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Molecular Characterization of ESBL and AmpC β-Lactamases among Blood Isolates of *Klebsiella pneumoniae* and *Escherichia coli*

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Authors' contributions

This work was carried out in collaboration between both authors. Author MPR designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript and managed literature searches. Authors MPR and BNH managed the analyses of the study and literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

The purpose of this study was to determine the mechanisms of third generation cephalosporins resistance among blood isolates of *Klebsiella pneumoniae* and *Escherichia coli*. A total of 549 isolates, *Klebsiella pneumoniae* (n=369) and *Escherichia coli* (n=180) were included. Antibiotic susceptibility pattern, phenotypic and genotypic detection of ESBL and AmpC production were carried out. The genetic environment surrounding bla_{CTX-M} gene was assessed for insertion sequences. The plasmid-mediated quinolone resistance (PMQR) and integron carriage rate of ESBL producers were studied. The clonality was assessed by pulsed-field gel electrophoresis. Also, the plasmids bearing the ESBL, AmpC genes were studied by incompatibility typing and conjugation assay. By phenotypic tests, *K. pneumoniae* (79.8%) and *E. coli* (74.1%) were ESBL producers. Similarly, *K. pneumoniae* (70.5%) and *E. coli* (76.8%) were AmpC producers. By PCR, *K. pneumoniae* (68%) and *E. coli* (62%) carried ESBL genes. $bla_{CTX-M-15}$ was the prevalent type

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(98.1%). The linkage of ISEcp1 with *bla*$_{CTX-M-15}$ gene was found in 92.5% of the *bla*$_{CTX-M-15}$ genes. *bla*$_{CMY-2}$ was present in 57% isolates. PFGE showed no clonal relatedness however; replicon typing revealed that ESBL genes were carried on 5 different replicon types, IncA/C being the commonest type present in this region. Also, PMQR was found in 19.3% of the ESBL producers. As high as 21.5% of class 2 integron was noted for first time from this region. A high prevalence of ESBL and AmpC genes was noticed. The clonal diversity, transferability of *bla*$_{CTX-M}$ plasmids suggest a higher incidence and wider distribution of ESBL and AmpC producing bacteria existing in the South India.

Keywords: Enterobacteria; multidrug resistance; extended spectrum β-lacatamase; integrons; qnr genes; pulse field gel electrophoresis.

1. INTRODUCTION

Since the introduction of antimicrobial agents, the extent of resistance among *Enterobacteriaceae* has become an increasing problem. Many enterobacteria are opportunistic pathogens that easily acquire resistance mechanisms and genes, which make the situation threatening. Production of β-lactamases continues to be the leading cause of resistance to β-lactam antibiotics in Gram- negative bacteria [1].

Extended Spectrum β-lactamases (ESBLs) represent a major group of plasmid mediated β-lactamases (PMABL) currently being identified worldwide in large numbers and are now found in a significant percentage in *Escherichia coli* and *Klebsiella pneumoniae* strains, they have also been found in other members of Enterobacteriaceae family and in *Pseudomonas aeruginosa* [2]. The *bla*$_{CTX-M}$ enzymes are group of molecular class A ESBLs that exhibit an overall preference for cefotaxime and ceftriaxone. Being plasmid mediated these enzymes spread fast amongst various bacteria and is important in infection control, clinical and therapeutic implications. The *bla*$_{CTX-M}$ types seem to be particularly successful in terms of horizontal spread. More than 100 variants have been found until 1979 [3]. AmpC β-lactamase production is one of the mechanisms of resistance to β-lactam antibiotics in Gram negative bacteria and they have gained importance since the late 1970s. The first plasmid encoded AmpC β- lactamases was isolated in 1989 from a *K. pneumoniae*. Currently, there are over 30 known types [4].

Organisms producing ESBL and PMABLs such as *E. coli* and *Klebsiella* species are often associated with multidrug resistance, leaving a few therapeutic options. Infections caused by them are an emerging problem being increasingly reported worldwide. Dissemination of these organisms has important implications for hospital infection control and patient management [5]. They are also known to be responsible for nosocomial outbreaks, multidrug resistance and high rate of clinical failure among infected patients. The main mechanisms contributing to bacterial resistance are the horizontal transfer of resistance determinants. This will be mainly through integrons which will act as efficient vehicles for the transfer of resistance markers among unrelated bacterial populations [6].

Integron-mediated antimicrobial resistance represents a major mechanism for transfer of resistance traits within both Gram-negative and Gram-positive bacteria [7]. Drug resistant ESBL genes located on integrons like structures are being increasingly reported worldwide [8]. However, data on the occurrence of integrons and,/ESBLs among Enterobacteriaceae are very scarce in India.

A number of genetic mechanisms have apparently been involved in the acquisition and mobilization of ESBL genes. Insertion sequences *IS*26, *ISEcp1* and *ISCR*1 in association with class 1 integron structures, as well as phage related elements seem to have played a prominent role in these processes in acquisition of *bla*$_{CTX-M}$ genes [9]. Since, fluoroquinolones are used in the treatment of ESBL and AmpC producing bacterial infections, the presence of plasmid-mediated quinolone resistance (PMQR) determinants in these isolates is of clinical significance.

It is worth knowing how widespread or common the multidrug resistant ESBL producing bacteria. Of particular interest to this study were *K. pneumoniae* and *E. coli*, the most common Gram- negative rods isolated in laboratories. The incidence of ESBLs varies with geographical location and time [2]. In 2007, a high prevalence of 96% of ESBL producing *K. pneumoniae* among the isolates from blood has been documented from this centre [10]. However, that

study did not look into the occurrence of Plasmid encoded AmpC β-lactamases which are of equal importance. This prompted to study the molecular genetics of ESBL and PMABL genes in a collection of large number of blood isolates over a four year period from this region. Further, in order to identify the different mechanisms of third generation cephalosporin resistance, the same isolates of *K. pneumoniae* and *E. coli* were subjected to AmpC studies.

Like any other developing countries, the prevalence of ESBLs has widely been reported in several parts of India whereas there is a paucity of information regarding their genetics and clonality especially from the South. Considering these facts, the present study was carried out to determine the magnitude and current trends of the antimicrobial resistance, the types of ESBLs, PMABLs, their association with integrons and plasmid-mediated quinolone resistance with respect to blood isolates of *K. pneumoniae* and *E. coli.*

2. MATERIALS AND METHODS

2.1 Bacterial Isolates

This study was conducted in a major tertiary care, multi-speciality teaching hospital receiving patients from Puducherry union territory and the surrounding regions of Tamilnadu State (Latitude:11°56′01″N and Longitude:79°49′47″E). Consecutive isolates of *K. pneumoniae* and *E. coli* obtained from blood cultures were included. One isolate from each patient was included. Isolates which were sensitive to carbapenems were only included in this study.

2.2 Isolation of *K. pneumoniae* and *E. coli* in Blood Culture

Blood cultures were done using the automated BACTEC blood culture system. The isolates were identified by colony characteristics, Gram staining and by the standard bacteriological methods [11].

2.3 Antimicrobial Susceptibility Test

Antimicrobial susceptibility of the isolates to a panel of beta lactam antimicrobials and other broad spectrum antimicrobials were determined by standard Kirby Bauer disc diffusion. The following antimicrobial agents were used: ampicillin (10 µg), amoxyclav (20/10 µg), amikacin (30 µg), aztreonam (30 µg), cefepime (30 µg), cefoxitin (30 µg) cefpodoxime (10 µg), cefotaxime (30 µg), ceftazidime (30 µg), ceftriaxone (30 µg), ciprofloxacin (5 µg), nalidixic acid (30 µg), co-trimoxazole (1.25/23.75 µg), gentamicin (10 µg), piperacillin (100 µg), piperacillin/tazobactam (100/10 µg) and meropenem (10 µg) (HiMedia Laboratories Ltd, Mumbai. India). The disc diffusion technique and zone interpretation of each antimicrobial agent was performed in accordance with the CLSI guidelines [12]. Strains concomitantly resistant to ≥ 3 different antimicrobial classes were considered as multi-drug resistant (MDR). Suitable *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 2785 were used as controls.

2.4 Determination of Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentration of ampicillin, ceftazidime, ceftriaxone, cefotaxime, cefpodoxime, aztreonam, cefepime, cefoxitin, amikacin, gentamicin, ciprofloxacin, nalidixic acid and co-trimoxazole were determined by agar dilution method and /broth microdilution method as per CLSI guidelines [12].

2.5 Phenotypic Detection of ESBL Production

All the isolates were screened preliminarily for ESBL production. Isolates showing reduced susceptibility to one of the third generation cephalosporins (3GCs) and aztreonam by disc diffusion test were suspected to be ESBL producers and further confirmed by combination disc method using the cephalosporin discs (cefpodoxime 10 µg, cefotaxime 30 µg and ceftazidime 30 µg) and cephalosporin/ clavulanate combination discs (cefpodoxime/ clavulanate 10/10 µg, cefotaxime/ clavulanate 30/10 µg and ceftazidime/ clavulanate 30/10 µg). The diameters of inhibition zone were measured. The interpretations of results were as per CLSI [12]. *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 were used as ESBL negative and positive controls.

2.6 Phenotypic Detection of AmpC Production

2.6.1 Detection of chromosomal inducible AmpC production

Isolates that fell in the sensitivity range to third generation cephalosporins and cefoxitin in the

antimicrobial susceptibility test were subjected to disc antagonism test for the detection of chromosomal inducible AmpC production [13]. Known inducible AmpC positive *P. aeruginosa* from in-house was used as the positive control and *E. coli* ATCC 25922 were used as the negative control.

2.6.2 Detection of plasmid mediated or de-repressed AmpC production

Isolates showing reduced susceptibility to cefoxitin by disc diffusion method (≤18 mm) and/ or broth microdilution method (≥32 µg/ml) were screened as putative AmpC producers. They were tested for de-repressed AmpC production by AmpC disc test [14] and were further confirmed by modified three dimensional test [15]. Quality control was achieved by using known AmpC (*bla*$_{CMY}$ gene) positive isolate of *K. pneumoniae* (Kindly provided by Dr. John P Hays, Erasmus MC, University Medical Center, Rotterdam, The Netherlands).

2.7 Molecular Studies

2.7.1 PCR for ESBL genes

Total genomic DNA, which served as template in all PCR reactions were extracted from the isolates by 'boiling-lysis' method. A multiplex PCR was optimized for the simultaneous detection of the ESBL genes using primers targeting *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX-M}$ and *bla*$_{OXA-1}$ gene. Universal primer sets were used to detect *bla*$_{SHV}$ and *bla*$_{CTX-M}$ genes [16,17]. The primers used for the amplification of *bla*$_{TEM}$ and *bla*$_{OXA-1}$ genes were designed using Primer 3 program (http://frodo.wi.mit.edu/primer3/) based on the reference sequences from the GenBank accession no. AB194682 and GQ896560 respectively.

Amplification was performed on a Corbett Research thermal cycler (HP, USA) with a final reaction volume of 25 µl. The amplification conditions were standardized as initial denaturation at 95ºC for 15 minutes; followed by 30 cycles of 94^{o}C for 30 sec, 63ºC for 90 sec, 72ºC for 90 sec; and final elongation 72ºC for 7 mins with 1 cycle at 4ºC for holding. Suitable positive strain of *K. pneumoniae* (N09-008) containing all the four *bla* genes of interest (Kindly provided by Dr. Michael Mulvey, National Microbiology Laboratory, Canada) was used as the positive control and sterile MilliQ water, containing no DNA was used as negative control in each reaction set performed.

2.7.2 Genetic environment of the ESBL gene

Linkage of *bla*$_{CTX-M}$ genes with an insertion sequence *ISEcp*1 and *IS*26 like-elements were analyzed. As ESBL genes have been described downstream of *ISEcp*1 or part of a complex class 1 integron, the genetic environment upstream of the ESBL gene was investigated with forward primers for *ISEcp*1 and/ or *IS*26 element and the ESBL gene reverse primer [18, 19]. Amplification was done by initial denaturation at 94ºC for 5min; followed by 30 cycles of 94ºC for 25 s;52ºC for 40s and 72ºC for 50s; and final elongation at 72ºC for 6 min. For the positive isolates with *ISEcp*1 PCR, to determine the genetic environment of *bla*$_{CTX-M}$ genes, sequencing was performed using primers for *ISEcp*1 [20] combined with reverse primer (5'-AGTTTCCCCATTCCGTTTC -3') for *bla*$_{CTX-M}$ genes.

2.7.3 Genotypic detection of plasmid encoded AmpC β-lactamase (PMABL) genes

All isolates phenotypically identified as AmpC producers were subjected to a multiplex PCR to detect six family-specific AmpC genes carried on the plasmids such as *bla*$_{MOX}$, *bla*$_{FOX}$, *bla*$_{EBC}$, *bla*$_{ACC}$, *bla*$_{DHA}$ and *bla*$_{CIT}$ as described previously [21]. Suitable positive controls of *E. coli*, *K. pneumoniae* and *Enterobacter cloacae* strains were included (kindly provided by Dr. John Hays, Erasmus Medical College, The Netherlands). All isolates positive for *bla*$_{CIT}$ family were further amplified to detect the presence of *bla*$_{CMY-2}$ gene, which give a 323 bp product. The *bla*$_{CMY-2}$ gene specific primer set was designed by Primer 3 program using the GenBank reference sequence (accession number X91840).

2.8 DNA Sequencing

2.8.1 Sequencing of ESBL genes

All the PCR products were purified using Spin Column PCR Products Purification Kit (Bio Basic Inc., Canada). Sequencing of the β- lactamase gene PCR products was performed using both forward and reverse strands primers. Primers for sequencing *bla*$_{SHV}$ and *bla*$_{OXA-1}$ products were same as PCR primers. Additional primers were used for *bla*$_{TEM}$ and *bla*$_{CTX-M}$ PCR products [22,23].

2.8.2 Sequencing of *bla*$_{CMY-2}$ gene

The blaCMY-2 gene positive isolates were sequenced using specific primers designed and its homology was compared with the sequences from the NCBI database. All nucleotide sequences were analyzed using Lasergene software (DNA STAR Inc. Madison, USA) and compared with the NCBI sequence database using the BLASTn algorithm (http://www . ncbi.nlm.nih.gov/blast/).

The primers used are 5'-ACACTGATTGCGTCTGACG-3', 5'-AATATCCTGGGCCTCATCG-3',5'-TGCAACCATTAAAACTGGC-3'and 5'-TTGCTTTTAATTACGGAAC-3'.

2.9 Genetic Fingerprinting

Genomic DNA was prepared in agarose blocks and restriction digestion was carried out using 40U of *Xba*I (Fermentas) at 37ºC. PFGE was performed in 1% SeaKem Gold Agarose gel in 0.5 X TBE in a CHEF DR-III system (Bio-Rad, Hercules, Calif) as per the PulseNet protocol developed for *E. coli* [24]. Gel patterns were analyzed using BioNumerics software program, version 3.5 (Applied Maths, Austin, Texas). Interpretation of PFGE patterns was based on previously proposed criteria for restriction patterns produced by PFGE [25]. Cluster analysis was done with the unweighted pair-group method average (UPGMA), and DNA relatedness was calculated based on the Dice coefficient. Isolates were considered to be genetically related if their macrorestriction DNA patterns differed by fewer than three bands and the Dice coefficient of correlation was 85% or greater. *Salmonella* ser. Braenderup standard strain H9812 was used as the ladder.

2.10 Plasmid Incompatibility/Replicon Typing

A PCR-based replicon typing method for classification of plasmids occurring in members of the Enterobacteriaceae was used. The presence of individual plasmid types were determined by PCR screening of I1, FIA, FIB, FIIs, A/C, F_{repB}, K/B and B/O replicons. After initial optimization (simplex PCR), template DNA was amplified by multiplex PCR, with primers for I1, FIA, FIB, FIIs, A/C replicons.

Because of the high level of homology between K and B/O replicons, the same forward primer was used in both these simplex PCRs. Extraction of plasmid DNA was carried out using commercial kits (Bio Basic Inc., Canada) as per manufacturer's instructions. The primers were obtained from Ocimum Biosolutions, India. The reaction was performed in a final volume of 25 µl reaction mix. The primers and amplification conditions were as described earlier [26]. The amplified PCR products were loaded in 1% wt/vol. agarose gel prepared in TBE buffer and detected by ethidium staining after electrophoresis (BioRad, USA).

2.11 Conjugation Assay

Transferability of *bla*ESBL and PMABL gene was tested by broth mating assay using *E. coli* K12 J62-2 (lac^- F^- rif^+) strain as the recipient strain [27]. Pure isolated colony of the donor clinical strain taken from overnight growth plate was inoculated into 10 ml brain heart infusion (BHI) broth with a selective antibiotic (2-10µg/ml cefotaxime and / cefoxitin). Pure isolated colony of the donor clinical strain taken from overnight growth plate was inoculated into 10 ml brain heart infusion (BHI) broth without any antibiotic Cultures of the donor and the recipient strains were mixed in the ratio of 1:10.This mixed culture was incubated overnight at 37°C. Transconjugants were selected on MacConkey agar supplemented with rifampicin (2.5 mg/ml) and ceftazidime and /cefoxitin (2 µg/ml). 200 µl of the mixed culture after overnight incubation was spread on the selection plates with the help of the L-rod. The plates were incubated overnight at 37°C. The plates were then observed for the presence of the transconjugants, which were then biochemically identified. The drug resistant profiles and ESBL and / or PMABL genes of the transconjugants were analyzed as described previously.

2.12 Association of Integron Classes with ESBL Genes

A total of 172 ESBL positive (*K. pneumoniae*, n=106; *E. coli*, n=66) and a control group of 55 clinical non-ESBL (*K. pneumoniae*, n=30; *E. coli*, n=25) isolates selected randomly were included. The isolates were subjected to integrase PCR for the amplification of integron classes (class1, class2 and class3) using primers and conditions as described earlier [28,29]. Isolates positive for the IntI1 gene and/or IntI 2 gene classes were subsequently subjected to PCR for amplification of the class 1 and/or class 2 integron gene cassettes arrays. For class 1 integron positive

isolates, 5CS and 3CS primers for amplifying the integron variable region-containing gene cassettes were used. Similarly, for class 2 integron positive isolates, *attI*2-F and *orfX*-R primers were used. The primers *attI2*-F and *orfX*-R bind, respectively, to *attI*2 and to *orfX*, which is situated within Tn7 in a position just downstream of the cassette region [30,31]. PCR products were separated by electrophoresis on 0.8% (wt/vol) agarose gels and were visualized under UV light after staining with ethidium bromide.

2.13 Analysis of Plasmid Mediated Quinolone Resistance (PMQR) Determinants among ESBL Producers

As ESBL producers are often multidrug resistance, the molecular mechanisms of resistance to fluoroquinolones by production PMQR determinants were analyzed. A total of 278 isolates *K. pneumoniae* (201) and *E. coli* (77) that were ESBL producers and resistant to nalidixic acid or ciprofloxacin were included. The isolates were classified as high-level quinolone resistance (HL-QR), if found resistance to both nalidixic acid and ciprofloxacin by disc diffusion test. Nalidixic acid resistant or intermediate and ciprofloxacin susceptible isolates were classified as low-level quinolone resistance (LL-QR). MIC of nalidixic acid, ciprofloxacin determined by agar dilution method as described earlier were considered. All the quinolone resistant or intermediate strains were subjected to a multiplex PCR for the amplification of *qnr*A, *qnr*B and *qnr*S genes using the standard primers [32,33].

2.14 Statistical Analysis

SPSS 11.0 was used for statistical analysis. Chi-square test was used for comparison of categorical variables. Odds ratio (OR) and confidence intervals (CI) were calculated and a 'p' value less than 0.05 was considered statistically significant.

3. RESULTS

3.1 Bacterial Strains

Blood cultures were done for a total of 3,361 patients during the study period. A total of 549 isolates, which include *Klebsiella pneumoniae* (n=369) and *Escherichia coli* (n=180) sensitive to carbapenems were studied.

3.2 Antimicrobial Susceptibility Test

Among the total 369 *K. pneumoniae* tested, more than 50% were resistant to third generation cephalosporins (3GCs) and fourth generation cephalosporins (4GCs). Against β-lactam inhibitors, piperacillin/tazobactam (52.3%) were resistant and amoxicillin/clavulanate (36%). A high resistance of cefoxitin (76.4%) and nalidixic acid (70%) was observed. Similarly, amongst the total *E. coli* (180), nearly (53%) were resistant to third generation cephalosporins, cefepime (36.1%) and piperacillin/tazobactam (52%). Also, *E. coli* was resistant to ciprofloxacin (55%). The percentage distribution of antimicrobial resistance profile of *K. pneumoniae* and *E. coli* is shown in Fig. 1.

3.3 Phenotypic Detection of ESBL Production

Among the total 549 isolates tested, 422 (76.8%) met the criteria for potential ESBL producers in the screening test. Out of the 369 *K. pneumoniae*, 283 (76.6%) and among 180 *E. coli*, 139 (77.2%) were resistant to one of the third generation cephalosporins and were suspected to be ESBL producers. By combination disc test, among the total 422 screen positive isolates, 329 (77.9%) were confirmed as ESBL producers. Out of the 283 screen positive *K. pneumoniae*, 226 (79.8%) and among 139 *E .coli*, 103 (74.1%) were confirmed to be ESBL producers phenotypically.

K. pneumoniae, 21 (7.4%) and *E. coli*, 8 (5.7%) shows some synergistic effect between the clavulanic acid and the cephalosporin disc. However, these isolates were not confirmed as ESBL positive (borderline) since the difference in the zones of inhibition was <5 mm.

In the phenotypic detection of ESBL producers using different 3GC substrates, cefpodoxime (CPD) screened 97.8% of the isolates followed by cefotaxime (CTX), 90.5% and CAZ (ceftazidime), 85.1%. In the confirmatory test using CPD/CLA, CTX/CLA and CAZ/CLA combinations, 100% ESBL producers were detected when all three are used (Table 1).

3.4 Antimicrobial Susceptibility of ESBL Producers

The antimicrobial resistance was significantly higher in ESBL producers than in non-ESBL producers ($p<0.05$). More than (75%) of ESBL

producing *K. pneumoniae* and *E. coli* were resistant to third generation cephalosporins. In *K. pneumoniae*, against piperacillin/ tazobactam (71.2%) of ESBL producers and (22.3%) of non-ESBL producers were resistant. The isolates exhibited high level of multidrug resistance pattern. Among *E. coli,* (66.9%) and (67.9%) of ESBL producers were resistant to ciprofloxacin and piperacillin/tazobactam respectively. The comparison of antimicrobial resistance patterns of ESBL and non-ESBL producing *K. pneumoniae* and *E. coli* is shown in Table 2.

3.5 Determination of Minimum Inhibitory Concentration

Among ESBL producing *K. pneumoniae*, MIC of cefpodoxime ranges from 32 to >512 µg/mL, with a maximum of 53 isolates showing MIC of 128 µg/mL. Similarly, for amikacin MIC ranges from 32 to >512 µg/mL with 37 isolates showing MIC of 512 µg/mL. For *E. coli,* MIC of ciprofloxacin ranges from 16 to 512 µg/mL with a maximum of 22 isolates showing MIC 64 µg/mL.

3.6 Phenotypic Detection of AmpC Production

3.6.1 Detection of inducible chromosomal AmpC

All the isolates that were susceptible to one of the third generation cephalosporins by disc diffusion test were tested for inducible AmpC. None were positive, thus showing the absence of chromosomal inducible AmpC enzymes.

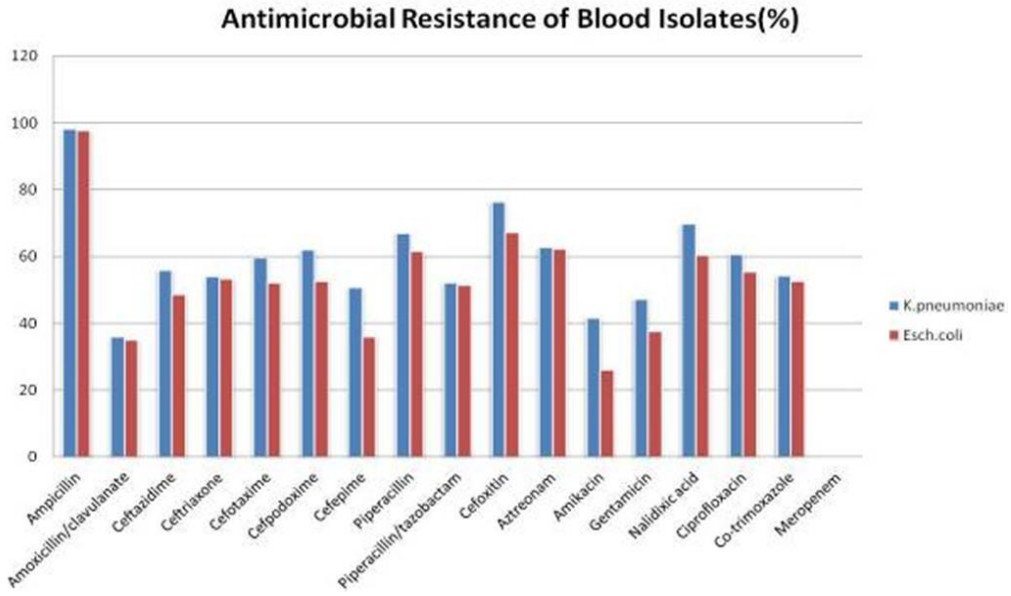

Fig. 1. Antimicrobial resistance profile of blood isolates of *K. pneumoniae* and *E. coli* during the study period

Table 1. Comparison of different third generation cephalosporins substrates in phenotypic testing of ESBLs

Total no. of ESBL positives, (n)	Positive, no. (%)							
	Screening test			Disk confirmatory test				
	CPD	CTX	CAZ	CTX/CLA	CAZ/CLA	Both CTX/CLA and CAZ/CLA	CPD/CLA	CPD/CLA, CTX/CLA and CAZ/CLA
K. pneumoniae (226)	226(100)	207(91.5)	193(85.3)	205(90.7)	193(85.3)	205(90.7)	225(99.5)	226(100)
E.coli (103)	99(96.1)	91(88.3)	87(84.4)	88(85.4)	80(77.6)	88(85.4)	103(100)	103(100)
Total (329)	322(97.8)	298(90.5)	280(85.1)	293(89)	273(82.9)	293(89)	328(99.6)	329(100)

CPD- cefpodoxime, CTX- cefotaxime, CAZ-ceftazidime, CLA-clavulanate

Table 2. Antibiotic resistance profile of ESBL and non-ESBL producing *K. pneumoniae* and *E. coli*

Antimicrobial agent	No. (%) of resistant strains			
	ESBL producers		Non-ESBL producers	
	K. pneumoniae (n=226)	*E. coli* (n=103)	*K. pneumoniae* (n=143)	*E. coli* (n=77)
Ampicillin	226 (100)	103 (100)	137 (95.8)	73(94.8)
Amoxycillin/clavulanate	101(44.6)	48(46.6)	32 (22.3)	15(19.4)
Ceftazidime	189(83.6)	80 (77.6)	18(12.5)	8(10.3)
Ceftriaxone	171(75.6)	84(81.5)	29(20.2)	12(15.5)
Cefotaxime	213(94.2)	91(88.3)	8(5.5)	3(3.8)
Cefpodoxime	220(97.3)	95(92.2)	9(6.2)	0(0)
Aztreonam	221(97.7)	99(96.1)	11(7.6)	13(16.8)
Piperacillin	193 (85.3)	81(78.6)	54 (37.7)	29(37.6)
Piperacillin/tazobactam	161(71.2)	70(67.9)	32(22.3)	23(29.8)
Cefepime	182(80.5)	63(61.1)	6(4.1)	2(2.5)
Cefoxitin	173 (76.5)	63(61.1)	109(76.2)	58 (75.3)
Meropenem	0(0)	0(0)	0(0)	0(0)
Amikacin	122(53.9)	38(36.8)	32(22.3)	9(11.6)
Gentamicin	136(60.1)	51(49.5)	39 (27.2)	17(22)
Ciprofloxacin	183(80.9)	69(66.9)	41(28.6)	31(40.2)
Nalidixic acid	201(88.9)	77(74.7)	57(39.8)	32(41.5)
Co-trimoxazole	162(71.6)	73(70.8)	39(27.2)	22(28.5)

3.6.2 Detection of plasmid de-repressed AmpC

Isolates with the zone diameter ≤ 18mm by disc diffusion and/ or MIC (≥32 µg/mL) were defined as cefoxitin resistant. Of the 549 isolates screened, 403 (73.4%) (*K. pneumoniae* n=282 (76.4%), *E. coli* n=121(67.2%) were found to be resistant to cefoxitin. They were considered as putative AmpC producers and were subjected to further analysis.

Out of the 549 isolates, 403(73.4%) were cefoxitin resistant. Among them 292 (72.4%) showed AmpC production phenotypically. Among the 369 *K. pneumoniae* screened 282 (76.4%) were cefoxitin resistant. Of them, 199 (70.5%) were AmpC producers and 83 (29.4%) were non-AmpC producers. Similarly, out of the 180 *E. coli* screened, 121(67.2%) were cefoxitin resistant and among them 93 (76.8%) were AmpC positive and 28 (23.1%) were AmpC negative. Using M3D test, all the AmpC producers (n=292) were detected. However, AmpC disc test detected 241 (82.5%) of them. The distribution of AmpC producers in the hospital was documented. The highest number of AmpC producers were from NICU with 43 (14.7%) isolates, followed by pediatrics ward 40 (13.6%), 33 (11.3%) from PICU and surgery wards each. Nearly, 9% were recovered from medicine, CCU and urology wards. The lowest rate of AmpC producers were from ortho with 1% of the isolates.

Of the 549 isolates that were included in the study, co-production of AmpC β-lactamases and ESBLs was noticed in 198 (36%) isolates with an incidence in *K. pneumoniae* (41%; n=152) and *E. coli*, (25.5%; n=46).

3.7 Molecular Characterization of the ESBL Genes

All the 422 isolates which were resistant to one or more 3GC were subjected to a multiplex PCR to identify the molecular genetics of ESBLs. Among them, 280 (66.3%) strains carried ESBL genes with a prevalence of bla_{CTX-M} in 81.7% (n=229), bla_{TEM}, 75.7% (n=212), bla_{SHV}, 71.4% (n=200) and bla_{OXA-1}, 32.1% (n=90). The percentage distribution of ESBL genes among the PCR positive isolates is shown in Fig. 3. The gel picture of the multiplex PCR is shown in Fig. 2(a).

Multiple beta-lactamase gene combinations were identified in 81.4% of strains (n= 228), with 18.9% of strains containing two genes (bla_{TEM} and bla_{CTX}, n =5; bla_{TEM} and bla_{SHV}, n =24; bla_{CTX-M} and bla_{SHV}, n=14; bla_{CTX-M} and bla_{OXA-1}, n=10). The distribution of *bla* ESBL genes among 280 PCR positive strains is shown in Table 3.

In *K. pneumoniae,* 283 screen positive isolates were subjected to PCR, of them 193(68.1%) carried ESBL gene. Similarly, among the 139 *E. coli* screened, 87 (62.5%) were positive for ESBL gene. Among them, 68(78.1%) carried

bla$_{CTX-M}$, 78(89.6%) *bla*$_{TEM}$, 65 (74.7%) *bla*$_{SHV}$ and 22 (25.2%) *bla*$_{OXA-1}$ (Table 3).

3.7.1 Sequencing of ESBL genes

Nucleotide sequencing was performed for randomly selected 55 ESBL gene positive isolates (*K. pneumoniae*, n=30; *E. coli*, n=25) choosing isolates from all the four gene combinations. Sequencing of the *bla*$_{CTX-M}$ showed that two variants; 54 (98.1%) were *bla*$_{CTX-M-15}$ and 1 (1.8%) *bla*$_{CTX-M-27}$, thus belonging to the CTX-M -1 group and Group 4. Among *bla*$_{SHV}$ genes; 49 (89%) were *bla*$_{SHV-12}$ and 6 (10.9%) *bla*$_{SHV-2a}$. Sequencing of *bla*$_{TEM}$ genes showed; 52 (94.5%) were *bla*$_{TEM-1}$ and 3 (4.5%) *bla*$_{TEM-12}$ variants. The *bla*$_{OXA-1}$ showed 100% similarity with the GenBank sequences.

3.7.2 Genetic environment of the *bla*$_{CTX-M-15}$ gene

A total of 40 *bla*$_{CTX-M-15}$ producers were tested. The insertion sequence *ISEcp*1 was identified in 37 (92.5%) of the isolates. No *IS*26 element was found. Among them, sequencing of the *ISEcp*1-*bla*$_{CTX-M-15}$ gene was done for six isolates. All showed the linkage of the *ISEcp*1 with the *bla*$_{CTX-M-15}$.

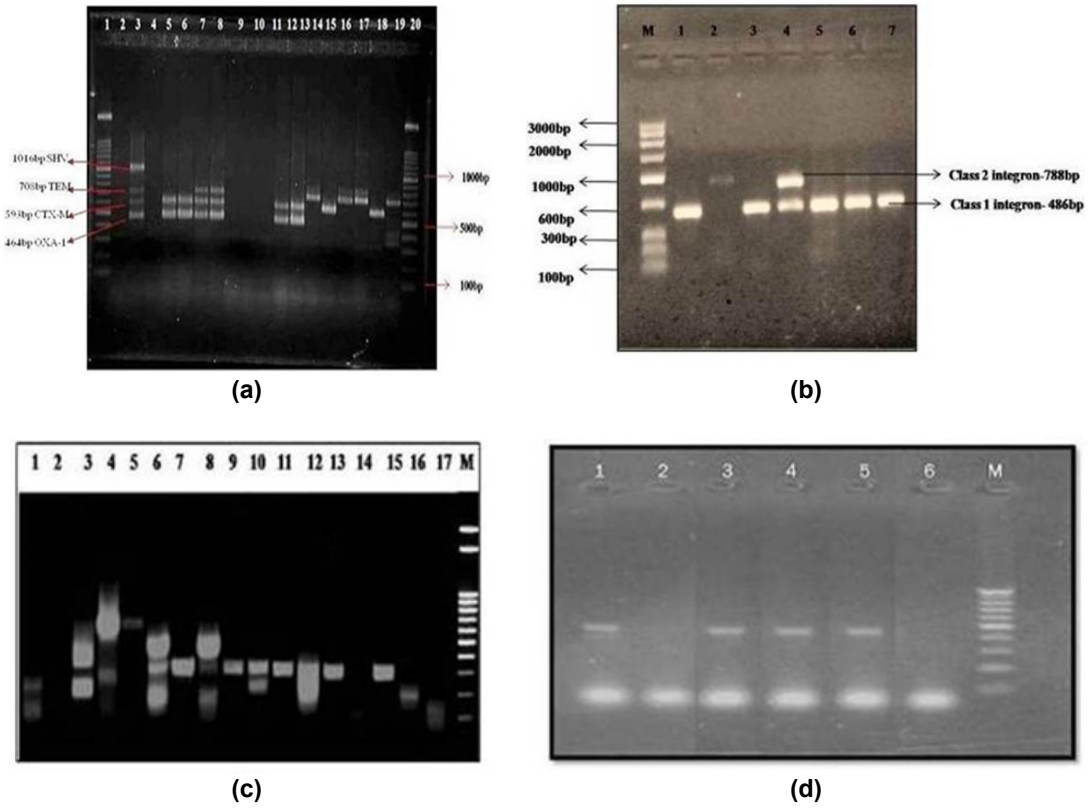

Fig. 2. (a) Gel picture of ESBL multiplex PCR: ***(Lane 1 & 20- DNA standard; Lane 2-blank, negative control ;Lane 3-ESBL positive control (bla$_{SHV}$, bla $_{TEM}$, bla $_{CTX-M}$, bla $_{OXA-1}$); Lane 4,9,10, 11- test negatives; Lane 5,6,12,13- bla$_{CTX-M}$ and bla $_{OXA-1}$; Lane 7 & 8- bla$_{CTX-M,}$ bla$_{TEM}$ and bla $_{OXA-1}$; Lane 14,16,17 & 19- bla$_{TEM}$ and Lane 15,18- bla$_{CTX-M.}$)***

(b) Gel picture result of integrase PCR: ***(Lane M- DNA ladder 3Kb, Lane 1, 3, 5, 6 and 7 - 486bp Class 1 integron positives. Lane 2- 788bp Class 2 integron positive, Lane 4- both class 1 and class 2 integrons positive)***

(c) Gel picture of class 1 integron gene cassettes: ***(M- 3Kb DNA ladder, Lanes 1-17 Amplified gene cassettes of Class 1 integron)***

(d) Gel picture of *aac(6')-Ib-cr* quinolone resistant determinant: (*M-100bp DNA ladder; Lanes 1,3,4,5- 483bp aac(6')-Ib-cr gene positive; Lanes 2, 6- negative test)*

Table 3. Distribution (%) and combination of *bla*ESBL genes among PCR positive *K. pneumoniae* and *E. coli* isolates

ESBL gene(s)	*K. pneumoniae* (n=193)		*E. coli* (n=87)		Total isolates (n=280)	
	n	%	n	%	n	%
bla_{CTX-M}	23	11.9	6	6.8	29	10.3
bla_{TEM}	9	4.6	8	9.1	17	6
bla_{SHV}	6	3.1	0	0	6	2.1
bla_{CTX-M} and bla_{TEM}	3	1.5	2	2.2	5	1.7
bla_{CTX-M} and bla_{SHV}	14	7.2	0	0	14	5
bla_{CTX-M} and bla_{OXA-1}	7	3.6	3	3.4	10	3.5
bla_{TEM}, bla_{SHV} and bla_{OXA-1}	4	2.0	0	0	4	1.4
bla_{TEM} and bla_{SHV}	13	6.7	11	12.6	24	8.5
bla_{SHV}, bla_{TEM} and bla_{CTX-M}	57	29.5	38	43.6	95	33.9
bla_{SHV}, bla_{CTX-M} and bla_{OXA-1}	9	4.6	0	0	9	3.2
bla_{TEM}, bla_{CTX-M} and bla_{OXA-1}	16	8.2	3	3.4	19	6.7
bla_{CTX-M}, bla_{TEM}, bla_{SHV} and bla_{OXA-1}	32	16.5	16	18.3	48	17.1

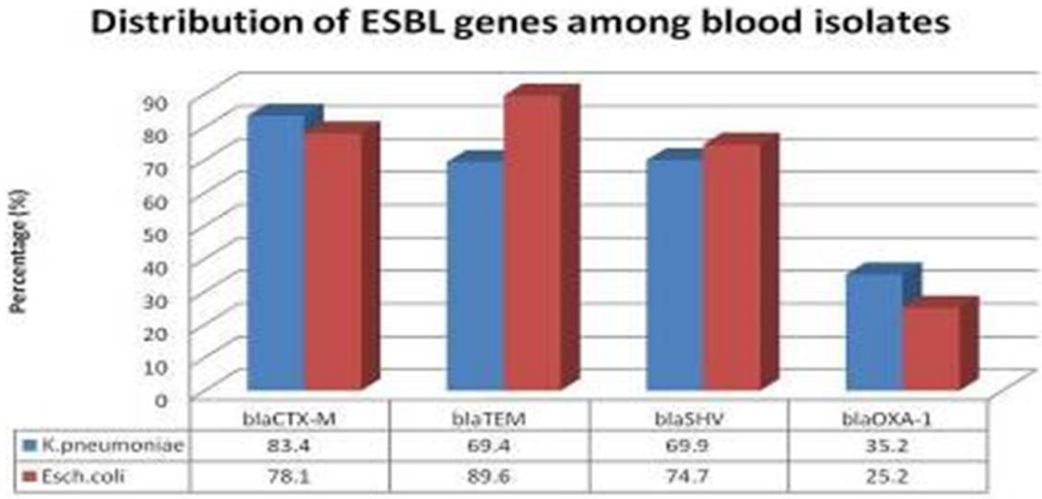

Fig. 3. Distribution of *bla*ESBL genes among the PCR positive isolates

3.8 Molecular Characterization of Plasmid mediated AmpC β-lactamase (PMABL) Genes

All putative AmpC producers were subjected to multiplex PCR for the simultaneous detection of the six AmpC plasmid encoded genes. Results showed that, among the 292 phenotypically positive AmpC producers, PMABL genes were present in 91 (31.1%) of the isolates. Thus, among the 549 isolates studied, PMABL genes were carried by 16.5% of the isolates. bla_{EBC} and bla_{FOX} genes were completely absent in both the genera. The distribution of the PMABL genes among the test isolates were shown in Table 4.

Table 4. Distribution of plasmid mediated AmpC β-lactamase genes among *K. pneumoniae* and *E. coli*

Plasmid encoded AmpC gene type	*K. pneumoniae* (n=55) No. of isolates (%)	*E. coli* (n=36) No. of isolates (%)	Total isolates (n=91)
DHA	30 (54.5%)	5 (13.8%)	35(38.4%)
CIT	22 (40%)	30 (83.3%)	52(57.1%)
ACC	1 (1.8%)	0 (0%)	1(1%)
EBC	0 (0%)	0 (0%)	0(0%)
MOX	2 (3.6%)	1 (2.7%)	3 (3.2%)
FOX	0 (0%)	0 (0%)	0(0%)

All the 52 isolates that were CIT family genes positive by multiplex PCR were subjected to bla_{CMY-2} gene specific PCR and found to be 100% positive. Sequencing of the CMY-2 PCR amplicons was performed in 10 positive isolates (5 *K. pneumoniae* and 5 *E. coli*) which showed, consensus sequences with the GenBank reference sequence (accession number X-91840).

3.9 Analysis of Genetic Relatedness of ESBL Producers

PFGE was conducted to determine if clonal spread plays a role in the dissemination of ESBL and producing isolates in this hospital. Since $bla_{CTX-M-15}$ was the prevalent variant found, forty $bla_{CTX-M-15}$ gene positive isolates of *K. pneumoniae* representing different resistance profiles were chosen for typing. Restriction was successful in 39 isolates. *Xba*I digestion was found to produce 9–19 fragments ranging in size from 20 to 380 kb. By UPGMA analysis, the 39 isolates belonged to 3 major clusters of *K. pneumoniae* with no relationship between PFGE type and ward was found as shown in Fig. 4.

Replicon typing was performed for forty $bla_{CTX-M-15}$ producing isolates of *K. pneumoniae*. PCR revealed that the $bla_{CTX-M-15}$ bearing plasmids were distributed among the following five incompatibility groups: IncL/M- 10 (25%), IncFIA-6 (15%), IncA/C- 19 (47.5%), IncI1-3(7.5%) of the isolates. Multiple replicon types of IncFIA/IncFIB were identified among 2 (5%) of the bla_{CTX-M}-bearing plasmids (Fig. 4).

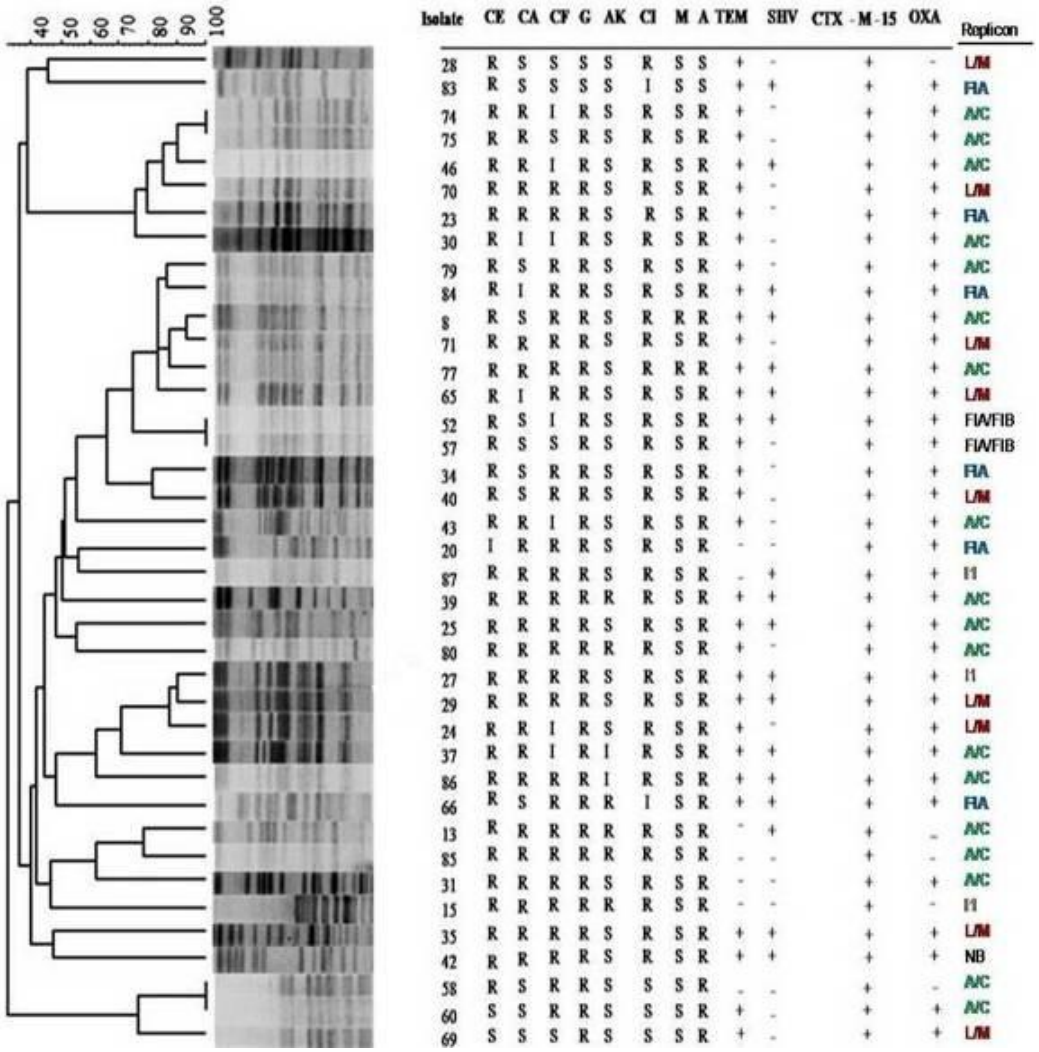

Fig. 4. PFGE dendrogram of $bla_{CTX-M-15}$ producing *K. pneumoniae*

Similarly, plasmid replicon typing was performed for 20 representative isolates of *bla*$_{CTX-M-15}$ producing *E. coli* chosen from different resistance profile. The replicon types were; IncL/M- 6 (30%), IncFIA-3 (15%), IncA/C-11(55%)

Incompatibility typing was also performed for the 52 CIT-positive isolates. Results showed that, the CMY bearing plasmids were distributed among 3 incompatibility groups: IncA/C, 28 (52.8%); IncL/M, 17(32.6%) and IncI1, 7 (14.5%).

3.10 Conjugation Studies

Conjugation studies were performed on randomly selected 12 *bla*$_{CTX-M-15}$ producing and 12 *bla*$_{CMY-2}$ isolates. Transconjugation occurred in 10 (83.3%) of the *bla*$_{CTX-M-15}$ producing isolates and in 12(100%) of the *bla*$_{CMY-2}$ producers. Antimicrobial susceptibility pattern of the transconjugants were same as the clinical isolates, thus revealing the resistance transferred. The genotypes of the transconjugants showed all had *bla*$_{CTX-M}$ and *bla*$_{CMY}$ genes.

3.11 Association of Integron with ESBL Genes

The carriage of integrons was detected in a group of 172 ESBL positive isolates of different gene combinations and in 55 ESBL negative isolates. Among the 172 ESBL positive isolates, 121(70.3%) carried class 1 integron, 37 (21.5%) carried class 2 integron while, 18(10.4%) harbored both class 1 and class 2 integrons. None of the isolates were positive for class 3 integron.

Among the 55 non-ESBL isolates, 13(23.6%) carried class 1 integron and none were positive for class 2 or class 3. Class 1 integrons were more frequently found among ESBL producing isolates than among non-ESBL ($P < 0.05$). The gel picture of integrase PCR is shown in Fig. 2(b).

Of the 106 ESBL positive *K. pneumoniae* tested, 73 (68.8%) carried class 1, 24 (22.6%) carried class 2 and 11 (10.3%) carried both classes. Similarly, among the 66 ESBL positive *E. coli* tested, 48 (72.7%), 13 (19.6%) carried class 1 and 2 integrons respectively. Nearly 11% of *E. coli* simultaneously carried both integron types (Table 5).

Gene cassettes amplification of the class 1 and 2 integron regions revealed products from 0.1 to 2kb, part of them were single band, while other were multiple bands (2 to 5 bands) thus revealing the number of resistant gene cassettes. Fig. 2(c) shows the amplified gene cassettes of class 1 integron.

A high rate of *bla*$_{CTX-M}$ genes when present alone and co-present with other *bla* genes carried the integrons. However, no *bla*$_{TEM}$ genes when present alone carried integrons. Table 6 shows the integron carriage among different ESBL genes.

3.12 Analysis of PMQR among ESBL Producers

Of the 549 total study isolates, 278 ESBL producing isolates resistant to nalidixic acid were analyzed. MIC of nalidixic acid ranged from ≥32 to ≥256 µg/mL. Of them, 252 were also resistant to ciprofloxacin with MIC ranged from 16 to ≥128 µg/mL.These isolate were classified as HL-QR. Twenty six isolates resistant to nalidixic acid and susceptible to ciprofloxacin were classified as LL-QR.

PCR for the detection of PMQR genes were performed for 186 (HL-QR, 160; LL-QR, 26) isolates. Among the 186 quinolone resistant strains, a total of 36 (19.3%) possessed PMQR genes. Screening for the *aac(6')-Ib-cr* gene showed that, a maximum of 27 (14.5%) isolates carried the gene, Fig.2(d). Among the *qnr* determinants, *qnrB* was present in 5(2.6%) followed by, *qnrS* in 3 (1.6%) and *qnrA* gene in

Table 5. Frequency of integrons among ESBL positive and ESBL negative isolates

	ESBL positive (n=172)		ESBL negative (n=55)	
	***K. pneumoniae* (n=106)**	***E. coli* (n=66)**	***K. pneumoniae* (n=30)**	***E. coli* (n=25)**
Class 1 integron	73 (68.8%)	48 (72.7%)	7 (23.3%)	6 (24%)
Class 1 cassettes	57 (53.7%)	32 (48.4%)	4 (13.3%)	3 (12%)
Class 2 integron	24 (22.6%)	13 (19.6%)	-	-
Class 2 cassettes	16 (15%)	9 (13.6%)	-	-
Both class 1 and 2	11 (10.3%)	7 (10.6%)	-	-

Table 6. Integron carriage rate (%) among different ESBL genes

ESBL gene (s)	Total no. of isolates (n=172)		
	Class 1 integron	Class 2 integron	Both classes
	n (%)	n (%)	n (%)
bla$_{CTX-M}$	19 (15.7%)	3 (8.1%)	3 (16.6%)
bla$_{TEM}$	0 (0)	0 (0)	0 (0)
bla$_{SHV}$	1 (0.8%)	0 (0)	0 (0)
bla$_{CTX-M}$ and *bla*$_{TEM}$	11 (9%)	5 (13.5%)	0 (0)
bla$_{CTX-M}$ and *bla*$_{SHV}$	14 (11.5%)	3 (8.1%)	2 (11.1%)
bla$_{CTX-M}$ and *bla*$_{OXA-1}$	5 (4.1%)	6 (16.2%)	2 (11.1%)
bla$_{TEM}$, *bla*$_{SHV}$ and *bla*$_{OXA-1}$	9 (7.4%)	1 (2.7%)	1 (5.5%)
bla$_{TEM}$ and *bla*$_{SHV}$	3 (2.4%)	0 (0)	0 (0)
bla$_{SHV}$, *bla*$_{TEM}$ and *bla*$_{CTX-M}$	7 (5.7%)	11 (29.7%)	3 (16.6%)
bla$_{SHV}$, *bla*$_{CTX-M}$ and *bla*$_{OXA-1}$	16 (13.2%)	2 (5.4%)	1 (5.5%)
bla$_{TEM}$, *bla*$_{CTX-M}$ and *bla*$_{OXA-1}$	12 (9.9%)	4 (10.8%)	2 (11.1%)
bla$_{CTX-M}$, *bla*$_{TEM}$, *bla*$_{SHV}$ and *bla*$_{OXA-1}$	24 (19.8%)	2 (5.4%)	4 (22.2%)
Total	121 (70.3%)	37 (21.5%)	18 (10.4%)

Table 7. Prevalence (%) of PMQR gene determinants among ESBL producers

Quinolone resistance	PMQR genes (n=36)			
	***qnr*A**	***qnr*B**	***qnr*S**	***aac(6')-Ib-cr***
HL-QR (n=160)	1(0.6%)	4(25%)	3(1.8%)	21(13.1%)
LL-QR (n=26)	-	1(3.8%)	-	6(2.3%)
Total (n=186)	1(0.5%)	5(2.6%)	3(1.6%)	27(14.5%)

1(0.5%) isolate. Nearly, 2.3% of LL-QR isolates carried the aac(6')-Ib-cr determinant. The rate of PMQR genes among ESBL producers is shown in Table 7. The gel picture of *aac(6')-Ib-cr* is shown in Fig. 2(d).

4. DISCUSSION

The frequency of ESBL-producing organisms differs significantly from institution to institution in accordance with geographical location [34,35]. During the last two decades, ESBL producing Gram-negative bacilli have emerged as a major problem in many settings [36]. In India, reported frequency of ESBL producing *K. pneumoniae* and *E. coli* in the past decade, is between 6-87%, with most studies reporting prevalence rate of around 50% and upwards [34,35,37,38] during this period. In the present study, *K. pneumoniae* (79.8%) and *E. coli* (74.1%) were found to be ESBL producers. This high frequency of ESBL production may be due to the fact that most of the isolates were recovered from hospitalized patients.

Earlier in 2007, ESBL producing *K. pneumoniae* (96%) among the isolates from blood was reported from this centre [10]. Whereas in the present study the frequency of ESBL production in *K. pneumoniae* is relatively less in number, the reason being the number of isolates included in the former.

In the screening test done as per the CLSI, we have experienced that cefpodoxime was a good indicator of ESBL production followed by cefotaxime, and ceftazidime. Cefpodoxime alone could pick up all the ESBL producing *K. pneumoniae* whereas in *E. coli* it picked up 96.1% of the ESBL producing strains. This finding is similar to that of Thomson 2001 [39], where cefpodoxime was found to be a more reliable indicator.

In the confirmatory test using three combination of cephalosporin, cephalosporin/clavulanate discs, cefpodoxime/clavulanate had better detection rate. However, all the three combinations should be used for optimum detection of ESBL production. This is in agreement with various studies where, use of more than two screening agents for screening increased the chance of detecting ESBL producers in screening as well as confirmatory tests [34,40].

Resistance to third generation cephalosporins ranges in *K. pneumoniae* (62%) and *E. coli* (53%). At least 52% of isolates were resistant to one of the third generation cephalosporins in this study. For several years in India, a lot of Gram negative organisms have been reported to have high antimicrobial resistance to cephalosporins [38,41] and this assertion was confirmed in this study. This resistance is most often mediated by ESBLs and/or AmpC enzyme production.

The antimicrobial resistance pattern of ESBL producers is high compared to the non-ESBL producers ($p<0.05$). The reason could be due to the excessive use of third generation cephalosporins, the most commonly used antibiotic class in hospitals; which might exert selective pressure for development of resistance especially among ESBL producers. Similar high resistant profiles against third generation cephalosporin were noted in other studies [42]. In a study from Kashmir, a high prevalence of 97.5% isolates of ESBL producers were reported to be resistant to third generation cephalosporin and 99.2% to fourth generation cephalosporin [43].

In addition to increasing resistance to cephalosporins, resistance to other commonly used antibiotics such as fluoroquinolones was noted. In 2004, Safdar et al. [44] reported that, ciprofloxacin and norfloxacin have high antimicrobial activity against third generation cephalosporin resistant Enterobacteriaceae and was very effective against isolates from blood stream infections. This statement is not true in this study, where, there has been a prominent resistance to ciprofloxacin among *K. pneumoniae* (80.9%) and *E. coli* (66.9%) seen. It is noteworthy that among non-ESBL producers, *K. pneumoniae* (28.6%) and *E. coli* (40.2%) were resistant. The possible reason may be due to the abuse of quinolones for third generation cephalosporin resistant strains due to the fact that ciprofloxacin has low toxicity and is an inexpensive drug [45]. In a SMART study, 73.4% of fluoroquinolones resistance was documented among ESBL producers from India [46].

For ESBL producing strains, however, whilst the high rate of resistance to other agents such as the fluoroquinolones is a concern, the aminoglycosides remain the next choice for treatment. In this study, *K. pneumoniae* (60%) and *E. coli* (49.5%) were resistant to gentamicin. The high level resistance observed may be due to emergence of ESBL producers and the co-carriage of plasmids which also harbor gene encoding resistance to several aminoglycosides [45]. Further, gentamicin sulfate containing antiseptic solution and creams are commonly used in the hospital environment and also by general public in the treatment of burn infections and skin lesions. This may have also resulted in the selection of gentamicin resistant determinants among the Gram negative isolates. Unlike gentamicin, the parenteral drug, amikacin was very effective *invitro* against both *K. pneumoniae* and *E. coli* and it is also resistant to many of the enzymes that inactivate gentamicin. Studies shown amikacin was the most active drug for ESBL infections [47]. However, in this study, *K. pneumoniae* (59.3%) and *E. coli* (36.8%) of ESBL producing isolates were resistant to amikacin. This may be due to the continued use of combination therapy involving cephalosporins and aminoglycosides for the treatment of resistant Gram-negative bacilli in our hospital. For ampicillin, irrespective of the ESBL profile >95% of the isolates were resistant.

Isolates that were resistant to all or most of the first-line drugs were tested against the second-line drugs, carbapenems. Meropenem was found to be very effective against both *K. pneumoniae* and *E. coli invitro* (100%). All the ESBL producing isolates were susceptible to meropenem. After carbapenems, amikacin was found to be the active class of antibiotics. These results are in agreement with another study from South India where meropenem (100%) followed by amikacin (82.6%) susceptibility was recorded [48].

In this study, among the third generation cephalosporin resistant strains, 93 (22%) were non-ESBL producers. This would suggest the existence of AmpC producers among them. Combination with clavulanic acid bringing it back to completely susceptible level would indicate an ESBL alone. However, there are reports where ESBL screening tests are positive but the confirmatory tests are negative due to the coexistence of both ESBLs and plasmid-mediated AmpC β-lactamases [39].

Resistance to 3GCs is often mediated by the acquisition of ESBLs or by AmpC β-lactamases. The possible alliance of AmpC β-lactamases in the mediation of 3GC resistance was also analyzed. Resistance to cefoxitin was measured as the key factor and the isolates were screened and characterized for plasmid mediated AmpC β-lactamases (PMABLs) production. Though laborious, modified 3D test detects 8.2% of the AmpC producers more compared to the AmpC disc test and was found to be sensitive.

The phenotypic tests only presumptively identify the presence of an ESBL and cannot differentiate ESBL types. In this study, the multiplex PCR used was found to be a rapid method which allows detection of four β-lactamase genes in less than 24 hours.

The results show that isolates having both *bla*$_{TEM}$ and *bla*$_{SHV}$ genes were more common than *bla*$_{TEM}$ or *bla*$_{SHV}$ alone. Among the single gene carrier isolates the proportion of *bla*$_{TEM}$ was higher than *bla*$_{SHV}$ in *K. pneumoniae.* In a study from Lucknow [49], of the ESBL producing *Klebsiella* spp. isolates, 26.5% had both *bla*$_{TEM}$ and *bla*$_{SHV}$ genes, 48.4% had *bla*$_{TEM}$ alone and 20.3% had SHV gene alone. Presence of *bla*$_{TEM}$ gene in ESBL producing *Klebsiella* spp. was more common than SHV genes, and this result is in agreement with the present study.

In this study, *K. pneumoniae* (83.4%) and *E. coli* (78.1%) possessed *bla*$_{CTX-M}$ gene. *bla*$_{CTXM}$ gene alone was present in 10.3% of the ESBL producers revealing that *bla*$_{CTX-M}$ was the most common gene and present either alone or in combination with other ESBL genes.PCR sequencing showed that *bla*$_{CTX-M-15}$ was the predominant type in 98.1% isolates from this region. Interestingly, in this study, a single isolate was found to be *bla*$_{CTX-M-27}$ variant, which is the first report from South India.

Also, among the phenotypically positive AmpC producers, PMABL genes were present in *K. pneumoniae* (27.6%) and *E. coli* (38.7%). Non-plasmid-derived AmpC activity was present in 57(61.2%) strains of *E. coli*, and was presumed to originate from hyperproduction of endogenous AmpC enzyme. From this region, *bla*$_{CIT}$ genes were predominant among *E. coli* (83.3%) and *bla*$_{DHA}$ gene predominant in *K. pneumoniae* (54.5%). Sequencing of the CIT-like PCR amplicons showed all were of CMY-2 type. To our knowledge this is the first report of plasmidic AmpC from South India.

A coincidental finding was the identification of a relatively large number of cefoxitin- resistant isolates among the same pool of ESBL screened isolates, *K. pneumoniae* (76.5%) and *E. coli* (61%). However, the molecular studies provide evidence that only 16.5% of this cefoxitin resistance was due to PMABL production. PCR should be performed to confirm PMABLs in *E.coli* because the phenotypic test is less specific due to the presence of chromosomal AmpC. We have reported earlier on the molecular characterization of PMABL genes from India, wherein the other types existing in different parts of the country are *bla*$_{MOX}$ and *bla*$_{ACC}$ types [50].

PFGE was conducted to determine if clonal spread plays a role in the dissemination of ESBL producers in this setting. Typing results indicate that a substantial heterogeneity exists among the isolates. It was evident based on PFGE data that the spread of ESBL producers in this region was polyclonal and not due to the clonal spread of a single strain, thus revealing their complexicity.

Plasmid typing revealed that, five different replicon types were associated with ESBL genes. Replicon IncA/C is the predominant type circulating among the ESBL producers, followed by IncL/M in this region. Two strains carrying both IncFIA/IncFIB were noted. It is interesting to found that, even among the same replicon types, the antimicrobial resistance pattern varies. The presence of ESBL genes and more specifically *bla*$_{CTX-M-15}$ on plasmids of multiple replicon types demonstrates considerable mobility of the ESBL loci and their availability in nature. *ISEcp*1 was found to be located upstream of *bla*$_{CTX-M-15}$ in this study. In addition, the *ISEcp*1 element is also implicated in the expression of the *bla*$_{CTX-M}$ gene as it serves as a strong promoter [9]. Thus, it is shown that, the ESBL genes were located on diverse plasmids in which *bla*$_{CTX-M-15}$ were located downstream of the mobile insertion element *ISEcp*1 that contributes to their ease of mobilization, expression and rapid spread.

Characterization of the plasmids bearing *bla*$_{CMY-2}$ revealed that both genetically similar and different IncI1, IncA/C and IncL/M plasmids are largely responsible for the spread of *bla*$_{CMY-2}$ across the hospital.The presence of *bla*$_{CMY-2}$ on plasmids of multiple replicon types demonstrates considerable mobility of the *bla*$_{CMY-2}$ loci. These results also demonstrate the association of *bla*$_{CMY-2}$ β-lactamase gene with similar IncL/M, IncA/C plasmid backbones that carried ESBLs. IncI1 and IncA/C plasmids have been previously associated with *bla*$_{CMY}$ genes in the USA, United Kingdom and Italy [26].

Analysis of integron-encoded integrases indicated an incidence of 70.3% of class 1 integron among ESBL-producing isolates in this region. Class 2 integron was found in 21.5% isolates and no class 3 integron-encoded integrase was detected. In a study by Bhattacharjee et al. [51] from India, the frequency of integrons among ESBL positive *K. pneumoniae* was 92% which is higher than 68.8% among *K. pneumoniae* from our centre. As high as 21.5% of class 2 integron noted for the first time from our region, in compared to 9% reported by Bhattacharjee et al. [51]. However, compared to the 40% integron positivity among

non-ESBL producers reported, in our study only 23.6% non-ESBL isolates carried integrons.

The co-existence of both class 1 and a class 2 type integron was noted in 10.4% isolates. This increases the higher risk of multidrug resistant gene transfer rates. Integron-positive isolates were more likely to be multiresistant than integron-negative isolates [52]. Multiresistant integrons are considered to be important contributors to the development of antibiotic resistance among Gram-negative bacteria [30,31]. In our study, high prevalence of class 1 integron contributed to the multiresistance in most isolates and the integron positivity was high in ESBL producers than in non-ESBL producers ($P<0.05$).

The previously described wide dissemination and the increase of the class 1 integrons rate among nosocomial isolates of Enterobacteriaceae during the last decade [29,53] was confirmed in this study. Except the bla_{TEM} when present alone, all the other ESBL genes carried integrons. This might be because, most of these isolates were of bla_{TEM-1}, which is a narrow spectrum ESBL type. As many ESBLs and integrons are on conjugative plasmids, horizontal spread by conjugation might be a major mechanism for their dissemination.

The amplified gene cassette of class 1 and class 2 integrons reveals the number of different drug resistant determinants present. However, they were not further sequenced which we found as a drawback in this analysis. Thirty two (26.4%) isolates containing the *IntI*1 gene failed to produce an amplicon using primers 5'CS and 3'CS. This was probably due to the lack of a 3' conserved segment or the variable region was too long to be amplified in these isolates. Conversely, the simultaneous presence of the same class 1 integron types among ESBL and non-ESBL isolates recovered from this same hospital might indicate a wide dissemination of some specific structures in which integrons are located, which needs to be analyzed further in future.

A high percentage of resistance to quinolones was noted among the ESBL producers. Of the 186 isolates tested, 19.3% posses the PMQR determinants. To our knowledge, this is the first report on PMQR among ESBL producing isolates from India. The prevalence of *aac(6`)-Ib-cr* among the *E. coli* and *K. pneumoniae* was 14.5%. This finding was comparable to other studies; where prevalence of *aac(6`)-Ib-cr* was 11.3% among ciprofloxacin- and/or tobramycin-resistant *E. coli* and *Klebsiella* spp. clinical isolates from Canada [54] and 9.9 % among ESBL-producing *E. coli* and *K. pneumoniae* isolates from six provinces in China [55].

PMQR determinants have always been reported to confer only low level of resistance or just reduced susceptibility to nalidixic acid and fluoroquinolones [32]. Whereas, the most important mechanism for acquiring high levels of quinolone resistance has constantly developed by the acquisition of chromosomal point mutations in the QRDRs of target genes particularly gyrA and parC [56].

Nevertheless, this study shows that, the *qnr* positive plasmids also harbored bla_{CTX-M} ESBLs, which is an important concern as the spread of bla_{CTX-M} apparently spreads the quinolone resistance also rapidly by conjugation. If the plasmids that carry *qnr* and bla_{CTX-M} spread rapidly, it is likely that the quinolones as antimicrobials may be a thing of past. This study shows that PMQR mechanisms co-exists and is emerging among the ESBL isolates. The higher rates of ciprofloxacin resistance appear to be a warning sign that this class needs to be used with greater caution and more wisdom.

5. CONCLUSION

In conclusion, this study reveals that, increases in cephalosporin resistance among *E. coli* and *K. pneumoniae* was linked and often mediated due to increases in the emergence and spread of ESBL and/or AmpC producers and they are multidrug resistant. Integrons have a major role in their dissemination. The clonal diversity of the isolates and the transferability of *bla*CTX-M plasmids suggest a higher incidence and wider distribution of ESBL and AmpC producing bacteria existing in South India.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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