Chapter Four

Materials and Methods
4. MATERIALS AND METHODS

4.1 Study period

The present study was conducted for a period of 36 months encompassing three years starting from January 1993 to December 1995. A total of 2511 patients suffering from acute secretory diarrhoea admitted to the Infectious Diseases Hospital, Calcutta were examined during the study period under analysis. The clinical symptoms of patients suffering from acute watery diarrhoea were noted in a standard proforma after admission to the hospital. The manifestations recorded were i) consistency and nature of the stool specimens, i.e. watery, bloody or mucoid type, ii) frequency of purging, iii) abdominal cramps, iv) vomiting, v) fever and vi) degree of dehydration i.e., mild, moderate or severe.

4.2 Bacteriology

4.2.1 Collection and transportation of clinical specimens

Stool specimens from the diarrhoea patients were collected using sterile catheter in sterile McCartney bottles. Rectal swabs were taken from patients from whom stool could not be obtained using sterile cotton-tipped swab sticks which were introduced in Cary-Blair transport medium. Stool specimens were processed within 1 h of collection since the Infectious Diseases Hospital is located adjacent to the Microbiology laboratory of NICED

4.2.2 Selective isolation of V.cholerae

Thiosulphate-citrate bile salts-sucrose agar (TCBS; Eiken, Japan) was used as the selective medium for the isolation of V.cholerae. Stool samples were inoculated on TCBS plates using sterile cotton-tipped swab sticks and then streaked for colony isolation 250 μl of the neat stool sample was also introduced into sterile 2 ml of alkaline peptone water (bactopeptone 1%, NaCl 1%, pH 8.5) used as an enrichment medium and incubated at 37°C
for 18 h. The inoculated plates (directly from stool samples or from enrichment medium) were incubated at 37°C for 18 to 24 h and subsequently examined for the growth of typical *V. cholerae*-like colonies appearing as round, yellow sucrose fermenting colonies, with elevated centres.

### 4.2.3 Presumptive identification of *V. cholerae*

The multitest medium devised by Kaper (1979) was used for presumptive screening of *V. cholerae*. The formula of the multitest medium is given below and the reactions are based on the principles of Triple sugar iron and Kligler's iron agar (Kaper 1979).

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteose peptone</td>
<td>5</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>3</td>
</tr>
<tr>
<td>Tryptone</td>
<td>10</td>
</tr>
<tr>
<td>Arginine hydrochloride</td>
<td>5</td>
</tr>
<tr>
<td>Glucose</td>
<td>1</td>
</tr>
<tr>
<td>Inositol</td>
<td>10</td>
</tr>
<tr>
<td>Arabinose</td>
<td>10</td>
</tr>
<tr>
<td>Sodium thiosulphate</td>
<td>0.5</td>
</tr>
<tr>
<td>Ferric ammonium citrate</td>
<td>0.5</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5</td>
</tr>
<tr>
<td>Bromo-cresol purple</td>
<td>0.02</td>
</tr>
<tr>
<td>Agar</td>
<td>15</td>
</tr>
</tbody>
</table>

pH was adjusted to 6.7, the boiled medium was dispensed into test-tubes (4 ml/tube) and then autoclaved at 121°C for 12 minutes.

*V. cholerae* being glucose positive, inositol and arabinose negative, arginine dihydrolase negative, and anaerogenic yields an alkaline slant over an acid butt in the
multitest medium.

Oxidase test: Growth from the multitest medium was used to check for oxidase reaction using the oxidase reagent.

<table>
<thead>
<tr>
<th>Oxidase reagent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetramethyl-p-phenylenediamine</td>
</tr>
<tr>
<td>Isoamyl alcohol</td>
</tr>
</tbody>
</table>

The above reagent was stored in an amber coloured bottle to protect from direct light in the refrigerator. Using a sterile wooden tooth pick or a platinum wire, a sweep of the growth of the test strain from the multi-test medium was introduced on a filter paper moistened with oxidase reagent. A deep purple colouration that appeared instantaneously indicated a positive reaction.

4.2.4 Preservation of strains

All the strains identified as *V.cholerae* were preserved as stabs in nutrient agar (Difco, USA) supplemented with 0.5% NaCl and kept at room temperature in the dark until used.

4.3 Biochemical characterization

4.3.1 Strains

Extensive characterization was performed for 50 strains of *V.cholerae* O139 to understand if these strains shared similar biochemical and physiological traits like that of *V.cholerae* O1. In addition, biochemical characterization of *V.cholerae* strains was performed by using a standardized identification system
4.3.2 API 20E system

API20E is a commercially available identification kit (Biomerieux, France) for Enterobacteriaceae and other Gram-negative rods. The identification was carried out in two steps.

i) Performing 20 biochemical tests which are miniaturized in the form of 20 microtubes containing dehydrated substrates. These, when inoculated with the bacterial suspension, and incubated to initiate the metabolism of the substrates will reveal the result either spontaneously or with the addition of reagents.

ii) Interpretation of the results is obtained by referring to the identification table and the analytical profile index.

Composition of media and reagents used
Suspension medium: sterile saline (0.85% NaCl)

<table>
<thead>
<tr>
<th>Tryptophane diaminase reagent (TDA)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferric chloride</td>
<td>3.4 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Indole reagent (IND)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paradimethylaminobenzaldehyde</td>
</tr>
<tr>
<td>Isoamyl alcohol</td>
</tr>
<tr>
<td>HCl 37%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Voges Proskauer reagent (VP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP1;</td>
</tr>
<tr>
<td>Potassium hydroxide</td>
</tr>
<tr>
<td>Distilled water</td>
</tr>
<tr>
<td>VP2;</td>
</tr>
<tr>
<td>Alpha naphthol</td>
</tr>
<tr>
<td>Ethanol</td>
</tr>
</tbody>
</table>
Preparation of the inoculum and inoculation of the strip:

i) The test strain was grown on nutrient agar plate and a single, well isolated colony was inoculated into 5 ml of sterile saline and mixed well to achieve a homogenous bacterial suspension.

ii) Using a sterile pasteur-pipette, tubes and cupules of tests CIT (sodium citrate utilization), VP (Voges Proskauer) and GEL (Kohn’s Gelatin) were filled up with the bacterial suspension. The tubes and not the cupules of the other tests were also filled up with the bacterial suspension.

iii) Sterile mineral oil was overlaid to create an anaerobic atmosphere in the tests ADH (Arginine dihydrolase), LDC (Lysine decarboxylase), ODC (Ornithine decarboxylase), URE (Urease) and \( \text{H}_2\text{S} \) (sodium thiosulphate).

iv) Approximately 5 ml of water was distributed into the honey combed wells of the test-strip tray to create a humid chamber.

v) The incubation tray containing the test strip was kept at 37°C for 18-24 h.

Reading of the strip:

All the spontaneous reactions were recorded on the report-sheet. The tests which required the addition of reagents were performed and interpreted as given below:

**VP Test**: 1 drop each of VP1 and VP2 reagents were added in the VP test cupule. After 10 minutes, a bright pink or red colour indicated a positive reaction.

**TDA test**: Addition of 1 drop of TDA reagent in the TDA cupule followed by the development of a dark-brown colour indicated a positive reaction.

**IND test**: 1 drop of Indole reagent was added in IND cupule and after 2 minutes, development of a red ring indicated a positive reaction.

The results were read, interpreted and recorded, using the interpretation table.
The 20 binary pieces of information thus obtained from the biochemical profile were condensed and transformed into a code of numerical profile which enabled an easy transcription of all the results obtained for the test organism and then compared with those listed in the analytical profile index, using which the organism was finally identified.

4.3.3 **Susceptibility to 2,4 diamino-6, 7-diisopropyl pteridine (150 μg)**

A 4 h preculture of the test-strain in 2 ml of tryptic soy broth was prepared and a loop of this was inoculated on nutrient agar plate containing 150 μg per ml of 2,4 diamino-6,7-diisopropyl pteridine (Sigma). After overnight incubation at 37°C, growth was checked and recorded.

4.3.4 **Polymyxin B sensitivity test**

15 μg/ml of polymyxin B sulphate (Sigma) was added to nutrient agar. The test strain was inoculated into tryptic soy broth and a 2 h culture was streaked on the nutrient agar plate containing polymyxin B. 569B, a classical *V.cholerae* O1 Inaba and MAK757 an ElTor O1 *V.cholerae* Ogawa were used as the negative and positive control strains, respectively. After incubating the plates at 37°C for 24 h, the results were recorded.

4.3.5 **Lysis by Mukherjee’s ElTor phage 5 and classical phage IV**

The test-strain was inoculated into 2 ml tryptic soy broth and 100 μl of 2 h preculture was spread on nutrient agar plate to obtain a thick bacterial lawn. With a 2 mm loop, Mukherjee’s ElTor phage 5 and classical phage group IV was applied on separate plates of the bacterial lawn. After overnight incubation at 37°C, the results were recorded. *V.cholerae* strains of classical biotype are susceptible to classical phage IV but are resistant to ElTor phage 5, whereas ElTor *V.cholerae* strains are resistant to classical phage IV, but are susceptible towards ElTor phage 5.
4.4 Serological characterization of *V. cholerae*

4.4.1 Polyvalent and monospecific O1 antisera

Strains of *V. cholerae* were serogrouped by performing slide agglutination tests using polyvalent O1 and monospecific Ogawa and Inaba antisera prepared at the National Institute of Cholera and Enteric Diseases, Calcutta. Strains were checked first for agglutination with polyvalent O1 antiserum and those which agglutinated with the above were tested with Ogawa and Inaba antisera for further serological characterization.

4.4.2 O139 antiserum

*V. cholerae* strains which did not agglutinate in the O1 antiserum were checked with monoclonal O139 antiserum prepared at this Institute (Garg et al., 1994).

4.4.3 Serogrouping of *V. cholerae* non-O1 non-O139

*V. cholerae* strains which did not agglutinate with either O1 antiserum or with O139 antiserum were assumed to belong to the non-O1 non-O139 serogroups. These strains were sent to Dr. T. Shimada, National Institute of Health, Tokyo, Japan for further confirmation and determination of the O-serogroup of the individual strain using the somatic serogrouping scheme developed for *V. cholerae*.

4.5 Detection and quantification of cholera toxin by Bead-Enzyme-linked immunosorbent assay (ELISA)

4.5.1 Preparation of anti-CT IgG

i) Immunization of rabbit

Before initiation of immunization, about 5 ml of blood from the rabbit was taken from the ear vein to extract preimmune serum. The rabbit was subsequently immunized by giving two subcutaneous injections and two intramuscular injections. The subcutaneous injection was given by pulling a fold of skin on the side of the neck while the intramuscular injection was
given in the hind muscle. The following protocol was used to immunize the rabbit:

<table>
<thead>
<tr>
<th>Day</th>
<th>Antigen solution</th>
<th>Adjuvant</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>50 µg CT in 2 ml PBS</td>
<td>2 ml Freund complete adjuvant</td>
</tr>
<tr>
<td>14</td>
<td>40 µg CT in 1 ml PBS</td>
<td>0.5 ml Freund complete adjuvant + 0.5 ml Freund incomplete adjuvant</td>
</tr>
<tr>
<td>28</td>
<td>40 µg CT in 2 ml PBS</td>
<td>-</td>
</tr>
</tbody>
</table>

ii) Collection of sera

On seventh day after the last booster dose the rabbit was bled to collect as much blood as possible. The sera were then separated. The immune sera were stored with sodium azide (0.01%, w/v, Sigma) at 4°C until use.

iii) Examination of Immune serum titer

Titer of the immune serum was examined by Ouchterlony immunodiffusion test (Ouchterlony, 1968). One percent agarose slab gels of one or two mm thickness were prepared in PBS, on glass slides. Wells were punched in agarose gels. The pure cholera toxin was added into the central well and in the surrounding wells different dilutions of immune serum were added. The gel was placed in a moist chamber at 37°C for 24 h. After 24 h, gels were checked for precipitin bands. If a precipitin band comes above the 1:32 dilution, it could be inferred that the rabbit was adequately immunized.

iv) IgG Purification

From the stock of immune serum, 5 ml was taken for purification of IgG. To 5 ml of immune serum, 0.90 gm of Na₂SO₄ was added so that concentration of Na₂SO₄ was 0.18 gm/ml and then stirred gently for 30 mins. After 30 minutes, the immune serum was
centrifuged at 10,000 rpm for 10 minutes. The supernatant was discarded and the precipitate was solubilized in 5 ml of 17.5 mM sodium phosphate buffer (pH 6.3). Then this solution was introduced into a cellulose tubing (dialysis bag) and dialysed against 17.5 mM phosphate buffer which was changed once and also kept stirred at all times. Serum was then taken out from cellulose tubing using a pasteur pipette. After this, 25 μl of the sample was diluted with 975 μl of 17.5 mM phosphate buffer (pH 6.3) and absorbance was taken at 280 nm and rest of the sample was introduced into a DE52 column. DE52 column purified anti-CT IgG was then used for coating of beads.

v) Coating of beads

Polyesterene beads were washed with triple distilled water till the beads were absolutely clean and blot dried on a Whatman filter paper. The washed beads were then immersed in 0.1 M sodium phosphate buffer (pH 7.5) containing anti-CT IgG (50 μg/ml) and 0.1% NaN₃ and stored overnight in this buffer. On the next day, the beads were washed with 0.01 M sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl, 0.1% BSA and 0.1% NaN₃ and stored in the above buffer at 4°C.

4.5.2 Protocol of the Bead ELISA

To detect CT by bead-ELISA technique in stool specimens or in culture supernates, 0.25 ml of the sample preparation was mixed with an equal volume of 10 mM bicine [N,N-bis (2-hydroxyethyl) glycine; Sigma] buffer (pH 8.3) containing 0.1 M NaCl, 0.1% bovine serum albumin (BSA) and 0.1% sodium azide in glass test tubes (13 x 10 mm). Anti-CT immunoglobulin G coated bead was introduced into the mixture which was incubated at 37°C for 1 h in a water bath and subsequently washed twice with double distilled water. The bead was then incubated with 0.5 ml of anti-rabbit IgG conjugated with horse radish peroxidase (400 ng/ml) in 10 mM phosphate buffer containing 100 mM NaCl (pH 7.0) and 2% bovine
serum albumin. After incubation, the bead was washed three times in double distilled water and transferred to a fresh test tube. Peroxidase activity was determined by incubating the bead at 30°C for 1 h with 0.6 ml of 28 mM 3', 3', 5', 5'-tetramethyl benzidine in 0.1 M sodium acetate buffer (pH 5.5) containing 3 mM EDTA, followed by addition of 0.2 ml of 0.02% H₂O₂. The reaction was stopped by adding 0.2 ml of 4N H₂SO₄, and the resulting yellow colour intensity was measured at 450 nm by using a spectrophotometer (DU-64; Beckman Instruments, USA).

4.5.3 Quantification of CT by Bead-ELISA

Whenever a batch of stool samples or cell-free culture supernates were assayed by bead-ELISA, six different dilutions of purified CT (Sigma) were also run concurrently as positive controls. On the basis of the results, a standard curve was plotted, by which the minimum CT detection ability of the test and also the amount of CT present in each ml of the test sample could be determined.

The following concentrations of CT were prepared from a stock solution of CT (0.5 mg/ml) using bicine buffer as the diluent.

<table>
<thead>
<tr>
<th>Dilutions</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amount of CT/ml</td>
<td>10 ng</td>
<td>2.5 ng</td>
<td>625 pg</td>
<td>156 pg</td>
<td>39 pg</td>
<td>10 pg</td>
</tr>
<tr>
<td>of bicine buffer</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

4.5.4 Absorption assay using anti-CT IgG

For absorption of CT produced by the strain of *V.cholerae* non-O1 non-O139 which produced CT, a dilution of the sample exhibiting an optical density (OD) below 1.2 was selected. The sample was preincubated at 37°C for 1 h with 10 µg of rabbit anti-CT IgG per ml. From earlier experiments, it was determined that 10 µg of anti-CT IgG per ml can
completely absorb 500 ng of pure CT per ml (Ramamurthy et al., 1992). Subsequently, the coated beads were introduced into 0.5 ml of the above described incubation and the bead-ELISA was performed as described previously.

4.5.5 Preparation of stool samples for detection of cholera toxin

Rice watery stool samples, after bacteriological processing, were transferred into fresh tubes and centrifuged at 8,000 x g for 15 min at 4°C. The clear supernatant was stored in separate tubes at -20°C until use.

4.6 Evaluation of factors promoting optimal production of cholera toxin by Vibrio cholerae O1 and O139

4.6.1 Bacterial strains

Three well characterized strains of V. cholerae (i) SG24 belonging to the serogroup O139 Bengal isolated during the early stages of the O139 epidemic in Calcutta, (ii) CO550 belonging to the serogroup O1, serotype Ogawa and to the ElTor biotype isolated after the O139 epidemic in Calcutta and (iii) 569B which is a standard O1 Inaba strain belonging to the classical biotype were used in this study.

4.6.2 Media used

Strains were inoculated in 2 ml volumes of six different media namely;

a) AKI (in g/L) [Iwanaga and Yamamoto, 1985]

\begin{align*}
\text{Bactopeptone} & \quad 15 \\
\text{Yeast extract} & \quad 4 \\
\text{NaCl} & \quad 5 \\
\text{NaHCO}_3 & \quad 3 \\
pH & \text{was adjusted to 7.4.}
\end{align*}

b) Brain heart infusion broth (BHI)
c) Casamino acid-yeast extract broth (CAYE) [g/L] (Kusama and Craig, 1970)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casamino acid</td>
<td>20</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>6</td>
</tr>
<tr>
<td>K$_2$HPO$_4$</td>
<td>8.7</td>
</tr>
<tr>
<td>NaCl</td>
<td>2.5</td>
</tr>
<tr>
<td>Glucose</td>
<td>2.5</td>
</tr>
<tr>
<td>MnCl$_2$</td>
<td>0.0078</td>
</tr>
<tr>
<td>MgSO$_4$</td>
<td>0.103</td>
</tr>
<tr>
<td>FeCl$_3$</td>
<td>0.0083</td>
</tr>
</tbody>
</table>

pH was adjusted to 8.0.

d) Casamino acid-yeast extract broth supplemented with 90 µg/ml of lincomycin (CAYE-L) (Yamamoto et al. 1981). After preparing the CAYE medium, it was autoclaved. Upon cooling of the medium to 50°C, 90 µg/ml lincomycin (Sigma) was added from a lincomycin stock solution (300 mg/ml).

e) Tryptic soy broth (TSB)

f) Yeast extract peptone (YEP) [g/L] (Iwanaga and Kuyyakanond, 1987)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bactopeptone</td>
<td>15</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>4</td>
</tr>
<tr>
<td>NaCl</td>
<td>5</td>
</tr>
</tbody>
</table>

pH was adjusted from 7 to 7.5.

All media and ingredients were from Difco Laboratories, Detroit, Michigan, USA.

4.6.3 Cultural conditions

The strains were cultured under two growth conditions (stationary and shaking) and
at two different incubation temperatures (30°C and 37°C) for 18 h. Culture supernatants were collected by centrifugation (Sorvall, USA) at 5,000 x g for 15 minutes. The supernatants were stored at -20°C until used.

4.6.4 Quantification of cholera toxin

CT was quantified by the bead-enzyme linked immunosorbent assay as described earlier (Section 4.5.3).

4.7 DNA probes for detection of virulence specific genes of *V.cholerae*

4.7.1 Description of DNA probes used

CT - *EcoRI* digested 554 base pair fragment of the plasmid, pKTN901 (Kaper *et al.* 1988) coding for the A1 subunit of CT was used to screen all the *V.cholerae* O1, O139 and non-O1 non-O139 strains for the presence of the *ctxA* gene.

*ZOT* - The 2.6 kb *PstI-XbaI* fragment upstream of the *ctxAB* genes from a clinical isolate of *V.cholerae* O1 strain GP14 was cloned into pUC119 (pMZNI) and sequenced. A gene probe specific for the *zot* gene was constructed from pMZNI. The 0.79 kb *Scal-AccI* fragment located in the coding frame of the *zot* gene was blunted and inserted into pUC119 digested with *HindIII* and *SmaI* (pMZP11). pMZP11 was then digested with *EcoRI* and *PstI* and the *EcoRI-PstI* fragment was isolated and used as a specific probe for the *zot* gene. The *zot* gene probe was constructed by Karasawa *et al.* (1993) and made available for this study by Professor Yoshifumi Takeda, International Medical Center of Japan, Tokyo, Japan.

*Ace* - An *EcoRI-HindIII* fragment of PKK2 which contains a 355-pb *NruI-AccII* fragment of the *ace* gene (Kurazono *et al.*, 1995) was used as the specific DNA probe for the *ace* gene.
4.7.2 Preparation of antibiotics and media

i) Ampicillin

A 25 mg/ml stock solution of the sodium salt of ampicillin (Sigma) in water was made, sterilized by filtration through 0.22 μm Millipore filter and stored in aliquots at -20°C. For plates: The autoclaved Luria Bertani agar medium was allowed to cool to 48°C before adding ampicillin to a final concentration of 35-50 μg/ml. Plates containing ampicillin was stored at 4°C and used within 1 or 2 weeks.

ii) Chloramphenicol

Chloramphenicol (Sigma) was dissolved in dehydrated ethanol at a concentration of 34 mg/ml and stored at -20°C. For plates: The autoclaved Luria Bertani (LB) agar medium was allowed to cool to 48°C and chloramphenicol was added to a final concentration of 10 μg/ml. The plates were stored at 4°C and used within 1 to 4 days.

Composition of Luria Bertani medium g/L

Bacto tryptone (Difco) 10
Bacto Yeast extract (Difco) 5
NaCl 10
pH was adjusted to 7.5.

4.7.3 Preparation of solutions

1. Solution I

Glucose (Sigma) 50 mM
EDTA (Sigma) 10 mM
Tris-HCl, pH 8.0 (Sigma) 25 mM

Autoclaved at 10 lbs pressure for 10 min and stored at 4°C.
2. Solution II

NaOH (Merck) 0 2N
SDS (SRL) 1%

Freshly prepared, kept at room temperature

3. Solution III

CH₃COONa (Merck) 3 M

pH adjusted to 4.5 with glacial acetic acid. Autoclaved and stored at 4°C.

4. Tris-EDTA (TE) buffer

Tris-HCl, pH 8.0 (SRL) 10 mM
EDTA (Sigma) 1 mM

Autoclaved and kept at room temperature

5. Lysozyme

A stock solution of 50 mg/ml of lysozyme (Sigma) was prepared in water, dispensed into aliquots and stored at -20°C.

6. RNase

Pancreatic RNase (RNase A, Sigma) was dissolved at a concentration of 10 mg/ml in 10 mM Tris-HCl (pH 7.5) and 15 mM NaCl, heated to 100°C for 15 min and allowed to cool slowly at room temperature, dispensed into aliquots and stored at -20°C.

7. Electrophoresis buffer Tris-Acetate (TAE, 50X)

Tris base (SRL) 2 M
EDTA (Sigma) 0.05 M

pH adjusted to 8.0 with 57.1 ml of glacial acetic acid (Merck).
8. Phenol-buffer saturation

Phenol (Sigma) was melted at 68°C and 8-hydroxyquinoline (Sigma) was added to a final concentration of 0.1%. The melted phenol was then extracted several times with an equal volume of buffer 1.0 M Tris-HCl, pH 8.0 followed by 0.1 M Tris-HCl (pH 8.0) and 0.2% β-mercaptoethanol (Sigma), until the pH of the aqueous phase was >7.6. The phenol solution was stored at 4°C under equilibration buffer for periods of upto 1 month.

9. Ethidium-bromide

1 gm of ethidium bromide (Sigma) was added to 100 ml of water and stirred on a magnetic stirrer for several h to ensure that the dye has dissolved. The container was wrapped with aluminium foil.

10. Elution buffer

NaCl (Sigma) 1.2 M
Tris-HCl (pH 8.0, Sigma) 10.0 mM
EDTA (pH 8.0, Sigma) 1 mM

11. 20x SSC

NaCl (Sigma) 3 M
CH₃COONa (SRL) 0.3 M
pH adjusted to 7.0. Sterilized by autoclaving

12. Denhardt's solution (50x)

Ficoll (Sigma) 5 g
Polyvinyl pyroldine (Sigma) 5 g
BSA (Fraction V, Sigma) 5 g
H₂O 200 ml
Filtered thorough a disposable ‘Nalgene filter’ and dispensed as 25 ml aliquots and stored at -20°C.

13 Components used for nick translation

Solution A: 0.2 mM dCTP
0.2 mM dGTP
0.2 mM dTTP
500 mM Tris-HCl (pH 7.8)
50 mM MgCl₂
100 mM 2-mercaptoethanol

Solution B: DNA fragment concentration 100 ng/ml

Solution C: 0.4 U/μl DNA polymerase I
40 pg/μl DNase I
50 mM Tris-HCl (pH 7.5)
5 mM Mg acetate
1 mM 2-mercaptoethanol
0.1 mM phenylmethylsulphonylfluoride (PMSF)
50% v/v glycerol
100 μg/ml nuclease free BSA

Solution D: Stop buffer, 300 mM disodiumethylenediaminetetraaceticacid (pH 8.0).

4.7.4 Preparation of plasmid DNA

Plasmid DNA was isolated by the alkaline lysis method (Birnboim and Doly, 1979). A single bacterial colony was inoculated into 100 ml of Luria Bertani medium containing the appropriate antibiotic and incubated at 37°C on a rotary shaker (200 rpm). Bacterial cells were harvested by centrifugation at 12,000x g for 10 minutes at 4°C. The medium was
aspirated and the pellet resuspended in 2 ml of ice cold solution I by vigorous shaking. To the bacterial suspension, lysozyme (10 mg/ml) was added and kept on ice for 30 minutes. Next, freshly prepared Solution II was added (twice the volume of Solution I i.e., 4 ml) and mixed by inverting the tube. The tube was kept on ice for 5 minutes. Solution III was added (3 ml) and the contents in the tube was mixed by inverting the tube rapidly. The tube was kept on ice for 1 h after which it was centrifuged at 12,000x g for 15 minutes at 4°C. The pellet was discarded and to the supernatant, ice cold ethanol (2 volumes) was added and tubes stored at -70°C for 1 h. The precipitated plasmid DNA was recovered by centrifugation at 12,000x g for 15 minutes at 4°C and rinsed with 70% ethanol. The tube was inverted on paper towel and the pellet was air dried. The pellet was dissolved in TE (500 µl) containing DNase free pancreatic RNase (20 µg/ml) and was incubated at room temperature for 30 minutes. An equal volume of 1.6 M NaCl containing 1.3% w/v polyethylene glycol was mixed well with the plasmid DNA and kept on ice for 1 h. The plasmid DNA was recovered by centrifugation at 12,000x g for 5 minutes at 4°C in a microfuge (Kubota). The supernatant was removed by aspiration and the pellet of the plasmid DNA was dissolved in 400 µl of TE, extracted once with equal volume of phenol/chloroform and once with chloroform. The aqueous phase was transferred to a fresh microfuge tube to which 40 µl of 3 M sodium acetate (pH 7.0) was added and mixed well. The DNA was ethanol precipitated, rinsed with 70% ethanol, air dried and dissolved in 100 µl of TE and the concentration of DNA was determined spectrophotometrically (1 OD260 = 50 µg of plasmid DNA/ml).

4.7.5 Purification of probe fragment

Digestion of the plasmid DNA with appropriate restriction endonucleases released the insert which was purified by running a 1% agarose gel in 1x TAE buffer at 60 V for 120 minutes. Restriction enzymes were used according to the recommendation of the suppliers
The fragment was electroeluted to DEAE-81 cellulose paper (Whatman). If the size of the insert was less than 1000-bp (DEAE-81 was prepared by soaking the uncharged paper for 3 h in 2.5 M NaCl, washed several times with distilled water and stored in TE buffer at 4°C) To elute the DNA from DEAE-81 paper, elution buffer was added and incubated in a 37°C water bath with occasional vortexing for 1 h. The eluent containing the DNA fragment was ethanol precipitated at -70°C overnight and after centrifugation the pellet was rinsed with 70% ethanol and redissolved in TE buffer.

If the size of the insert was more than 1000-bp, it was purified by gene clean (Bio-Rad). The desired DNA band from the ethidium bromide stained agarose gel was sliced and placed into a microfuge tube. The sample was centrifuged for several seconds to bring down the gel slice and the volume of this slice was estimated. Based on this volume, 3 volumes of Prep-A-gene binding buffer was added and agitated gently to dissolve the agarose. Next, Prep-A-gene matrix was added (1 μl/0.2 μg DNA) and the tube was kept at room temperature for 5 to 10 minutes with occasional flicking. The DNA containing Prep-A-gene matrix was pelleted by centrifuging for 30 seconds in a micro-centrifuge. The supernatant was aspirated and the pellet was rinsed twice by resuspending it in an amount of binding buffer equivalent to 50 times the amount of added matrix. The supernatant was discarded and the pellet was rinsed thrice with 50x matrix volume of prepared wash buffer (one volume of 95-100% ethanol was added to Prep-A-gene wash buffer before use). After the last wash, the pellet was air dried. To elute the bound DNA, the Prep-A-gene matrix pellet was resuspended in 1 pellet volume of elution buffer and incubated at 40°C for 5 minutes, centrifuged, and the supernatant transferred to a clean tube. This was repeated twice. At this point the DNA fragment was labeled.
4.7.6 Radioactive labelling

The purified DNA fragment was labeled \textit{in vitro} with $\alpha^{32}$P deoxynucleotide triphosphate (BRIT, Bombay) by nick translation (BRL, Nick Translation System).

The following reagents were added in a 1.5 ml microfuge tube placed on ice, and mixed briefly.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution A</td>
<td>5 $\mu$l</td>
</tr>
<tr>
<td>Solution B</td>
<td>10 $\mu$l</td>
</tr>
<tr>
<td>Hot dATP</td>
<td>2 $\mu$l</td>
</tr>
<tr>
<td>$\text{H}_2\text{O}$</td>
<td>27 $\mu$l</td>
</tr>
<tr>
<td>Solution C</td>
<td>5 $\mu$l</td>
</tr>
</tbody>
</table>

The tube was incubated in a 15°C water bath for 60 minutes after which the reaction was stopped with 5 $\mu$l of Solution D.

Labeled DNA was purified by Sephadex G50 (Pharmacia, Uppsala, Sweden, particle size 10-40 $\mu$m) chromatography. The double stranded labeled DNA was denatured as described earlier and used as probe.

4.7.7 Preparation of colony blots

The test organisms were inoculated on nitrocellulose membranes (Schleicher & Schuell, Keene, Germany) overlaid on LB agar plates and incubated at 37°C, overnight. The membranes containing the freshly grown strains were placed successively on top of 3 mm paper (Whatman) soaked with denaturation solution (0.5 M NaOH, 1.5 M NaCl) for 7 minutes, twice on neutralization solution (0.5 M Tris-HCl, pH 7.2, 1.5 M NaCl) for 4 minutes each and finally on 2x SSC. The membranes were air dried, baked at 80°C for 2 h to immobilize the DNA to the membrane and stored in a vacuum desiccator.
4.7.8 Hybridization

The baked nitrocellulose membranes were incubated at 65°C for 2 h in the following hybridization solution: 4x SSC, 5x Denhardt's solution, 0.02% nuclease free bovine serum albumin Fraction V (Sigma) and fragmented heat denatured 100 μg/ml Salmon sperm DNA. The filters were then transferred to fresh hybridization solution containing 10^6 cpm of heat denatured labeled DNA and incubated at 65°C for 16 to 18 h.

4.7.9 Washing and signal detection

After hybridization, the filters were washed twice at 65°C in 2x SSC containing 0.1% sodium dodecyl sulfate (SDS, SRL) for 20 minutes each, twice in 0.2x SSC with 0.1% SDS for 20 minutes each, rinsed in 0.2x SSC at room temperature and air dried. The filter were wrapped in Saran Wrap, exposed to X-Omat-R X-Ray filter to obtain autoradiographic image.

4.8 Multiplex PCR

4.8.1 Description of primers

Three primer pairs for ctxA (Shirai et al., 1991), tcpA (classical biotype) [Keasler and Hall, 1993] and tcpA (ElTor biotype) [Keasler and Hall, 1993] were used for the multiplex PCR assay. The primer sequences along with the resulting amplicon size are given in the Table.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Amplicon size</th>
</tr>
</thead>
<tbody>
<tr>
<td>ctxA</td>
<td>5'-CTCAGACGGGATTTGTAGGCACG-3'</td>
<td>301-bp</td>
</tr>
<tr>
<td></td>
<td>5'-TCTATCTCTGTAGCCCTATTACG-3'</td>
<td></td>
</tr>
<tr>
<td>tcpA</td>
<td>Classical</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5'-CACGATAAGAAACCGGTCAAGAG-3'</td>
<td>617-bp</td>
</tr>
<tr>
<td></td>
<td>5'-ACCAATGCAACGCAGATGGAGC-3'</td>
<td></td>
</tr>
<tr>
<td>tcpA</td>
<td>ElTor</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5'-GAAGAAGTTTGTAAAAGAAGAAGACAC-3'</td>
<td>471-bp</td>
</tr>
<tr>
<td></td>
<td>5'-GAAAGGACCTCTTTTCACGTTC-3'</td>
<td></td>
</tr>
</tbody>
</table>
4.8.2 Preparation of reaction mixture

The following were added to each 100 µl PCR mixture: 10 µl of Mg-free 10x amplification buffer (500 mM KCl, 100 mM Tris-HCl [pH 9.0], 0.1% TritonX-100), 8 µl of 25 mM MgCl₂, 2 µl each of 2mM dATP, dTTP, dGTP and dCTP; 50 pmol each of the primers and 2.5 U of Taq DNA polymerase (Takarashuzo, Otsu, Japan).

4.8.3 PCR assay

PCR was carried out in 0.5 ml microcentrifuge tubes, with 43.5 µl of the PCR mixture described above and 6.5 µl of LB broth (Difco) culture of the test strains heated at 94°C for 5 minutes. The solution was overlaid with a drop of sterile mineral oil (Sigma) and PCR was performed in an automated thermocycler (FTS 320 Corbett Research, NSW, Australia). PCR amplification was performed for 30 cycles, and the cycling conditions were as follows: denaturation at 94°C for 1 min 30 sec, annealing at 60°C for 1 min 30 sec and extension at 72°C for 1 min 30 sec. A reagent blank (containing all the components of the reaction mixture and water instead of broth containing template DNA), VC20 (V.cholerae O1 ElTor Ogawa) and 569B (V.cholerae O1 Classical Inaba) were run as controls. Amplified products from PCR were electrophoresed on 1% agarose gels and were stained with ethidium bromide. A 1-kb molecular size ladder (Gibco BRL, Gaithensburg, MD) was run with each gel.

4.9 Detection of cell-free factors of V.cholerae non-O1 non-O139 which evokes morphological changes in tissue culture cells

4.9.1 Cell lines used and their maintenance

Chinese hamster ovary (CHO) and HeLa cell lines were used in the assay. Continuous cell line stocks of these were obtained from the Department of Microbiology, Kyoto
Disposable tissue culture flasks and microtiter plates (Costar, USA) were used. CHO and HeLa cells were grown and maintained in Dulbecco’s Modified Eagle Medium (DMEM) or Eagle’s minimum essential medium (MEM) (GIBCO, USA) which were supplemented with 10% horse serum (GIBCO) to prepare 10 DMEM and 10 MEM, respectively. Penicillin G (Sigma) and Streptomycin sulphate (Sigma) were the antibiotics used. Humidified 5% CO₂ and 37°C temperature were maintained during the growth of the cell lines.

After the growth of CHO and HeLa cells in the form of a monolayer, the medium was aspirated and 5 ml of 0.05% Trypsin-EDTA solution (Sigma) was added to the flask and incubated at 37°C for 5 minutes. The trypsinized cells were dislodged and washed with the medium by centrifugation at 800 rpm for 5 minutes. The cells were introduced in 75 cm² tissue culture flask containing the appropriate medium and incubated as described above.

4.9.2 Preparation of tissue culture media

**DMEM (Dulbecco’s modified eagle medium)/MEM**  
(Eagle’s minimal essential medium)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM/MEM powder (GIBCO)</td>
<td>10 gm</td>
</tr>
<tr>
<td>Streptomycin sulphate (Streptomycin sesquisulfate, Sigma)</td>
<td>0.1 gm</td>
</tr>
<tr>
<td>Penicillin - G (Benzyl penicillin, potassium salt, Sigma)</td>
<td>0.0633 gm</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>2 gm</td>
</tr>
<tr>
<td>Triple distilled water</td>
<td>900 ml</td>
</tr>
<tr>
<td>Trypsin-EDTA solution</td>
<td></td>
</tr>
<tr>
<td>Trypsin powder</td>
<td>0.5 gm</td>
</tr>
</tbody>
</table>
Ethylenediamine
Tetra acetate disodium salt (Sigma) 0.2 gm
NaHCO₃ 2 gm
Hank’s balance salt solution (GIBCO) 1 litre

Both tissue culture media and trypsin solution were filtered through 0.22 μm, cartridge filter (Sterivex-G, Millipore Co., USA).

4.9.3 Preservation of the cell lines

Cryopreservation medium was prepared by mixing dimethyl sulphoxide (Sigma) and horse serum in a 1:9 ratio (v/v). Cells grown in 75 cm² flask (Corning) with healthy, confluent growth were trypsinized and washed with 10 DMEM or 10 MEM depending on the cell lines used. The cells were centrifuged at 800 rpm for 10 minutes. The pellet was resuspended in an appropriate volume of the cryopreservation medium, kept in an ice bath and dispensed in 1 ml aliquots in sterile cryovials (Nunc), kept at -70°C overnight with each cryovial wrapped in bubble paper to permit slow cooling and next day transferred into a can containing liquid nitrogen.

4.9.4 Preparation of cell free culture filtrates

Tryptic soy broth (Difco), containing 0.6% yeast extract (TSB-YE) and AKI broth were used for cultivation of the test strains at 37°C for 24 h in shaking condition. After centrifugation (5000 rpm for 20 min at 4°C), the culture supernate was filtered (0.2 μm disposable filter; Millipore) and the cell-free culture filtrate was kept in a sterile test tube and was used for the tissue culture assay.

4.9.5 Toxin assay

A confluent layer of CHO and HeLa cells grown for 3 days was removed from the tissue culture flask and 200 μl of the cell suspension was added to each of the wells of 96-
well tissue culture plates (Nunc) and incubated as described earlier to obtain a monolayer.
After aspirating the medium from each well, appropriate medium supplemented with 2% horse serum was added and incubated for further 2 h. Fifty μl of the cell-free culture supernatant was added and the cytotoxic and cytotoxic changes were recorded after 24 h. Fifty μl of the unnoculated broth medium and 50 μl of pure cholera toxin (100 μg/ml) were added in separate wells as negative and positive controls, respectively.

4.10 Antimicrobial susceptibility

4.10.1 Preparation of culture

Test strains were plated on TCBS (Eiken, Japan) and incubated at 37°C for 18-24 h. Single colony was picked and inoculated into 3 ml of Tryptic Soy Broth (Difco). The broth was incubated at 37°C for 2 h to obtain moderate turbidity. 100 μl of the broth culture was introduced on a dry Mueller Hinton Agar (Difco) plate and was spread using a glass spreader on the entire surface area of the plate. Using sterile fine forceps, antimicrobial discs (Span Biotech) were placed on the agar plate, at least 15 mm away from the edge, at equal distances and sufficiently separated from each other to avoid the overlapping of the zones of inhibition. The plates were kept at room temperature for 30 minutes (prediffusion time) and then incubated at 37°C for 18 h.

4.10.2 Antibiotics used

The antibiotics used in the study are listed below along with their symbol, strength and interpretation of the diameters of the zones of inhibition.
<table>
<thead>
<tr>
<th>Antibiotic used</th>
<th>Symbol</th>
<th>Strength (µg/disc)</th>
<th>Interpretation of the diameter of zone of inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Resistant mm or less</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>A</td>
<td>10</td>
<td>13</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>C</td>
<td>30</td>
<td>12</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>Cf</td>
<td>5</td>
<td>15</td>
</tr>
<tr>
<td>Co-trimoxazole</td>
<td>Co</td>
<td>25</td>
<td>10</td>
</tr>
<tr>
<td>Furazolidone</td>
<td>Fz</td>
<td>100</td>
<td>14</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>G</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>Na</td>
<td>30</td>
<td>13</td>
</tr>
<tr>
<td>Neomycin</td>
<td>N</td>
<td>30</td>
<td>12</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>Nx</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>S</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>T</td>
<td>30</td>
<td>14</td>
</tr>
</tbody>
</table>

4.10.3 Interpretation of results

At the end of the incubation period the diameters of the zones of inhibition were measured to the nearest millimeter. The size of the inhibition zone at which the organism was considered resistant, intermediate or sensitive is mentioned in the above table. Strains showing intermediate zones of inhibition were interpreted as resistant to that drug.

4.10.4 Statistical analysis

The Mantel-Haenszel chi-square, Yates correction and Fisher extract chi-square tests (Kirkwood, 1988) were employed to compare and assess year-wise significance in increase or decrease of drug resistance to *V. cholerae* O1, O139 and non-O1 non-O139.
4.11 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

4.11.1 Strains used

The following well characterized *V. cholerae* O1 and O139 strains isolated during the O139 epidemic from different parts of India at different time periods were used for the lipopolysaccharide (LPS) and outer membrane protein (OMP) preparations.

<table>
<thead>
<tr>
<th>Strain No.</th>
<th>Serogroup</th>
<th>Place of isolation</th>
<th>Period of isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td>VC20</td>
<td>O1</td>
<td>Calcutta</td>
<td>May, 1992</td>
</tr>
<tr>
<td>SG24</td>
<td>O139</td>
<td>Calcutta</td>
<td>November, 1992</td>
</tr>
<tr>
<td>MO3</td>
<td>O139</td>
<td>Madras</td>
<td>December, 1992</td>
</tr>
<tr>
<td>BLO2</td>
<td>O139</td>
<td>Bangalore</td>
<td>May, 1993</td>
</tr>
<tr>
<td>NPO10</td>
<td>O139</td>
<td>Nagpur</td>
<td>June, 1993</td>
</tr>
<tr>
<td>AHO4</td>
<td>O139</td>
<td>Ahmedabad</td>
<td>July, 1993</td>
</tr>
<tr>
<td>LUO10</td>
<td>O139</td>
<td>Ludhiana</td>
<td>August, 1993</td>
</tr>
</tbody>
</table>

The following well characterized *V. cholerae* O139 strains isolated from different countries were used for the lipopolysaccharide (LPS) and outer membrane protein (OMP) preparations.

<table>
<thead>
<tr>
<th>Strain No.</th>
<th>Serogroup</th>
<th>Place of isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td>AL1852</td>
<td>O139</td>
<td>Bangladesh</td>
</tr>
<tr>
<td>SG24</td>
<td>O139</td>
<td>India</td>
</tr>
<tr>
<td>SRL1</td>
<td>O139</td>
<td>Sri Lanka</td>
</tr>
<tr>
<td>NP44</td>
<td>O139</td>
<td>Nepal</td>
</tr>
<tr>
<td>TH492</td>
<td>O139</td>
<td>Thailand</td>
</tr>
<tr>
<td>MYAO2</td>
<td>O139</td>
<td>Myanmar</td>
</tr>
</tbody>
</table>
4.11.2 Preparation of lipopolysaccharide (LPS)

a) Pure cultures of the above strains were plated on TCBS agar and single colonies were inoculated in 2 ml TSB and incubated overnight at 37°C in shaking condition.

b) Broth cultures were washed thrice with 10 mM PBS and the OD value at 540 nm was adjusted to 0.6. 1.5 ml of this cell suspension was pelleted and 50 μl of sample buffer was added followed by boiling in water bath at 100°C for 10 minutes. 10 μl of 25% proteinase K was added and kept at 60°C for 2 h. The preparation was kept in cold till use.

4.11.3 Preparation of Outer membrane protein (OMP)

OMP from bacterial cells were prepared by the method of Filip et al (1973). The *V. cholerae* O1 and O139 strains were grown in 1 liter tryptic soy broth at 37°C for 18 h under shaking conditions (100 rpm) in an orbital shaker. The cells were harvested by centrifugation at 8000 rpm for 10 minutes and washed twice in 100 mM HEPES (N-[2 hydroxyethyl]piperazine N'-[2-ethanesulfonic acid] [Sigma] pH 7.0) buffer and finally suspended in 50 ml of the same buffer. Cells were then disrupted in an ultrasonic disintregator (MICROSON, Cell Disrupter) at 4°C with intermitent bursts of one minute. The intact cells were removed by low speed centrifugation (6000 rpm) for 10 minutes at 4°C. The crude envelope fractions were collected from the supernatant by centrifugation at 1,05,000x g (Sorval) for 1 h at 4°C. The pellet containing crude envelope fraction was treated with 0.5% (weight/volume) N-Lauryl sarcosine sodium salt (Sigma) to selectively solubilize the inner membrane part. The insoluble outer membrane protein fraction were recovered as pellet by centrifugation at 10,000x g (Sorval) for 1 h at 4°C. The pellet was resuspended in HEPES buffer and stored at -20°C until use.

Outer membrane proteins were estimated by the Bicinchoninic acid (BCA) protein
assay reagent (Pierce, Rockford, USA)

Composition of reagents:

i) Reagent A • Sodium carbonate, sodium bicarbonate, BCA detection reagent and sodium tartrate in 0.2 N NaOH.

ii) Reagent B • 4% copper sulphate solution.

Preparation of working reagent

Working reagent was prepared by mixing 50 parts of reagent A to 1 part of reagent B.

Protocol

i) A set of protein standards of known concentration by diluting the bovine serum albumin standard solution were prepared.

ii) 0.1 ml of standard as well as protein (OM) samples were taken in appropriately labeled test tubes. For blank, tube contained 0.1 ml of distilled water.

iii) To each tube, 2 ml of working reagent was added and mixed by vortexing and incubated for 30 minutes at 37°C. After incubation, all tubes were cooled to room temperature.

iv) The absorbance was measured at 562 nm in Beckman spectrophotometer and a standard curve was plotted using the net absorbance versus BSA concentration. The protein concentration of the OMP preparations were determined from the standard curve.

4 11.4 Preparation of reagents for SDS-PAGE

Solution A

Acrylamide bisacrylamide solution

29.2 gms of acrylamide (Sigma) + 0.8 gms of N,N’-methylene-bis-acrylamide
(Sigma) dissolved in triple distilled water, the final volume was made to 100 ml.

Solution B

Resolving buffer : 1.5 (M) Tris-HCl, pH 8.8

18.16 gms of Tris + 0.4 gms sodium dodecyl sulphate was dissolved in 60 ml of triple distilled water and pH was adjusted to 8.8 using HCl. The final volume was made to 100 ml with triple distilled water.

Solution C

Stacking buffer : 0.5 (M) Tris-HCl, pH 6.8

6.050 gms of Tris + 0.4 gms of SDS were dissolved in 60 ml of triple distilled water and pH was adjusted to 6.8 with HCl. The final volume was made to 100 ml with triple distilled water.

Solution D

A 10% solution of ammonium persulphate was prepared freshly before every use.

TEMED : N,N,N',N'-tetramethyl ethylene diamine (Sigma)

Running buffer : 0.025 (M) Tris; 0.192 (M) glycine, 0.1% SDS, pH 8.3

Dissolved 0.028 gms of Tris + 14.413 gms glycine + 1 gm SDS in 500 ml of triple distilled water, the pH was adjusted to 8.3 with HCl and the volume was made upto 1000 ml.

Sample buffer:

1.6 ml of 0.5(M) Tris (pH 6.8) + 1.0 ml of glycerol + 2.0 ml of 10% SDS + 0.2 ml of 1% bromophenol blue + 1 ml β-mercaptoethanol + 3.4 ml of triple distilled water.

Fixative

50 ml methanol + 10 ml acetic acid + 40 ml of triple distilled water.
Staining solution:

- Coomassie brilliant blue R250 (Sigma) 0 25%
- Methanol 50%
- Acetic acid 10%

Destainer:

- Methanol 25%
- Acetic acid 5%

4.11.5 Procedure for electrophoresis

The LPS preparation of various serogroups of *V. cholerae* were resolved by using 14% acrylamide-bisacrylamide separating gel while OMP preparations were resolved in 12.5% acrylamide-bisacrylamide separating gels according to the method described by Laemmli (1970). The gel recipes are described below:

The discontinuous SDS-PAGE was performed

<table>
<thead>
<tr>
<th>Solution</th>
<th>Resolving gel (12.5%)</th>
<th>Resolving gel (14%)</th>
<th>Stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>7.5 ml</td>
<td>8.4 ml</td>
<td>0.9 ml</td>
</tr>
<tr>
<td>B</td>
<td>4.5 ml</td>
<td>4.5 ml</td>
<td>-</td>
</tr>
<tr>
<td>C</td>
<td>-</td>
<td>-</td>
<td>1.5 ml</td>
</tr>
<tr>
<td>D</td>
<td>0.07 ml</td>
<td>0.07 ml</td>
<td>0.018 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.01 ml</td>
<td>0.01 ml</td>
<td>0.006 ml</td>
</tr>
<tr>
<td>Water</td>
<td>6 ml</td>
<td>5.1 ml</td>
<td>3.6 ml</td>
</tr>
</tbody>
</table>

Gel apparatus : Mini slab (AE6400, ATTO)

Running condition . Stacking gel at 60 v (constant voltage)

Resolving gel at 120 v (constant voltage)
Running buffer : as above

4.11 6 Silver staining for LPS:

After running the gel and treating overnight with the fixative solution, the gel was washed briefly with triple distilled water. Oxidation was carried out by keeping the gel for 7 minutes under shaking conditions in 0.78% periodic acid and then washed for 1 h with several changes of water. The gel was impregnated with silver solution for 10 minutes (28 ml of 0.1N NaOH and 2 ml of concentrated NH₃ solution were mixed). The silver nitrate solution (1.0 gm AgNO₃ dissolved in 5 ml of water) was added dropwise. As the silver nitrate solution is added, a precipitate forms, changes colour and then dissolves. When all of the silver nitrate had been added, stirring was stopped and the final volume was made to 150 ml with water and the solution was added to the gel.

The gel was washed for 30 minutes with frequent changes of water and then the gel was transferred to the developing solution (0.05 gms citric acid + 0.5 ml of 37% formaldehyde + 200 ml of water) The gel was gently agitated constantly and the developing solution was replaced as soon as it became cloudy. When sufficiently stained, the gel was washed extensively with water.

4.12 Immunoblot analysis

4.12.1 Preparation of reagents for immunoblotting

Transfer buffer

1.45 gms of Tris + 0.725 gms glycine + 0.0925 gms SDS + 50 ml of methanol

The final volume was made to 250 ml by triple distilled water.

Tris buffer saline (TBS)

1.21 gms of Tris + 4.38 gms of NaCl; pH was adjusted to 7 4 using 1(N) HCl. The
final volume was made to 1000 ml using triple distilled water

**TBS-T**

500 µl of Tween 20 (Sigma) was added to 1 litre of TBS to make a final concentration of 0.05%.

**Carbonate buffer** (0.1 M NaHCO₃, 1.0 mM MgCl₂, pH 9.8)

0.84 gms of NaHCO₃ and 0.0203 gms MgCl₂.6H₂O were dissolved in approximately 80 ml of distilled water, pH was adjusted to 9.8 using 5(N) NaOH and final volume was made to 100 ml with distilled water.

**Antibody probe**

Ascitic fluid of the 4A3 clone producing anti-O139 IgG2a monoclonal antibody was used as the antibody probe.

**4.12.2 Procedure for immunoblotting**

The technique of immunoblotting was similar to that described by Towbin *et al.* (1979). After separating the proteinase K treated LPS electrophoretically on SDS-PAGE as described previously, these were transferred to nitrocellulose paper (pore size 0.45 µm, Bio-Rad, USA) using a transblot apparatus (Bio-Rad, Richmonds, USA) keeping the gel on the cathodal side and nitrocellulose on the anodal side, in the transfer buffer at constant voltage of 60 volts for 6 h at 4°C. After preparing a nitrocellulose blot of the samples, it was developed as follows:

a) Extra binding sites were blocked using 3% BSA in TBS for 2 h in shaking condition.

b) Washed the blot several times with TBS-T and TBS.

c) Incubated with the antibody probe diluted 1.50 in 1.5% BSA in TBS for 2 h in shaking condition.
d) Washed the blot thoroughly with TBS-T and TBS.

e) Reincubated with anti-mouse IgG conjugated with alkaline phosphatase at a 1:5000 dilution (Jackson ImmunoResearch Laboratories, Inc., USA) in 1.5% BSA in TBS for 45 minutes.

f) Washed with several changes of TBS-T and TBS.

g) Stained for alkaline phosphatase activity by using p-nitro blue tetrazolium chloride (NBT) and 5-Bromo-4 chloro-3 Indonyl phosphate p-toluidne salt (BCIP) [3.75 mg of BCIP + 250 µl of N,N-dirnethyl formamide (DMF) and 7.50 mg of NBT + 175 µl of DMF + 75 µl of triple distilled water were mixed and 25 ml of bicarbonate buffer was added].

h) After sufficient staining, the blot was washed several times in water and stored between sheets of paper.

4.13 Aquatic microcosm study

4.13.1 Selection of strains

Two well characterized strains of *V. cholerae* (i) SG24 belonging to the serogroup O139 Bengal isolated during the early stages of the O139 epidemic in Calcutta and (ii) VC20 belonging to the serogroup O1, serotype Ogawa and to the ElTor biotype isolated before the O139 epidemic in Calcutta were used in this study. The two strains were maintained as stabs in nutrient agar.

4.13.2 Collection of different types of water for preparation of microcosm

Microcosm study was performed in the laboratory using water from three different sources such as river, lake and tap to assess the length of survival of the O1 and O139 serogroups. River water was collected from the river Ganges, lake water from Subhash...
Sarovar, Calcutta and tap water from the NICED, Calcutta.

4.13.3 Procedure of microcosm study

Microcosms were prepared using 250 ml bottles (Wheaton) containing 100 ml autoclaved river water (Ganges), lake water (Subash Sarobar) and tap water. Single colony of *V. cholerae* O1 and O139 picked from TCBS plate was transferred to 5 ml LB broth and incubated at 37°C in an incubator with shaking (150 rpm) for 18 h. 500 μl of each broth culture was transferred to 100 ml LB broth (pH 7.2) in 250 ml conical flasks and incubated for 6 h at 37°C in an incubator with shaking. Cells were collected by centrifugation at 1000x g for 15 mins at room temperature and washed two times with sterile physiological saline (0.85% NaCl) to minimize the amount of available nutrients from the broth and ultimately resuspended in 3 ml of saline. Microcosms were inoculated with 1 ml of resuspended bacterial suspension and incubated at room temperature without shaking. Samples were aseptically removed periodically for bacterial enumeration from the each microcosm bottle.

4.13.4 Method of enumeration

After introducing the bacterial inoculum, serial dilutions of the sample from each microcosm bottle was prepared. Neat and serial dilutions of the samples from each microcosm bottle were spread plated on bile salt agar (BSA) plate (1% bile salt, 0.5% NaCl and nutrient agar). The plates were incubated at 37°C and colony counts were made 24 h later using a colony counter and expressed as colony forming units. Samples from each microcosm was taken on day 0, 2, 4, 8, 16, 32, 64, 70, 80, 90 and 100.

4.13.5 Examination of colonies for virulence genes

The parent *V. cholerae* O1 and O139 strains used in the microcosm study were toxigenic and possessed an intact virulence cassette. To examine whether the strains retained their virulence cassette intact after prolonged period of time in the microcosm, 25 separate
colonies of each strain from different microcosms were taken on day 70 for DNA hybridization and multiplex PCR assay which were performed as described earlier (Sections 4.7 and 4.8).