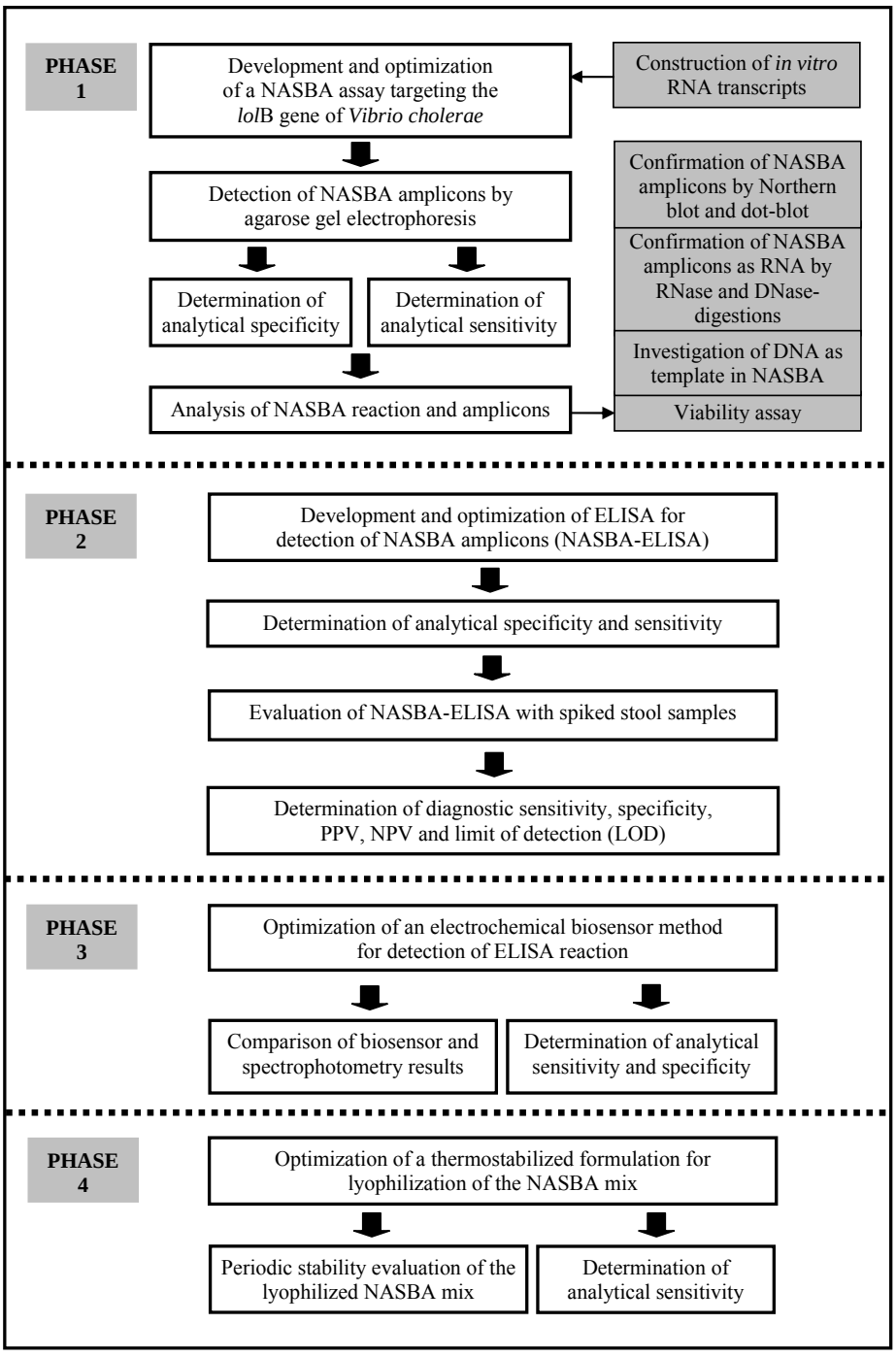
Phase 3: Development of an electrochemical biosensor detection for cholera NASBA ELISA assay

9. To optimize detection of the NASBA-ELISA signal with an electrochemical biosensor
10. To compare the biosensor results with spectrophotometric measurements

Phase 4: Optimization of a thermostabilized NASBA mix

10. To optimize the enzymes and enzyme stabilizer concentrations for thermostabilization of the NASBA mix
11. To determine the stability of the thermostabilized NASBA mix stored at different temperatures over a certain period of time

1.14 Overview of the study



CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Bacterial strains

2.1.1.1 Reference strains

Six *V. cholerae* strains comprising of O1, O139 and non-O1, non-O139 serogroups were used as reference strains for optimization and evaluation of the sensitivity of the NASBA assay. Six other *Vibrio* species strains and 29 enteric pathogen strains were used as reference strains for evaluating the specificity of the NASBA assay. The *V. cholerae*, *Vibrio* spp. and 10 enteric pathogen strains were obtained from Prof. Mitsuaki Nishibuchi, Centre for Southeast Asia Studies, Kyoto University, Japan as a gift. The remaining 19 enteric pathogen strains were obtained from the culture collection of the Department of Microbiology and Parasitology, Universiti Sains Malaysia, Malaysia. Details of the reference strains used in this study are given in table 2.1.

2.1.1.2 Clinical isolates

For evaluation of the NASBA assay using spiked stool samples, 110 well-characterized strains of *V. cholerae* isolated from clinical samples were obtained from Centre for Southeast Asia Studies, Kyoto University, Japan; Communicable Diseases Hospital, Thandiarpettai, Chennai, India; Hospital Universiti Sains Malaysia (HUSM) and General Hospital Kota Bharu (GHKB), Kota Bharu, Kelantan; National Public Health Laboratory (MKAK), Ministry of Health, Sungai Buloh; and Institute for Medical Research (IMR), Selangor, Malaysia.

In addition, 45 well-characterized strains of other *Vibrio* species and various enteric pathogens isolated from clinical samples were also obtained from Centre for Southeast Asia Studies, Kyoto University, Japan; Hospital Universiti Sains Malaysia (HUSM), Kota Bharu, Kelantan and Institute of Medical Research (IMR), Selangor, Malaysia.

All 41 reference strains were also included in the evaluation of the NASBA assay using spiked stool samples as these were originally isolated from clinical samples. Details of the clinical isolates used in the study are given in table 2.2.

2.1.1.3 Growth and maintenance of bacterial strains

All bacterial strains were preserved and maintained as glycerol stock in LB broth with 15% glycerol and kept at -70°C. For experimental purposes, the bacterial strains were revived from glycerol stocks by inoculating a loopful of culture into LB broth and incubated overnight at 37°C. For *V. cholerae* strains, the overnight cultures were then subcultured onto TCBS agar while for other enteric bacteria, the overnight cultures were subcultured onto LB agar and incubated overnight at 37°C. *V. cholerae* appeared as yellow, convexed and mucoid colonies on TCBS agar. A single colony was picked with a sterile wire loop, streaked onto LB agar and incubated overnight at 37°C again. These cultures were used to perform the NASBA assay.

2.1.2 Culture Media

Culture media were prepared using molecular biology or biotechnology grade chemicals. Distilled water was used for preparing media solutions. The appropriate media were autoclaved at 121°C, 15 psi for 15 minutes. Sterility check was performed by incubating the media at 37°C overnight. Sterile agar media were stored at 4°C while broth and other liquid media were stored at room temperature. The list of culture media used in this study is given in appendix A.

Table 2.1: Details of the reference strains used in this study

Reference strains	Source	Serogroup or species	No. of strains
Vibrio cholerae (n = 6)	Centre for Southeast Asia Studies, Kyoto University, Japan	*V. cholerae*, O1 Classical	2
		V. cholerae, O1 El Tor	2
		V. cholerae, O139 Bengal	1
		V. cholerae, non-O1, non-O139	1
Other *Vibrio* spp. (n = 6)	Centre for Southeast Asia Studies, Kyoto University, Japan	*V. cincinnatiensis*	1
		V. fluvialis	1
		V. furnissii	1
		V. mimicus	1
		V. parahaemolyticus	1
		V. vulnificus	1
Enteric pathogens (n = 29)	Centre for Southeast Asia Studies, Kyoto University, Japan	*Aeromonas hydrophila*	1
		Escherichia coli	2
		Pleisiomonas shigelloides	1
		Salmonella enteritidis	1
		Salmonella typhi	1
		Shigella boydii	1
		Shigella dysenteriae	1
		Shigella flexneri	1
		Shigella sonnei	1
	Department of Microbiology and Parasitology, Universiti Sains Malaysia	*Acinetobacter* spp.	1
		Acinetobacter baumanii	1
		Bacillus cereus	1
		Bacillus subtilis	1
		Burkholderia pseudomallei	1
		Citrobacter freundii	1
		Enterobacter cloacae	1
		Klebsiella spp.	1
		Klebsiella pneumoniae	1
		Morganella morganii	1
		Proteus mirabilis	1
		Proteus vulgaris	1
		Providencia stuartii	1
		Pseudomonas aeruginosa	1
		Salmonella spp.	1
		Salmonella para typhi A	1
		Salmonella para typhi B	1
		Serratia marsescens	1
		Yersinia enterocolitica	1
		TOTAL	**41**

Table 2.2: Details of the clinical isolates used in this study

Clinical isolates	Source	Serogroup or species	No. of isolates
Vibrio cholerae (n = 116)	Hospital Universiti Sains Malaysia (HUSM), Kelantan, Malaysia	*V. cholerae*, O1	8
	General Hospital, Kota Bharu (GHKB), Kelantan, Malaysia	*V. cholerae*, O1	17
	National Public Health Laboratory, Ministry of Health, Sungai Buloh, Selangor, Malaysia	*V. cholerae*, O1	6
	Institute for Medical Research (IMR), Selangor, Malaysia	*V. cholerae*, O1 *V. cholerae*, O139 Bengal *V. cholerae*, non-O1, non-O139	55 1 3
	Communicable Diseases Hospital, Thandiarpettai, Chennai, India	*V. cholerae*, O139 Bengal	9
	Centre for Southeast Asia Studies, Kyoto University, Japan	*V. cholerae*, O1 *V. cholerae*, O139 Bengal *V. cholerae*, non-O1, non-O139	14 2 1
Other *Vibrio* spp. (n =12)	Centre for Southeast Asia Studies, Kyoto University, Japan	*V. cincinnatiensis* *V. fluvialis* *V. furnissii* *V. mimicus* *V. parahaemolyticus* *V. vulnificus*	1 3 2 2 1 2
	Institute for Medical Research (IMR), Selangor, Malaysia	*V. vulnificus*	1
Enteric pathogens (n = 68)	Centre for Southeast Asia Studies, Kyoto University, Japan	*Aeromonas hydrophila* *Escherichia coli* *Pleisiomonas shigelloides* *Salmonella enteritidis* *Salmonella typhi* *Shigella boydii* *Shigella dysenteriae* *Shigella flexneri* *Shigella sonnei*	1 4 1 1 1 1 1 1 1

Table 2.2 (continued)

Clinical isolates	Source	Serogroup or species	No. of isolates
Enteric pathogens (n = 68)	Department of Microbiology and Parasitology, Universiti Sains Malaysia	*Acinetobacter* spp.	1
		Acinetobacter baumannii	1
		Bacillus cereus	1
		Bacillus subtilis	1
		Burkholderia pseudomallei	1
		Citrobacter freundii	2
		Enterobacter cloacae	1
		Escherichia coli	1
		Klebsiella spp.	1
		Klebsiella pneumoniae	1
		Morganella morganii	1
		Proteus vulgaris	1
		Pseudomonas aeruginosa	2
		Salmonella enteritidis	1
		Salmonella typhi	1
		Serratia marsescens	1
		Shigella boydii	1
		Staphylococcus aureus	1
		Streptococcus group G	1
	Institute for Medical Research (IMR), Selangor, Malaysia	*Aeromonas hydrophila*	1
		Acenitobacter baumanii	1
		Bacillus cereus	1
		Burkholderia pseudomallei	1
		Enterococus faecalis	1
		Klebsiella spp.	1
		Klebsiella pneumoniae	1
		Proteus mirabilis	1
		Providencia stuartii	1
		Salmonella spp.	4
		Salmonella enteritidis	1
		Salmonella para typhi A	5
		Salmonella para typhi B	3
		Salmonella typhi	4
		Shigella boydii	2
		Shigella dysenteriae	2
		Shigella flexneri	1
		Shigella sonnei	2
		Staphylococcus aureus	1
		Yersinia enterocolitica	1
		TOTAL	**196**