5. MATERIALS AND METHODS

5.1 STUDY DESIGN

The present study will be undertaken among all patients diagnosed with diarrhea. Over a period of 12-18 months, stool specimen of all patients reporting to the hospital with diarrhea will be collected after taking the informed consent. Those specimens positive for *Escherichia coli* will be assessed further and rest will be excluded.

**Type of study:** Cross-sectional study

**Duration of study:** 12-18 months

**Place of study:** Rama Medical College, Hospital & Research Centre, Kanpur, U.P.

**Inclusion Criteria:**

The present study will include all subjects (children under the age of 5 years) suffering from diarrhea who report to the hospital, both admitted as well as outpatient.

**Exclusion Criteria:**

1. Diarrhea patients not infected with *E. coli* (confirmed by microscopy)
2. Patients who were on antibiotics in last 15 days before reporting to the hospital
3. Patients who did not give consent to participate
5.2 SAMPLE SIZE

A minimum of 196 samples would be needed. This calculation of the sample size is based on the assumption that the prevalence of diarrheagenic \textit{E. coli} in stool samples of children suffering from diarrhea is about 15%.

Formula\(^7\): \[ n = \frac{(1.96)^2 p \times q}{P^2} \]

Assumptions:

\begin{align*}
\text{Precision (P)} &= 5.00\% \\
\text{Prevalence (p)} &= 15.00\% \\
q &= 100 - p = 85\% \\
n &= \frac{(1.96)^2 (0.15) (0.85)}{(0.05)^2} \\
&= \frac{3.84 \times 0.15 \times 0.85}{0.0025} \\
&= 0.4896 / 0.0025 \\
&= 195.84 \approx 196
\end{align*}

Estimated sample size \( n \geq 196 \). A total of 200 samples would be targeted to achieve the desired sample size.

**Age Limit** – Children with diarrhea under 5 years of age will be included in the study.

5.3 PROCEDURE

5.3.1 Laboratory diagnosis

Laboratory diagnosis of diarrheagenic \textit{E. coli} depends upon the type of stool, frequency and other clinically related symptoms like nausea, vomiting, abdominal cramps & fever > 38°C.

5.3.2 Samples Considered

Stool samples from each patient, including watery stool (within 24 hours), mucous, purulent blood stool, where the patients had not taken antibiotics will be considered.
5.4 INVESTIGATION OF STOOL SAMPLES

5.4.1 Collection

Fresh stool samples obtained from outpatient or in-patients (hospitalized) will be collected in a wide mouthed sterile container (properly labeled) on the day of presentation.

5.4.2 Staining

We will make slide from a stool sample thin smear that will be stained by Gram Staining method for studying characteristic morphology of E.coli and will then examine in oil immersion at 100x.

5.4.3 Culture

Fecal samples will be seeded on Eosin methylene blue agar and MacConkey Agar and Sorbitol MacConkey Agar incubate on 37°C for 24 hours.61

5.4.4 Biochemicals

Colony of E.coli isolated from culture media after 24 hours/48 hours of incubation will be verified by Standard Biochemical Tests i.e. Triple Sugar Iron, Indole, Methyl Red/ Voges-Proskauer, Citrate, Urease, and Mannitol fermentation.72

5.4.5 DNA Extraction

The DNA Extraction Kit will extract DNA in all the positive samples.

5.4.6 Polymerase Chain Reaction (PCR)
5.4.6.1 Detection of *E.coli* strain by Multiplex PCR

The Multiplex PCR assays would be standardized for the detection of five types of DEC. The reference strains will be cultured on Mac.Conkey agar. A sweep of about five *E.coli* like colonies will be used by PCR. The DNA will be isolated from colonies by suspending the colonies in 50μl of deionized water. The suspension will be boiled for 10 min at 95°C and centrifuged at 10,000 g for 10 min. The supernatant will then be used as the DNA template. The DNA templates will be subjected to multiplex PCR with specific primers (Table 2), for the detection of the following: eaeA for the structural gene of intimin of EPEC and EHEC, hylA for the plasmid encoded enterohemolysin of EHEC, bfpA for the structural gene of the bundle forming pilus of EPEC, elt and stlA for the enterotoxins of ETEC, ial for the invasion plasmid found in EIEC, CVD432 for the nucleotide sequence of the EcoR1-Pst DNA fragment of EAEC.73

The primer will be selected on the basis of similar studies done earlier. The multiplex PCR assays will be tested with several PCR cycling protocols. The reaction mixture containing optimized protocol will be carried out with a 50μl mixture containing 10mM Tris-HCL (8.3), 50mM KCl, 2.0mM MgCl2, a 2mM concentration of each deoxynucleoside triphosphate, 2 U of Hot start Taq DNA polymerase, 5μl of the DNA template, 0.5μM of each of the primers i.e. CVD432 for EAEC isolates, elt and stlA for ETEC isolates and hlyA and eaeA for EHEC isolates. The cycling conditions will be as follows: 95°C for 1 min, 55°C for 1 min, 72°C for 1 min and 72°C for 5 min. The protocol used will be similar to that used in multiplex PCR except that the primers used will be ial for EIEC isolates and eaeA and bfpA for EPEC isolates.

The thermo cycling conditions would be as follows: 95°C for 1 min for one cycle followed by 35 cycles of 94°C for 1 min, 57°C for 1 min, 72°C for 1 min and 72°C for 5 min. The PCR products (10μl) will be analyzed by gel electrophoresis with 2.0% (w/v) agarose gels. The DNA bands would be visualized and photographed under UV light after staining the gel with ethidium bromide.
### PCR Primers used in the Multiplex PCR Assays for the detection of virulence genes of Diarrheagenic *E.coli* (Table 2)

<table>
<thead>
<tr>
<th>Reference strain</th>
<th>Primers (5´ to 3´)</th>
<th>Target gene</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPEC</td>
<td>TGATAAGCTGCAGTCGAATCC CTGAACCACTCGTAACGGC CACCGTTACCAGGTGTA GTTGCCGCTTCAGCAGGAGT</td>
<td>eaeA, bfpA</td>
<td>229, 450</td>
</tr>
<tr>
<td>ETEC</td>
<td>CTCTATGTGCACACGGAGCC CATACTGATTGCCGAAT TCTTTCCCCTCTTTTAGTCAGTC CCGCACAGGCAGGATTAC</td>
<td>elt, Stla</td>
<td>322, 170</td>
</tr>
<tr>
<td>EAEC</td>
<td>CTGGCGAAAGACTGTATCAT CAATGTATAGAATAATCCGCTTT</td>
<td>CVD432</td>
<td>630</td>
</tr>
</tbody>
</table>

### 5.4.6.2 Expected *E.coli* strain PCR results

A positive PCR test should give a 252 bp DNA fragment that would appear as an intense band on an ethidium bromide-stained 2.0% agarose gel. The molecular size of the band can be verified by comparing the migration to that of a DNA molecular size marker (100 bp ladder DNA) using the same gel. On the contrary, a negative PCR test will not produce any visible bands in the ethidium bromide-stained agarose gel.

### 5.4.6.3 Advantages of PCR

PCR is a highly sensitive and specific molecular biology technique for the detection of target DNA in various clinical specimens. The Multiplex PCR assays would be also used for detecting DEC directly from the stool samples that were positive for DEC. The multiplex
PCR assays would be found to be effective for direct detection of DEC in stool samples. The assays correctly identified 100% of the DEC strains directly in stool samples.\textsuperscript{74}

5.4.7 Antibiotic susceptibility testing

Antimicrobial susceptibility testing would be performed by the disk diffusion method according to the Clinical and Laboratory Standards Institute guidelines.\textsuperscript{75} Approximately 4mm thick Mueller-Hinton agar would be prepared from a dehydrated base according to the manufacturer’s recommendations. The inoculums for antimicrobial susceptibility testing of bacterial isolates will be prepared from fresh pure cultures. The cell suspensions of the bacterial isolates will be prepared in sterile Mueller-Hinton broth. A cell suspension equal to a density of 0.5 McFarland turbidity standard will be used for inoculation. When the proper density is achieved, a cotton swab would be dipped into the bacterial suspension excess fluid will be removed by pressing and rotating the swab against the wall of the tube. The swab will be used to inoculate the entire surface of the Mueller-Hinton agar plate three times, rotating the plate 60 degrees between each inoculation. The inoculum would be allowed to dry before placing the disks on the plates. Drying usually takes only a few minutes, and should take no longer than 15 minutes. After the plate is dry, the antimicrobial disks will be placed on the plates.

Sterile forceps will be used to place the disks on the Mueller Hinton agar and taped gently to ensure that they adhere to the agar. Diffusion of the drug in the disk begins immediately; therefore, once a disk contacts the agar surface, the disk will not be moved. The plates will be incubated in an inverted position for 18–24 hours at 37°C. After overnight incubation, the diameter would be measured of each zone of inhibition with a ruler or a caliper. In all measurements, the zones of inhibition will be measured as the diameters from the edges of the last visible colony, and the results recorded in millimeters (mm). Inhibition zone diameters will be compared to the reference list of the manufactures.

Antimicrobial agents tested would be ampicillin (10 mcg), Amoxycillin/Clavulanic Acid (30 mcg), Cefoxitin (30 mcg), Cefoperazone/sulbactum (30 mcg), ceftazidime (30 mcg),
ceftazidime/Clavulanic Acid (30 mcg), co-trimoxazole (25 μg), amikacin (30 mcg), piperacillin/Tazobactam (30/6 mcg), Ertapenem (10 mcg), Imipenem (10 mcg), Meropenem (10 mcg), Colistin (10 mcg), Tigecycline (15 mcg), Ceftriaxone (30 mcg), Cefepime (30 mcg), Gentamycin (10 μg), Netilmicin (10 mcg), Aztreonam (30 mcg), Levofloxacin (5 mcg) (HiMedia Laboratories Co., India). The *E. coli* strain ATCC 25922 would be included as a quality control in all assays.

5.5 STATISTICAL ANALYSIS

Data will be analyzed using SPSS version 22 software. Percentages would be compared using a Pearson chi-square test for dependent samples or Fisher’s exact test when appropriate.