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# Genetic determination and characterization of extended spectrum β-lactamase producing *Escherichia coli* and *Klebsiella pneumoniae* in a tertiary care hospital, India

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Extended spectrum beta lactamase (ESBL) producing *Escherichia coli* and *Klebsiella pneumoniae* are known to cause nosocomial outbreaks which emerged as one of the world's extreme health threats in past two decades. In this context, the present study was aimed to isolate multi drug resistant *E. coli* and *K. pneumoniae* and evaluated the ESBL production. The samples were collected from district Govt. hospital, Ariyalur, Tamil Nadu, India in the period of September 2014 to September 2015 and a total of 1400 nosocomial isolates were isolated. All the isolates were subjected to antibiotic susceptibility testing by Kirby–Bauer disc diffusion method. *E. coli* (n = 160) had shown high antibiotic resistance pattern to Amikacin, Ceftazidime and Nalidixic acid while *K. pneumoniae* (n = 110) had shown high antibiotic resistance pattern to Ceftazidime and Nalidixic acid. Based on the phenotypic confirmatory test, 163 (60.4%) isolates (n = 89, *E. coli* and n = 74, *K. pneumoniae*) were ESBL producer. ESBL-positive isolates were screened for the presence of ESBL encoding $bla_{TEM}$, $bla_{SHV}$, $bla_{CTX\text{-}M\text{-}1}$, $bla_{NDM\text{-}1}$, $bla_{IMP1}$ and $bla_{GES}$ resistance genes by multiplex polymerase chain reaction (mPCR). Among the ESBL producing genes, $bla_{CTX\text{-}M\text{-}1}$ was the highest (90.8%) prevalence followed by $bla_{TEM}$ (77.3%), $bla_{GES}$ (19%) and $bla_{NDM\text{-}1}$ (3.1%) alone or together. The present study concluded that the highest prevalence of ESBL producing MDR *E. coli* and *K. pneumoniae* with multiple resistance genes demand for new therapeutic options.

**Keywords:** *Escherichia coli,* extended spectrum beta lactamase, *Klebsiella pneumoniae,* multidrug resistance

## Introduction

Extended spectrum beta lactamase (ESBL) producing Gram negative bacteria have rapidly spread worldwide in last two decades which remain a public health concern[1]. Nowadays, nosocomial infections caused by multidrug resistant (MDR) enterobacteriaceae and its associated diseases significantly increased mortality and morbidity[2-3]. However, ESBL producing MDR *Escherichia coli* and *Klebsiella pneumoniae* infections have emerged as one of the world's greatest health threats because of resistance to the third generation cephalosporin and has become a major problem presently[4].

ESBLs are plasmid mediated bacterial enzymes that confer resistance to utmost beta-lactam antibiotics, including third and fourth-generation cephalosporins except cephamycins and carbapenems. These enzymes generate serious therapeutic challenge to clinicians due to its limited therapeutic options which are mainly caused by MDR *E. coli and K. pneumoniae*[5-6]. The ESBL-encoding genes are plasmid encoded mostly TEM-, SHV-, and CTX-M-type enzymes and are commonly produced by *E. coli* and *K. pneumoniae*[7-8]. During 1990s, ESBL-producing organisms were labeled mainly as members of the TEM- and SHV-β-lactamase families in *E. coli* and *K. pneumoniae* causing nosocomial outbreaks. The rapid spread of highly virulent ESBL producing bacteria, particularly *E. coli* and *K. pneumoniae* to Asia and all over the world is now an important alarm for the development of new therapies against such infection[9]. The emergence of ESBL producing MDR bacteria, particularly *E. coli* and *K. pneumoniae* are now important alarm for the development of therapies against bacterial infection. ESBLs are more prevalent in *E. coli* and *K. pneumoniae* than any other Gram negative bacterial species and the occurrence of infections caused by ESBL producing strains have been widely reported[10].

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Several risk factors that are associated with these ESBL producing *E. coli* and *K. pneumoniae* microbial infection include advanced age and patients' previous comorbidities (such as neoplasia, renal failure, immunosuppression, etc.), long hospital stay, use of invasive devices (urinary catheters, venous catheters, endotracheal tubes) and previous therapy with wide spectrum antibiotics[9,11-12]. However, the genomic level studies elucidating the prevalence of ESBL producing MDR *E. coli* and *K. pneumoniae* in hospitalized patients in Tamilnadu is scanty. Hence, the present study was aimed to determine the prevalence of ESBL producing MDR *E. coli* and *K. pnuemoniae* in Govt. hospital, Ariyalur, Tamil Nadu, India.

## Materials and Methods

### Sample and Data Collection

The study was executed for a period of 12 months (September 2014 to September 2015) at Ariyalur district Govt. hospital, Tamil Nadu. A total of 1400 clinical samples comprising of feces (120), sputum (105), nasal swabs (118), cerebrospinal fluid (102), urine (324), pus (135), wound swab (205), endotracheal aspirate (185) and cervical swab (106) were collected from long stay patients, ICU, surgical wards and neonatal wards. All the clinical specimens were quickly transported to the laboratory for isolation of microbial pathogen. Using standard methods described by Al-Amin *et al*[13] the pathogenic *E. coli* and *K. pneumoniae* were selectively isolated and identified from the clinical specimens.

### Antibiotic Susceptibility Testing

Antimicrobial susceptibility test was performed using the Kirby-Bauer disk diffusion method as per the Clinical and Laboratory Standards Institute (CLSI) guidelines[14] with the following set of antibiotics (Hi-media, Mumbai): amikacin (30 µg), gentamicin (10 µg), cotrimoxazole (25 µg), tetracycline (30 µg), ceftazidime (30 µg), cefotaxime (30 µg), cefoperazone (75 µg), ciprofloxacin (5 µg), ofloxacin (5 µg), piperacillin/tazobactam (100/10 µg), imipenem (30 µg) and nalidixic acid (30 µg). All the antibiotic discs (Hi-Media, Mumbai) were impregnated onto bacterium inoculated, dried surface of a 150 mm Muller Hinton agar plate, and incubated at 37$^{o}$C for 24 hours. After the incubation the zone of bacterial clearance was measured and the sensitivity was compared using Hi-Media standards.

### ESBL Production Assay

All *E. coli* and *K. pneumoniae* isolates from the clinical specimens were tested for ESBLs production. The ESBL producing MDR strains were determined by the phenotypic confirmatory test according to the Clinical and Laboratory Standards Institute (CLSI)[14]. A confirmatory test for ESBL-production was performed using the double-disk (combined-disk) method. The zones of inhibition of each isolate were tested on Mueller-Hinton agar plates against the disks containing 30 µg of ceftazidime only and in a combination with 10 µg of clavulanic acid respectively. As per the standards the ESBL phenotypes were classified, briefly, the zone of inhibition produced off by combination disc was more than 5 mm larger than that produced by the corresponding antimicrobial disc without clavulanic acid.

### DNA Extraction

All the positive ESBL isolates (n = 163) were used for genomic DNA extraction. Genomic DNA from the bacterial isolates was extracted by modified STE (Sucrose Tris EDTA) method with slight modification[15].

### Multiplex Polymerase Chain Reaction (mPCR)

All mPCR reactions were performed in a 25 µl volume containing 2.5 µl of 10X PCR buffer, 2.0 µl of 1.24 mM dNTPs, 1.0 µl of 10.0 pmol each primer, 0.2 µl of 5 U *Taq* polymerase and 3.0 µl of template DNA. The nuclease free water was used to bring the reaction mixture to 25 µl. The target genes of $bla_{\text{TEM}}$[16], $bla_{\text{SHV}}$[16], $bla_{\text{CTX-M-1}}$[16], $bla_{\text{IMP1}}$[17], $bla_{\text{NDM-1}}$[18] and $bla_{\text{GES}}$[19], primer sequences, PCR conditions and amplified product sizes are given in the Table 1. The reference strain of *K. pneumoniae* ATCC 1705 was used as a positive control and the same reaction mixture except the DNA template was considered as negative control. The amplified PCR products were separated on a 0.9% agarose gel, stained with ethidium bromide (0.5 µg/ml) and the gel images were taken using a gel documentation system (UVI tech, Cambridge)[20].

## Results

A total of 1400 clinical samples were isolated from Ariyalur district Govt. hospital, Tamil Nadu and out of 1400 clinical samples, 376 Gram negative bacteria were isolated. They were 160 *E. coli* (42.55%), 110 *K. pneumoniae* (29.26%), 50 *Pseudomonas aeruginosa* (13.3%), 34 *Enterbacter aerogenes* (9.04%), 15 *Acinetobacter baumannii* (3.99 % and 7 *Proteus* sp. (1.86%). Around 55.62% (n = 89) *E. coli* and 67.27% (n = 74) *K. pneumoniae*

isolates showed ESBL positive (Table 2). The susceptibility tests were carried out by disc diffusion method. ESBL producing *E. coli* isolates showed high resistance to amikacin, ceftazidime and nalidixic acid (100%) whereas ESBL producing *K. pneumoniae* isolates were highly resistant to ceftazidime and nalidixic acid (100%). The antibiotic resistance pattern of *E. coli* and *K. pneumoniae* were shown in the Table 3.

The phenotypic confirmatory test of ESBL producers were analysed for all the isolates. The production of ESBL was confirmed by phenotypic confirmatory test and the distribution of 270 isolates, 163 (60.4%) isolates were observed ESBL positive in which *E. coli* isolates were in peak followed by *K. pneumoniae* (Table 3). The remaining 39.63% (107 of 270) isolates were non ESBL producers. Among the 270 isolates, 160 (59.25%) showed *E. coli*, of which 56% were from male and 44% from female patients. Although, 110 (40.74%) showed *K. pneumoniae*, of which 48% were from male and 52% from female patients (Table 4).

All the 163 isolates were subjected to genotypic detection of ESBL encoding resistant genes by mPCR analysis and the results are presented in Figure 1. Among 163 isolates, the highest percentage of ESBL production was seen in *E. coli* (n = 89) followed by *K. pneumoniae* (n = 74). Among the 163 isolates, $bla_{\text{CTX-M-1}}$ gene was more prevalent in 90.8% (148 out of 163) followed by $bla_{\text{TEM}}$ gene 77.3% (126 out of 163), $bla_{\text{GES}}$ 19% (31 out of 163) and $bla_{\text{NDM-1}}$ 3.1% (5 out of 163).

## Discussion

Extended spectrum beta lactamase producing Gram-negative bacteria, particularly *E. coli* and *K. pneumoniae*, are now widespread nosocomial pathogens which cause a variety of infections such as septicemia, urinary tract infection, hospital-acquired pneumonia, intra-abdominal abscess, brain abscess, and device-related infections[9]. Kumar *et al*[21] reported that in India, the incidence of ESBL mediated resistance was observed among 60 – 68% of clinical pathogens that were isolated from major hospitals.

Table 1 — List of PCR primer pairs sequence used for the detection of antibiotic resistance genes

| Primer direction | Locus | Primer sequence | Amplicon (bp) | PCR condition | | Reference |
|---|---|---|---|---|---|---|
| | | | | Temp (°C) | T (sec) | |
| $bla_{TEM}(+)$<br>$bla_{TEM}(-)$ | TEM | GAGTATTCAACATTTCCGTGTC<br>TAATCAGTGAGGCACCTATCTC | 800 | 56 | 30 | [16] |
| $bla_{SHV}(+)$<br>$bla_{SHV}(-)$ | SHV | ATGCGTTATATTCGCCTGTG<br>TTAGCGTTGCCAGTGCTC | 867 | 52 | 60 | [16] |
| $bla_{CTX-M-1}(+)$<br>$bla_{CTX-M-1}(-)$ | CTX-M-1 | GCGTGATACCACTTCACCTC<br>TGAAGTAAGTGACCAGAATC | 300 | 50 | 60 | [16] |
| $bla_{IMP1}(+)$<br>$bla_{IMP1}(-)$ | IMP1 | GGCAGTCGCCCTAAAACAAA<br>TAGTTACTTGGCTGTGATGG | 737 | 51 | 60 | [17] |
| $bla_{NDM-1}(+)$<br>$bla_{NDM-1}(-)$ | NDM-1 | GGTGCATGCCCGGTGAAATC<br>ATGCTGGCCTTGGGGAACG | 660 | 61 | 60 | [18] |
| $bla_{GES}(+)$<br>$bla_{GES}(-)$ | GES | GTTTTGCAATGTGCTCAACG<br>TGCCATAGCAATAGGCGTAG | 371 | 48 | 60 | [19] |

Table 2 — Distribution of ESBL producing MDR *E. coli* and *K. pneumoniae*

| Specimen | Distribution of nosocomial isolates | | ESBL producing MDR strains | |
|---|---|---|---|---|
| | *E. coli* | *K. pneumoniae* | *E. coli* | *K. pneumoniae* |
| Feces (n = 120) | 17 (14.17%) | 5 (4.17%) | 10 (8.33%) | 3 (2.5%) |
| Sputum (n = 105) | 18 (17.14%) | 6 (5.71%) | 12 (8.0%) | 4 (3.81%) |
| Nasal swabs (n = 118) | 17 (14.41%) | 15 (12.7% | 8 (6.78%) | 10 (8.47%) |
| Cerebrospinal fluid (n = 102) | 11 (10.78%) | 12 (11.76%) | 3 (2.94%) | 2 (1.96%) |
| Urine (n = 324) | 38 (11.73%) | 20 (6.17%) | 14 (4.32%) | 12 (3.7%) |
| Pus (n = 135) | 12 (8.89%) | 14 (10.37%) | 7 (5.19%) | 10 (7.41%) |
| Wound swab (n = 205) | 25 (12.2%) | 13 (6.34%) | 20 (9.76%) | 12 (5.85%) |
| Endotracheal aspirate (n = 185) | 12 (6.5%) | 10 (5.41%) | 8 (4.32%) | 9 (4.86%) |
| Cervical swab (n = 106) | 10 (9.43%) | 15 (14.15%) | 7 (6.6%) | 12 (11.32%) |
| Total (n = 1400) | 160 (11.42%) | 110 (7.86%) | 89 (6.36%) | 74 (5.29%) |

Table 3 — The antibiotic resistance pattern of *E. coli* *K. pneumoniae*

| Antibiotics | Drug resistant pathogens (%) | |
|---|---|---|
| | *E. coli* (n = 160) | *K. pneumoniae* (n = 110) |
| Amikacin | 160 (100) | 72 (65.45) |
| Gentamicin | 154 (96.25) | 50 (54.54) |
| Tetracycline | 158 (98.6) | 44 (30.9) |
| Ceftazidime | 160 (100) | 110 (100) |
| Ciprofloxacin | 68 (42.5) | 93 (84.54) |
| Nalidixic acid | 160 (100) | 110 (100) |
| Ofloxacin | 135 (84.4) | 31 (28.2) |
| Co-trimaxazole | 145 (90.6) | 86 (78.2) |
| Doxycycline hydrochloride | 151 (94.4) | 91 (82.73) |
| Piperacillin/ tazobactam | 87 (54.4) | 38 (34.54) |
| Impinenem | 30 (18.75) | 21 (19.1) |
| Cefoperazone | 126 (78.75) | 107 (97.3) |

Table 4 — Demographic characteristics of the patient population of ESBL producing MDR *E. coli* and *K. pneumoniae*

| Age group | Distribution of nosocomial isolates (*E. coli*) | | Total | Distribution of nosocomial isolates (*K. pneumoniae*) | | Total |
|---|---|---|---|---|---|---|
| (years ) | Male | Female | | Male | Female | |
| <10 | 16 | 30 | 46 | 10 | 14 | 34 |
| 10-60 | 34 | 30 | 64 | 20 | 24 | 44 |
| > 60 | 14 | 36 | 50 | 17 | 15 | 32 |
| Total | 64 | 96 | 160 | 47 | 53 | 110 |

In the present study, *E. coli* (59.25%) is the most common cause of nosocomial infections followed by *K. pneumoniae* (47.74%). Because, *E. coli* usually present in the human intestine and it is an opportunistic pathogen to become resistant when host factors that alter susceptibility to infection[22]. Furthermore, ESBL production was found to be 163 out of 270 (60.4%). The similar study conducted in India by Akram *et al*[23] reported that the *E. coli* showed high antibiotic resistance pattern followed by *K. pneumoniae*. Among 270 isolates, ESBL producing MDR *E. coli* and *K. pneumoniae* were mostly isolated from urine followed by ICU and surgical ward. Chauhan[24] confirmed that maximum number of ESBL isolates were obtained from urine sample (74.0%) followed by pus sample (14.6%), surgery ward (21.9%) and ICUs (17.6%), respectively. Pathak and Pokharel[25] reported the prevalence of ESBL producing isolates varies geographically and the prevalence of ESBL is clearly increasing gradually. In many parts of world, 10-40% of strains of *K. pneumoniae* and *E. coli* express ESBLs. Agrawal *et al*[26] had reported the prevalence of the ESBL producers to be 6% to 87% in his previous studies from India.

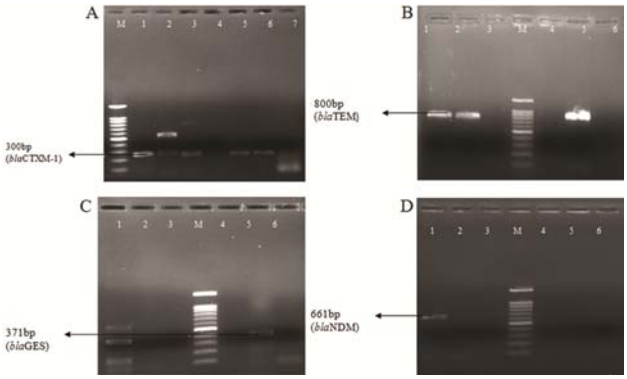


Fig. 1 — Agarose gel electrophoresis of multiplex polymerase chain reaction (mPCR) products. A. mPCR analysis for identification of $bla_{\text{CTX-M-1}}$ gene resistant strains. Lane M –DNA marker (100 bp), Lane 1: *K. pneumoniae* - Reference strain (positive control); Lane 2: other tested strain, Lane 3-6: *E. coli* and *K. pneumoniae* isolates, Lane 7: Nuclease free water (negative control); B. mPCR analysis for identification of $bla_{\text{TEM}}$ gene resistant strains. Lane 1: *K. pneumoniae* - Reference strain (positive control); Lane 2, 3, 4, 5: *E. coli* and *K. pneumoniae* isolates, Lane M - DNA marker (100 bp), Lane 6: Nuclease free water (negative control); C. mPCR analysis for identification of $bla_{\text{Ges}}$ gene resistant strains. Lane 1: *K. pneumoniae* - Reference strain (positive control), Lane 2-5: *E. coli* and *K. pneumoniae* isolates, Lane M –DNA marker (100 bp), Lane 6: Nuclease free water (negative control); D. mPCR analysis for identification of $bla_{\text{NDM-1}}$ gene resistant strains. Lane 1: *K. pneumoniae* - Reference strain (positive control); Lane 2-5: *E. coli* and *K. pneumoniae* isolates, Lane M - DNA marker (100 bp); Lane 6: Nuclease free water (negative control).

In the present study**,** molecular detection and characterization of ESBL producing $bla_{\text{CTXM-1}}$ gene was more prevalent in 90.8% (148 out of 163) followed by $bla_{\text{TEM}}$ gene 77.3% (126 out of 163), $bla_{\text{GES}}$ 19% (31 out of 163) and $bla_{\text{NDM-1}}$ 3.1% (5 out of 163) respectively. Molecular detection and characterization of ESBL producing MDR *E. coli* and *K. pneumoniae* pathogens confirmed that $bla_{\text{CTX-M-1}}$ gene was more prevalent among common ESBL genotypes and this resistant gene is found to be more dissemination among ESBL producing *Enterobacteriaceae*. The mechanism of ESBL gene transfer is successively transmitted to the next progeny via vertical gene transfer or other bacteria through horizontal gene transfer process, making their treatment more difficult[27]. Canton *et al*[28] confirmed the mobilization of $bla_{\text{CTX-M-1}}$ genes via transposons onto plasmids has guided to the successful dissemination and adaptation among the bacterial clones. The $bla_{\text{GES}}$ and $bla_{\text{NDM-1}}$ are also plasmid encoded genes however it was found to be low (19% and 3.1%, respectively) in our study, might be the recent emergence of such genes in the

hospital environment. Apart from antibiotic resistance, ESBL-plasmid-carriage may also enhance adaptive virulence of ESBL-producing *E. coli*[29]. Similar study has done by Sima *et al*[30] reported that ESBL-producing strains revealed that $bla_{\text{CTX-M-1}}$ genes were the most frequent ESBL types (74%), followed by $bla_{\text{TEM}}$ (67%) and $bla_{\text{SHV}}$ (45%).

In the present study, the age group of 0-10 were found to be more susceptible to ESBL (Table 4) and the prevalence of MDR *E. coli* and *K. pneumoniae* were observed that more organisms were isolated from female compare to men. This may be due to low immunity. Similar study has done by Schoevaerdts *et al*[31] reported that a two-thirds of the patients carrying ESBLs were elderly adults aged over 65 years and who presented a large variety of co-morbidities.

From our findings, the abundance of the ESBL producers was high because of less hygiene, poor knowledge in disease management or treatment, inadequate facilities for diagnosis those MDR pathogens. In this context, some guidelines were reported by Ikeda *et al*[32] that propose general standard precautions for the infection at all medical facilities, particularly infection by MDR organisms, including ESBL-producing bacteria.

## Conclusion

The present study highlight the prevalence of third generation cephalosporins resistant nosocomial isolates with multiple ESBL gens which plays an important role in conferring resistance of *E. coli* and *K. pneumoniae* strains to β-lactam antibiotics. The frequency of $bla_{\text{CTX-M-1}}$, $bla_{\text{TEM}}$, $bla_{\text{GES}}$, and $bla_{\text{NDM-1}}$ genes in the nosocomial isolates highlights an urgent need to develop valuable strategies for the prevention and control of diseases.

## Ethical Approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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