Distribution of CTX-M, TEM, SHV Beta-lactamase Gene among the *Klebsiella pneumoniae* Clinical Isolates from Tertiary Care Centre in Palakkad, Kerala

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**Abstract**

Resistance against the routinely used antibiotics has reached a worrying level globally. Extended spectrum β-lactamases (ESBLs) production is the major mechanism of antimicrobial resistance. These ESBLs bacteria are resistant to penicillin, cephalosporins, monobactams. TEM1&2, CTX-M, SHV are the main ESBLs genes present in *Klebsiella pneumoniae*, which is produced by the alteration of amino acid in the active site. The aim of this study is to determine the prevalence of ESBL genes such as *bla*<sub>TEM</sub> 1&2, *bla*<sub>CTX-M</sub>, and *bla*<sub>SHV</sub>. The present study was carried out from April 2019 to September 2019, a total of 121 *K. pneumoniae* isolates were collected and subjected to phenotypic study. Among these 19 isolated was ESBL positive, genes (*bla*<sub>SHV</sub>, *bla*<sub>TEM</sub>, *bla*<sub>CTX-M</sub>) were detected by conventional PCR method. *bla*<sub>TEM</sub> (100%) was the predominant gene detected flowed by CTX-M (68.42%) and SHV (57.89%). The highest level of antimicrobial resistance towards ampicillin (93.4%) followed by ceftriaxone (28.9%), cefotaxime (24.8%) and ciprofloxacin (22.3%). However, ESBL-producing isolates were showed resistance to ampicillin (100%) followed by ceftazidime (94.74%), cefotaxime (89.47%), amikacin and amoxicillin-clavulanic acid (68%). Antimicrobial resistance of bacteria is due to the genes, especially extended spectrum beta lactamase, which is widely found in members of Enterobacteriaceae. Nevertheless, there is a paucity of studies regarding the distribution of ESBL in *K. pneumoniae* in Palakkad Dist., Kerala. Hence the aim of the current study determines the distribution of ESBL genes in ESBL producing *K. pneumoniae* isolated from various clinical samples.

**Keywords:** *K. pneumoniae*, Antimicrobial Resistance, ESBL, Genotype
INTRODUCTION

Beta-lactam antibiotics are versatile antibiotics used for a broad spectrum of infections. The emergence of antimicrobial resistance is a significant threat in today's society. The members of the Enterobacteriaceae family are commonly occurring bacteria in the environment. Klebsiella pneumoniae is one of the frequently isolating bacteria coming under this family. Resistance to various antibiotics is quite a concern because of the ESBL and carbapenem resistance of K. pneumoniae, which is repeatedly reported from different parts of the world. It is a causative agent of health care infections and community acquiring infections. The most common diseases are Urinary tract infections, respiratory tract infections, and wound infections, which now act as co-pathogen in Covid-19 cases.

K. pneumoniae is a well-studied organism due to its multidrug resistance. The ESBL is an enzyme produced by bacteria, acting on the beta-lactam ring. B-lactam antibiotics are widely used worldwide; they bind to the penicillin-binding protein and inhibit the biosynthesis of the bacterial cell wall. Nevertheless, these bacteria produce a β-lactamase enzyme, which acts on β-lactam ring and develops resistance to beta-lactam antibiotics. The β-lactamase is classified by Ambler into four classes, A, B, C & D in this. Class A, C & D have serine enzymes in the active site and Zinc-Metallo enzyme in the case of class B.

ESBL gene is the essential resistance present in Enterobacteriaceae; it is encoded by blaTEM, blaCTX-M, and blaSHV genes, and of these, CTX-M is common. These plasmid-mediated genes are associated with other resistance genes, resulting in a co-resistance that includes aminoglycosides and tetracycline. The first ESBL resistance in K. pneumoniae was detected in Europe in 1980 and later spread to other areas. The distribution of ESBL resistance varies from one institution to another, one place to another. The ESBL resistance is a global concern; it leads to treatment failure, increased hospital stay, and a financial burden on humans. So the early detection of ESBL will help the clinicians with therapy. Hence, this study aims to detect the ESBL K. pneumoniae and the distribution of ESBL genes present in K. pneumoniae, isolated from different clinical samples.

MATERIALS AND METHODS

The study was conducted from April 2019 to September 2019 in the Department of Microbiology, Karuna Medical College, Palakkad, Kerala. A total of 121 isolates of K. pneumoniae from different clinical samples were subjected to phenotypic studies.

The bacterial isolates were identified by biochemical reactions according to the standard protocol. All the K. pneumoniae isolates were phenotypically tested for ESBL production by double disc synergy test.

Antimicrobial Susceptibility Testing

All ESBL-positive isolates were tested for antimicrobial susceptibility by the Kirby Bauer disc diffusion method according to CLSI guidelines. The discs used for susceptibility test, Ampicillin (10µg), cotrimoxazole (25µg), amoxyclave (10µg), cefotaxime (30µg), ceftriaxone (30µg), ceftazidime (30µg), ciproflaxacin (5 µg), amikacin (30µg), gentamicin (10µg), imipenem (10 µg) and tetracycline (30 µg), (Himedia, Mumbai).

ESBL Detection by Double Disc Synergy Test

The ESBL detection of all isolates is performed by double disc synergy test using cefotaxime (30 µl) and cefotaxime clavulanic acid (30 µl + 10 µl) combination according to CLSI guidelines. The K. pneumoniae isolates were tested with cefotaxime and ceftazidime disc with and without clavulanic acid are placed 25 mm apart from each other in Muller-Hinton agar (Himedia). The zone of inhibition >5mm in clavulanic acid combination is interpreted as ESBL positive.

Genotypic Method for the Detection of ESBL Gene

Phenotypically confirmed ESBL producing K. pneumoniae isolated were tested genotypically by conventional PCR. CTX-M, TEM 1&2, and SHV gene specific primers were used for performing PCR test. The plasmid DNA isolation was performed by using Thermo scientific TM Gene JET Plasmid Kit, according to manufacturers' instruction.
ESBL genes such as CTX-M Group 1, TEM 1 & 2, SHV were amplified individually by using specific primers (Table 1).

The amplification mixture final volume 15µl containing, Thermo master mix 8 µl, primer mix 1µl, deionized water 5 µl and DNA template 1 µl. The PCR amplification condition of each gene displayed in Table 2, 3 & 4. After amplification the PCR product were loaded in 1.5% agarose gel. After the run the band visualized on gel documentation system (Bio Rad gel doc XR+) and took the photographs.

**Statistical Analysis**

All the data were analyzed using SPSS version 21 (IBM corporation/ Armonk, Newyork/ USA). The chi-square test and Fisher’s exact test evaluated the antimicrobial resistance patterns of ESBL and non-ESBL. ‘P’ value less than or equal to 0.01 was considered statistically significant.

**RESULTS**

In the present study, 121 *K. pneumoniae* were isolated from different clinical samples; among them, 58 (47.9%) were females, and 63 (52.1%) were males (Table 5).

The distribution of isolates in different specimen, 56 (46.3%) urine, 37 (30.6%) sputum, 23(19%) pus, 2 (1.7%) blood and 1(0.8%) each from secretion tip, tissue and ascetic fluid (Table 6). Out of these 121 isolates, 19 were phenotypically positive for ESBL production (Figure 1). The distribution of ESBL positive isolates distribution among various clinical samples is shown in table 6, and among the clinical samples, ESBL prevalent in Urine 8 (14.3%), followed by sputum 5(13.51%) and pus 4 (17.4%).

The antimicrobial resistance of ESBL and non-ESBL are shown in Figure 2. The percentage of resistance to cephalosporins in ESBL producing *K. pneumoniae* and non-ESBL producing *K. pneumoniae* were 89% and 13% for cefotaxime, 95% and 8% for ceftazidime, 84% and 19% for ceftriaxone. However, the ESBL and non-ESBLs were susceptible to carbapenem such as imipenem (100%).

The comparison of the antimicrobial resistance pattern of ESBL and non-ESBL is shown in Table 7. The association between antimicrobial resistance and ESBL production was statistically significant for Cotrimoxazole (<0.001), cefotaxime (<0.001), Ceftazidime (<0.001), ceftriaxone (<0.001), amikacin (<0.001), amoxiclav (<0.001), gentamicin (<0.001), tetracycline (<0.001), ciprofloxacin (<0.001), cefoxitin (0.002). The antimicrobial susceptibility of ESBL isolates revealed that the highest resistance was against

| Gene                  | Primers                                                                 |
|-----------------------|-------------------------------------------------------------------------|
| TEM 1 & 2             | Forward Primer: 5'-CATTTYCGTGTCCGCTCTATTC-3'  
                       | Reverse Primer: 5'- CGTTCATCCATGTTGCGTAC-3'                              |
| SHV                   | Forward Primer: 5'- ATCCCGCAGATAAACCCACCA-3'  
                       | Reverse Primer: 5'- ACGGCTCTGAGCAATTACACAC-3'                            |
| CTX-M Group 1         | Forward Primer: 5'- TTAGGAARTGTCGCCCTGAC-3'                            
                       | Reverse Primer: 5'- CGATATCGTGGTGTGGTCCAT-3'                            |

**Table 2. Protocol for TEM 1 & TEM 2 Amplification**

| Step                  | Temp. | Time  |
|-----------------------|-------|-------|
| Initial denaturation  | 95°C  | 5 minutes |
| Denaturation          | 94°C  | 30 seconds |
| Annealing             | 62°C  | 40 seconds |
| Extension             | 72°C  | 1 minute |
| Go to step 2-4 for 35 times |       |         |
| Elongation            | 72°C  | 10 minutes |

**Table 3. Protocol for SHV gene amplification**

| Step                  | Temp. | Time  |
|-----------------------|-------|-------|
| Initial denaturation  | 95°C  | 5 minutes |
| Denaturation          | 94°C  | 30 seconds |
| Annealing             | 55°C  | 45 seconds |
| Extension             | 72°C  | 1 minute |
| Go to step 2-4 for 35 times |       |         |
| Elongation            | 72°C  | 10 minutes |

Table 1. Primers used for PCR

| Gene                  | Primers                                                                 |
|-----------------------|-------------------------------------------------------------------------|
| TEM 1 & 2             | Forward Primer: 5'-CATTTYCGTGTCCGCTCTATTC-3'  
                       | Reverse Primer: 5'- CGTTCATCCATGTTGCGTAC-3'                              |
| SHV                   | Forward Primer: 5'- ATCCCGCAGATAAACCCACCA-3'  
                       | Reverse Primer: 5'- ACGGCTCTGAGCAATTACACAC-3'                            |
| CTX-M Group 1         | Forward Primer: 5'- TTAGGAARTGTCGCCCTGAC-3'                            
                       | Reverse Primer: 5'- CGATATCGTGGTGTGGTCCAT-3'                            |
ampicillin (100%), followed by ceftazidime (94.74%) and cefotaxime (89.47%). All isolates were susceptible to imipenem.

All phenotypically ESBL positive isolates were genotypically tested for the detection of resistance genes such as bla\textsubscript{SHV}, bla\textsubscript{TEM} 1 & 2, and bla\textsubscript{CTX-M}. Out of this beta-lactamase (bla) genes, all 19 (100%) isolates showed bla\textsubscript{TEM} 1 & 2, followed by bla\textsubscript{CTX-M} 13 (68.42%) and bla\textsubscript{SHV} 11 (57.89%) (Figure 3-5). Among the nineteen \textit{K. pneumoniae} isolates, 4 (21.05%) isolates possess only bla\textsubscript{TEM} 1 & 2 ESBL gene. No isolates carry bla\textsubscript{CTX-M} and bla\textsubscript{SHV} genes alone (Table 8).

**DISCUSSION**

Antimicrobial resistance is a significant concern in the health care system. ESBL production is considered one of the antimicrobial resistance mechanisms in the family \textit{Enterobacteriaceae}.\textsuperscript{9} ESBLs are the plasmid-coded genes, which provide resistance to third-generation cephalosporins, and monobactam (aztreonam)\textsuperscript{10} is considered an essential factor for the emergence of antimicrobial resistance.

Antimicrobial resistance in \textit{K. pneumoniae} was noted as an important problem in the health

### Table 4. Protocol for CTX-M gene Amplification

| Step                        | Temp. | Time  |
|-----------------------------|-------|-------|
| Initial denaturation        | 95°C  | 5 minutes |
| Denaturation                | 94°C  | 30 seconds |
| Annealing                   | 58°C  | 45 seconds |
| Extension                   | 72°C  | 1 minute |
| Go to step 2-4 for 35 times|       |        |
| Elongation                  | 72°C  | 10 minutes |

### Table 5. Gender wise distribution of \textit{K. pneumoniae} isolates

| Gender | Frequency | Percent |
|--------|-----------|---------|
| Female | 58        | 47.9    |
| Male   | 63        | 52.1    |
| Total  | 121       | 100.0   |

**Figure 1.** ESBL detection by double disc synergy test (CTX- Cefotaxime and CEC-Cefotaxime clavulanic acid; CAZ-Ceftazidime and CAC-Ceftazidime clavulanic acid)

**Figure 2.** Antimicrobial resistance in ESBL and Non-ESBL producing \textit{K. pneumoniae}

AMR % of Non-ESBL

AMR % of ESBL

AMP- Ampicillin, COT- Cotrimoxazole, CTX- Cefotaxime, CAZ- Ceftazidime, CTR- Ceftriaxone, AK- Amikacin, AMC- Amoxicillin-Clavulanic acid, GEN- Gentamicin, TE- Tetracycline, IPM- Imipenem, CIP- Ciprofloxacin, CX- Cefoxitin
care setting worldwide. In the present study, all isolates showed low susceptibility to ampicillin (6.61%) and a high-level susceptibility to imipenem (100%). This suggests ampicillin is inadequate for empirical treatment unless combined with other suitable drugs. In the current study, ESBL K. pneumoniae are resistant to amikacin (68%), gentamicin (53%), cotrimoxazole (47%), and ciprofloxacin (58%). The leading cause of antimicrobial resistance is the overuse of antibiotics, which is one of the factors contributing to the emergence of resistant bacteria in the community. Carbapenem is a high-level antibiotic used for the treatment of resistant organisms. The current study showed that all isolates are susceptible to imipenem. This follows Ghafourian et al. and Uddin et al.

The distribution of ESBL-producing isolates varied between the geographical regions. In the present study, 19 (15.45%) ESBL K. pneumoniae were isolated phenotypically. This report follows the study from Hyderabad, India, conducted by Kumar et al., showed 19% of ESBL in K. pneumoniae. A study from India reported that 25-84% was ESBL occurrence in different institutional studies. Another study from North India reported 52.27% of ESBL Klebsiella pneumoniae isolated from various clinical samples. A Study from China reported that ESBL producers' isolation rate varies between 25-40%. Another study from Israel and Spain showed 40% ESBL prevalence in K. pneumoniae. Some other studies recorded a high incidence rate of ESBL-producing isolates, Feizabedi et al., reported 69.7% of K. pneumoniae were ESBL positive from Iran, and a study by Lal et al., noticed 97.1% of ESBL producers from India and another study from north India indicated 66.7% of K. pneumoniae were ESBL positive. Turkey (60%), Brazil (45.4%), Western Pacific (24.6%), Netherlands (22.6%), and Iran (44 -74%) have reported the highest rate of ESBL prevalence. The percentage of isolation of ESBL producers varies with different factors such as prolonged hospitalization, antibiotic use and policy, the hygiene of hospital personnel, and disinfection of ICU. Some studies reported a low isolation rate in different geographical regions. The distribution of ESBL-producing isolates varied between the geographical regions. In the present study, 19 (15.45%) ESBL K. pneumoniae were isolated phenotypically. This report follows the study from Hyderabad, India, conducted by Kumar et al., showed 19% of ESBL in K. pneumoniae. A study from India reported that 25-84% was ESBL occurrence in different institutional studies. Another study from North India reported 52.27% of ESBL Klebsiella pneumoniae isolated from various clinical samples. A Study from China reported that ESBL producers' isolation rate varies between 25-40%. Another study from Israel and Spain showed 40% ESBL prevalence in K. pneumoniae. Some other studies recorded a high incidence rate of ESBL-producing isolates, Feizabedi et al., reported 69.7% of K. pneumoniae were ESBL positive from Iran, and a study by Lal et al., noticed 97.1% of ESBL producers from India and another study from north India indicated 66.7% of K. pneumoniae were ESBL positive. Turkey (60%), Brazil (45.4%), Western Pacific (24.6%), Netherlands (22.6%), and Iran (44 -74%) have reported the highest rate of ESBL prevalence.

Table 6. Distribution of K. pneumoniae among the clinical sample

| No. | Samples       | No. of isolates (N=121) | Percentage (%) | No. of ESBL positive isolates |
|-----|---------------|-------------------------|----------------|-------------------------------|
| 1.  | Urine         | 56                      | 46.3           | 8 (14.3)                      |
| 2.  | Sputum        | 37                      | 30.6           | 5 (13.51)                     |
| 3.  | Pus           | 23                      | 19.0           | 4 (17.4)                      |
| 4.  | Blood         | 2                       | 1.7            | 0 (0)                         |
| 5.  | Secretion tip | 1                       | 0.8            | 1 (100)                       |
| 6.  | Tissue        | 1                       | 0.8            | 1 (100)                       |
| 7.  | Ascitic fluid | 1                       | 0.8            | 0 (0)                         |
| 8.  | Total         | 121                     | (100)          | 19                             |

Figure 3. PCR amplification image of CTX-M gene in K. pneumoniae after 1.5% agarose gel electrophoresis
level of ESBL prevalence in *K. pneumoniae*, 17%-24-25 and 12%.26

The ESBL production is due to the mutation in TEM-1 & 2 and SHV 1 genes.27 In the current study, *bla* _TEM_ (100%) was the most prevalent gene in ESBL producers, followed by CTX-M (68.42%) and SHV (57.89%). A similar result was found in the study conducted by Ferreiral et al.28 in this *bla* _TEM_ (100%), CTX-M (72%), and SHV (96%). The ESBL genes were present in all ESBL positive isolates, but the prevalence of this gene varies. The previous reports from north India showed that 73% of *bla* _TEM_ and 55% of *bla* _CTX-M_ were the most dominant ESBL genes present in PGIMER and AIIMS, respectively. Another study from Lucknow also reported 75% of *bla* _TEM_ in ESBL *K. pneumoniae_.29 Samanje et al.,30 in their study, found that 95% of ESBL *K. pneumoniae_ isolates harbor the *bla* _TEM_ gene, and 90% CTX-M were detected. On the other hand, over the past decade, CTX-M was the predominant gene reported in many Asian countries in *K. pneumoniae_.31 A study

### Table 7. Comparison of antibiotic resistance patterns of ESBL and Non ESBL producing *K. pneumoniae*

| Antibiotic | Negative (n=102) | Positive (n=19) | X² | P      |
|------------|------------------|----------------|----|--------|
| AMP        | Resistant 94      | 19             | 1.596 ns | 0.354 ns (Fisher’s Exact test) |
|            | Susceptible 8     | 0              |     |        |
| COT        | Resistant 8       | 9              | 20.72** | <0.001 |
|            | Susceptible 94    | 10             |     |        |
| CTX        | Resistant 13      | 17             | 46.54** | <0.001 (Yates correction) |
|            | Susceptible 99    | 2              |     |        |
| CAZ        | Resistant 8       | 18             | 66.63** | <0.001 (Yates correction) |
|            | Susceptible 94    | 1              |     |        |
| CTR        | Resistant 19      | 16             | 33.39** | <0.001 |
|            | Susceptible 83    | 3              |     |        |
| AK         | Resistant 8       | 13             | 40.98** | <0.001 |
|            | Susceptible 94    | 6              |     |        |
| AMC        | Resistant 12      | 13             | 31.36** | <0.001 |
|            | Susceptible 90    | 6              |     |        |
| GEN        | Resistant 10      | 10             | 21.29** | <0.001 |
|            | Susceptible 92    | 9              |     |        |
| TE         | Resistant 8       | 12             | 35.52** | <0.001 |
|            | Susceptible 94    | 7              |     |        |
| IMP        | Resistant 0       | 0              | -   | -      |
|            | Susceptible 102   | 19             |     |        |
| CIP        | Resistant 16      | 11             | 16.46** | <0.001 |
|            | Susceptible 86    | 8              |     |        |
| CX         | Resistant 17      | 10             | 11.951** | 0.002  |

** Significant at 0.01 level; ns Non significant

AMP- Ampicillin, COT- cotrimoxazole, CTX- cefotaxime, CAZ- ceftazidime, CTR- ceftriaxone, AK- amikacin, AMC- amoxicillin-clavulanic acid, GEN- gentamicin, TE- tetracycline, IPM- imipenem, CIP – ciprofloxacin, CX- cefoxitin

### Table 8. Overview of ESBL genotype among *K. pneumoniae* isolates

| A. Single ESBL gene | No. of positive | Percentage (%) |
|---------------------|-----------------|----------------|
| *bla* _CTX-M_       | 0               | 0              |
| *bla* _TEM 1&2_     | 4               | 21.05%         |
| *bla* _SHV_         | 0               | 0              |

| B. Two ESBL genes   | Percentage (%)  |
|---------------------|-----------------|
| *bla* _CTX-M_ and _bla_ SHV_ | 0               |
| *bla* _CTX-M_ and _bla_ TEM_   | 4               | 21.05%         |
| *bla* _TEM_ and _bla_ SHV_     | 2               | 10.53%         |

| C. Three ESBL genes  | Percentage (%)  |
|---------------------|-----------------|
| *bla* _CTX-M_ , _bla_ TEM_ and _bla_ SHV_ | 9               | 47.37          |
from Malaysia reported that the most prevalent ESBL gene in \textit{K. pneumoniae} isolates was CTX-M (91.3%).\textsuperscript{32} A study from Nepal reported that 78.9% of \textit{K. pneumoniae} contains the CTX-M gene.\textsuperscript{33} However, a study from the middle east detected that 93.75\% (30/32) TEM and 87.5\% (28/32) SHV gene was found in ESBL \textit{K. pneumoniae}.\textsuperscript{34} The prevalence of the ESBL gene depends on various factors like the time of the study, the number of isolates, and geographical area.

The current study showed the presence of multiple genes in a single isolate. This follows the other studies.\textsuperscript{35} Nevertheless, the combination of ESBL genes is different in the different studies.
The current study reported that the co-existence of bla\textsubscript{TEM} and bla\textsubscript{CTX-M} was 21.05% (4/19), and bla\textsubscript{TEM} and bla\textsubscript{SHV} was 10.53% (2/19). A study conducted by Bora et al.\textsuperscript{36} observed that 27.58% of \textit{K. pneumoniae} isolates pose bla\textsubscript{TEM} and bla\textsubscript{CTX-M} together. In the present study, the presence of three genes was 47.37%. National Institute of hygiene in Lome, Togo, conducted a study from 2013-2015 that reported the presence of 3 genes in \textit{K. pneumoniae} 59.26% (32/54).\textsuperscript{37} However, another study conducted by Manoharan et al.\textsuperscript{38} observed that 42.6% of \textit{K. pneumoniae} isolates have all three genes TEM, SHV, and CTX-M. The co-existence of these three genes and some other antimicrobial resistance mechanisms may increase the antimicrobial resistance towards the last choice antibiotics like carbapenems and contribute to a high level of antimicrobial resistance.

**CONCLUSION**

ESBL is the most crucial antimicrobial resistant mechanism, and the high level of high resistance is consistent with the presence of the resistance gene in this study. Determining ESBL-producing bacterial isolates is essential in controlling infection and epidemiological surveillance studies. The current study reported that most ESBL-producing isolates are resistant to cephalosporins. The ESBL genes TEM 1 & 2, CTX-M, and SHV, these three genes are present in most cases. Only imipenem was active against the ESBL-producing \textit{K. pneumoniae}. The knowledge, identification, and updating of the antimicrobial resistance among the common pathogens are helpful for the appropriate treatment of infections. Monitoring ESBL production using phenotypic and genotypic methods is recommended to benefit infection control.

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**CONFLICT OF INTEREST**

The authors declare that there is no conflict of interest.

**AUTHORS’ CONTRIBUTION**

All authors listed in this study have made a substantial direct and intellectual contribution to the work and approved it for publication.

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**DATA AVAILABILITY**

All datasets generated during the study are included in the manuscript.

**ETHICS STATEMENT**

Not applicable

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