



## Genetic diversity study of various $\beta$ -lactamase-producing multidrug-resistant *Escherichia coli* isolates from a tertiary care hospital using ERIC-PCR

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**Background & objectives:** The prevalence of multidrug-resistant (MDR) *Escherichia coli* isolates producing  $\beta$ -lactamase enzyme is a growing problem across the globe. Strain typing is an epidemiologically important tool not only for detecting the cross transmission of nosocomial pathogens but also for determining the source of infection. The present study was conducted to understand the clonal relationship among various  $\beta$ -lactamase-producing MDR *E. coli* isolates using enterobacterial repetitive intergenic consensus (ERIC) polymerase chain reaction (PCR).

**Methods:** A total of 41 MDR *E. coli* isolates were randomly collected from various clinical samples and processed. Isolated organisms were tested for antibiotics resistance pattern. Phenotypic detection of metallo  $\beta$ -lactamases (MBL) was carried out by the imipenem-ethylenediaminetetraacetic acid disc diffusion/double-disc synergy test. AmpC enzyme production was tested by a modified three-dimensional extract test.

**Results:** Almost all isolates were found sensitive to colistin. A high percentage of drug resistance was observed in these isolates against ceftazidime (100%), cefotaxime (100%), cefepime (100%), ofloxacin (97.56%), amoxicillin/clavulanic acid (97.56%) and norfloxacin (85.36%). Of the 41 isolates, ESBL producers were found to be predominant, *i.e.*, 22 (53.65%), followed by AmpC (6, 14.63%) and MBL (5, 12.19%).

**Interpretation & conclusions:** At 60 per cent similarity cut-off value, the dendrogram analysis showed that there were a total of 14 unique clusters of ERIC (CL-1 - CL-14) within the 41 *E. coli* isolates, which revealed the genetic diversity existing between them.

**Key words**  $\beta$ -lactamase - enterobacterial repetitive intergenic consensus polymerase chain reaction - *Escherichia coli* - multidrug resistance

*Escherichia coli* is well known to be a universal commensal flora in humans as well as in several animal species but is also reported to be one of the

most common enterobacterial species which causes extra-intestinal infections in these hosts<sup>1</sup>. Clinicians often recommend  $\beta$ -lactam group of antibiotics as an

empirical treatment of infections due to *E. coli*<sup>2</sup>. The members of *Enterobacteriaceae* family are mostly associated with the hospital-acquired infections such as gastrointestinal, urinary tract and pyogenic lesions, of which the commonly isolated species is the *E. coli*<sup>3</sup>. Increasing prevalence of multidrug-resistant *E. coli* has been a global public health issue that brings in undesirable hindrance in the diagnosis of infecting agents and treatment options<sup>4</sup>.

The frequent occurrence of high-level antibiotic resistance is through the transfer of large plasmids which can acquire numerous genes for multiple  $\beta$ -lactamase resistance from different classes of bacteria<sup>5</sup>.  $\beta$ -lactamase production by several Gram-negative and Gram-positive organisms is perhaps the most important single mechanism of resistance to  $\beta$ -lactam agents<sup>3</sup>. In addition, resistance to broad-spectrum  $\beta$ -lactams, mediated by extended spectrum  $\beta$ -lactamases (ESBL), metallo  $\beta$ -lactamases (MBL) and AmpC  $\beta$ -lactamases (AmpC) enzymes has been rising problem worldwide<sup>6</sup> and has become a major concern as they bring about limitations in therapeutic options, result in treatment failure and are fast propagating throughout the world<sup>7</sup>.

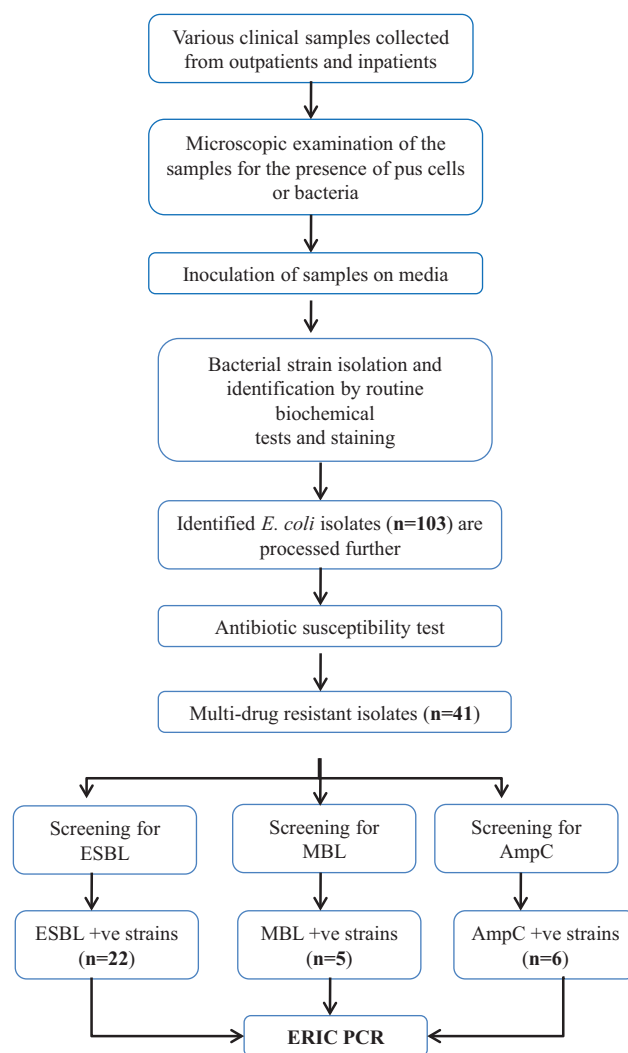
Strain typing is an epidemiologically important tool not only for detecting the cross transmission of nosocomial pathogens but also for determining the source of infection<sup>8</sup>. Available sub-typing methods for *E. coli* include pulsed-field gel electrophoresis (PFGE), plasmid profiling, ribotyping and polymerase chain reaction (PCR)-based typing methods such as arbitrary primed PCR, repetitive extragenic palindromes and enterobacterial repetitive intergenic consensus (ERIC)<sup>9</sup>. However, the simplicity, ease of operation, low per isolate cost and limited need for specialized laboratory equipment favours the use of ERIC-PCR over other methods<sup>10</sup>. The present study was carried out to determine the genetic diversity of different  $\beta$ -lactamase producing multidrug-resistant (MDR) *E. coli* isolates using ERIC-PCR in Odisha in a hospital setup.

### Material & Methods

The antibiotics used in this study were purchased from Hi-media, Mumbai. Molecular biology reagents were purchased from Thermo Scientific, India. All other chemicals and reagents were of analytical grade purchased from Hi-media. Thermal cycler and gel documentation system were used for the phylogenetic analyses of the isolates were from Bio-Rad, India.

The present study was carried out from June 2015 to February 2016 in the department of Microbiology, IMS & SUM Hospital, Bhubaneswar, Odisha. During the study period, a total of 103 non-repeated *E. coli* isolates were obtained from various clinical samples (pus, wound, urine, blood, tracheal aspirate). All isolates were identified morphologically and biochemically by standard procedures. Of the 103 *E. coli* isolates, 41 (39.8%) were selected for this study as these isolates were multidrug resistant. Details of the sample collection and their processing are presented in the flow chart (Fig. 1).

**Antimicrobial susceptibility testing:** By using Kirby-Bauer's disc diffusion method as per Clinical Laboratory Standards Institute (CLSI) guidelines, antimicrobial



**Fig. 1.** Flow chart showing study design. ESBL: Extended spectrum  $\beta$ -lactamase, MBL: Metallo  $\beta$ -lactamase, ERIC-PCR: Enterobacterial repetitive intergenic consensus polymerase chain reaction, AmpC: Class C Cephalosporinase.

susceptibility was determined<sup>11</sup>. Antibiotics used were ceftazidime (30  $\mu$ g) (CAZ), cefotaxime (30  $\mu$ g) (CTX), cefepime (30  $\mu$ g) (CFM) (Cephalosporin), piperacillin/tazobactam (100/10  $\mu$ g) (PIT), amoxicillin/clavulanic acid (30/10  $\mu$ g) (AMC) (Penicillin combinations), cefoperazone/sulbactam (75/30  $\mu$ g) (CFS), ceftazidime/clavulanic acid (30/10  $\mu$ g) (CAC) (Cephalosporin combinations), ofloxacin (5  $\mu$ g) (OF), norfloxacin (5  $\mu$ g) (NX) (Quinolones/fluoroquinolones), amikacin (30  $\mu$ g), gentamicin (10  $\mu$ g) (Aminoglycosides), co-trimoxazole (25  $\mu$ g) (COT) (Sulphonamides), nitrofurantoin (300  $\mu$ g) (NIT) (Nitrofurans), imipenem (10  $\mu$ g) (IPM), meropenem (10  $\mu$ g) (MRP) (Carbapenem), colistin (10  $\mu$ g) (CL) (Polypeptides) and tigecycline (15  $\mu$ g) (TGC) (Others). However, COT (25  $\mu$ g) and NIT (300  $\mu$ g) were used only in case of urine samples.

*Screening for metallo  $\beta$ -lactamases (MBL) production:* MBL producing *E. coli* strains were suspected when the isolates were resistant to IPM/MRP. Phenotypic detection of MBL was carried out by the IPM-ethylenediaminetetraacetic acid disc diffusion/double-disc synergy test as described previously<sup>12</sup>.

*Detection of extended spectrum  $\beta$ -lactamases (ESBL) & AmpC  $\beta$ -lactamase:* Bacteria with resistance or with decreased susceptibility (intermediate by CLSI criteria) to the third generation cephalosporins were tested for ESBL production by NCCLS confirmatory test as described previously<sup>12</sup>. AmpC enzyme production was tested by a modified three-dimensional extract test as described previously<sup>12</sup>.

*Enterobacterial repetitive intergenic consensus polymerase chain reaction:* ERIC-PCR<sup>13</sup> was carried out to obtain DNA fingerprints of the *E. coli* isolates. Colony PCR was carried out using the primers ERIC-IR: 5'-ATGTAAGCTCCTGGGGAATCAC-3' and ERIC-2: 5'-AAGTAAGTGACTGGGGTGAGCG-3'<sup>14</sup>. A 25  $\mu$ l of the reaction mixture was prepared by taking 12.5  $\mu$ l double-distilled sterile water, bacterial colony (template DNA), 3.0 mM MgCl<sub>2</sub> and 200  $\mu$ M dNTP (each), 0.5  $\mu$ M primer and 1.0 unit of Taq Polymerase (Merck, Germany). The reaction mixture was initially denatured for three minutes at 94°C, subjected to 40 cycles of denaturation at 94°C for one minute, annealed at 48°C for one minute, extended at 72°C for two minutes, finally extended at 72°C for 10 min and finally soaked to 4°C. The amplified PCR products were analyzed using 1.5 per cent agarose gel electrophoresis.

Gel was stained with ethidium bromide and visualized by ultraviolet transilluminator.

## Results

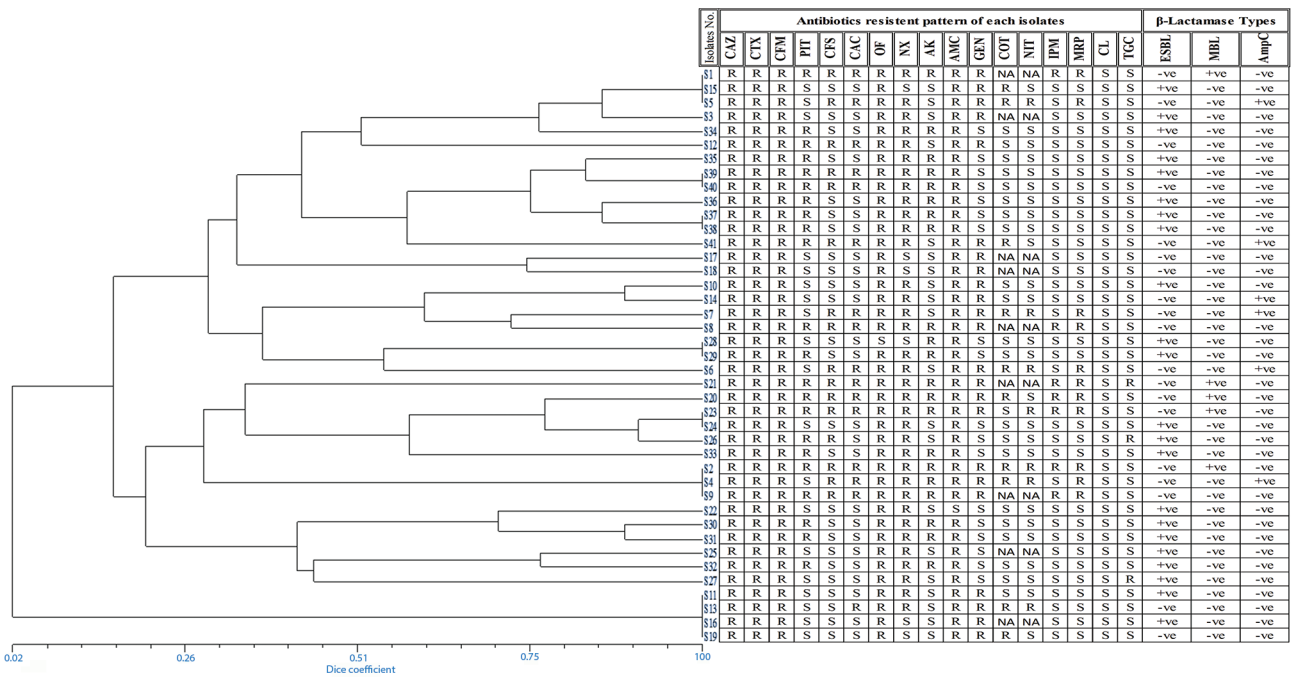
MDR *E. coli* isolates showing resistance to two or more groups of antibiotics and producing any one of the  $\beta$ -lactamases (ESBL, MBL and AmpC) were included. The majority of isolates (30: 73.17%) were from urine samples, and the rest were from other body fluids.

The antibiotic sensitivity pattern of the MDR *E. coli* isolates revealed that the maximum resistance was observed against CAZ (100%), CTX (100%), CFM (100%), OF (97.56%), AMC (97.56%) and NX (85.36%). Lower resistivity to a range of 39-54 per cent, was observed in  $\beta$ -lactamase inhibitors CFS, CAC and PIT except AMC. The lowest rate of resistance was observed in TGC (7.31%), IPM (17%), MRP (26.82%) and NIT (22%) (data not shown). It was found that all the MDR  $\beta$ -lactamase producing *E. coli* isolates are sensitive to CL. Among all the  $\beta$ -lactamases, ESBL were found to be higher in number, *i.e.*, 22 (53.65%), followed by AmpC 6 (14.63%) and MBL 5 (12.19%) (Fig. 2).

The genetic diversity analysis of 41 non-repeated randomly collected  $\beta$ -lactamase-producing *E. coli* isolates was carried out by ERIC-PCR fingerprint method using ERIC-IR and ERIC-2 primers. The fingerprints obtained from the ERIC typing of the 41 MDR *E. coli* isolates showed a DNA banding profile consisting of amplified bands ranging from 1 to 7 having size 150 bp to 2500 bp (Fig. 3A & B). At 60 per cent similarity cut-off value of the dendrogram analysis showed that there were a total of 14 unique clusters of ERIC (CL-1 to CL-14) within the 41 *E. coli* isolates, exhibiting genetic diversity (Fig. 4). Only five clusters (CL-1, CL-2, CL-6, CL-8 and CL-14) represented four or more isolates while the remaining nine clusters contained less number of isolates varying from 1 to 3. Some isolates showing similar ERIC profile, four from CL-1 (S19, S16, S11 and S13), three from CL-8 (S15, S1 and S5) and three from CL-12 (S9, S2 and S4), which indicated clonal similarities between the isolates.

## Discussion

The development of MDR due to the production of  $\beta$ -lactamases in Gram-negative bacteria has become a major problem worldwide, which limits the treatment option and increases the hospital cost, the rate of morbidity and mortality. The sources of infection



**Fig. 2.** Phylogenetic relationships among *Escherichia coli* isolates generated using UPGMA based on dice coefficients method derived from analysis of the ERIC-PCR profiles. Antibiotic resistance profile and β-lactamase type are also shown. R: Resistant, S: Sensitive, NA: Not applied, +ve: Positive, -ve: Negative.

and spreading pattern may generally be understood by analyzing the clonal similarity among the isolates collected from any hospital setup using specific molecular marker.

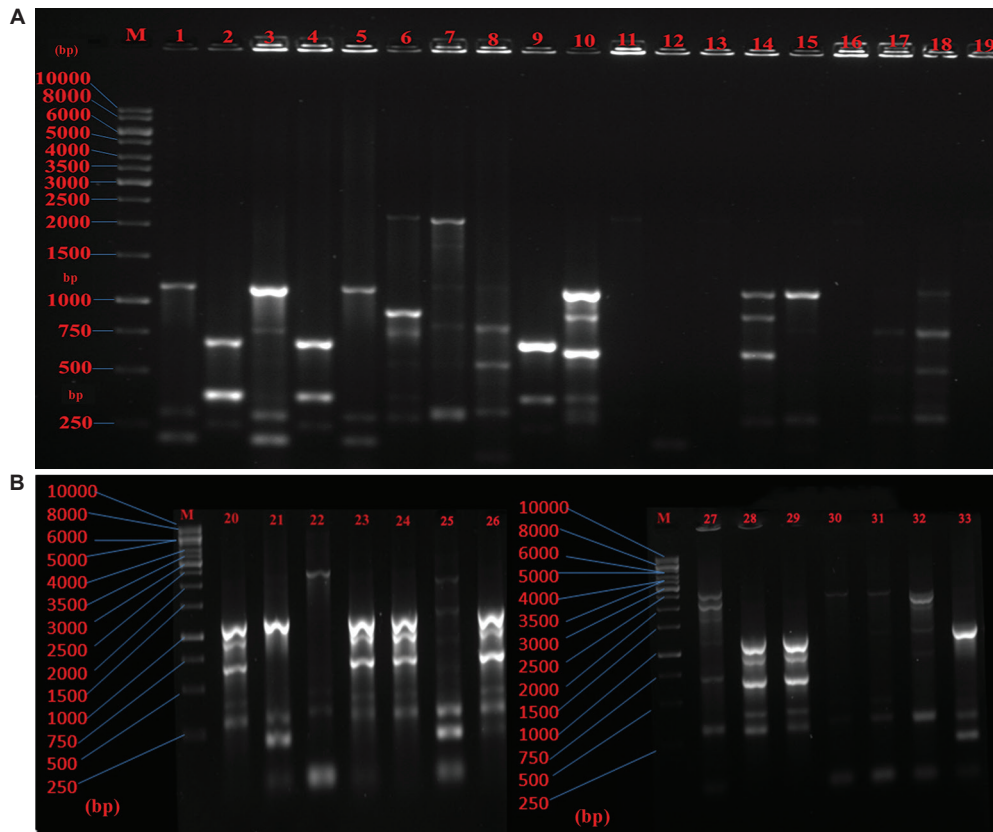
In the present study, the maximum number of isolates (73.17%) were from urinary tract infections (UTI) which corroborated with several other studies described previously<sup>15-17</sup>. The reason being *E. coli*, the most prevalent causative organisms of UTI is solely responsible organism for more than 80 per cent of the infections<sup>18</sup>.

In our study, the rate of resistance to CAZ (100%) and AMC (97.56%) were much higher when compared with other studies from Malaysia<sup>19</sup> and China where it was found to be 28 per cent for CAZ and 84 per cent AMC<sup>20</sup>. CFM showed 100 per cent resistance in our study, which was much higher when compared with another study<sup>19</sup> reporting only two per cent. Carbapenem group of antibiotics, such as IPM and MRP showed resistance at 17 and 26.82 per cent, respectively, in our study, but Adwan *et al*<sup>21</sup> showed the rate of resistance to IPM to be 22 per cent in *E. coli* and obtained no MRP resistance isolates. A study by Rezai *et al*<sup>22</sup> reported high CL resistant (82%) in ESBL-producing uropathogenic *E. coli*. However, the present study showed complete (100%) susceptibility to CL.

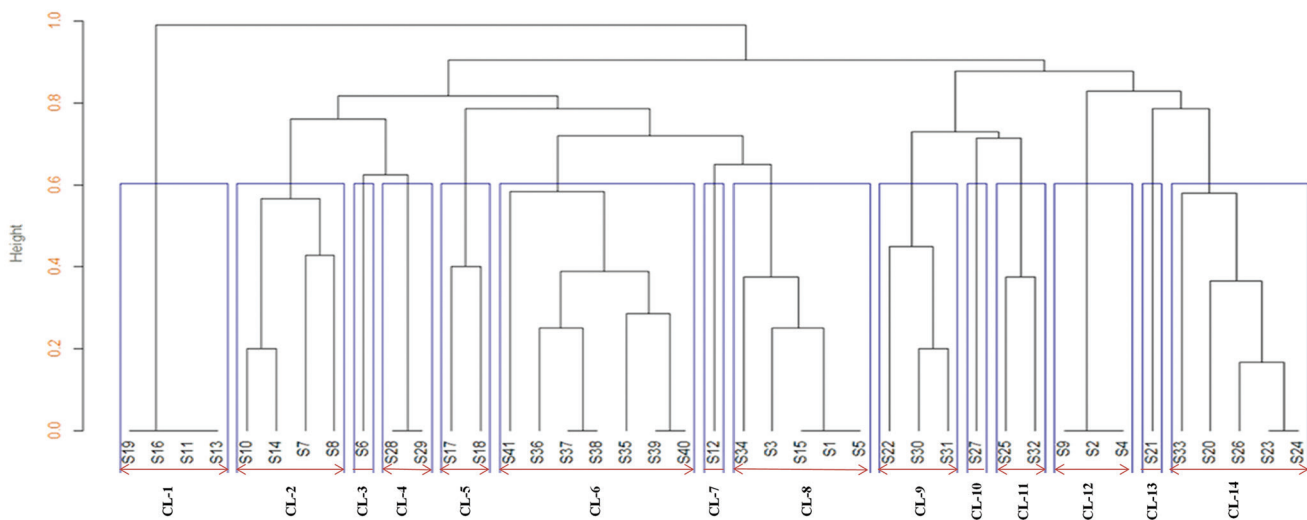
The prevalence of ESBL-producing *E. coli* varies from country to country and from centre to centre. Our study showed the prevalence of ESBL was 53.65 per cent, which was higher than the study from Sudan with 30.2 per cent ESBL producers in *E. coli*<sup>23</sup>. It has been shown that the prevalence of ESBL-producing *E. coli* is highest in India (60%), followed by Hong Kong (48%) and Singapore (33%)<sup>24</sup>. The prevalence of ESBL-producing isolates of *E. coli* was reported to be 13.3 per cent in Lebanon<sup>25</sup>, 9.2 per cent in Korea<sup>26</sup>, 10.3 per cent in Arabia<sup>27</sup> and 17 per cent in Turkey<sup>28</sup>, which was much low in prevalence when compared to India<sup>29,30</sup>. In the present study, we found 14.63% AmpC β-lactamase producers which were lower when compared with the studies from Chennai (in children under five) and Kolkata (37.5 and 47.8 per cent, respectively)<sup>31,32</sup>. We found 12.19 per cent were MBL producers, which was lower as compared with the study by Bora *et al*<sup>33</sup> from Nepal with a higher incidence of MBL-producing *E. coli* (18.98%) from different clinical samples.

In our study 14 unique clusters of ERIC were observed in 41 MDR *E. coli* isolates at a 60 per cent cut-off value analysis and exhibited genetic heterogeneity, but Adwan *et al*<sup>21</sup> reported 11 unique clusters of ERIC in 41 MDR *E. coli* which showed genetically diverse.





**Fig. 3.** (A) Agarose gel electrophoresis of enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC-PCR) generated DNA fingerprints of 1-19 *Escherichia coli* isolates. M: Marker (1 kb), (B) Agarose gel electrophoresis of ERIC-PCR generated DNA fingerprints of 20-33 *Escherichia coli* isolates. M: Marker (1 kb).



**Fig. 4.** Dendrogram of *Escherichia coli* isolates at 60 per cent similarity cut-off value analysis, it showed 14 unique clusters. CL: Cluster.

On the basis of ERIC-PCR fingerprint, the isolates included in our study were genetically diverse. This is the most expected outcome as the isolates were randomly collected from different sources and

from different samples, which demonstrate that, the transmission might have occurred from clones of different origin. These findings corroborated with other studies<sup>21,34,35</sup>.

In the present study, some isolates had similar ERIC profile, four from CL-1 (S19, S16, S11 and S13), three from CL-8 (S15, S1 and S5) and three from CL-12 (S9, S2 and S4) showed genetically homogeneous profile. Similar report was given by Loncaric *et al*<sup>6</sup> analyzing bacterial isolates from migratory and resident population of Rooks birds (*Corvus frugilegus*) from Austria. From this study, we observed that there is no correlation of the clonally similar strains with their respective antibiotic sensitivity nor with pattern the  $\beta$ -lactamase enzymes they produced. Loncaric *et al*<sup>6</sup> demonstrated that the spread of antibiotic resistance not only due to the dissemination of different  $\beta$ -lactamases but also due to clonal transmission.

In the present investigation, only a few isolates showed conserved banding profile on the basis of ERIC analyses, which indicated the rapid dissemination of similar clonal groups. A similar report was given by Durmaz *et al*<sup>4</sup> on ESBL-producing quinolone-resistant clinical *E. coli* isolates. Shakil *et al*<sup>7</sup> reported clonal similarity among four blaCTX-M-15-positive *E. coli* isolates from NICU patients by ERIC-PCR and assigned them to be temporarily responsible for clonal outbreak. From the present study, it can be concluded that ERIC-PCR-based strain typing of  $\beta$ -lactamase producing MDR *E. coli* collected from various clinical samples exhibited a considerable amount of clonal heterogeneity. The occurrence of a few clonally similar groups among these isolates could be either due to the spread of antibiotic resistance through dissemination of different  $\beta$ -lactamases or due to clonal transmission. However, lack of association of these clonally similar isolates with their respective antibiotic sensitivity pattern or with the  $\beta$ -lactamase enzyme production in our study rules out above possibility, indicating antibiotic selection pressure to be one of the major reasons which need to be validated further.

The sample size of the present study was small. Multicentric study with larger sample size, however, may help better in establishing the genetic diversity existing among the *E. coli* isolates in a region.

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**Conflicts of Interest:** None.

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