FINAL REPORT OF UGC PROJECT

Entitled

PREVALENCE OF ESBL PRODUCING ESCHERICHIA COLI FROM FECAL ISOLATES AND ITS CORRELATION WITH PUTATIVE VIRULENCE DETERMINANTS

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Summary of the project

**Background & Objective:** Diarrheagenic *Escherichia coli* (DEC) is associated with early death of children in developing countries and are being identified now as an important evolving pathogen. The objective of this study was to assist in determining the prevalence rates of the multidrug resistant *E. coli* expressing β-lactamase enzymes, as residents of gut flora in paediatric patients suffering from diarrhea and in healthy children and perform multiplex PCR for simultaneous detection of three categories of DEC in two sets of PCR reactions using seven virulent genes.

**Materials and Methods:** During 3-year study period 700 stool samples were collected from the community children with diarrhea, non diarrheal hospitalized children and compared with control group. *E. coli* was isolated and was also subjected to antimicrobial testing by disc diffusion method. Screening and confirmation of β-lactamases were done by phenotypic methods. DNA was extracted from the isolates and the extracted DNA was used as a template for multiplex PCR. The presence of the following genes depicted the various DEC: *elt* and *est* for ETEC, *eagg* and *east* for EAEC, and *eae*, *eae* and *bfpA* for EPEC.

**Results:** 100 *E.coli* were isolated from outpatient, 90 isolated from inpatient and 40 isolated from control groups out of the total samples collected. Out of 230 (32.86%) *E.coli* isolated, virulent genes of DEC were detected in 203 (88.26%) samples by multiplex PCR. Overall, *elt* and *est* were detected in 8% and 33% of specimens; EPEC typical and atypical were detected in 17.29% and 28.71% respectively. *bfpA* were detected in 24.71% specimens; EAgg was detected in 36.14% and *east* in 14.29% specimens.

β-lactamase production was observed in 101(84.16%) isolates. 64.16% isolates were ESBL producers, 24.16% were MBL producers and 16.66% were AmpC producers. Co-existence of
ESBL with MBL was observed in 10.83% isolates MBL with AmpC in 6.66% and ESBL with AmpC was observed in 4.16% isolates.

**Conclusion:** Multiplex PCR is a rapid method for the simultaneous detection of 7 virulent genes of DEC at a time and it will provide a platform in understanding the diarrheal diseases in a more improved manner. Dissemination of ESBL, AmpC and MBL producing clones resulting from movement of patients between community to hospital and intra and inter-hospital not only promotes epidemic spread without any borders but also limits availability of treatments options for infections caused by such organism. Thus, there is an urgent need to promote a rational use of antibiotics, both in hospital and in the community.
Detailed Report:

Origin of the research problem

Infectious diarrhea is a common complaint among patients seeking medical advice. Despite progress in diagnosis and treatment, it remains one of the leading causes of morbidity and mortality worldwide. \(^1\)\(^2\) *Escherichia coli* is the most important etiologic agent of childhood diarrhea and represents a major public health problem in developing countries. \(^3\) It causes 1.8 million childhood deaths in children fewer than five years of age \(^4\) and in India, diarrhea is the third most common cause of death in this age group. \(^5\) Some children in developing countries experience over a dozen episodes of diarrhea in their first year. Repeated and persistent diarrhea in young children contributes to significant cognitive and growth impairment that can impact school performance and development. \(^6\)\(^7\)\(^8\) Diarrhea makes a greater contribution to malnutrition and growth impairment than other common infections and *Escherichia coli* diarrheas may be even more detrimental than rotavirus infections in this regard. \(^6\)\(^9\)\(^10\) The diverse range of diarrheagenic *E. coli* pathogenic types means that children may be subject to repeated infection by different subtypes without immune protection. Moreover low birth weight, premature rupture of membrane, and indiscriminate use of antibiotics to treat childhood diarrhea may be individual risk factors for *E.coli* diarrhea.

Moreover the widespread reports of different mechanisms of drug resistance in *E.coli* are a cause of great concern. Not only does this complicate treatment of different infections caused by it but also enhances the risks of transmission of these traits into bacteria of the same or other genus. Since *E.coli* is a normal commensal of the human intestinal tract, this increases the chance of fecal carriage of drug resistant strains and their subsequent spread. The increasing importance of fecal carriage of *E.coli* as a source of drug resistant strain associated outbreaks has been widely
reported. A drug resistant strain causing diarrhea is another dangerous scenario, which would grossly limit our treatment options in severe cases. Global tourism has contributed to a change in the environmental niche of such pathogens and thus has led to the emergence complication in management of such cases.

At present a number of lacunae exist in identification of pathogenic *E.coli*. Commercial *E.coli* anti-sera is not only very expensive but also very cumbersome to prepare in-house. *E.coli* is a frequent causative agent of acute diarrhea in children less than 5 years is an assumption that is not always true and is a pragmatic alternative to reduce the burden on diagnostic laboratories. Since the virulence factors of pathogenic *E.coli* are many, individual detection of each of them by commercial systems entails huge cost, cell-culture facility and animal systems which are not accessible to every diagnostic laboratory.

The potential threat of spreading of drug resistance via transferrable genetic elements is complemented by the ability of *E.coli* to remain as intestinal commensal bacteria. The significance of detection of enteric carriage of resistant *E.coli* lies in the fact that such situation in a hospital poses serious health threat requiring the implementation of surveillance and control measures for nosocomial infections. Such data is of immense importance in formulating the treatment protocol and hospital antimicrobial policy as well as outbreak control.

This study attempts to improve the diagnosis of DEC by detecting the virulence genes of enteropathogenic *E.coli* (EPEC), enterotoxigenic *E.coli* (ETEC) and enteroaggregative *E.coli* (EAggEC) by developing a multiplex PCR. The multiplex PCR assay in which several PCR primers are combined with the aim of detecting one of several different diarrheagenic *E. coli* pathotypes in a single reaction. This assay will thus allow categorization of DEC strain in a single reaction tube from an overnight stool culture.
Moreover the significance of detection of enteric carriage of resistant *E.coli* lies in the fact that such situation in a hospital poses serious health threat requiring the implementation of surveillance and control measures for nosocomial infections. Such data is of immense importance in formulating the treatment protocol and hospital antimicrobial policy as well as outbreak control. There lies the relevance of the current study.

**Brief objective of the project:**

1. To identify the role of DEC as a common cause of diarrhea amongst children less than 5 years admitted in hospital as well in the community in developing countries and its antibiotic resistance patterns including ESBL, AmpC & MBL.

2. To estimate the percentage multidrug resistant E.coli carriage as a normal gut commensal and correlate with the usage of antimicrobials amongst the under 5 years of age group.

3. To study the host pathogen interaction by amplifying putative virulence markers: *eae, bfpA, elt, est, east* genes and EFA and EAgg plasmids.

**Methodology:**

700 consecutive patients less than 5 years of age presenting with acute diarrhea at Guru Teg Bahadur Hospital were enrolled in the study after obtaining pre-informed consent from patients guardians and ethical clearance from Institutional Ethics Committee.

The study population comprised three groups.

**OPD group** included 300 children ≤5yrs with diarrhea for <72 h duration (not receiving any antibiotics) attending the outpatient department of our hospital.
**IPD group** consisted of children non-diarrheal group included 300 children ≤5yrs hospitalized and receiving antibiotic (oral or intravenous) for 72 h or more for reasons other than diarrhea

**Control group** represented healthy controls of 100 children below 5 years of age not suffering from diarrhea or any other disease.

Fresh stool samples were collected and inoculated on media as per standard laboratory methods, and *E. coli* was identified based on standard biochemical reactions.

Antibiotic susceptibility profile were done on Muller Hinton agar plates by Kirby-Bauer disc diffusion method and interpreted as per CLSI guidelines. The following antibiotics (from Hi-Media Laboratories, BD Diagnostics Pvt Ltd, India) would be included in the panel to be tested:

- Ampicillin (10 μ g)
- Amoxicillin – Clavulanic acid (20/10 μ g)
- Piperacillin – Tazobactam (100/10 μ g)
- Ceftazidime (30 μ g)
- Ceftazidime-Clavulanic acid (30/10 μ g)
- Tetracycline (30 μ g)
- Imipenem (10 μ g)
- Cotrimoxazole (1.25/23.75 μ g)
- Nalidixic acid (30 μ g)
- Norfloxacin (10 μ g)
- Gentamicin (10 μ g)
- Amikacin (30 μ g)

Where-ever *E.coli* considered as a putative agent from such stool samples:

I. ESBL, AmpC and MBL will be detected

II. multiplex PCR will be done to study their virulence factors. This will be done for isolates of *E.coli*.

**Phenotypic methods for screening and confirmation of Beta lactamases**

**ESBL** - Phenotypic screening for ESBL production was performed on *E. coli* isolates. Recordings of the zone diameters of ceftazidime/cefotaxime/ceftriaxone/aztreonam were performed by disc diffusion test on Mueller Hinton Agar plates using CLSI guidelines. Any
isolate showing a reduced (resistant) zone diameter was included for confirmatory testing.

**Confirmation - Double Disk Synergy Test (DDST)** was performed on isolates resistant to ceftazidime/cefotaxime/ceftriaxone/aztreonam by placing disks of ceftazidime (30 μg) or cefotaxime (30 μg) discs at a distance of 20 mm from the ceftazidime+clavunate (20/10 μg) disc; a enhanced zone of inhibition ≥5mm towards ceftazidime/clavunate (20/10 μg) disc was considered as positive for ESBL production.\(^{13}\) *E. coli* ATCC 25922 was used as negative control and *K. pneumoniae* ATCC 700603 as positive control.

**AmpC** - For AmpC screening a 30ug cefoxitin disk was placed on Mueller Hinton agar plate with lawn culture of *E. coli*, zone diameters less than 18mm was selected for confirmation of AmpC production.

**Confirmation - AmpC disk test:** Tris-EDTA disk was rehydrated with 20 μl of saline and 4–5 colonies of the test isolate were inoculated on the disk. The inoculated disk was then placed beside a cefoxitin disk on a Mueller Hinton agar plate inoculated with a lawn of *E. coli* ATCC 25922 and incubated overnight. A positive test appears as a flattening or indentation of the cefoxitin inhibition zone near the test disk while a negative test has an undistorted zone.\(^{14}\)

**Boronic acid disk test method:** a 30ug cefoxitin disk was supplemented with 300ug phenyl boronic acid. An organism that demonstrated a defined increase(≥5mm) in zone diameter around the antibiotic disk with added inhibitor compound compared to that with antibiotic containing disk alone was considered to be AmpC producer.\(^{15}\)

**Disk approximation method** - Use of disk approximation technique to detect inducible AmpC activity was tested using ertapenem (10ug), cefoxitin (30ug) and amoxicillin-clavulanic acid disks (20/10ug) as the inducing substrates and ceftazidime (30ug) disk as the reporter substrate. Disks were placed at a distance of 20mm, and any obvious blunting or flattening of the zone of
inhibition between ceftazidime disk and the inducing substrates was interpreted as a positive result for AmpC.\textsuperscript{15,16}

\textit{E. coli} ATCC 25922 was used as negative control and \textit{E. cloaceae} BAA-1143 as a positive control

\textbf{MBL:} screening was performed in all isolates for imipenem/ ertapenem/ meropenem (10µg) resistance by disc diffusion method.

\textbf{Confirmation-Combined disc diffusion test:} Two 10 µg ertapenem (ERT) discs were placed at a distance of 20mm on a lawn culture of the isolate and 10 µl of 0.5 M EDTA solution was added to one ertapenem disc. A plain EDTA disc can also be placed separately on the same plate to detect its inhibitory effect on test isolates. The zone of inhibition around ERT discs alone and those with EDTA will be compared after 16-18 hours. An increase in zone size of at least 5 mm \(>/\) around the ERT-EDTA disc as compared to ERT disc alone was recorded as a positive result.\textsuperscript{17}

\textbf{Modified Hodge test:} An IMP/ERT disc (10 µg) was placed at the centre of the lawn of an overnight culture suspension of \textit{Escherichia coli} ATCC 25922, adjusted to one-tenth turbidity of the 0.5 McFarland standards. The test isolates was streaked heavily from the edge of the disc to the plate periphery. The presence of a clover-leaf shaped zone of inhibition was interpreted as production of MBL.\textsuperscript{18}

\textit{E. coli} ATCC 25922 as negative control and \textit{K. pneumoniae} ATCC/BAA-1705/1706 was used as positive control

\textbf{MBL E-test-} An E Test MBL strip containing a double sided seven-dilution range of IPM (4 to 256 µg/mL) and IPM (1 to 64 µg/mL) in combination with a fixed concentration of EDTA (AB Biodisc, Solna, Sweden) was placed on the dried surface of a lawn culture inoculation from a log
phase growth of test organism. The presence of MBL will be reflected by a reduction in the IMP MIC by ≥3 dilutions in the presence of EDTA or the presence of a phantom zone or deformation in the IMP ellipse.19

**DNA Extraction for multiple PCR:**

After isolation of E.coli, DNA was extracted from the isolated E.coli using a commercial kit (Real Biotech Corporation, Taiwan). The presence of the following genes depicted the various DEC: *elt* and *est* for ETEC, *eagg* and *east* for EAEC, atypical EPEC with *eae* and *eae + bfpA* for typical EPEC;

GAPDH was used as amplification internal quality control.

The following 7 primer sets (Table 1) were used to amplify the above mentioned genes and the amplicon products were separated by 1.5% agarose gel by electrophoresis with ethidium bromide and visualized under UV-light.

Each 0.2 ml tubes used for each set of multiplex PCR assay contained a total volume of 25 μl including 2.5μl buffer (10X), 1 μl dNTPs (200 μM), MgCL2 1 μl (1.5mM), 1 μL of each primer, forward and reverse (10 μM), 5 μl of the extracted DNA and nuclease-free water to make up the volume. All PCR reagents were purchased from Genei, Bengaluru, and amplification was performed on a thermocycler (Eppendorf).

An initial denaturation was performed at 94°C for 10 min which was followed by 35 amplification cycles of 40 s at 94°C and 30 s at 50°C and 55°C and 50 s at 72°C, and final extension of 7 min at 72°C. The amplified PCR products were analyzed by electrophoresis on 1% agarose gel, stained with ethidium bromide at 125 V and 15 mA current for 30 min. Molecular marker of 100 bp was used to determine the size of the amplicons. Uniplex PCR
(Fermentas, India) was also performed in DEC isolates that showed the presence of multiple genes, for the confirmation of mixed infection.

Table 1: Target genes & their primers & amplicon size

<table>
<thead>
<tr>
<th>E.coli type</th>
<th>Target gene or encoding region</th>
<th>Primer sequences (5'-3')</th>
<th>Amplicon (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETEC</td>
<td>elt</td>
<td>GCGACAAATTATACCGTGCT</td>
<td>708</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CCGAATTCTGTTATATATATGT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>est</td>
<td>ATTTTTA/CTTTCTGTATTA/CTCTT</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CACCCGGGTACAA/GGCAGGATT</td>
<td>190</td>
</tr>
<tr>
<td>EPEC</td>
<td>eae</td>
<td>AAACAGGTGAAACTGGTTCGCC</td>
<td>454</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CTCTGAGATTAAACCTCTGC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>bfpA</td>
<td>AATGGTGCTTGGCTTGCTGC</td>
<td>324</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GCCGCTTTATCCAACCTGGTA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EAF</td>
<td>CAGGGTAAAAAGAAGATGATAA</td>
<td>397</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TATGGGGGACCATGTATTATCA</td>
<td></td>
</tr>
<tr>
<td>EAggEC</td>
<td>EAgg</td>
<td>CTGGCGAAAGACTGTATCAT</td>
<td>630</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CAATGTATAGAAATCCGCTGT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>east</td>
<td>CACAGTGATATCCGAAGGCC</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CGAGTGACGGCTTTGTAG</td>
<td></td>
</tr>
</tbody>
</table>
Results:

Figure 1: Uniplex PCR of control genes

Uniplex PCR of control strain of EPEC, ETEC & EAEC
Lane 1: Marker 100bp; Lane 2: *eae*; Lane 3: *bfpA*
Lane 4: *elt*; Lane 5: EAgg; Lane 6: *eae*; Lane 7: *est*
Lane 8: EAF

Figure 2: Multiplex PCR of control genes

Multiplex PCR of positive control strains of EPEC, ETEC & EAEC
Lane 1: Marker 100bp; Lane 2: Set 1: *eae* (94bp) & *est* (190bp)
Lane 3: Set 2: *elt* (708bp), *eae* (454bp), EAgg (630bp), EAF (397bp), *bfpA* (324bp)
Figure 3: Multiplex PCR of different samples

In both Top & Down lane:
Lane 1 & 13 is 100bp marker;
Lane 2,3,4,5 are different OPD samples;
Lane 6,7,8,9 are different IPD samples;
Lane 10,11,12 are different control strains.
Table 2: Results of multiplex PCR in various study groups

<table>
<thead>
<tr>
<th></th>
<th>OPD n=300 (%)</th>
<th>IPD n=300 (%)</th>
<th>Control n=100 (%)</th>
<th>Total n=700 (%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>bfpA</td>
<td>90 (30)</td>
<td>30 (10)</td>
<td>41 (41)</td>
<td>173 (24.71)</td>
<td></td>
</tr>
<tr>
<td>bfpA+EAF+eae−</td>
<td>45 (15)</td>
<td>53 (17.67)</td>
<td>0</td>
<td>98 (14)</td>
<td></td>
</tr>
<tr>
<td>Typical EPEC (eae+bfpA−)</td>
<td>68 (22.67)</td>
<td>45 (15)</td>
<td>8 (8)</td>
<td>121 (17.29)</td>
<td></td>
</tr>
<tr>
<td>Atypical EPEC (eae+EAF−)</td>
<td>38 (12.67)</td>
<td>113 (37.67)</td>
<td>50 (80)</td>
<td>201 (28.71)</td>
<td></td>
</tr>
<tr>
<td>EPEC</td>
<td>241 (80.33)</td>
<td>241 (80.33)</td>
<td>99 (99)</td>
<td>581 (83)</td>
<td></td>
</tr>
<tr>
<td>elt</td>
<td>33 (11)</td>
<td>23 (7.67)</td>
<td>0</td>
<td>56 (8)</td>
<td></td>
</tr>
<tr>
<td>est</td>
<td>95 (31.67)</td>
<td>128 (42.67)</td>
<td>8 (8)</td>
<td>231 (33)</td>
<td></td>
</tr>
<tr>
<td>elt + est</td>
<td>100 (33.33)</td>
<td>30 (10)</td>
<td>0</td>
<td>130 (18.57)</td>
<td></td>
</tr>
<tr>
<td>ETEC</td>
<td>228 (76)</td>
<td>181 (60.33)</td>
<td>8 (8)</td>
<td>417 (59.57)</td>
<td></td>
</tr>
<tr>
<td>east</td>
<td>30 (10)</td>
<td>55 (18.33)</td>
<td>15 (15)</td>
<td>100 (14.29)</td>
<td></td>
</tr>
<tr>
<td>EAagg</td>
<td>125 (41.67)</td>
<td>70 (23.33)</td>
<td>58 (58)</td>
<td>253 (36.14)</td>
<td></td>
</tr>
<tr>
<td>EAagg+east</td>
<td>38 (12.67)</td>
<td>60 (20)</td>
<td>0</td>
<td>98 (14)</td>
<td></td>
</tr>
<tr>
<td>EAEC</td>
<td>193 (64.33)</td>
<td>185 (61.67)</td>
<td>73 (73)</td>
<td>451 (64.43)</td>
<td></td>
</tr>
</tbody>
</table>

The most frequent category of DEC detected was EPEC followed by EAEC and ETEC, as shown in Table 2.

The most common mixed infections observed in our study were EAEC, EPEC and ETEC in 210/700 (30%); EAEC and EPEC in 99/700 (14.14%) and EPEC and ETEC in 30/700 (4.29%)
isolates. Coexistence of all the three categories of DEC together was present in 120/300 isolates (40%) in OPD Group and 90/300 (30%) in IPD Group. In healthy controls, the coexistence of EAEC and EPEC occurred in 11/40 isolates (27%) [Table 3].

Table 3: Co-infection of diarrheagenic *Escherichia coli* among three groups

<table>
<thead>
<tr>
<th>Study Group</th>
<th>EAEC + EPEC</th>
<th>EPEC + ETEC</th>
<th>EPEC + ETEC + EAEC</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPD n=300 (%)</td>
<td>0</td>
<td>12 (4)</td>
<td>120 (40)</td>
</tr>
<tr>
<td>IPD n=300 (%)</td>
<td>72 (24)</td>
<td>18 (6)</td>
<td>90 (30)</td>
</tr>
<tr>
<td>Control n=100 (%)</td>
<td>27 (27)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total n=700 (%)</td>
<td>99 (14.14)</td>
<td>30 (4.29)</td>
<td>210 (30)</td>
</tr>
</tbody>
</table>

Table 4: Resistance (%) of all antibiotics

<table>
<thead>
<tr>
<th>Study Group</th>
<th>Amp</th>
<th>Cef</th>
<th>Ptz</th>
<th>Ceft</th>
<th>Az</th>
<th>Imp</th>
<th>Amika</th>
<th>Gnt</th>
<th>NA</th>
<th>Nor</th>
<th>Cip</th>
<th>Cot</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPD</td>
<td>1</td>
<td>90</td>
<td>88</td>
<td>99</td>
<td>100</td>
<td>17</td>
<td>14</td>
<td>42</td>
<td>96</td>
<td>82</td>
<td>90</td>
<td>95</td>
</tr>
<tr>
<td>IPD</td>
<td>16.7</td>
<td>12.5</td>
<td>66.7</td>
<td>100</td>
<td>100</td>
<td>16.7</td>
<td>54.2</td>
<td>54.2</td>
<td>70.83</td>
<td>87.5</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>100</td>
<td>12.5</td>
<td>100</td>
<td>25</td>
<td>100</td>
<td>50</td>
<td>75</td>
<td>100</td>
</tr>
</tbody>
</table>
Figure 4: Percentage Resistance of all antibiotics

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Percentage Resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amp: Ampicillin</td>
<td>OPD 10% IPD 20% CONTROL 30%</td>
</tr>
<tr>
<td>Cef: Cefotaxime</td>
<td>OPD 15% IPD 25% CONTROL 35%</td>
</tr>
<tr>
<td>Ptz: Piperacillin/tazobactam</td>
<td>OPD 20% IPD 30% CONTROL 40%</td>
</tr>
<tr>
<td>Ceft: Ceftazidime</td>
<td>OPD 25% IPD 35% CONTROL 45%</td>
</tr>
<tr>
<td>Az: Aztreonam</td>
<td>OPD 30% IPD 40% CONTROL 50%</td>
</tr>
<tr>
<td>Imp: Imipenem</td>
<td>OPD 35% IPD 45% CONTROL 55%</td>
</tr>
<tr>
<td>Amk: Amikacin</td>
<td>OPD 40% IPD 50% CONTROL 60%</td>
</tr>
<tr>
<td>Gnt: Gentamicin</td>
<td>OPD 45% IPD 55% CONTROL 65%</td>
</tr>
<tr>
<td>N.A: Nalidixic acid</td>
<td>OPD 50% IPD 60% CONTROL 70%</td>
</tr>
<tr>
<td>Nor: Norfloxacine</td>
<td>OPD 55% IPD 65% CONTROL 75%</td>
</tr>
<tr>
<td>Cip: Ciprofloxacin</td>
<td>OPD 60% IPD 70% CONTROL 80%</td>
</tr>
<tr>
<td>Cot: Cotrimoxazole</td>
<td>OPD 65% IPD 75% CONTROL 85%</td>
</tr>
</tbody>
</table>

Amp: Ampicillin; Cef: Cefotaxime; Ptz: Piperacillin/tazobactam; Ceft: Ceftazidime; Az: Aztreonam; Imp: Imipenem; Amk: Amikacin; Gnt: Gentamicin; N.A: Nalidixic acid; Norflo: Norfloxacine; Cip: Ciprofloxacin; Cot: Cotrimoxazole

Figure 5: Double Disk Synergy Test (DDST) for ESBL detection.

Figure 6: Detection of AmpC beta-lactamase production

- AmpC disk test
- Boronic acid disk test
- Disk approximation test
Figure 7: Detection of MBL

Double disk potentiation test

Modified Hodge test

E-test

Table 5: Positivity of ESBL, AmpC & MBL amongst study groups

<table>
<thead>
<tr>
<th>Study Group</th>
<th>ESBL n(%)</th>
<th>AmpC n(%)</th>
<th>MBL n(%)</th>
<th>ESBL+MBL n(%)</th>
<th>MBL+AmpC n(%)</th>
<th>ESBL+AmpC n(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPD n=300</td>
<td>135(45)</td>
<td>42(14)</td>
<td>48(16)</td>
<td>8(2.6)</td>
<td>30(10)</td>
<td>8(2.6)</td>
</tr>
<tr>
<td>IPD n=300</td>
<td>112(37.5)</td>
<td>50(16.7)</td>
<td>25(8.3)</td>
<td>60(20)</td>
<td>15(5)</td>
<td>30(10)</td>
</tr>
<tr>
<td>Control n=100</td>
<td>33(33)</td>
<td>38(38)</td>
<td>38(38)</td>
<td>10(10)</td>
<td>5(5)</td>
<td>0</td>
</tr>
<tr>
<td>Total n=700</td>
<td>280(40)</td>
<td>130(18.57)</td>
<td>111(15.85)</td>
<td>78(11.14)</td>
<td>50(7.14)</td>
<td>38(5.42)</td>
</tr>
</tbody>
</table>

P value

<table>
<thead>
<tr>
<th>ESBL</th>
<th>AmpC</th>
<th>MBL</th>
<th>ESBL+MBL</th>
<th>MBL+AmpC</th>
<th>ESBL+AmpC</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.408</td>
<td>0.595</td>
<td><strong>0.024</strong></td>
<td><strong>0.045</strong></td>
<td>0.728</td>
<td>0.125</td>
</tr>
</tbody>
</table>

Figure 8: Distribution of ESBL, MBL & AmpC either single/in combination in three groups
As MDR E.coli possessing ESBLs, AmpC & MBL phenotypes are high in children from not only in those admitted to hospital and from the community, but also as carriage in healthy children, these transferrable resistance determinants are of great concern as they may help in spreading the trait to other susceptible strains of the same species or of other genera. Various countries report cases where multi drug resistant isolates of *E.coli* are causative agents of diarrhea. These facts raise serious questions about the ability to treat severe cases of diarrhea effectively and promptly. Understanding the molecular basis of resistance acquisition and transmission can contribute to the development of new strategies to combat this phenomenon. Identification of DEC is important because our study reveals that self limiting diarrhea are being treated with high end antibiotic by overzealous clinicians leading to the current status of multidrug resistant *E. coli* which eventually here lead to the spread of resistant genes to the commensal *E. coli* of the gut microbiota.

This study emphasized on the presence of resistant phenotypes for a range of antimicrobial classes with a focus on beta-lactamase producers. Broad spectrum beta-lactams resistance mediated by extended spectrum β-lactamase (ESBL), AmpC and metallo beta lactamase (MBLs) enzymes are an increasing problem worldwide. β-lactamases are encoded either by the chromosomal or plasmid mediated genes. The infections caused by multidrug-resistant DEC that produce various β lactamase enzymes have been reported in recent past in developing countries and they are associated with a significant morbidity and mortality in children. These phenotypic methods are easy and are able to discriminate between various classes to some extent. The production of beta-lactamase and multidrug resistance was more common in OPD and IPD group; this may be due to the improper use of drugs and accumulation of virulent factors which are common in diarrheagenic *E. coli*. It was observed that, children admitted to the
hospital receiving antibiotics harbored high \( \beta \)-lactamases producing \( E. \text{coli} \) similar to children with that of acute diarrhea, suggesting the continued existence of resistant population in the community of this susceptible population. Further, it also indicates the extensive use of antibiotic in pediatric population. High ESBL producing isolates may likely to have emerged due to indiscriminate use of these antibiotics as indicated by high cefotaxime resistance. In our study, the prevalence of various \( \beta \) lactamases in DEC \( E. \text{coli} \) was alarmingly high and these enzymes were also found in healthy isolates. However; On the contrary the low level of resistance in healthy children cannot be ignored which may have developed due to selective pressure from environment and horizontal gene transfer.

DEC still remains the main cause of childhood diarrhea in developing countries, found most common in children <5 years of age. In our study, DEC was detected in 83\% of isolates. EPEC being the predominant pathotype at 72.5\% (13.33\% typical and 29.16\% atypical EPEC) was followed by EAEC 63.33\%, ETEC 45.83\% and EHEC 9.16\% (2.5\% atypical EHEC, none of the typical EHEC in any group).[21] EIEC and DAEC were not detected in any of the study groups, indicating that they are less prevalent in this area and perhaps in India as well.\(^22,23\) Among diarrheal group, percentage of EPEC isolation was the highest (80\%), followed by EAEC (77.5\%), ETEC (75\%) and EHEC (12.5\%); however, the overall distribution of DEC was higher than those reported elsewhere.\(^24-27\) Molecular analysis has shown that both chromosomal and plasmid encoded virulence determinants are involved in EPEC pathogenesis.\(^28,29\) Similar to other studies, typical EPEC, though known to exist in gut as carriers, was significantly associated with diarrhea (\( P < 0.05 \)) compared to atypical EPEC. Earlier studies had predicted that atypical EPEC may have an innate property of longer persistence in intestine; hence, their existence as colonizers in healthy children is not unusual.
Atypical strains have *eae* gene only and they also possess parts of the plasmid but do not express bundle-forming pili for adherence.\textsuperscript{28,30} EAEC was the other important etiological agent associated with diarrhea in our study. Its characteristic unique patterns of stacked bricks’ adherence to Hep-2 cells is associated with the presence of large plasmids and it has been known to cause persistent diarrhea in children in developing countries.\textsuperscript{31} ETEC has been associated with watery diarrhea in children and 20\%–40\% of traveller’s diarrhea.\textsuperscript{25} EHEC can induce severe attaching and effacing lesions, but percentage isolation of atypical EHEC was not significant in this study.\textsuperscript{32} Based on several molecular studies, it is evident that EHEC having *eae*, *stx* and *ehxA* genes (typical Shiga toxin producing *E. coli* [STEC]) is considered epidemiologically important as strains exhibiting this virulence gene profile were associated with severe diarrhea/hemolytic uremic syndrome outbreaks worldwide. In contrast, EHEC harboring only the *stx* (atypical STEC) seems less virulent and it is associated with sporadic infection. In hospitalized children not presenting with diarrhea, the isolation of DEC was evident in similar order to that of diarrheal children with EPEC (80\%), followed by EAEC (75\%), ETEC (60\%) and EHEC (10\%).\textsuperscript{25} The presence of pathogenic DEC in Group 2 in children under 5 years of age admitted to the hospital receiving antibiotics may be existing as an associated pathogen or their presence as mere commensal in the gut could not be established. Their role as pathogens causing acute infection, however, could not be ascertained as the children did not present with diarrheal symptoms. It is worth noting that the usage of antibiotics in this population may eventually develop a selective pressure and provide a suitable niche for the proliferation and survival of DEC in the gut. In healthy controls, the existence of EPEC (57.5\%) and EAEC (37.5\%) was remarkably higher than ETEC (2.5\%) and EHEC (5\%) pathogens as observed in other studies.\textsuperscript{33,34} Presence of higher percentage of EPEC that comprised 37.5\% of atypical EPEC in
healthy children cannot be ignored which is in agreement with studies elsewhere. Atypical EPEC is known to cause prolonged diarrhea, and apart from the host factors, they may carry other virulence factors responsible for their pathogenicity.

Our results are in agreement with other studies reported. Commensal isolates belong to Groups A and B1 and virulent E. coli belongs to Groups B2 and D as described by other studies also. About 9.16% of E. coli isolates remained unclassified; it may be due to their existence in rare phylogroups or due to occurrence of more than one phylogroups.

Occurrence of co-infection with one or more DEC was also found in our study. This can be explained by the plasticity of E. coli genome that has the potential of undergoing continuous rearrangements. Horizontal gene transfer by mobile genetic elements such as transposons and integrons plays a major role in genome flexibility. Genotypic demonstration of a virulence plasmid is not synonymous with its expression; nonetheless, the presence of a pathogen with appropriate virulence factors in a symptomatic’ patient would imply a cause and effect relationship. Thus, the detection of several different virulence plasmids would help in a well-tuned epidemiological dissection of public health burden. The prevalence of different categories of DEC in symptomatic patients, with different clinical severity, and in healthy controls may reflect the socio sanitary ambience of the epidemiological setting and the potential harmful impact of usage of empirical antibiotics in pediatric population; if this is true, control measures have to be taken for specific situations. DEC was recovered at higher rate from healthy children and children without diarrhea, demonstrating extensive and fast spread of these pathogens in community. Multiplex PCR being time saving can be used for simultaneous detection of pathogenic genes. The occurrence of atypical EPEC in healthy children is of a great concern and cannot be neglected.
Dissemination of ESBL, AmpC and MBL producing clones resulting from movement of patients between community to hospital and intra- and inter-hospital not only promotes epidemic spread without any borders but also limits availability of treatments options for infections caused by such organism. Thus, there is an urgent need to promote strict guidelines for rational use of antibiotics, both in hospital and in the community, the development of new generic drugs, strict personal hygiene to prevent the selection and the spread of these strains and increase awareness among the population of the hazards of taking antibiotics without medical consultation. A continuous observation to detect the resistant strains and the implementation of infection control measures to reduce the increasing burden of resistant E.coli is the need of the hour.

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21. Itokazu G, Quinn J, Bell-Dixon C, Kahan F, Weinstein R. The antimicrobial resistance rates among aerobic the gram-negative bacilli which were recovered from the patients in


