Chapter III

MATERIALS AND METHODS

Multidrug resistant enteropathogenic *Escherichia coli* associated with urinary tract infections
MATERIALS AND METHODS

3.1. COLLECTION OF URINE SAMPLE

Six hundred and fifty urine samples of patients with clinical symptoms of Urinary Tract Infection before administration of antimicrobial agent were collected from different parts of North-East India for the study. Sample collection procedures were clearly explained to the patients before sample collection as to open bag and to remove culture collection cup, thereby to unscrew the cap of the cup and to place the cup on counter with the inner side facing upside. Immense care was observed so that the patients did not touch the inside of the cup to avoid contamination.

For male patients special instructions were given to wipe the head of penis in a single motion with one towelette (s) and if not circumcised, to hold the foreskin back before cleansing. Then to urinate a small amount in to the toilet and to place the cup under stream and to replace cap on the cup immediately. Female patients were also instructed to clean the genital area and the collection method remained the same.
Materials and methods

Fig. 1. Right method of placing of the cap of the sterile urine collection cup for culture test

Fig. 2. Wrong method of placing of the cap of the sterile urine collection cup for culture test
3.2. SPECIMEN TRANSPORTATION (FOR HOME COLLECTION AND OUT STATION PATIENTS)

Urine samples in sterile urine culture containers were brought to the laboratory within one hour of collection under refrigerated temperature (2-8°C).

Specimen in Boric acid culture and preservative tubes (Bacton Dickenson Vacuitainer kit) were also used to collect specimen as it is stable upto 48 hours from the time of collection at 18-25°C or refrigerated temperature.

Procedure for use of urine culture vacutainer kit (kit used for home collection and for out station patients) (Fig.3) is as follows:
Fig. 3. Procedure for use of urine culture vacutainer kit

Fig. 3 (a) Cup and vacutainer

Fig. 3 (b) Sample and vacutainer

Fig. 3 (c) Holding position

Fig. 3 (d) Vacutainer after collection.
3.3. ISOLATION OF BACTERIA

3.3.1. Media

The various media used for isolation, purification, sensitivity and maintenance of *Escherichia coli* were as follows:

a) Media for primary isolation- Cystein lactose electrolyte deficient agar (CLED) and MacConkey’s Lactose agar (MLA)
b) Media for purification of culture- Eosine methylene blue agar (EMB)
c) Media for sensitivity test- Mueller Hinton agar (MHA)
d) Media for maintenance of culture- Nutrient agar (NA)

3.3.2. Procedure

3.3.2.1. Media inoculation

The organisms were isolated by standard bacteriological technique (Cruickshank *et al.* 1975). After collection of urine samples, samples were cultured on sterile MLA and CLED agar (Hi-Media) with calibrated inoculation loop (i.e. 0.01 ml of loop, one colony of 0.01 ml = 100 CFU/ml) in a bio-safety cabinet. Inoculated plates were incubated aerobically at 37°C for 24-48 hours.

3.4. IDENTIFICATION OF ORGANISM

The isolates were characterized and identified on the basis of the following criteria suggested by Cruickshank *et al.* (1975) and Edwards and Ewing (1986).
3.4.1. Colony characteristics

The size, shape, colour, form and overall appearance of the colonies on different media were noted.

3.4.2. Morphology of the organisms

Morphological characteristics like size, shape, arrangement and response of the organism to Gram’s staining were recorded. These were accomplished by preparing smears from fresh overnight culture and staining by Gram staining method.

3.4.3. Motility of the organism

The motility of the organism was determined by the hanging drop method (Cruickshank et al. 1975).

3.4.4. Purification of the organisms

The isolated colonies on MLA/ CLED agar plates were further purified by streaking on EMB agar. The inoculated plates were incubated at 37°C for 24-48 hours and observed for the characteristic metallic sheen. The typical colonies were picked up and inoculated on NA slants. The purified organisms were further subjected to biochemical tests for confirmation.
3.4.5. Biochemical reactions:

3.4.5.1. IMViC Test

3.4.5.1.1. Test for indole production

Tryptophan is an essential amino acid that can undergo oxidation by way of the enzymatic activities of some bacteria (e.g. *E. coli*). Conversion of tryptophan into metabolic products (indole, pyruvic acid and ammonia) is mediated by the enzyme tryptophanase. This ability to hydrolyze tryptophan with the production of indole is not a characteristic of all microorganisms and therefore serves as a biochemical marker. The presence of indole is detectable by adding Kovac’s reagent, which produces a cherry red layer. This colour is produced by the reagent, which is composed of p-dimethylamino benzaldehyde, butanol and hydrochloric acid. Indole is extracted from the medium into the layer by the acidified butanol component and forms a complex with the p-dimethylamino benzaldehyde, yielding the cherry red colour.

**Procedure**

One loopful of fresh, overnight culture broth is inoculated into 3ml of 2% peptone medium and incubated at 37°C for 48 hours. After incubation, 0.5ml of Kovac’s reagent was added into the culture medium and
Materials and methods
gently shaken. Cultures producing a red reagent layer following addition of Kovac’s reagent are indole positive. Absence of red colouration demonstrates that the substrate tryptophan was not hydrolyzed and indicates an indole negative.

3.4.5.1.2. Test for methyl red

Glucose is the major substrate oxidized by all enteric organisms for energy production. The end products of this process will vary depending on the specific enzymatic pathways present in the bacteria. In this test, the pH indicator methyl red detects the presence of large concentrations of acid end products. Although all enteric microorganisms ferment glucose with the production of organic acids, this test is of value in the separation of E. coli and Enterobacter aerogenes. Both these organisms initially produce organic acid end products during the early incubation period. The low acidic pH 4 is stabilized and maintained by E. coli at the end of incubation. However, E. aerogenes converts these acids to non-acidic end products such as 2,3-butanediol and acetoin, resulting in an elevated pH of about 6. The methyl red indicator in the pH range of 4 will turn red, which is indicative of a positive test. At a pH of 6, still indicating the presence of acid but with a lower hydrogen ion concentration, the indicator turns yellow and is a negative test.
Materials and methods

Procedure

One loopful overnight culture broth is inoculated in 3ml glucose phosphate broth and incubated at 37°C for 48 hours. After incubation 2 drops of methyl red solution was added. A positive reaction is indicated by red colour whereas a yellow colour indicates a negative test.

3.4.5.1.3. Tests for Voges-Proskauer reaction

The Voges-Proskauer test determines the capability of some organisms to produce non-acidic or neutral end products, such as acetylmethyl carbinol, from the organic acids that result from glucose metabolism. The reagent used in this test, Barritt’s reagent, consists of a mixture of alcoholic alpha-naphthol and 40% potassium hydroxide solution. Detection of acetylmethyl carbinol requires that this end product be oxidized to a diacetyl compound. This reaction will occur in the presence of the alpha-naphthol catalyst and a guanidine group that is present in the peptone of the MR-VP medium. As a result, a pink complex is formed, imparting a rose colour to the medium. *E. coli* gives a negative response to this test.

Procedure

One loopful of overnight culture is inoculated into 3ml of glucose phosphate medium and incubated at 37°C for 48 hours. After
Materials and methods

incubation, 0.2ml of α-naphthol and 0.6 of 40% KOH solution is added and the tubes are shaken. The tubes are observed for upto 2 hr for the appearance of a pink colour which indicates a positive test.

3.4.5.1.4. Test for citrate utilization

In the absence of fermentable glucose or lactose, some microorganisms are capable of using citrate as a carbon source for their energy. This ability depends on the presence of a citrate permease that facilitates the transport of citrate in the cell. Citrate is the first major intermediate in Kreb’s cycle and is produced by the condensation of active acetyl with oxaloacetic acid. Citrate is acted on by the enzyme citrase, which produces oxaloacetic acid and acetate. These products are then enzymatically converted to pyruvic acid carbon dioxide. During this reaction the medium becomes alkaline-(the carbon dioxide that is generated combines with sodium and water to form sodium carbonate, an alkaline product). The presence of sodium carbonate changes the bromothymol blue indicator incorporated into the medium from green to deep Prussian blue. Following incubation, citrate positive cultures are identified by the presence of growth on the surface of the slant, which is accompanied by blue colouration. Citrate negative cultures will show no growth and the medium will remain green.
Materials and methods

Procedure

One loopful overnight culture is streaked on the surface of a slant of Simmons citrate agar medium. Incubation was done at 37°C and the incubation period continued up to 4 days and any change in the colour of the medium was recorded.

3.4.5.2. Test for H₂S production (TSI agar test)

This test is based on the differences in the carbohydrate fermentation patterns and hydrogen sulphide production by various groups of intestinal organisms. To facilitate observation of carbohydrate utilization patterns, the TSI agar slants contain lactose and sucrose in 1% concentrations and glucose in a concentration of 0.1%, which allows for detection of the utilization of this substrate only. The acid-base indicator phenol red is also incorporated to detect carbohydrate fermentation that is indicated by a change in colour of the medium from red to orange-yellow in the presence of acids. The TSI agar medium also contains sodium thiosulfate, a substrate for hydrogen sulphide (H₂S) production and ferrous sulphate for detection of this colourless end product. Following incubation, only cultures of organisms capable of producing H₂S will show an extensive blackening in the butt because of the precipitation of the insoluble ferrous sulphide.
Materials and methods

Procedure

An overnight culture of the isolates was inoculated by stabbing into the butt and then streaking onto the slope of the TSI agar slant. It was incubated at 37°C and observed daily for up to 5 days. The presence or absence of blackening in the butt of the medium and production of acid or gas was recorded.

3.4.5.3. Catalase test

On contact with the colony, production of gas bubbles from the hydrogen peroxide solution indicates a positive reaction while absence of bubbles indicates a negative reaction.

3.5. COLONY COUNT

The presence of several species in culture is suggestive of contamination with faecal organisms. About 10,000/ml or more of a single species indicates infection of the bladder or kidney and counts of that order are taken to indicate clinically significant bacteriuria. (Malcolm et al., 2010).
3.6. MAINTENANCE OF CULTURE

After purification on EMB agar, *E. coli* colonies showed typical metallic sheen were selected and streaked on NA slants. The colonies were allowed to develop on the NA slants by incubating at 37°C for 24 hours. The slants were sealed with parafilm and stored in a refrigerator at 4°C. To maintain viability of the cultures, sub-culturing of cultures onto fresh NA was done after every 6 weeks.

3.7. SEROTYPING OF *Escherichia coli*

The organisms identified as *Escherichia coli* were serotyped at National Salmonella and Escherichia Centre, Central Research Institute, Kasauli (H.P.).

3.8. DRUG SUSCEPTIBILITY OF *Escherichia coli*

The antibiogram of the isolated organisms was determined by the disc diffusion test (Bauer et al. 1966) using commercially available antimicrobial discs (HiMedia, Mumbai) (Fig.4). The antimicrobial agents and their concentration per disc used in the test are presented in table 2.
# Materials and methods

Table 2: Antimicrobial agents used and their concentration per discs

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Antimicrobial agent</th>
<th>Symbol</th>
<th>Disc content</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Amikacin</td>
<td>AK</td>
<td>30 mcg</td>
</tr>
<tr>
<td>2</td>
<td>Amoxicillin/Clavulanic acid</td>
<td>AC</td>
<td>20/10 mcg (30 mcg)</td>
</tr>
<tr>
<td>3</td>
<td>Ampicillin</td>
<td>A</td>
<td>10 mcg</td>
</tr>
<tr>
<td>4</td>
<td>Cefazoline</td>
<td>CZ</td>
<td>30 mcg</td>
</tr>
<tr>
<td>5</td>
<td>Cefepime</td>
<td>CPM</td>
<td>30 mcg</td>
</tr>
<tr>
<td>6</td>
<td>Ceftazidime</td>
<td>CA</td>
<td>30 mcg</td>
</tr>
<tr>
<td>7</td>
<td>Cefuroxime sodium (parental)</td>
<td>CU</td>
<td>30 mcg</td>
</tr>
<tr>
<td>8</td>
<td>Chloramphenicol</td>
<td>C</td>
<td>30 mcg</td>
</tr>
<tr>
<td>9</td>
<td>Ciprofloxacin</td>
<td>CF</td>
<td>5 mcg</td>
</tr>
<tr>
<td>10</td>
<td>Co-trimoxazole</td>
<td>CO</td>
<td>10 mcg</td>
</tr>
<tr>
<td>11</td>
<td>Gentamicin</td>
<td>G</td>
<td>10 mcg</td>
</tr>
<tr>
<td>12</td>
<td>Imipenem</td>
<td>I</td>
<td>10 mcg</td>
</tr>
<tr>
<td>13</td>
<td>Levofloxacin</td>
<td>LE</td>
<td>5 mcg</td>
</tr>
<tr>
<td>14</td>
<td>Nalidixic acid</td>
<td>NA</td>
<td>30 mcg</td>
</tr>
<tr>
<td>15</td>
<td>Netilmicin</td>
<td>NT</td>
<td>30 mcg</td>
</tr>
<tr>
<td>16</td>
<td>Nitrofurantoin</td>
<td>NF</td>
<td>300 mcg</td>
</tr>
<tr>
<td>17</td>
<td>Norfloxacin</td>
<td>NX</td>
<td>10 mcg</td>
</tr>
<tr>
<td>18</td>
<td>Ofloxacin</td>
<td>OF</td>
<td>5 mcg</td>
</tr>
<tr>
<td>19</td>
<td>Piperclillin</td>
<td>PC</td>
<td>100 mcg</td>
</tr>
<tr>
<td>20</td>
<td>Piperclillin tazobactum</td>
<td>PT</td>
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<tr>
<td>21</td>
<td>Tetracycline</td>
<td>T</td>
<td>30 mcg</td>
</tr>
<tr>
<td>22</td>
<td>Ticarcillin</td>
<td>TI</td>
<td>75 mcg</td>
</tr>
<tr>
<td>23</td>
<td>Ticarcillin/Clavulanic acid</td>
<td>TC</td>
<td>75/10 mcg</td>
</tr>
<tr>
<td>24</td>
<td>Tobramycin</td>
<td>TB</td>
<td>10 mcg</td>
</tr>
</tbody>
</table>
Materials and methods

Fig. 4. Antimicrobial agents used
3.8.1. Preparation of inoculum

At least three to five well-isolated colonies of the same morphological type were selected from an agar plate and transferred into a tube containing 4 to 5 ml of a peptone broth. The broth culture was incubated at 35°C until it achieves or exceeds the turbidity of the 0.5 McFarland standard (usually 2 to 6 hours). The turbidity of the actively growing broth culture was adjusted with sterile saline or broth to obtain a turbidity optically comparable to that of the 0.5 McFarland standard.

3.8.2. Inoculation of test plates

Optimally, within 15 minutes after adjusting the turbidity of the inoculum suspension, a sterile cotton swab is dipped into the adjusted suspension. The swab was rotated several times and pressed firmly on the inside wall of the tube above the fluid level. This was done to remove excess inoculum from the swab. The dried surface of a Müller-Hinton agar plate was inoculated by streaking the swab over the entire sterile agar surface. This procedure was repeated by streaking two more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculum. As a final step, the rim of the agar was swabbed. The lid may be left ajar for 3 to 5
Materials and methods

minutes, but not more than 15 minutes, to allow for any excess surface moisture to be absorbed before applying the drug impregnated discs.

3.8.3. Application of discs to inoculated agar plates

The batteries of 24 antimicrobial discs (Table 2) were dispensed onto the surface of the inoculated agar plate. Each disc was pressed down to ensure complete contact with the agar surface. The discs were placed individually with the help of a forcep dipped in alcohol and flamed and were distributed evenly so that they were no closer than 24 mm from center to center. The plates were inverted and placed in an incubator set to 37°C within 15 minutes for 24-48 hrs after the discs were applied.

3.8.4. Reading and interpretation

The diameter of the zones of inhibition around each disc was measured to the nearest millimeter (mm), which included 6mm diameter of the disc using the Zone Reading Scale provided by the manufacturer of the discs. The results were interpreted using the zone-size interpretative table provided along with the discs.
Fig. 5. Zone-size interpretation by zone reading scale
3.9 DETECTION OF TOXIN GENES (stx1, stx2, est, elt and hlyA) BY PCR

3.9.1. Preparation of the template DNA

The template DNA was prepared by the boiling method and the procedure followed for the extraction of all the five genes were similar (Rahman, 2002). A loopful of pure culture of each isolate was incubated overnight in 5ml of TSB broth at 37°C. About 0.5ml of the broth culture was taken in a microcentrifuge tube and the bacterial cells were separated by centrifugation at 10,000 rpm for 5 min in a refrigerated centrifuge (Eppendorf) and washed twice with phosphate buffer saline (PBS, pH 7.4). The cells were finally suspended in 300μl of HPLC grade water in a microcentrifuge tube, gently vortexed and boiled for 10 min. After boiling, the cell suspensions were cooled in an ice bath and immediately tested for the presence of the stx1, stx2, est, elt and hlyA genes by PCR amplification using their specific primers.

3.9.2. PCR amplification of the toxin genes (stx1, stx2, est, elt and hlyA)

Details of the primers used for stx1, stx2, est, elt and hlyA genes, PCR conditions, etc are presented in Table 3. The primers are obtained from M/s. Bangalore GeNei, India. The detection of stx1 and stx2 gene by PCR was carried out as per the method described by Rahman (2002), while the elt and est genes detection were carried out as per the method described
by Osek (1999). The amplification reaction was carried out in a final volume of 25µl containing 12.5µl Master mix (Bangalore GeNei, India), 2.5µl Taq DNA polymerase, 200µM each of dATP, dCTP, dTTP and dGTP and PCR buffer, 5µl (1µM) each of forward and reverse primers and 2.5µl of template DNA. The PCR incubation was carried out in a thermocycler (Eppendorf) in 30 cycles.

3.9.3 Visualization of the PCR product

The PCR product was visualized in a horizontal submarine agarose gel electrophoresis as described by Sambrock et al. (1989). A 1% agarose gel containing 0.5µl/ml ethidium bromide in TAE buffer (1X) was used. About 5µl of PCR product was mixed with 2µl of gel loading dye (0.5% bromophenol blue) was loaded into the wells of the gel. A standard DNA marker 100bp DNA ladder (Bangalore GeNei, India) was also loaded into the wells with the gel loading dye. Electrophoresis was carried out at 70V for 60 min till the bromophenol blue of the gel loading buffer migrated more than 4/5th the length of the gel. At the end of electrophoresis, the gel was stained with ethidium bromide (0.5µl/ml) for 15 min and was visualized in the gel documentation system (Vilber Lourmat) and photographed.
Table 3: Primer sequence and PCR conditions used for detection of \textit{E. coli} enterotoxigenic genes

<table>
<thead>
<tr>
<th>Primer code</th>
<th>DNA sequence (5'-3')</th>
<th>Target gene</th>
<th>PCR conditions</th>
<th>No. of cycles</th>
<th>Amplified product (bp)</th>
<th>Reference</th>
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<tr>
<td>LP30</td>
<td>cag tta atg tcg tgg cga agg</td>
<td>stx1</td>
<td>Denaturation (°C/sec)</td>
<td>94/60</td>
<td>72/60</td>
<td>30*</td>
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<tr>
<td>LP31</td>
<td>cac cag aca atg taa ccc cgg ctg</td>
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<td>Annealing (°C/min)</td>
<td>55/60</td>
<td>59/90</td>
<td></td>
</tr>
<tr>
<td>LP43</td>
<td>atc cta ttc ccc gga gtt tac g</td>
<td>stx2</td>
<td>Extension (°C/min)</td>
<td>72/60</td>
<td>72/60</td>
<td></td>
</tr>
<tr>
<td>LP44</td>
<td>ggc tga ccc tgg cgg cgc ggc cgc cgc cgc cgc</td>
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</tr>
<tr>
<td>STa1</td>
<td>tct ttc ccc tct ttt agt cag aca ggc agg att aca aca aag</td>
<td>estl</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LT1</td>
<td>ggt gca gca gaa aaa gtt gta g</td>
<td>hlyA</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LT2</td>
<td>tct cgc ctg ata cgtg ttt ggt a</td>
<td></td>
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Materials and methods