# CHAPTER IV

# MATERIALS AND METHODS

## PART I : COLLECTION OF SPECIMENS

### 1. Clinical isolates.

The clinical isolates of *Vibrio parahaemolyticus* isolates from patients with diarrhea who admitted in King Chulalongkorn Memorial Hospital during 1999-2000 were received from Department of Microbiology, King Chulalongkorn Memorial Hospital.

### 2. Environmental isolates.

The environmental isolates of *Vibrio spp.* used in this study were collected in collaborated with the Department of Microbiology, Faculty of Veterinary Science, Chulalongkorn University in March and April 2000. All samples were randomly sampling from Chonburi and Suratthani province. Sediment samples were taken from shrimp pond and the gulf of Thailand, Shrimp samples, black tiger shrimp (*Penaeus monodon*), were taken from shrimp pond, Coastal water were taken

from shrimp pond and the gulf of Thailand and Shellfish samples, cockle (*Cerastoderma edule*), were taken from the gulf of Thailand. The collecting methods of specimen for all sample sorts are described as follows.

2.1) Shrimp samples (West and Cowell, 1984)

The hepatopancrease part was dissected and diluted 1:10 with 0.85% normal saline. The suspension was streaked on TCBS and incubated at 37°C, overnight. The green colonies were further examined by biochemical characteristic test.

2.2) Cockle samples (West and Cowell, 1984)

Cockle meat was diluted 1:10 with alkaline peptone water. The suspension was streaked on TCBS and incubated at 37°C, overnight. The green colonies were further examined by biochemical characteristic test.

2.3) Sediment samples (West and Cowell, 1984)

A 1 gram of sediment was diluted 1:10 with 9 ml of alkaline peptone water. The suspension was streaked on TCBS agar and incubated at 37°C, overnight. The green colonies were further examined by biochemical characteristic test.

2.4) Seawater samples (Palasuntheram and Selvarajah, 1980)

A 1 liter of seawater was filtered through Whatman NO.1 and then through millipore filter size 0.45 μ. The millipore filter membrane was inoculated into alkaline peptone water with 3% NaCl and incubated at 37°C, overnight followed by plating on TCBS agar. The green colonies were further examined by biochemical characteristic test.

## PART II : ISOLATION AND IDENTIFICATION

### 1. Biochemical characteristic test.

1.1) Media and reagents.

All isolates used in the study were identified by biochemical conventional method as described by Kim et al., 1999. All media and chemical reagents were allowed to equilibrate to room temperature prior to use. The name lists of media and reagents are shown in appendixes.

1.2) Culture preparation.

The preserved cultures at –70°C were enriched in alkaline peptone water (pH 8.6) at 37°C for 6-8 h before subculturing to non-selective media such as blood agar or nutrient agar.

1.3) Biochemical characteristic test.

23 biochemical characteristics tests were selected according to Kim et al., 1998. A single colony was picked and inoculated to testing media. The biochemical test lists and interpretation criteria for *Vibrio parahaemolyticus* are shown as described below.

1.3.1) Oxidase test

A 18-24 h growth colony from nonselective media is smeared with a sterile wooden applicator stick on a filter paper impregnated with oxidase reagent. *Vibrio parahaemolyticus* produces a dark purple color on the paper with in 10 seconds indicates a positive results.

1.3.2) Indole production

Inoculate peptone water + 1% NaCl and incubate for 24 h, 35°C. Add few drops of Kovac's reagent and shake well. *Vibrio parahaemolyticus* shows red color in the regent layer, which is indicated indole production.

1.3.3) Motility

Stab tubes of motility medium to a depth of about 5 mm. and incubate 24 h, 35$^{0}$C. *Vibrio parahaemolyticus* shows migration throughout the medium (tubid) which indicates a positive result.

1.3.4) Citrate Utilization

Streak the slope of Simmons' citrate medium surface and incubate 24 h, 35$^{0}$C. *Vibrio parahaemolyticus* shows original green color, which is indicated non-citrate utilized.

1.3.5) Methyl red reaction ( MR test )

Inoculate MR-VP medium and incubate 24 h, 35$^{0}$C. Add 2 drops of methyl red reagent then shake and examine. *Vibrio parahaemolyticus* produces positive MR reaction, which is shown by the appearance of red color at the surface. An orange or yellow color indicates negative result.

1.3.6) Voges-Proskauer ( VP test )

Inoculate MR-VP medium and incubate 24 h, 35$^{0}$C. Add 0.6 ml of VP-1 reagent then follow with 0.2 ml of VP-2 reagent, shake well. *Vibrio parahaemolyticus*

gives negative result which is shown by original color of reagent after 4 h.

1.3.7) TSI medium test

Inoculate a tube of Triple Sugar Iron agar by stabbing the butt and streaking the slope and incubate 24 h, 35$^{0}$C. *Vibrio parahaemolyticus* produces a purple (alkaline) slant and a yellow (acid) butt with no blackening of the butt, which is indicated hydrogen sulphide production.

1.3.8) NaCl requirement

Inoculate tryptone (1%) broth supplemented individually with 0, 3, 6, 8 and 10 % NaCl and incubate 24h, 35$^{0}$C then observe for turbidity. *Vibrio parahaemolyticus* grows in broth containing 3-8 % NaCl.

1.3.9) Decarboxylation reaction

Inoculate four tubes of Falkow's decarboxylation broth containing lysine, arginine, ornithine and control (basal medium). Add paraffin oil layer before tighten screw caps and incubate for 4 days, 35$^{0}$C. *Vibrio parahaemolyticus* produces purple color in broth containing amino acid lysine and orithine and yellow color in broth containing amino acid arginine and control.

1.3.10) Carbohydrate utilization

Inoculate bromthymol blue broth containing glucose, manitol, mannose, sucrose, lactose, arabinose, inositol and rhamnose then incubate 24 h, 35$^{0}$C. *Vibrio parahaemolyticus* produces a yellow color (acid production) in broth containing glucose, manitol, mannose and arabinose.

1.4) Interpretation criteria.

The result of all tests as noting above are interpreted according to Bergey's manual of determinative bacteriology 9$^{th}$ edition. The + symbol is noted as 90% or more strains are positive while the – symbol imply 90% or more of strains are negative.

## PART III : CULTURE PRESERVATION

### 1. Media for culture preservation.

Trypticase soy broth (BBL, Becton Dickinson and Company, Cockeysville, MD) containing 50% horse serum was used to preserve all culture strains in this study.

**2. Preservation method.**

All culture strains were grown entirely on tryptic soy agar (BBL, Becton Dickinson and Company, Cockeysville, MD) surface containing 1% NaCl at 37$^0$C. The overnight cultures were transferred in to micro-centrifuged tubes of 1 ml trypticase soy broth containing 50% horse serum and Mix-well suspensions were kept at –70 $^0$C until required as described by Ohashi et al. (1978)

## PART IV : REFERENCE BACTERIAL STRAINS

**1. *Vibrio spp.***

Reference strains of *Vibrio parahaemolyticus* ATCC 17802 and *Vibrio alginolyticus* ATCC 17749 were kindly provided by Prof. Takashi Aoki, Tokyo University of Fisheries, Tokyo, Japan and used as positive and negative control, respectively, in PCR amplification step.

**2. *Escherichia spp.* and *Pseudomonas spp.***

Reference strains of *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used for quality control of antimicrobial susceptibility test.

## PART V : ANTIMICROBIAL SUSCEPTIBILITY TEST

MIC determination by Etest was performed according to the manufacturer instructions (AB BIODISK, Solna, Sweden) as described below

### 1. Media and reagent.

Muller-Hinton agar (BBL, Becton Dickinson and Company, Cockeysville, MD) plates (100 mm) were used to perform Etest in this study. Ciprofloxacin Etest strips (AB BIODISK, Solna, Sweden) with concentration ranged from 0.002 to 32 μg/ml were stored in airtight container bedded with silica gel at $-20^{0}$C until required. The media plates and Etest strips must be allowed to reach room temperature prior to use.

### 2. Inoculum preparation.

The 3-5 single colonies of *Vibrio parahaemolyticus* with the same morphological type were selected from over night culture plates. The selected colonies were transferred to tryptic soy broth and incubated for 2-8 h at $35^{0}$C. Turbidity of cultures suspension were adjusted with sterile 0.85 saline or broth to equal the 0.5 Mcfarland turbidity standard.

**3. Inoculation of agar plate.**

Sterile cotton-tipped swabs were dipped and rotated into the inoculum suspension. The excess liquid was removed by rotating the swab against the side of the tube. Muller-Hinton agar plates were swabbed three times within 15 min of inoculum preparation by rotating the dish $60^0$ each time to ensure a distribution of inoculum.

**4. Application of strips.**

The inoculated agar plates were allowed to dry for approximately 10 minutes prior to apply Etest strips. After the Etest strip was placed on the agar surface, do not move or remove it or place it in another position. Air bubbles were removed by pressing gently on the strip with steriled forceps, beginning at the lower edge of the bubbles and moving up the concentration gradient of the strip toward the E, to make sure the whole length of the strip was completely contacted with the agar surface.

**5. Incubation.**

The agar plates were inverted and incubated within 15 min after the Etest strip was applied at $35^0$C, overnight in ambient-air incubator.

**6. Reading of MIC**

After the recommended incubation period, MICs were read only if sufficient growth is seen and the inhibition ellipse is clearly visible. The MICs were read where the ellipse intersects the scale and always read the end point at complete inhibition of all growth including hazes and isolated colonies.

**7. Interpretation of result.**

Since Etest comprises a continuous gradient as described in Etest technical guides (AB BIODISK, Solna, Sweden), MIC value in between two-fold dilutions can be categorized following NCCLS MIC breakpoints for ciprofloxacin; Susceptible $\leq$ 1 μg/ml; 1 $<$ Intermediate $<$ 4 μg/ml; Resistant $\geq$ 4 μg/ml. For any Etest MICs that fall between the two fold dilution values, the MICs will be read and interpreted as the next highest two fold dilution values. If there is no zone of inhibition, the MIC is reported as greater than the highest concentration of ciprofloxacin on the Etest strip (32 μg/ml). And if the zone of inhibition does not intersect the strip (zone is below the strip), the MIC is reported as less than the lowest concentration of ciprofloxacin (0.002 μg/ml).

**8. Quality control.**

The quality control strains for ciprofloxacin were used according to NCCLS recommendations and Etest technical guides.

- *Escherichia coli* ATCC 25922 ( MIC = 0.004-0.016 μg/ml)
- *Pseudomonas aeruginosa* ATCC 27853 ( MIC = 0.25-1 μg/ml)

## PART VI : CONFIRMATION OF *Vibrio parahaemolyticus* BY PCR.

**1. DNA extraction.**

*Vibrio parahaemolyticus* strains, which have 1 μg/ml of MIC values or more were grown at 37$^0$C in Luria-Bertani broth (LB broth) containing 1% NaCl on shaker 160 rpm, overnight. The1 ml of each culture was boiled for 5 min at 100$^0$C and centrifuged 13,000 rpm for 3 min at 4$^0$C. The supernatant was diluted with sterile distilled water 1:10 and used as PCR templates as described by Kim et al., 1998.

**2. Amplification of *gyrB* gene by PCR.**

2.1) Oligonucleotide primer

The oligonucleotide primers based on the nucleotide sequence data of the *Vibrio parahaemolyticus gyrB* gene were synthesized according to Venkateswaran et al., 1997.

Primer VP-1 (5'-CGGCGTGGGTGTTTCGGTAGT) and primer VP-2r (5'TCCGCTTCGCGCTCATCAATA) were purchased from GibcoBRL, (Life Technologies, Inc., Rockville, MD) and specific nucleotide sequences within 1.2-Kb *gyrB* gene of ciprofloxacin resistant *Vibrio parahaemolyticus* strains were amplified with primers VP-1 and VP-2r.

2.2) The PCR mixtures

PCR assay was carried out in 100 µl reaction mix containing 200 µM (each) dATP,dTTP, dCTP, dGTP; 10 µl of 10-fold diluted boiled overnight culture; 10 µl of Gene Tag buffer; 2.5 U Tag DNA polymerase; 50 mM $MgCl_2$;1 µM (each) primers. The amplification was performed by using 30 PCR cycles, each consisting of 1 min at 94 $^0$C, 1.5 min at 58 $^0$C, 2.5 min at 72 $^0$C, and a final extension step at 72 $^0$C for 7 min, to yield 285-bp amplicon, which was analyzed by agarose gel electrophoresis. The gel was stained with 0.1 µg of ethidium bromides per ml for 15 min, then visualized and photographed under UV transillumination using Chemi Doc (BIORAD Laboratories, USA).

# PART VII : DNA SEQUENCING ANALYSIS OF *gyrA* QRDR AND *parC* QRDR GENES

## 1. DNA extraction.

*Vibrio parahaemolyticus* strains, which have 1 μg/ml of MIC values or more were grown at 37°C in LB broth containing 1% NaCl on shaker 160 rpm, 1 ml of cultures were boiled for 5 min at 100°C and centrifuged 13,000 rpm at room temperature. Supernatant was diluted with sterile distilled water 1:10 and used as PCR template as described by Kim et al., 1998.

## 2. Amplification of *gyrA* QRDR and *parC* QRDR by PCR assay.

### 2.1) Oligonucleotide primers.

The oligonucleotide primers were generated according to the nucleotide sequence data of the *Vibrio parahaemolyticus gyrA* and *parC* QRDR gene as described by Okuda et al., 1999. All primers below were purchased from GibcoBRL, (Life Technologies, Inc., Rockville, USA).

QRDR of *gyrA*

| | |
|---|---|
| GYRAM1 | 5'-CGATTGGAACAAACCATATAAA-3' |
| GYRAM2 | 5'-CGGTGTAACGCATTGCCGCA-3' |

QRDR of *parC*

| | |
|---|---|
| PARM1 | 5'-CTTGGTCTTTCGGCATCAGC-3' |
| PARM2 | 5'-CTTCGGTATAACGCATTGCC-3' |

2.2) The PCR mixture.

PCR was carried out in 50 µl reaction mix containing 200 µM of each dNTPs; 30 µl of 10-fold diluted boiled overnight culture; 5 µl of Gene Tag buffer; 2.5 U Tag DNA polymerase; 0.2 µM of each primers. The amplification was performed by using 30 PCR cycles, each consisting of 30 sec at 95°C, 30 sec at 60°C, 2 min at 72°C. After amplification, 200-bp and 214-bp amplicon for the *gyrA* QRDR and the *parC* QRDR were analyzed by gel electrophoresis, the gel was stained with 0.1 µg of ethidium bromide per ml for 15 min, then visualized and photographed under UV transillumination using Chemi Doc (BIORAD Laboratories, USA).

**3. DNA sequencing.**

3.1) PCR purification

PCR products of 200 bp *gyrA* QRDR and 214 bp *parC* QRDR from amplification step were purified by QIAquick PCR Purification Kit (QIAGEN Inc., Chatworth, USA) as described by the manufacturer. The concentration of DNA were measured by spectrophotometer (BIO RAD, Smart Spec$^{tm}$ 3000, U.S.A) and approximately adjusted to 100 ng/µl for preparation of sequencing reaction.

3.2) Sequencing reaction preparation

Approximately 100 ng each of DNA sample was sequenced by using primer GYRM1 for *gyrA* QRDR and PARM1 for *parC* QRDR with ABI prism Big Dye terminator cycle sequencing ready reaction kit (Perkin Elmer Corporation, ABI prism, Applied Biosystem, Foster city, CA). The PCR assay was carried out in10 µl reaction mix containing 2 µl of purified PCR product; 1 µl of 5 *p*mole of each primer; 4 µl of sterile distilled water and 3 µl of Big Dye terminator as described by manufacturer. The sequencing cycle were performed using 25 cycles of

Denaturing step : 30 sec at 96°C

Annealing step : 10 sec at 55°C

Extension step : 4 min at 60°C

3.3) Ethanol-Sodium acetate precipitation

The PCR products were precipitated using 2 µl of 3M sodium acetate (NaOAc), pH 4.6 and 50 µl of 95% ethanol (EtOH) for each sequencing reaction as described by manufacturer (Perkin Elmer Corporation, ABI prism, Applied Biosystem, Foster city, USA). The precipitated DNA was subjected to automated sequence analysis on ABI prism 310 automated sequencer (Perkin Elmer Corporation, ABI prism, Applied Biosystem, Foster City, USA).

3.4) Sequencing analysis

Nucleotide sequences data of *gyrA* QRDR and *parC* QRDR were compared with *Vibrio parahaemolyticus* ATCC 17802 using WWWBlastScrvcr at National Ccntcr for Biotcchnology Information website ( http:// www.ncbi.nlm.gov/BLAST).