DEVELOPMENT OF GOLD NANOPARTICLES BASED IMMUNOSENSOR DIPSTICK FOR THE DETECTION OF CHOLERA TOXIN

By

ENGKU NUR SYAFIRAH BT ENGKU ABD RAHMAN

Dissertation submitted to the School of Health Sciences, Universiti Sains Malaysia as partial fulfillment of the requirements for the Degree of Bachelor of Health Sciences (Biomedicine)

MAY 2012

CERTIFICATE

This is to certify that the dissertation entitled **"Development of gold nanoparticles based immunosensor dipstick for the detection of cholera toxin"** is the bonafide record of research work done by Miss Engku Nur Syafirah bt Engku Abd Rahman during the period from July 2011 to May 2012 under my supervision.

ACKNOWLEDGEMENTS

I am grateful to many people and admit sincerely that without their help, advice and encouragement this final year project would not have been possible. I appreciated their moral support, friendship, humor and valuable ideas. The following people deserve mention for their efforts.

First and foremost, I would also like to express my gratitude to my supervisor; Dr Chan Yean Yean to her queries and comments which helped me maintains a continuous enthusiasm for this work. Her enthusiasm, boundless ideas, expertise, experience, criticism, encouragement and challenges were very much appreciated.

A token of appreciation to Ang Geik Yong, Yu Choo Yee, Low Kim Fatt, Sis Balqis and Sis Elina with whom I have had the pleasure of working during final year project session. Their good deal of advice and guidance throughout kept me going positively. The completion of this dissertation was a pleasant experience due to the continuous support and cooperation from my dear colleagues and course mates.

The demanding hours of thesis writing often takes me away from my beloved family especially on semester breaks and holidays. No words to express love and understanding bestowed on my mum, Norakmal Ibrahim your caring voice and moral supports were really therapeutic to my fretfulness. Also goes to my late dad, Engku Abd Rahman who has been encouraged me nonstop till his last days.

My siblings were tolerating my absence or inability to spend time with them in family activities especially during semester breaks. To my dear brother, Engku Ahmad Syahidi and little sister, Engku Nur Syazwani, I deeply appreciate your moral support and encouragement and I thank you all from the deepest part of my heart. Your encouragement and believing on me keeps me going.

There have been lots of memorable moments during my four years course as a Biomedicine student at health campus, USM. I would like to acknowledge all these individuals that have touched my life. This dissertation is especially dedicated to all of you. Thank you.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS, ABBREVIATION

Symbols/ Abbreviation	Meanings
CtxA	Cholera toxin A subunit
CtxB	Cholera toxin B subunit
V. cholerae	*Vibrio cholerae*
LB agar/ broth	Luria-Bertani agar/ broth
cAMP	cyclic adenosine monophosphate
kDa	kilo Dalton
zot	Zonula Occludens Toxin
ace	Accessory Cholera Enterotoxins
cep	Core-Encoded Pilin
orfU	Product of unknown function from *V. cholerae*
G_{M1}	Ganglioside G_{M1}, natural receptor for CT
CT	cholera toxin
ORS	Oral Rehydration Solution
APW	Alkaline Peptone Water
YEP	Yeast Extract Peptone
TCBS	Thiosulfate-Citrate-Bile salts-Sucrose
TSI	Triple Sugar Iron
KSI	Kligler's Iron agar
NaCl	Sodium chloride
NaOH	Sodium hydroxide
K_2CO_3	Potassium carbonate
mAb	Monoclonal antibody

BSA	Bovine serum albumin
PBS	Phosphate buffer saline
WHO	World Health Organization
CDC	Centers for Disease Control and Prevention
ELISA	Enzyme-Linked Immunosorbent Assay
CHO	Chinese hamster ovary
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
NC	Nitrocellulose membrane
CFU/ml	Colony-Forming-Unit per milliliter volume
OD_{525}	Optical Density at 525 nm
x g	Values of revolutions centrifugal force (RCF) in units of times gravity
rpm	revolutions per minute

ABSTRAK

PEMBANGUNAN DIPSTIK IMMUNOSENSOR BERDASARKAN NANOPARTIKEL EMAS UNTUK PENGESANAN TOKSIN KOLERA

Hidup di negara miskin dan sanitasi yang tidak baik sering dikaitkan dengan penyakit cirit-birit bawaan air seperti taun. Toksin kolera yang dihasilkan oleh strain toxigenik *Vibrio cholerae* merupakan etiologi kepada penghasilan najis beras berair yang menyebabkan kejutan dan dehidrasi yang teruk. Dalam kajian ini, percubaan telah dibuat untuk membangunkan asai dipstik untuk mengesan toksin kolera. Pengoptimuman pH dan jumlah antibodi salutan untuk konjugasi anti-toksin kolera ke atas koloid emas telah dilakukan dengan menggunakan zarah asai pemberbukuan. Kepekatan garis kawalan dan ujian telah dioptimumkan dan pelbagai agen penyekat telah diuji untuk mengelakkan pengikatan tidak spesifik pada membran pengesan. Kestabilan konjugat emas kering telah ditentukan dengan mengoptimumkan kepekatan koloid emas dan sukrosa. Proses pengkulturan bagi penghasilan toksin kolera telah dioptimumkan dengan menguji jenis media, keadaan kultur, jumlah isipadu media, masa pengeraman dan suhu pengkulturan. Toksin kolera tulen yang dicairkan telah digunakan untuk ujian sensitiviti manakala spesifisiti asai dinilai dengan menggunakan pelbagai jenis strain bakteria. Kajian menunjukkan bahawa 0.20 mg/ml antibodi kambing anti-mencit dan 5 µg/ml G_{M1} didapati merupakan kepekatan optimum untuk garis kawalan dan ujian, masing-masing. Tiga peratus BSA telah didapati sebagai reagen penghalang yang optimum. Konsistensi yang optimum dalam pembebasan emas koloid ditunjukkkan oleh emas koloid ditambah dengan 3% BSA dan 10% sukrosa dalam 2 mM PBS, masing-masing. Isipadu 10 ml media YEP dikultur dengan menggunakan kaedah AKI-SW selama 20 jam pada 37°C telah didapati menjadi kaedah terbaik untuk menghasilkan toksin kolera. Had pengesanan sensitiviti dipstik ialah 10 ng/ml manakala spesifisiti ialah 100% dengan tiada keputusan tidak sah. Oleh itu, asai dipstik membenarkan perbezaan antara strain *V. cholerae* toxigenik dan

bukan toxigenik melalui pengesanan toksin kolera yang dapat membantu pengawasan kes-kes taun.

ABSTRACT

DEVELOPMENT OF GOLD NANOPARTICLES BASED IMMUNOSENSOR DISPTICK FOR THE DETECTION OF CHOLERA TOXIN

Living in poverty stricken and poor sanitation countries often linked to water-borne diarrheal disease like cholera. The cholera toxin produced by toxigenic *Vibrio cholerae* strains are the etiology that results in profuse secretion of rice watery stool, leads to severe dehydration and shock. In this study, attempts were made to develop a dipstick assay for cholera toxin detection. Optimization of pH and amount of coating antibody for colloidal gold-anti-cholera toxin conjugation was done using particle flocculation assay. The control and test line concentrations were optimized and various blocking agents were tested to prevent the non-specific bindings on detection membrane. The stability of the dried gold conjugate was determined by optimizing the concentration of colloidal gold and sucrose. The culture for cholera toxin production was optimized by testing with different types of media, culture conditions, media volume, incubation time and temperature. A serially diluted pure cholera toxin was used for sensitivity test while specificity of dipstick was evaluated using others bacterial strains. Study showed that 0.20 mg/ml of goat anti-mouse and 5 µg/ml of G_{M1} were found to be the optimum concentrations for control and test lines, respectively. Three percent BSA was found to be the optimal blocking reagent. The optimum consistency in colloidal gold release was showed by colloidal gold added with 3% BSA and 10% sucrose in 2 mM PBS, respectively. The 10 ml volume of YEP broth culture using AKI-SW method for 20 hours at 37°C were found to be the best method for cholera toxin production. The detection limit of dipstick sensitivity was found to be 10 ng/ml while specificity was found to be 100% with no invalid results. Thus, the dipstick assay permits rapid differentiation between toxigenic and non-toxigenic *V. cholerae* strains via cholera toxin detection that helps in surveillance of cholera cases.

CHAPTER 1: INTRODUCTION

1.1 History of cholera

Cholera was known in the classical world and becomes a major infectious disease in the recent past with a global increase in its incidence. During the fifth pandemic in 1883, *Vibrio cholerae* has been described and identified as the causative agent of cholera (Koch, 1884). In cholera history timeline, 7 pandemics had struck the world since 1817 till the present day. Six pandemics of cholera were proposed by Pollitzer: first pandemic between 1817 and 1823; second pandemic between 1829 and 1851; third pandemic between 1852 and 1859; fourth pandemic between 1863 and 1851; fifth pandemic between 1881 and 1896; and sixth pandemic between 1899 and 1923 which has been responsible by the classical biotype (Pollitzer *et al.*, 1959).

However, this classical biotype has been completely displaced worldwide (Samadi *et al.*, 1983) with the seventh and current cholera pandemic causative agent since 1961, the El Tor biotype (Wachsmuth *et al.*, 1994). According to previous studies, cholera infections had spread out from Southeast Asia including Malaysia to Asian mainland (Barua and Greenough, 1992; Kaper *et al.*, 1995). It also cause large-scale outbreaks in the Middle East like Arabian Peninsula, Syria, Jordan as well as sub-Saharan West Africa in 1970 (Cohen *et al.*, 1971; Goodgame and Greenough, 1975; Kaper *et al.*, 1995).

Nevertheless, in 1992 at India, the emergence of new serogroup associated with cholera, *V. cholerae* O139 has raised the concern that this may be the beginning of the eighth pandemic of cholera (Bhattacharya *et al.*, 1993a). At first, the responsible culprit was referred to non-O1 *V. cholerae* because it did not agglutinate with O1 antiserum (agglutination occurs with O1 antiserum indicates the O1 strains). Further studies had exposed that the organism belongs to new serogroup known as O139 Bengal after the area where the strains were first isolated (Bhattacharya *et al.*, 1993b).

1.2 Descriptions of *V. cholerae*

1.2.1 General features

V. cholerae, a member of family *Vibrionaceae* is a facultatively anaerobic, oxidase-positive and gram negative bacterium. With 1.4 – 2.6 μm in length, it is non-spore forming curved rod that is capable of reducing nitrates and fermentative metabolism. This bacterium is motile by means of a single polar flagellum (WHO, 1993). The pathogenesis of cholera is well known due to the ingestion of contaminated food and water. The massive, profuse secretion of rice watery diarrhea is the characteristic of cholera caused by cholera toxin (CT) produced by toxigenic strains of *V. cholerae*. The major serogroups of *V. cholerae* consists of 3 types: O1, O139 Bengal and non-O1/ non-O139.

V. cholerae of the O1 serogroup is commonly associated with epidemic and pandemic cholera due to CT production and considered as toxigenic strain. This serogroup can be further sub-divided into three serotypes: Ogawa, Inaba and Hikojima. Serotypes of Ogawa and Inaba are commonly found compared with Hikojima that is rarely isolated. *V. cholerae* O1 also can be divided into two biotypes: Classical and El Tor which all combinations can be found, i.e, classical strains which are Ogawa or Inaba and El Tor strains which are Inaba or Ogawa (Kaper *et al.*, 1995).

V. cholerae of the O139 Bengal was found in the early 1993 when the first report of a new epidemic severe cholera-like disease appeared in eastern India and Bangladesh. Initially, this organism was referred to the non-O1 *V. cholerae* as it did not agglutinate in the O1 antisera, however, further investigations done had revealed that it does not belong to the O1 serogroups but to a new serogroup, which was given the designation O139 and a synonym "Bengal" in recognition of the origin of this strain (Albert *et al.*, 1993). Generally, this organism appears to be the hybrid of the O1 strains and the non-O1 strains but it is indistinguishable from the typical El Tor *V. cholerae* O1 strains (Rhine and Taylor, 1994).

In recent years, all the isolates of *V. cholerae* from the biochemical tests that were negative for the O1 serogroup and O139 serogroup were referred to as "non-O1/ non-O139 *V. cholerae*". Due to the O139 emergence, now one might refer to O2 until O138 as non-epidemic *V. cholerae* because majority of these strains did not produce the CT. These organisms commonly found in estuarine environments and they are usually isolated from sporadic diarrhea cases which has been caused by contaminated shellfish and also

isolated from extraintestinal infections including wounds, ear, sputum, urine and cerebrospinal fluids (Hughes *et al.*, 1978; Morris and Black, 1985).

1.2.2 Mode of cholera toxin action

A dynamic 4.5 kb core region termed as the virulence cassette (Trucksis *et al.*, 1993), has been identified in toxigenic strains of *V. cholerae* but not in the non-toxigenic strains. It is known to carry at least six genes, including the *ctxAB*, *zot* [encoding for zonula occludens toxin (Fasano *et al.*, 1991)], *ace* [encoding for accessory cholera enterotoxins (Trucksis *et al.*, 1993)], *cep* (encoding for core-encoded pilin) and *orfU* (encoding the product of unknown function).

The CT consists of A and B subunits with specific functions. The 5 identical B subunit serves to bind the holotoxin to the eukaryotic cell receptor which is the small intestine and the single A subunit possess a specific enzymatic function which acts intracellularly (Kaper *et al.*, 1995). The B subunit consists of 103 amino acids with a subunit weight of 11.6 kDa whereas the A subunit has weight of 27.2 kDa which proteolytically cleaved to yield two polypeptide chains, A1 and A2 components (Spangler, 1992). These are known as the major toxin causing the severe diarrhea cholera-like disease. The *zot* gene function is to increase the permeability of the small intestine mucosa by an effect on the structure of the intestinal tight junctions (Fasano *et al.*, 1991) while the *ace* affects the ion transport channels in the intestinal epithelium.

The main receptor for CT binding is the ganglioside G_{M1} which interact with the B subunit of CT and can be found on the epithelial cells of small intestines. The binding of CT to the epithelial cells is enhanced by neuraminidase produced by *V. cholerae* and leads to greater fluid secretion (Holmgren *et al.*, 1975). The B subunits of CT bind to G_{M1} ganglioside receptors on epithelial cells of intestinal mucosa and right after attachment, the A subunit and A2 subunit are cleaved which later on facilitating the entry of A1 subunit component into the epithelial cells. The A1 component stimulates the adenylate cyclase enzyme production which is responsible for the cyclic AMP (cAMP) production. The increment levels of cAMP leads to disruption of the electrolytes active transportation across the cell membrane which later on prevents the fluid absorption and results in fluid secretion into the small intestine. When the volume of fluid entering the colon from small intestine is greater than the reabsorptive capability, diarrhea occurs (CDC, 2010).

1.2.3 Clinical manifestation and treatment of cholera

Cholera gravis is the most severe manifestation of cholera infection which infects only minority of people. From previous studies, it showed that infections with classical strains are generally more severe than El Tor strains. The incubation period of cholera can range from several hours to five days, depends on the inoculum size. The transmission of cholera is mainly by the fecal–oral route which means, commonly from contaminated water or food consumption. The cases are occasionally seen in people who have eaten raw or undercooked shellfish, particularly oysters from contaminated waters (Morris, 2003).

The treatment for cholera cases is similar regardless of the serogroups types, the difference is only the severity of the illness itself. The key to cholera therapy is by supplying an adequate rehydration until the disease has run its course that takes one to five days in the absence of antimicrobial therapy. In severe cases, intravenous infusion of fluid is usually given or also by oral rehydration solution (ORS) for rehydration purpose which serve as the first line therapy (Morris, 2003). Antimicrobial agents play a secondary line therapy by decreasing the severity of illness and the duration of excretion of the organism (Kaper *et al.*, 1995).

1.3 Diagnosis of cholera

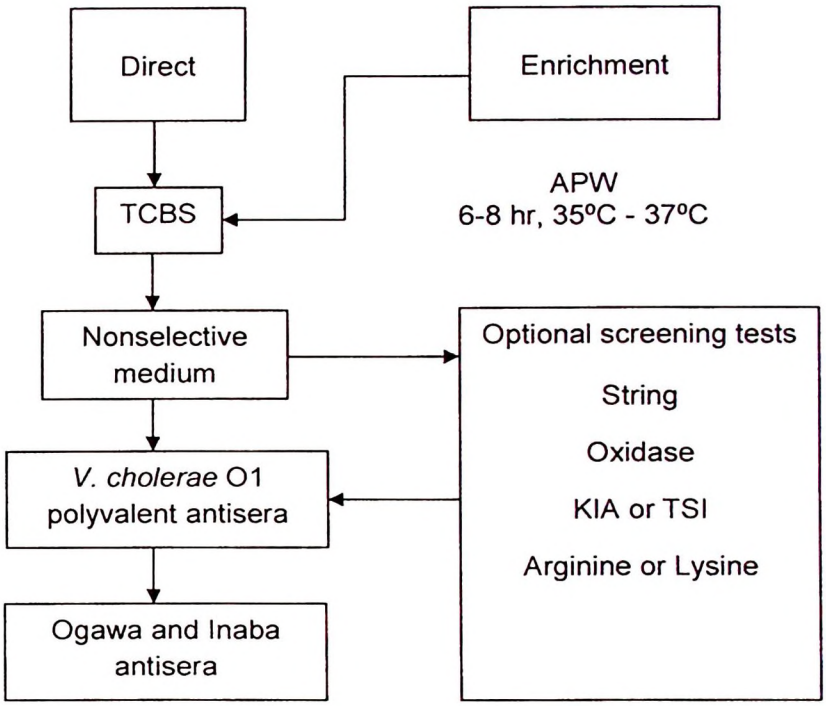
1.3.1 Detection of *V. cholerae* in laboratory

The conventional method that commonly used in laboratory is vibrios isolation that involves the collection of stool samples in the early of illness (before antimicrobial therapy given) in the alkaline peptone water (APW) in order to recover the low levels of vibrios, particularly from formed stools. Watery rice-like diarrhea usually does not need the enrichment media since the concentration of vibrios is so high, 10^7 to 10^8 per ml of liquid feces (Kaper *et al.*, 1995).

V. cholerae isolation is usually done by using thiosulfate-citrate-bile salts-sucrose (TCBS) agar. The sucrose-fermenting *V. cholerae* isolates are detected on TCBS as a large, yellow and smooth colonies (Gangarosa *et al.*, 1968). Slide serology test is usually sufficient for a primary identification of *V. cholerae* O1. However, it may be advantageous to screen with biochemical tests if the isolation of vibrios with TCBS is not sufficiently

selective to inhibit competitors from *Enterobacteriaceae*, *Aeromonas* and *Pseudomonas* (CDC, 2010; See **Figure 1.1**).

Types of biochemical screening tests that are commonly done are oxidase test, triple sugar iron (TSI) or Kligler's iron agar (KSI), Arginine or Lysine and String test (CDC, 2010). The oxidase test can rule out the non-vibrios like *Enterobacteriaceae* because it gives positive result for *V. cholerae* (Bagchi *et al.*, 1993). Whereas, the KSI and TSI rule out the *Pseudomonas* sp. and certain *Enterobacteriaceae* spp. Lysine is generally more helpful than Arginine for screening out the *Vibrio* spp. (Kaper *et al.*, 1995) and the String test is useful to rule out the non-*Vibrio* spp. especially *Aeromonas* spp. Other characteristics of *V. cholerae* from biochemical tests results are positive for Indole production (Minami *et al.*, 1991; Bagchi *et al.*, 1993), positive for Methyl Red (Kaper *et al.*, 1995), Citrate test and negative for Voges-Proskauer test (Feeley, 1965).



Adopted from: Laboratory methods for the Diagnosis of *Vibrio cholerae* from IV section: Isolation of *Vibrio cholerae* from fecal specimens. Page 21. Figure IV-4.

Figure 1.1: Procedure for recovery of *V. cholerae* from fecal specimens (CDC, 2010)

Serological identification of *V. cholerae* O1 or O139 strains are done due to its rapid and specific way of identifying the strains compared with biochemical tests listed above. Although identifying the serogroup and serotype of *V. cholerae* isolates are unnecessary for cholera treatment but this information may be important for epidemiologic and public health.

Currently, presence of somatic O antigen on *V. cholerae* that has been identified is more than 130 serogroups but only the serogroup O1 so far is associated with serious epidemic and pandemic cholera whereas serogroup O139 is associated with epidemic cases (Albert *et al.*, 1993; Ramamurthy *et al.*, 1993). Other serogroups may be related with severe diarrhea but mostly they do not agglutinate with O1 antisera. The serotype identification is based on agglutination in antisera to type-specific O antigens (CDC, 2010).

The O antigen of *V. cholerae* O1 strain consists of three factors designated A, B and C; the A factor may be the D-perosamine homopolymer but the B and C factors remains unknown (Stroeher *et al.*, 1992). The difference among the subtypes are quantitative; the Inaba strains produce only A and C antigens while Ogawa produce A and B antigens and small amount of C. Whereas Hikojima subtype contains all three antigens, thereby reacting with both Inaba and Ogawa strains but its rarely found (Kaper *et al.*, 1995).

1.3.2 Detection of cholera toxin (CT)

There are several methods to detect the CT including tests for toxin activity, toxin antigens and toxin coding genes but they are not routinely done for the identification of *V. cholerae* because the ease of identifying this species by conventional methods. The selection of a specific assay depends on the training, experience and facilities available in the laboratory. There are three type of assays that are commonly used; bioassays (animal methods and tissue culture methods), immunoassays (ELISA) and DNA-based assays (PCR) (CDC, 2010; See **Figure 1.2**).

Rabbit ileal loop, infant rabbit assay and rabbit skin tests are known as the animal methods. They are usually used to study the mechanisms of action of CT via injections of CT into ligated segments of intestine of rabbits that causes accumulation of fluid. However, this method not only causes excessively stress to the animals but is also time-consuming, cumbersome and difficult to standardize as well as expensive (Burrows and Musteikis, 1966). As well known tissue culture method is very sensitive and reproducible which explains the extensively used assay for CT detection (CDC, 2010). However, tissue culture method requires skilled personnel, special reagents and facilities to perform the work. Detection of CT using tissue culture, Y1 mouse adrenal (Y1) and Chinese hamster ovary (CHO) cell cultures are commonly used. When the supernatant containing CT is added to culture cells, it stimulates the adenylate cyclase which elevates the intracellular concentration of cAMP. The increased amounts of cAMP lead in morphological response onto the cells which can be seen under microscope like CHO cells elongates and Y1 cells become rounded (Donta *et al.*, 1974; Sack and Sack, 1975).

Assay	Sensitivity (per ml)	Type of assay	Specific target of assay	Sample tested
Rabbit ileal loop	30 ng	Bioassay	Stimulation of fluid accumulation	Culture supernatant
Infant rabbit assay	250 – 500 ng	Bioassay	Stimulation of fluid accumulation	Broth culture or supernatant
Rabbit skin test	0.1 – 3.5 ng	Bioassay	Permeability factor	Culture supernatant
Y1 mouse adrenal cells	10 pg	Bioassay	Accumulation of cAMP	Culture supernatant
Chinese hamster ovary cells	10 pg	Bioassay	Accumulation of cAMP	Culture supernatant
G_{M1}-ELISA	10 pg	Immune	B subunit [b]	Culture supernatant
Co-agglutination	50 ng [a]	Immune	B subunit [b]	Culture lysates
Reverse passive latex agglutination	1 – 2 ng	Immune	B subunit [b]	Culture supernatant
DNA probe	Detects *ctx* gene	Genetic	*ctx* gene	DNA (colony biot)
PCR	Detects *ctx* gene	Genetic	*ctx* gene	DNA (crude cell lysates)
[a] Sensitivity for detection of cholera toxin using antiserum to *E. coli* heat-labile enterotoxin [b] B-subunit of cholera molecule				

Adopted from: Laboratory methods for the Diagnosis of *Vibrio cholerae* from VII section: Detection of cholera toxin. Page 64. Table VII-1.

Figure 1.2: Commonly used methods for detection of CT (CDC, 2010)

Enzyme-linked immunosorbent assay (ELISA) is a well-known immunoassay and has been used as diagnostic tool due to its high sensitivity. The discovery of G_{M1} ganglioside as the natural receptor for CT has led to development of ganglioside-capture enzyme-linked immunosorbent assay (G_{M1}-ELISA) which used culture supernatants added into microtiter plate wells coated with G_{M1} ganglioside. The toxin bound to the G_{M1} receptors are then detected by adding the antiserum to CT followed by enzyme-conjugated antiglobulin antibody (CDC, 2010). Molecular tests which identify pathogenic microorganisms based on DNA sequences have many applications in diagnostic and public health microbiology. In polymerase chain reaction (PCR), the Taq DNA polymerase enzyme is used to amplify multiple copies of a specific DNA sequences that can then be detected on agarose gel (CDC, 2010). The toxigenicity of *V. cholerae* can be tested using PCR and primers that specifically amplify only the CT genes. Since PCR can synthesize DNA segments directly from sample, the presence of this bacterium can be determined without culturing the organism. Moreover, PCR gives more sensitive results compared with immunochromatographic assay and culture method but costly and requires skilled personnel to perform it (Huang *et al.*, 2009).

In comprising between conventional methods (animal methods and tissue culture methods) and current technology (immunoassays and DNA-based assays), the latest technology provides more advantages in terms of high specificity and sensitivity. For instance, the use of PCR avoids the difficulties encountered with *V. cholerae* strains which do not express CT at detectable levels but possess the *ctx* genes. Nevertheless, the current methods require specific training in molecular methods and more expensive reagents compared with conventional ones.

1.4 Dry surface immunoassay

In recent years, the dipstick assay has been indicated as a suitable medical diagnostic instrument which can provide rapid and reliable results in remote settings. In the literature, this dipstick assay is also known as lateral flow technology (LFT), lateral flow devices (LFD), lateral flow biosensor, immunochromatographic (IC) tests, dry surface immunoassays and dipstick tests (Kumar and Sinha, 2011; Yu *et al.*, 2011). Components of dry surface immunoassays comprised of sample pad, conjugate pad, detection membrane which commonly used is nitrocellulose (NC) membrane, and absorbent pad.

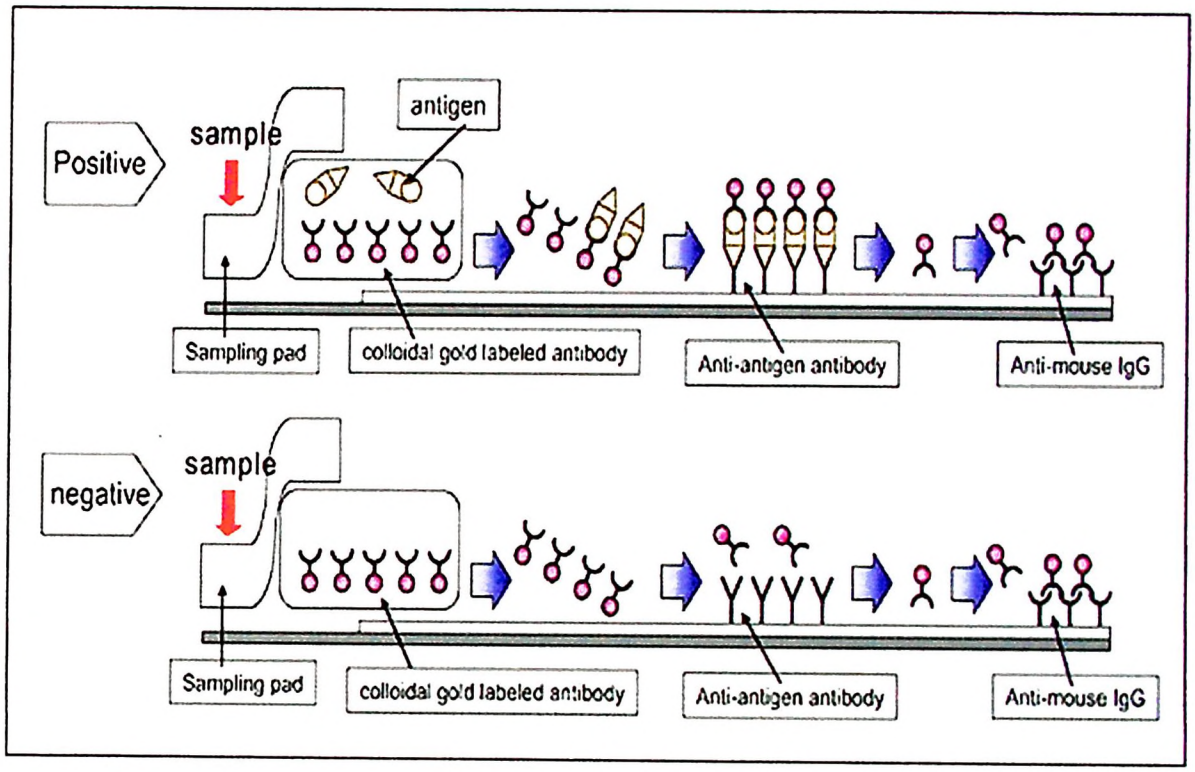
1.4.1 Dry surface immunoassay principle

The dry surface immunoassay is based on the principles of immunochromatography, similar to sandwich ELISA method but differs in which the reaction is carried out on the chromatography paper instead of microtiter wells. For dipstick assay system, two kinds of specific antibodies or protein receptors against the antigens are used. They are immobilized on the detection membrane (test line and control line) and the other is labeled with colloidal gold, dispensed on the conjugate pad and completed by attaching the sample pad at the end of the membrane (Biological laboratory, 2007).

When sample is applied to the sample pad, the antigen present in the sample forms an antigen-antibody complex with the colloidal gold conjugates as the sample migrates by capillary action diffuses through the conjugate pad and rehydrates the gold conjugates. The gold conjugates complexed then moves towards the capture target where it becomes immobilized and concentrated, producing a distinct signal in form of red line that indicates

positive for the antigen presence in the sample. The capture target situated known as the test line.

The second line (control line) formed on the membrane by trapping the excessive colloidal gold conjugates, indicates the test is complete and the result produced is true positive (Chandler *et al.*, 2000). Since the sample migrates through the membrane very fast, it makes it possible to detect the presence or absence of antigen within 10 – 15 minutes and the intensity of the line determines the amount of antigen presence (Paek *et al.*, 2000; Biological laboratory, 2007; See **Figure 1.3**; Zhang *et al.*, 2009).



Adopted from: Immunochromatographic assay. Figure of Principal of immunochromatography kit section.

Figure 1.3: Principle of dipstick assay for positive detection and negative detection of analytes (Biological laboratory, 2007)

1.5 Rationale of study

In recent years, cholera illness has become endemic in a large number of geographical areas around the world with immense global implications especially in poor undeveloped countries with improper sanitation (Tuteja *et al.*, 2007). In previous years, it had caused series of pandemic catastrophe which directly explains that it is a life-threatening disease caused by *V. cholerae* serogroups O1 and O139 (WHO, 1993).

Laboratory diagnosis of cholera consists of conventional methods which depends on the isolation and identification of the agent by biochemical tests, that are costly, time-consuming and of course tedious works. In addition, the confined facilities available in small laboratories for isolation and identification purpose make the detection of this organism difficult (WHO, 1974). Thus, it is therefore important to detect this agent as quickly as possible in clinical and environmental specimens, so that appropriate monitoring and effective preventive measures can be undertaken before the next cholera outbreak occurs (Tuteja *et al.*, 2007).

Consequently, studies show that efforts have been made to develop simple yet specific rapid diagnostic tests that would allow the detection of *V. cholerae* in the field and some has shown various degrees of success such as the Cholera SMART kit which showed 100% specificity and 96% sensitivity compared with conventional methods (Hasan *et al.*, 1994).

CT consists of *CtxA* and *CtxB* gene which is the virulence factor that distinguishes between toxigenic and non-toxigenic strains (Trucksis *et al.*, 1993; WHO, 1993). Presence of *CtxAB* gene indicates the vibrios are toxigenic to human and there are more than 95% of O1 and O139 serogroups of *V. cholerae* found to be toxigenic (Faruque *et al.*, 2003). Moreover, the current format of dipstick assay cannot specifically detect the toxigenic *V. cholerae* strains and therefore, another step would be needed to detect the CT production (Bhuiyan *et al.*, 2003). Since the cholera intervention depends on the toxigenicity and severity of the case, thus an urge to develop a specific and rapid diagnostic test to differentiate between the strains is a must rather than detect the organism itself.

Therefore, in this study an immunosensor dipstick incorporated with gold-nanoparticles for the detection of CT will be developed which carries a potential in simultaneous detection between toxigenic and non-toxigenic strains of *V. cholerae*. This dipstick assay is able to aid in surveillance in cholera cases as well as in clinical diagnostic in future.

CHAPTER 2: OBJECTIVES

The objectives of this research project are as follows:

General objective:

Development of an immunochromatography (ICT) dipstick for the detection of CT.

Specific objectives:

1. To conjugate the anti-CT monoclonal antibodies on gold nanoparticles
2. To optimize the parameters for the development of the dipstick for CT detection
3. To optimize the culture conditions for CT production
4. To evaluate the performance of the immunosensor towards CT

2.1 Overview of study

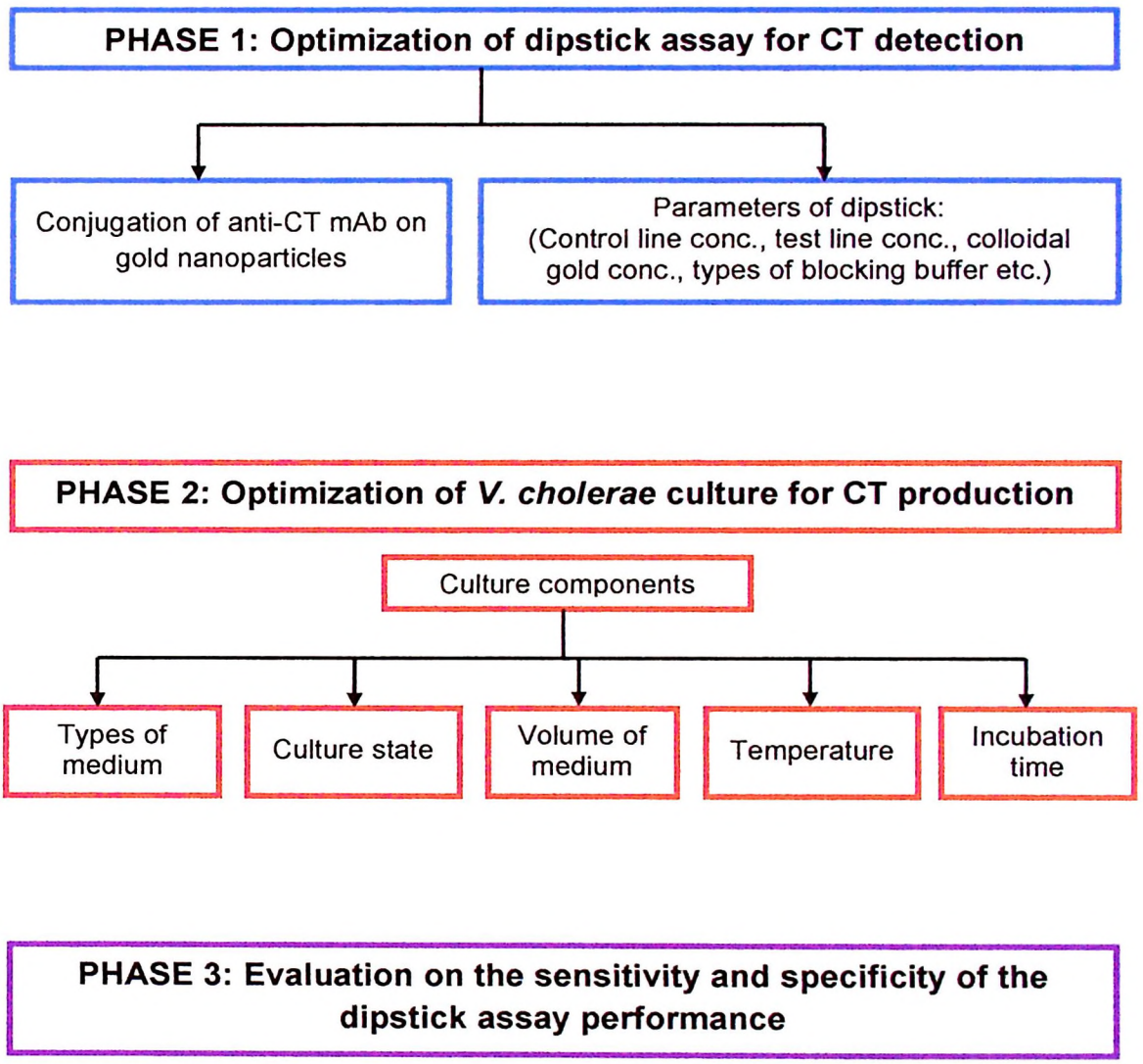


Figure 2.1: Dipstick assay and culture optimization plan

CHAPTER 3: MATERIALS AND METHODS

3.1 Materials

All the chemical reagents, media, laboratory equipments, bacteria strains and consumables used in this research project are listed in Table 3.1 until 3.4.

Table 3.1: List of chemical reagents and media

No	Name	Supplier
1	Sodium chloride (NaCl)	Amresco®, USA
2	Sodium hydrogen carbonate ($NaHCO_3$)	AnalaR®, USA
3	Potassium carbonate (K_2CO_3)	Sigma-Aldrich®, USA
4	Bacto™ Yeast Extract	BD (Becton & Dickinson), USA
5	Peptone from casein	Merck Chemicals, Germany
6	Agar-agar	Merck Chemicals, Germany
7	Phosphate Buffered Saline (PBS)	Amresco®, USA
8	Sucrose	Sigma-Aldrich®, USA

Table 3.2: List of laboratory equipments

No	Name	Company
1	BioPhotometer	Eppendorf, Germany
2	Centrifuge 5424	Eppendorf, Germany
3	Vortex mixer	IKA®, China
4	VersaMax microplate reader	Molecular Devices Inc, USA
7	Eureka Auto dry box	Taiwan Dry Tech Corp, Taiwan
8	Electronic balance	A&D Company, Japan
9	Cyber pH meter	Eutech Instruments, Singapore
10	Thermolyne Cimarec 2 Hot plate/ stirrer	Scientific Support, USA
11	Incubator 37°C	Memmert, Germany
12	Dry oven 56°C	BINDER Inc, USA
13	Incubator shaker	New Brunswick Scientific, USA
14	Water deionizer	ELGA, USA

Table 3.3: List of consumables, instruments and antibodies

No	Name	Company
1	Goat anti-Mouse IgG (H+L), Polyclonal	Thermo Fisher Scientific, USA
2	Monosialoganglioside G_{M1}, from bovine brain	Sigma-Aldrich®, USA
3	Anti-cholera toxin β-monoclonal antibody from mouse IgG	Pierce Antibodies, USA
4	Colloidal gold nanoparticles (40nm)	Heron Diagnostic, Thailand
5	Bovine Serum Albumin (BSA)	Amresco®, USA
6	96 Well ELISA microplate (Flat bottom)	Greiner bio-one, Germany
7	Syringe (20cc)	BD (Becton & Dickinson), USA
8	Filter (Minisart®)	Sartorius Stedim Biotech, France
9	Cellulose fibre sample pad (ref:CFSP173000)	Millipore™, USA
10	Glass fibre conjugate pad (ref:GFCP083000)	Millipore™, USA
11	Laminated nitrocellulose membrane card (ref:HF135MC100)	Millipore™, USA

Table 3.4: List of bacteria strains

No	Name	Supplier
1	*V. cholerae* El Tor Ogawa strain (J3321)	Archive sample
2	*V. cholerae* El Tor (UVC 1342)	Archive sample
3	*V. cholerae* Classical (J2127)	Archive sample
4	*V. cholerae* O139 (J2876)	Archive sample
5	*V. cholerae* non-O1/ non-O139 (J2119)	Archive sample
6	*Enterococcus sp.* (LMG 16192)	Archive sample
7	*Staphylococcus aureus* (ATCC 25923)	Archive sample
8	*Salmonella sp.* Braenderup strain (H9812)	Archive sample

3.2 Methods

3.2.1 Optimization of colloidal gold-anti-CT mAb conjugation

Determination of the optimal pH and concentration of coating monoclonal antibody (mAb), the anti-CT for colloidal gold-anti-CT mAb conjugation was carried out according to Yu *et al.* (2011). The selection of optimal pH for colloidal gold-anti CT mAb conjugate preparation was performed by adding 40 µg/ml of anti-CT into 1 ml of colloidal gold solution adjusted to pH 6.0, 7.0, 8.0, 9.0 and 10.0 using 0.2 M potassium carbonate (K_2CO_3), respectively. After 15 minutes of incubation at room temperature, 60 µl of 10% sodium chloride (NaCl) was added into the mixture and incubated for another 15 minutes. The OD_{525} (optical density at 525 nm) of the solution before and after the addition of 10% NaCl were measured. Any color changes occurred to the solution after addition of NaCl was recorded. The amount of decrease in absorbance after the addition of 10% NaCl versus pH of the colloidal gold solution was also plotted into graph.

Similarly, the determination of the optimal concentration of anti-CT mAb required to stabilize the colloidal gold was carried out by adding increasing concentration of anti-CT (1.25 µg/ml, 2.5 µg/ml, 5.0 µg/ml, 10.0 µg/ml, 20.0 µg/ml and 40.0 µg/ml) into 1 ml of colloidal gold with adjusted pH 9.0. After 15 minutes of incubation, 60 µl of 10% NaCl was added into the solution. The OD_{525} measurement before and after addition of 10% NaCl were recorded together with color changes that occurred on colloidal gold solution after addition of NaCl.

3.2.2 Preparation of colloidal gold-mAb conjugates

The colloidal gold-anti-CT mAb conjugates were prepared by adding 10.0 µg/ml of anti-CT mAb into 10 ml of colloidal gold solution (pH 9.0). The colloidal gold mixture was then mixed gently using belly dancer at room temperature for 1 hour. Then, 400 µl of 1% bovine serum albumin (BSA) was added to block any unreacted sites on the gold colloids and the mixture was incubated at room temperature for another 1 hour. Finally, the colloidal gold-anti-CT mAb conjugate was centrifuged at 8944 x g (10000 rpm) for 30 minutes and the supernatant was taken out, washed with wash buffer; 0.01 M phosphate buffer saline (PBS) (pH 8.0) added with 1% BSA (pH 8.0), for 3 times, respectively. After the last wash, the loosely packed sediment was resuspended in 400 µl of suspension buffer (0.01 M PBS (pH 8.0) containing 1% BSA and 0.05% sodium azide) and stored at 4°C before further use.

3.2.3 Optimization of dipstick assay components

3.2.3.1 Optimization of control line

Determination of optimal concentration of goat anti-mouse (control line) was carried out by testing on increasing concentration of goat anti-mouse (0.25 mg/ml, 0.20 mg/ml, 0.15 mg/ml and 0.10 mg/ml) from 2 mg/ml stock solution of goat anti-mouse antibody. The function of the control line is to capture the excess of colloidal gold-anti-CT mAb conjugates. The control line was lined up by manual pipetting with 1 µl of volume in the middle of NC membrane followed by 1 hour incubation in dessicator (17.5% humidity) for drying. Meanwhile, 3% BSA was prepared from 25% BSA stock as the blocking reagent in

order to prevent non-specific binding on the NC. After an hour, the NC membrane was blocked with 16.5 µl of 3% BSA followed by at least 1 hour of incubation in dessicator before further testing.

Evaluation of the control line functionality was done as follows: A 5 µl of 0.01 M PBS (pH 7.4) followed by 10 µl of colloidal gold conjugates and 130 µl of 0.01 M PBS were pipetted into micro wells of ELISA plate. After that, the NC strip immobilized with goat anti-mouse antibody was dipped into each of the wells for 3 mins. As the NC strip was dipped in first well, the PBS solution moves upwards by capillary action. Immediately afterwards, the NC strip was placed into 10 µl of colloidal gold conjugates for another 3 mins. Finally, the NC strip was dipped into 130 µl of 0.01 M PBS in order to wash out the remaining gold colloidal conjugates on the membrane via capillary action. As the flow goes, the NC strip was visualized for detection of red line signifying the presence of control line due to colloidal gold conjugates captured by goat anti-mouse immobilized on NC membrane.

3.2.3.2 Optimization of test line

Determination of optimal concentration of monosialoganglioside G_{M1} (test line) was carried out by testing on decreasing concentration of G_{M1} (1.0 mg/ml, 0.75 mg/ml, 0.50 mg/ml, 0.25 mg/ml, 0.15 mg/ml, 0.05 mg/ml, 25 µg/ml, 5.0 µg/ml and 2.5 µg/ml) from 2 mg/ml stock solution of G_{M1}. This test line function is to capture the CT that has been complexed with colloidal gold-anti-CT mAb conjugates during migration process which provides the yes/no result for CT presence. The test line was manually lined up with 1 µl of volume in the middle of NC membrane as well as 1 µl of 0.20 mg/ml goat anti-mouse as the control line, followed by 1 hour incubation for drying. The distance between each line was 5 mm.