CHAPTER IV
METERIALS AND METHODS

1. Detection Limit of Chole-Dot Test Kits on *V. cholerae* O1 and O139 Whole Cell Homogenate

V. cholerae O1 (O17SR) and *V. cholerae* O139 (VCMO45) whole cell homogenates were used to assess the limit of detection of Chole-Dot test kits. Three µl of two-fold serial dilutions of each whole cell homogenate were dotted onto nitrocellulose membrane (NCM; BioRad, Germany) in duplicate. All the membranes containing antigen dots were tested by dot-ELISA using instructed manual provided with the test kits (**section 4**).

2. Sample Size Calculation

The sample size for the study was calculated by the formula of Danial (1991):

$$N = z^2_{\alpha}pq/d^2$$

p = the rate of patients who were infected by *V. cholera* O1 at Bamrasnaradura Infectious Disease Institute, Nonthaburi, Thailand (Feb, 1998) was 11.31%. (p = 0.1131 and q = 1-p = 1- 0.1131 = 0.8869)

d = error allowance is 0.05 (the product of reliability coefficient and standard error) or one half the desired interval.

z_{α} = the standard normal deviate for a two-tailed α , where $(1-\alpha)$ is the confidence level (since $\alpha = 0.05$ for a 95% confidence level, $z_{\alpha} = 1.96$).

N = the total number of samples required

$$N = (1.96)^2 \times 0.1131 \times 0.8869/ (0.05)^2 = 154.10$$

Sample size based on a desired ability to detect *V. cholerae* O1 rate of 11.31% with the power of 80%. The required sample size was 154.

The sample size in this study should be at least two times of the sample size derived from the statisticalculation at least 308 samples would be used.

3. Culture, Isolation and Identification of *Vibrio* spp., *V. Cholerae* O1, and *V. Cholerae* O139 in Clinical Samples

Rectal swab samples were collected from patients with watery diarrhea who admitted at Bamrasnaradura Infectious Disease Institute (BIDI), Nonthaburi Province, Thailand. Each sample was tested in duplicate for O1 and O139 antigens by dot-ELISA using the Chole-Dot test kits on two different occasions but using the same lot of reagents.

From individual diarrhea patient admitted in Bamrasnardura Infectious Diseases Institute, Nonthaburi Province, Thailand, three rectal swabs were collected. One rectal swab was placed in the Cary-Blair medium (Oxoid, England) and was used for recovery of enterobacteriaceae. One swab was placed in selenite F broth and was used for *Salmonella* and *Shigella* isolation. One swab was put in alkaline peptone water (APW) and was used for recovery of *Vibrio* spp. and related bacteria including *Aeromonas* and *Plesiomanas.* The identification and isolation of enterobacteriaecae, *Vibrionaceae* and *Campylobacter* spp. in each sample were performed by a skilled laboratory bacteriologist at the institute. *V. cholerae* O1 and O139 were isolated according to method of Mindy *et al.* (WHO, 2003) as shown in **Figure 3**.

After the rectal swab was adequately enriched in the APW, aliquots were plated onto TCBS (Eiken, Japan) agar by a bacteriologist at the institute, the remaining APW was collected and subsequently transport in an icebox to the Faculty of Allied Health Sciences, Thammasat University (Rangsit Campus), Pathumthani, where detection of *V. cholerae* O1 and O139 antigens by specific MAb-based dot-ELISA were performed. Each APW was divided into two portions. One portion was boiled for 30 min and kept at -70 °C for subsequent antigen detection. Another portion was kept at -20 °C for further use in PCR analysis. For MAb-based dot-ELISA, three microliters of each boiled-rectal swab sample in alkaline peptone water was spotted on two nitrocellulose sheets in respective duplicate and air dried.

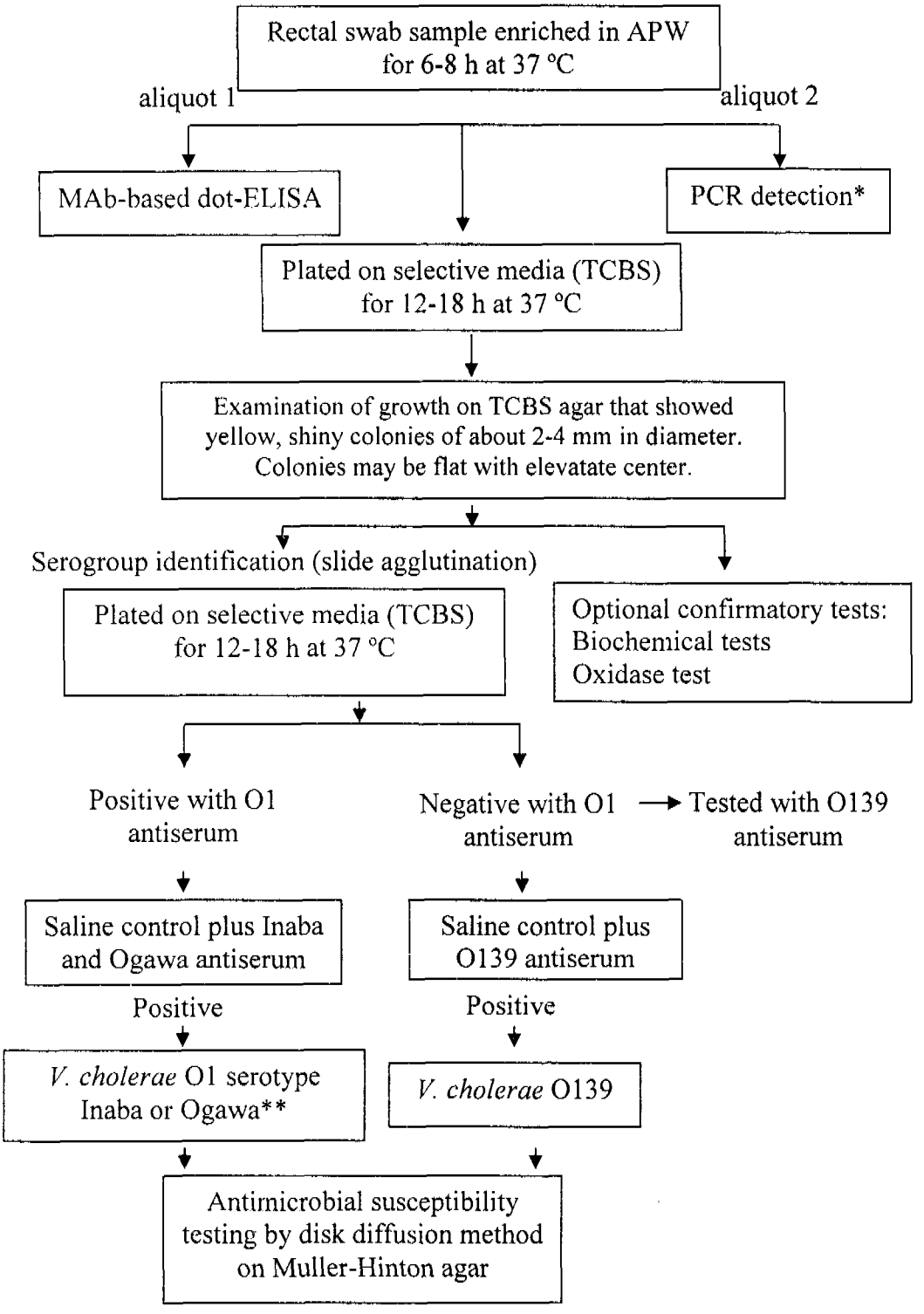

Figure 3 Flowchart for isolation and identification of *Vibrio cholerae* from rectal swabs Mindy *et al.* (WHO, 2003).

* When results of dot-ELISA and culture method were unconformed

** suspect Hikojima isolates were sent to National reference laboratory for confirmation

4. Chole-Dot Procedure for Detection of *V. cholerae* O1 and *V. cholerae* O139

Each Chole-Dot antigen test kit consists of

1. Starter kit; each starter kit contains 2 plastic boxes (1 test box and 1 control box), antibody-enzyme cobjugate, substrate, microcentrifuge tube.
2. Test kit; each test kit contains; monoclonal antibodies (MAb for *V. choleare* O1 and MAb for *V. cholerae* O139 (Chaicumpa *et al.*, 1995, 1998), blocking solution, washing buffer A (100 ml of stock solution). Working solution of washing buffer A is prepared by mixing 10 ml of the stock solution provided in the test kit with 240 ml distilled water. Washing B, control pieces, 2 pieces of 80 x 110 mm nitrocellulose membrane (NCM). On each NCM, there are 100 small squares, and 2 pieces of filter paper. The test procedures as given in the instruction manual of the test kits were followed.

The samples for this test were obtained from alkaline peptone water enriched rectal swabs. The cnriched samples were boiled for 30 min in order to (1) eliminate the background of non-specific reaction, (2) to release outer membrane antigens and (3) to disinfect the sample. Three microliters of each sample was applied onto each square area on two separate NCM strips, which were then used as "test strip" and "control strip". The strips were allowed to dry at room temperature (25 °C) for ten min. The test NCM strip and the control piece with positive and negative antigens (provided in the kit) were placed into the test plastic box. The control strip was sepalately placed into the control box. An adequate volume of blocking solution was poured into the test box and control box to cover the strips. The boxes were placed on rocking platform at 25 °C for 10 min. After the NCMs were washed three times with working buffer A for five min each, control NCM strip was kept in the washing buffer A while the test NCM strip and control piece were submerged in specific MAb. After incubating at 25 °C for 20 min all strips were washed as described above and then they were incubated with enzyme conjugates (dilution 1:3,000 in washing buffer A). Both strips and control piece were washed again with the buffer A after 30 min incubation and wash once with washing buffer B provided in the test kit. All NCMs were then placed in a substrate solution (diluted 1:2 in washing buffer B). The enzyme-substrate reaction was allowed to take place for 5 min in the dark. The reaction was stopped by rinsing the membrane with distilled water and aired dried.

The results must be read after the NCM strips were dried. Results were interpreted by observing the test sample areas on test strip in comparisom with the same sample area on the control strip NCM. The positive spot appeared as purplish-blue color on the membrane which could be clearly distinguished from the non-specific brown color or clear area of the control strip. The control piece, positive control and negative control must showed correct appearance.

5. Polymerase Chain Reaction (PCR)

Extensive study of the molecular basis of cholera pathogenesis has revealed that the expression of the most important virulence factors, namely CT and Tcp, is coordinated by a unique regulatory system. This regulatory system consists of three transcriptional activators, ToxT and TcpP located within the *Vibrio cholerae* pathogenicity island (VPI), and the third, ToxR, located within the ancestral *Vibrio* chromosome. The AraC-like transcriptional activator ToxT directly activates both the *ctx* and *tcp* gene clusters. *V. cholerae* strains lacking ToxT make no CT and Tcp and are avirulent (Champion *et al.*, 1997). In this study, *ctx* and *tcp* primers were used to identify *V. cholerae* in case of unconformed results occurred between the culture method and dot-ELISA for detecting *V. cholerae*. The primer sequences and PCR conditions of *ctx*A, *ctx*B and *tcp*A as described previously by Mitra *et al.* (2001), Keasler and Hall (1993), Rivera *et al.* (2001), and Varela *et al.* (1993, 1994) were used to identify these virulence genes of *V. cholerae*.

5.1 Preparation of DNA templates

The alkaline peptone water enriched-rectal swab sample (~0.8 ml) was centrifuged at 20 × *g* for 5 min at 25 °C (to get rid of debris). Then 0.5 ml of supernatant was transferred to a new tube. The sample was centrifuged at 12,000 × *g* for 10 min at 4 °C. The sediment was resuspended with 100 µl of DW and 200 µl of 5% Chelex solution (**Appendix C**) was added. The mixture was then mixed by vortexing and incubated at 56 °C for 30 min. It was subjected to boiling for 8 min. The preparation was then centrifuged at 12,000 × *g*, for 3 min and the supernatant was precipitated with absolute ethanol. The supernatant of 200 µl volume was added to absolute ethanol solution (500 µl) and 20 µl of 3 M sodium acetate (pH 4.2). The precipitation was kept at -20 °C overnight or at -70 °C for 1 h. DNA was pelleted by

centrifugation at 12,000 × *g*, for 5 min; the pellet was washed once with 70% ethanol and air-dried. It was resuspended in 50 µl of TE buffer and kept -20 °C until use.

5.2 Primer sets

The DNA primers specific for cholera toxin (*ctxA* and *ctxB*) gene of *V. cholerae* O1 and O139, the gene encoding toxin-coregulated pili (Tcp), the intestinal colonization factor, major subunit (*tcpA*), and the ToxR regulator gene (*toxR*) as shown in **Table 4** were used in this study. The sizes of amplicons were 301, 460, 620, 451 and 779 base pairs (bp) for *ctxA, ctxB, tcpA* (Classical biotype, El Tor biotype) and *toxR*, respectively.

5.3 PCR reaction mixture

PCR reaction mixture at the total volume of 25 µl contained 0.4 µM of each primer (*ctxA, ctxB, tcpA,* or *toxR* primers), 200 mM of each dNTP, 1x PCR reaction buffer (100 mM Tris-HCl (pH 8.8), 500 mM KCl, 0.8% Nonidet P40) (Fermentous, Lithuania), 1.5 mM $MgCl_2$ (Fermentous, Lithuania), 1 U *Taq* DNA polymerase (Fermentous, Lithuania), and 3 µl of Chelex 100 extracted-sample.

5.4 PCR amplification

Amplification was accomplished in an Eppendorf (Masterthermal gradient) thermal cycler, according to the following parameters (30 cycles): initial denaturation at 94 °C for 5 min; denaturation at 94 °C for 1.30 min; annealing at 60 °C for 1.30 min (for *ctxA*, *tcpA*, and *toxR*, but for *ctx* B used 55 °C instant); extention at 72 °C for 1.30 min; and final extention at 72 °C for 5 min.

5.5 Detection of the PCR products

PCR products (amplicons) were detected by agarose electrophoresis at 100 V for 60 min. Ten µl of the PCR amplicons were separated in a 1.5% agarose. Then the gel was stained with ethidium bromide solution (0.5 µg/ ml) for 10 min, washed with distilled water and examined under UV light using UVP transilluminator (Bio Doc-It, England). The sizes of *ctxA*, *ctxB*, *tcpA* (classical and El Tor), and *toxR* amplicons were 301 bp, 460 bp, 620 bp, 451 bp and 779 bp, respectively. The presence of respective genes was defined with the presence of a visible band at the expected

locations, while the sample which did not show visible specific amplicon was regarded as negative.

6. Data Analyses

6.1 Statistical analysis

The method of Galen (1980) was used for calculating the diagnostic specificity, diagnostic sensitivity and accuracy of the dot-ELISA in comparison with the results of bacterial culture method. Samples with unconformed results between the two tests were subjected to PCR for *ctx*, and *tcp*A for confirmation. The table and formula for calculating test agreement was shown below.

Results of MAb-based dot-ELISA	Culture method or PCR results		Total
	+	-	
+	TP(a)	FP(c)	TP + FP (p_2)
-	FN(b)	TN(d)	FN + TN(q_2)
Total	TP + FN(p1)	FP + TN(q1)	TP + FP + TN + FN (N)

The true positive (TP) is the number of *V. cholera* O1 and O139 infected patients (culture or PCR positive) correctly classified by the dot-ELISA

The false positive (FP) is the number of non-*V. cholerae* O1 and O139 infected patients (culture or PCR negative) but classified as positive reaction by the dot-ELISA

The true negative (TN) is the number of non-*V. cholerae* O1 and O139 infected patients (culture negative or PCR) correctly classified by the dot-ELISA

The false negative (FN) is the number of *V. cholerae* O1 and O139 infected patients (culture positive or PCR) but classified as negative reaction by dot-ELISA.

The diagnostic sensitivity is the probability that the dot-ELISA is positive when the culture or PCR for *V. cholerae* O1 and O139 is positive. It can be expressed in percentage as shown in the following formula:

$$(TP / TP + FN) \times 100$$

The diagnostic specificity is the probability that the dot-blot ELISA is negative when the disease is not present as tested by the culture method or PCR. It can be expressed as shown in the following formula:

$$(TN / TN + FP) \times 100$$

The efficacy (accuracy) of the dot-blot ELISA is the percentage of patients correctly classified as *V. cholera* O1 and O139 infection and non-*V. cholerae* O1 and O139 infection by the dot-ELISA as compared with the culture method or PCR. It can be expressed as shown in the following formula:

$$(TP + TN / N) \times 100$$

6.2 Kappa coefficient (κ)

Kappa coefficient (κ) (Cohen, 1960) was used to correlate detection of *V. cholera* O1 and O139 by dot-ELISA and culture method or PCR. The Kappa coefficient value was derived from the following formula:

$$\text{Kappa coefficient } (\kappa) = \frac{2(ad-bc)}{P_1q_2 + P_2q_1}$$

Where $a = TP$, $b = FN$, $c = FP$, $d = TN$

$p_1 = TP + FN$, $q_1 = FP + TN$, $p_2 = TP + FP$, $q_2 = FN + TN$

N = Total number of samples

Landis and Koch (1977) have characterized the different ranges of values of kappa coefficient (κ) with respect to the degree of agreement of the two test. The definitions of these values are expressed as the following:

Value of κ	Strength of agreement between the two tests
<0.20	Poor
0.21 - 0.40	Fair
0.41 - 0.60	Good
0.61 - 0.80	Very good
0.81 - 0.99	Excellent
1.00	Perfect

Table 4 Primers for amplification of *Vibrio cholerae* genes

Target	Primers and sequences	Amplicon size (bp)	Reference
ctxA	5'-CTC AGA CGG GAT TTG TTA GGC ACG-3' (forward) 5'-TCT ATC TCT GTA GCC CCT ATT ACG (reverse)	301	Mitra *et al.* (2001) Keasler *et al.* (1993)
ctxB	5'-GGT TGC TTC TCA TCA TCG AAC CAC-3' (forward) 5'-GAT ACA CAT AAT AGA ATT AAG GAT-3' (reverse)	460	Mitra *et al.* (2001)
tcpA (classical and El Tor)	5'-CAC GAT AAG AAA ACC GGT CAA GAG-3' (forward) 5'-CGA AAG CAC CTT CTT TCA CAC GTT G-3' (reverse) 5'-TTA CCA AAT GCA ACG CCG AAT G-3' (reverse)	620, 451	Rivera *et al.* (2001)
toxR	5'-CCT TCG ATC CCC TAA GCA ATA C-3' (forward) 5'-AGG GTT AGC AAC GAT GCG TAA G-3' (reverse)	779	Rivera *et al.* (2001)