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Occurrence of ESBL and AmpC Beta-Lactamase Producers Among *Klebsiella pneumoniae* Clinical Isolates – A Study from Chennai, Tamil Nadu, India

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Abstract:

- ***Introduction:***
  The emergence of Extended Spectrum Beta-Lactamases (ESBLs), AmpC β-lactamases and their co-existence among the members of Enterobacteriaceae has been found to be alarmingly high and poses newer diagnostic and treatment challenges.

- ***Materials and Methods:***
  In this study, we investigated both ESBL and AmpC β-lactamases in clinical isolates of *Klebsiella pneumoniae* (64). The detection of ESBL and AmpC β-lactamases was performed based on screening and confirmatory tests. The isolates that screened positives were phenotypically confirmed by the double disc synergy test and the Cefoxitin-Cloxacillin Double disk synergy test. Conventional PCR was performed to screen for the presence of ESBL-encoding gene *bla*TEM and the AmpC-encoding gene *bla*MOX.

- ***Result:***
  The most common method used was DDST, which detected 50% of ESBL producers, followed closely by ESBL screening at 42.19%. Cefoxitin screening and the Cefoxitin-Cloxacillin Double Disk Synergy Test each accounted for 12.5% of the cases. ESBL gene was detected in 29 (46.7%) isolates. None of the isolates showed positive for *bla*MOX genes.

- ***Conclusion:***
  The Cefoxitin resistance was found to be a discriminative parameter in detecting the AmpC-producing strains. The high positive rate of ESBLs and AmpC beta-lactamases production in isolates calls for the need for strong intervention to minimize further occurrence and spread of such resistance.

Keywords: *Klebsiella Pneumoniae, ESBL, AmpC, Drug Resistance.*

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I. INTRODUCTION

Multidrug-resistant Organisms (MDROs) usually show resistance to one agent in three or more classes. It is emerging globally as a serious problem. Bacteria gain resistance through mutations, horizontal gene transfer, or the improper use of antibiotics [1]. In recent times, Drug resistance has posed a very serious threat due to the acquisition of resistance

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to multiple antibiotics by Gram-negative bacteria like *Klebsiella pneumoniae* (*K. pneumoniae*). The various resistance mechanisms include extended-spectrum beta-lactamase (ESBL) production, efflux mechanism and porin deficiency are responsible for the multidrug resistance [2].

Gram-negative bacilli can also develop resistance, particularly through AmpC β-lactamase enzymes encoded by AmpC genes, either chromosomally or via plasmids. Plasmid-mediated AmpC beta-lactamase is prevalent in Gram-negative bacilli and also resists various classes of antibiotics, which has led to significant therapeutic challenges and limited treatment options [3]. AmpC β-lactamases are enzymes that belong to the Class C enzymes, confer resistance to β-lactam antibiotics, and also to their inhibitors like clavulanic acid. Class A enzymes, ESBLs, are sensitive to these inhibitors. However, plasmid-mediated AmpC beta-lactamase can be masked by ESBLs, leading to inaccurate reporting and therapeutic failures [4].

Chromosomal AmpC beta-lactamase production can be enhanced by gene mutations, which typically cause organism resistance, and its activity can be increased through induction. The potential development of Plasmid-mediated AmpC beta-lactamases may have occurred due to the transfer of chromosomal AmpC genes onto plasmids [5]. Unlike chromosomal AmpCs, plasmid-mediated AmpC β-lactamases are not induced and are usually associated with extensive drug resistance. High levels of chromosomally mediated AmpC β-lactamases are also produced in *Klebsiella pneumoniae* [6].

While certain clinically significant organisms (such as *Salmonella* species, *Proteus mirabilis*, and *Klebsiella* species) have minimal chromosomal expression, others (such as *Escherichia coli*) lack chromosomally encoded AmpC [7]. For AmpC detection, there are no authorized standards from the Clinical Laboratory Standards Institute (CLSI) or any other source. When an organism produces enough AmpC β-lactamase, it usually tests positive to the ESBL screening test but fails the confirmatory tests that require more clavulanic acid sensitivity [8]. This study aimed to detect the epidemiology of ESBL and AmpC producers among *Klebsiella pneumoniae* strains.

II. MATERIALS AND METHODS

➢ *Bacterial Isolates and Antibiogram*

A total of *K. pneumoniae* (n=64) isolates from various infections were included. Bacterial identification was done by biochemical methods and other standard microbiological techniques. Antimicrobial susceptibility testing was done with the following antibiotics: amikacin (30 µg), gentamicin (10 µg), amoxicillin-clavulanic acid (20/10 µg), cefotaxime (30 µg), cefuroxime (30 µg), ceftazidime (30 µg), cefepime (30 µg), imipenem (10 µg), meropenem (10 µg), ertapenem (10 µg), ciprofloxacin (5 µg), and norfloxacin (10 µg) and by doing this, the antimicrobial resistance profiles are identified (CLSIM100-Ed34, March 2024).

➢ *Phenotypic Characterization of ESBL and AmpC β-Lactamase Enzymes*

- *Detection of ESBL Producers*

The phenotypic tests were performed on multidrug-resistant isolates to confirm ESBL and AmpC β-lactamases production.

- *ESBL Screening*

ESBL-producing *Klebsiella pneumoniae* isolates were screened for cephalosporin antibiotics, including cefuroxime and ceftazidime. The isolates showing resistance to cephalosporin antibiotics are presumptively identified as ESBL producers.

- *Confirmation of ESBL Production*

Double disc synergy test (DDST) is a confirmatory test to detect Extended-Spectrum Beta-Lactamase (ESBL). It is based on the inhibitory effect of clavulanate against the hydrolysis of the beta-lactam ring of the antibiotics by the beta-lactamases produced by *K. pneumoniae*. The lawn is made on the surface of the MHA plate with the test organism. The cephalosporin antibiotics alone (Ceftazidime) and in combination with the Clavulanate are dispensed. Then the plates were incubated and observed for the difference in the zone of inhibition. If the difference of the combination disc of ≥5 mm is present when compared with the cephalosporin alone, it is considered ESBL positive [9].

➢ *Detection of AmpC Producers*

- *Cefoxitin Screening*

The Cefoxitin disk diffusion method is done to screen the presence of AmpC beta-lactamase enzymes. This test is based on that the organisms possessing AmpC beta-lactamase to confer resistance against the cephamycin antibiotics, Cefoxitin. The test isolates are swabbed onto the surface of the MHA plate and the Cefoxitin antibiotic is dispensed and the plates are incubated. On observation, if the zone diameter is ≤18mm, it is considered as Cefoxitin resistant and a potential AmpC producer [10].

- *Confirmation of AmpC Production*

The Cefoxitin Cloxacillin-Double Disc Synergy Test (CC-DDST) relies on the inhibitory effect of the beta-lactam inhibitor, Cloxacillin on *K. pneumoniae*. The isolates were swabbed onto the surface of the Muller Hinton Agar and cephamycin antibiotic, Cefoxitin is dispensed alone and in combination with the Cloxacillin. The plates are incubated at 37°C for 18 to 24 hours. The zone diameters are observed and if the difference in the zone of inhibition of the combination disk is 4mm larger when compared to the cefoxitin, it is considered to be an AmpC producer.[11]

- *Quality Controls*

AmpC beta-lactamase-producing organism *Enterobacter cloacae* subspecies BAA-1143 was used as a positive control. Non-AmpC beta-lactamase-producing organism *Escherichia coli* ATCC 25922 was used as a negative control.

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➢ *Molecular Detection of Drug Resistance Genes*

Beta-lactamase genes were analyzed by molecular analysis. Multiplex PCR was performed for the amplification of drug resistance genes, including the ESBL-encoding gene *bla*TEM and the AmpC-encoding gene *bla*MOX. PCR was performed in a DNA thermal cycler (Biometra, Germany) with a final volume. The DNA was extracted from the bacterial culture that was incubated overnight. The nucleic acid of *K. pneumoniae* isolates was extracted using the phenol-chloroform method. A 50-µL reaction mixture containing two microliters of total DNA was collected. The PCR and master mix were configured in compliance with the cycling conditions and usual method of the kit.

The amplicon bands on a 2% agarose gel that was run at 60-90 V for 30 to 45 minutes were examined using gel electrophoresis and noticed under ultraviolet (UV) light. The gel doc system (Bio Era, India) was used to complete the gel documentation. The sizes of the PCR products were determined by comparing them to the molecular size markers of a typical DNA ladder. (GeneRuler 100 bp Plus DNA ladder; Thermo Fisher Scientific, Vilnius, Lithuania) [13,14,15].

Table 1 Primers Used for Screening Drug-Resistant Genes.

Target Gene	Primers	Size (bp)	References
bla_{TEM}	CATTTCCGTGTCGCCCTTATTC	800	[12] Verma.S et al., 2022
	CGTTCATCCATAGTTGCCTGAC		
bla_{MOX-1}	F: GCAACAACGACAATCCATCCT	895	[13] Varsha Rani Gajamer et al., 2019
	R: GGGATAQGCGTAACTCTCCCAA		

III. RESULTS

In this study, a total of 64 isolates of *K. pneumoniae* were evaluated for the presence of ESBL and AmpC beta-lactamase enzymes by phenotypic screening and genotypic characterization along with antimicrobial susceptibility testing. The antibiotic resistance pattern of the *Klebsiella pneumoniae* isolates is presented in Table 2.

The isolates were confirmed as ESBL (Extended-Spectrum beta-lactamase) producers according to CLSI guidelines, as they were resistant to all beta-lactam antibiotics, third- and fourth-generation cephalosporins. The isolates were differentiated from non-ESBL producers by a double disc synergy test, based on the susceptibility of the cephalosporin (Ceftazidime) with or without clavulanate.

The AmpC-producing isolates showed positive results in cefoxitin screening and CC-DDST, and these tests also helped characterize their resistance pattern. When screening with Cefoxitin, the isolates exhibited reduced zone of inhibition of diameter (≤18mm), indicating potential resistance, while the remaining isolates showed sensitivity to the cefoxitin antibiotic. This screening test provided a preliminary indication of isolates producing the beta-lactamase enzymes.

Table 2 Results of Antibiogram of *K. Pneumoniae* Isolates

S. No	Antibiotics	Sensitive %	Intermediate %	Resistant %
1.	Amoxyclav (AMC)	38.40	5.12	56.48
2.	Amikacin (AK)	21.62	11.90	66.48
3.	Gentamicin (GEN)	35.80	10.40	53.80
4.	Imipenem (IMP)	24.30	5.20	70.50
5.	Meropenem (MRP)	42.70	5.12	52.18
6.	Ertapenem (ETP)	23	6.83	70.17
7.	Cefotaxime (CTX)	25.60	9.40	65
8.	Cefuroxime (CXM)	22.20	8.50	69.30
9.	Ceftazidime (CAZ)	45.38	4.12	50.50
10.	Cefepime (CPM)	45.20	10.20	44.60
11.	Ciprofloxacin (CIP)	36.70	1.80	61.50
12.	Norfloxacin (NX)	23.90	6.83	69.27

The cefoxitin-cloxacillin double disc synergy test (CC-DDST) was performed to differentiate between AmpC-mediated resistance and other mechanisms, such as the production of ESBLs. This test evaluates the ability of cloxacillin to enhance the inhibitory effect of cefoxitin on AmpC beta-lactamase-producing isolates. The presence of a zone of inhibition around the cloxacillin-containing disc adjacent to the cefoxitin disc indicates the inhibition of AmpC activity, which confirms the presence of the AmpC beta-lactamase.

ESBL screening detected in 27 strains, making up 42.19% of the total. The Disc Diffusion Susceptibility Test (DDST) was the most commonly used confirmatory method, accounting for 50% of the isolates with 32 instances. Cefoxitin screening and Cefoxitin-Cloxacillin Double disk synergy test both had the same frequency, with 8 positive isolates each, representing 12.5% of the total *K. Pneumoniae* tested (Table 3). Overall, these phenotypic tests provided comprehensive insights into the antimicrobial resistance profiles of the isolates, enabling the detection and

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characterization of the AmpC beta-lactamase-mediated resistance and ESBL.

Table 3 Phenotypic Results of ESBL and AmpC Producers Among *Klebsiella pneumoniae* Isolates

Detection Methods	**Positive isolates**	
	No	**%**
ESBL Screening	27	42.19
DDST	32	50
Cefoxitin Screening	8	12.5
Cefoxitin-Cloxacillin Double disk synergy test	8	12.5

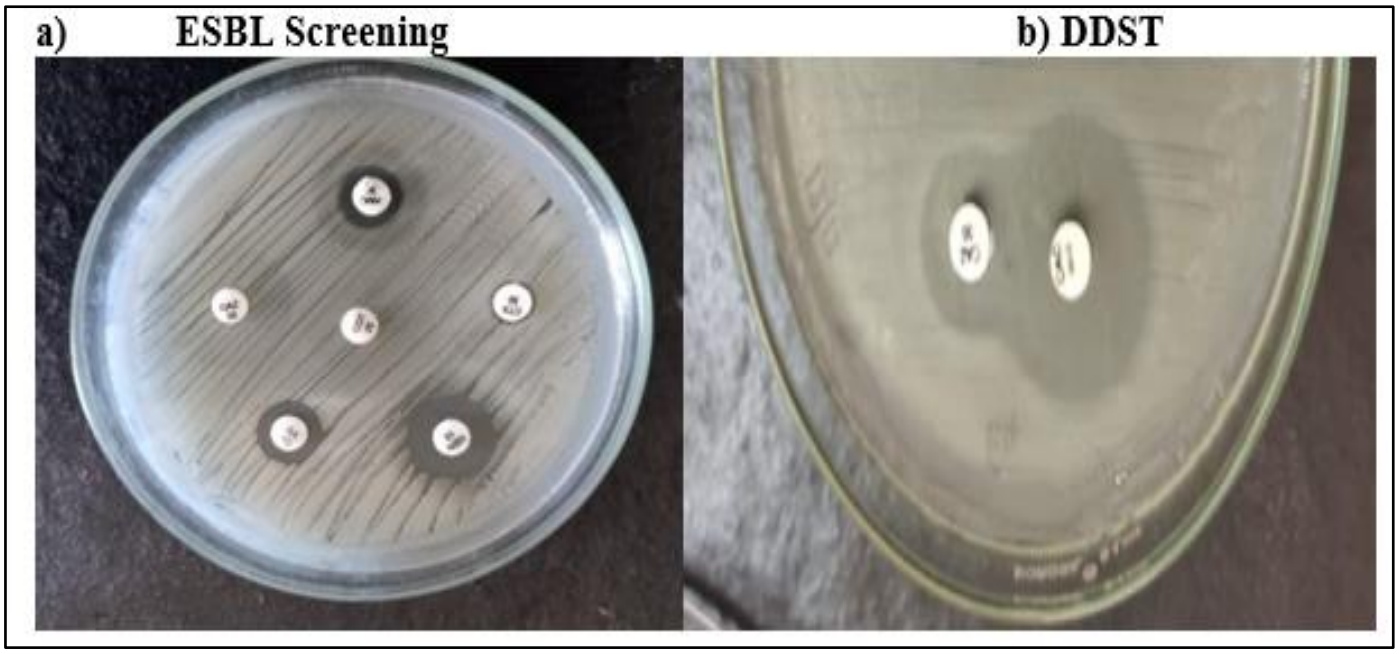

Fig 1 Phenotypic Screening and Confirmatory Test Results

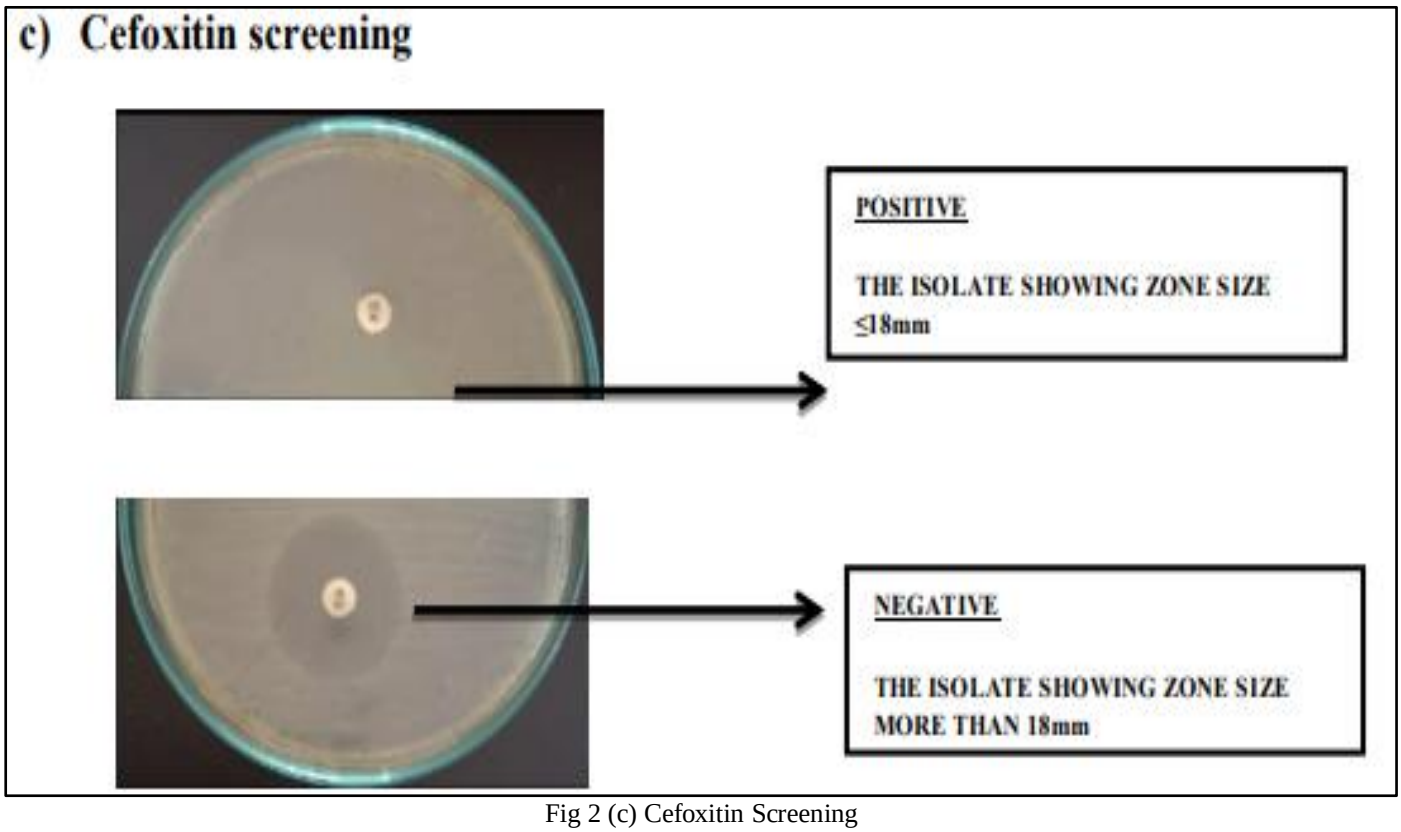

Fig 2 (c) Cefoxitin Screening

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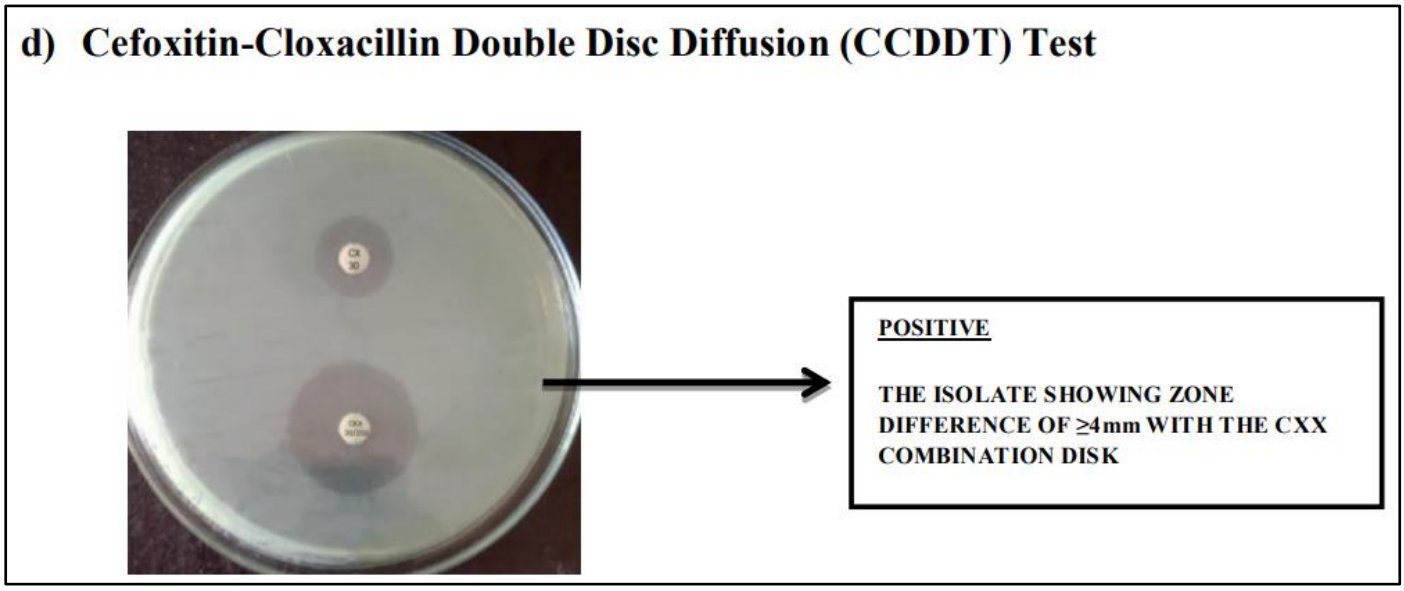

Fig 3 (d) Cefoxitin-Cloxacillin Double Disc Diffusion (CCDDT) Test

- The isolate showing resistance to cephalosporins
- The isolate showing a zone difference of ≥5mm with the CAC combination disk
- The isolate showing resistance to cefoxitin
- The isolate showing zone difference of ≥4mm with the CXX combination disk

Multiplex PCR was performed for the amplification of drug resistance genes, including the ESBL-encoding gene *bla*TEM and the AmpC-encoding gene *bla*MOX. PCR was performed. The Multiplex PCR was performed to detect, including the ESBL-encoding gene *bla*TEM and the AmpC-encoding gene *bla*MOX. The PCR-based examination of the plasmid-encoded AmpC beta-lactamase genes among the *K. pneumoniae* of the confirmed phenotypes revealed the presence of *bla*TEM (46.7 %) and *bla*MOX gene was not detected.

➢ *Statistical Analysis*

Data were collected in Microsoft Excel and result was analyzed and expressed in frequency and percentage.

IV. DISCUSSION

Our investigation into antibiotic resistance yielded a significant finding in detecting class A and class C beta-lactamase enzymes, based on the resistance of *Klebsiella pneumoniae* to cephalosporins with or without clavulanic acid and cephamycin antibiotics.

In the current investigation, among the 12 different antibiotics used against *K. pneumoniae*, the highest resistance was reported for imipenem (70.50%), followed by ertapenem (70.17%), cefuroxime (66.30%), and norfloxacin (69.27%). The maximum percentage of sensitivity was found in ceftazidime (45.38%), cefepime (45.20%), followed by ciprofloxacin (35.2%), meropenem (42.70%) and amoxyclav (38.40%).

A study by Khan, R. *et al*. (2023) showed susceptibility to tazobactam, amikacin, piperacillin, and meropenem, among other antibiotics [13]. However, they demonstrated high resistance to ticarcillin, amoxicillin, vancomycin (vancomycin testing was done to evaluate possible cross-resistance or unconventional susceptibility patterns among Enterobacterales species, even though CLSI guidelines do not have specific breakpoints for them, which is crucial to identify potential emerging trends or resistance mechanisms), and chloramphenicol.

Another study conducted by Kaur Gill M. *et al*. (2019) reported a high degree of resistance to aminoglycosides and fluoroquinolones. 96% of the samples tested positive for ciprofloxacin, compared to 85% for amikacin and 89% for gentamicin [14].

The present study found 42.19 % and 50 % of ESBL producers by screening method and DDST, respectively. Tekele et al. (2020) found a higher percentage of ESBL-producing among GNB was identified in *K. pneumoniae* 56.1% (n = 23/41) which is comparable with our results [15]. Notably, the ESBL detection by the DDST revealed a positive rate of 50%, and the AmpC producers accounted for approximately 12.5%. The findings are in line with the study reported a similar prevalence rate, along with emphasizing the growing challenge in treating the UTI.

Kumuda Arumugam *et al*. (2025) used the disc diffusion method, 128 isolates (63%) of *E. coli* and 75 isolates (37%) of *K. pneumoniae* produced ESBL, whereas the combination disc test revealed putative AmpC in 103 isolates (51%) of *E. coli* and 120 isolates (59%) of *K. pneumoniae* [16].

The current study detected 46.7 % of ESBL gene, *bla*TEM among the ESBL-positive isolates. Bora, Arijit et al. (2014) identified 73.58% of E. coli and 67.24% of *K. pneumoniae* that produce ESBL by phenotypic methods [17].

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Nevertheless, PCR amplification revealed that each of the original isolates that tested positive for ESBL had one or more ESBL genes. The most common of the three ESBL genotypes was identified as *bla*TEM in *K. pneumoniae* (77.58%) and *bla*CTX-M in *E. coli* (88.67%) ESBL-producing isolates. The majority of isolates that produce ESBL have several ESBL genes. Rani Kumari Sah *et al*. (2024) found ESBL genes (blaTEM and blaCTX-M) in 49.3% and 54.8% of the population, respectively [18]. When comparing CDM to PCR, the overall accuracy of CDM in detecting the blaCTX-M gene was 55.26%. The prevalence of blaTEM is higher than the current study. Ebtisam S. Mohamed *et al.* (2020) identified 311 ESBL producers out of 440 Enterobacteriaceae isolates by phenotyping testing [19]. ESBL genes were found in 308 isolates. The *bla*TEM gene was also found to be highly prevalent (189 isolates,60.7%).

Our study results showed that 8 (12.5%) isolates were AmpC producers by both cefoxitin screening and confirmatory Cefoxitin-Cloxacillin Double disk synergy test. Indrani Gogoi *et al*. (2024) detected 56.07% of AmpC producers. While Mohammad Hossein Haddadi *et al*. (2023) observed that 56.57% (43/76) of AmpC producers by cefoxitin disc diffusion method, which is greater than our study results [20]. Dhanashree P Inamdar *et al*. (2020) tested 140 isolates of *K. pneumoniae* for AmpC production. They reported AmpC producers in 16 (20.1%) and 38 (47.5%) isolates by using cefoxitin screening and Cefoxitin Cloxacillin Double disc Synergy (CC-DDS) methods, respectively [21].

In our study, *bla*MOX gene was not found in any of the AmpC-positive isolates. Zhijun Zhu et al. (2021) investigated 54 clinical isolates of *Klebsiella pneumoniae* for beta-lactamase resistant genes and none of the isolates carried *bla*MOX gene, which is on par with our study [22]. Soha A. El-Hady 2015 detected 92% (74) of AmpC genes [23]. They reported the majority of the isolates had MOX group genes (including CYM-1) (73.9%), CIT group genes (including CMY-2) (56.5%), and FOX group genes (including FOX-1) (30.4%).

V. CONCLUSION

In conclusion, this study offers an in-depth exploration of the detection and characterization of ESBL and AmpC beta-lactamase in *Klebsiella pneumoniae*. By meticulously isolating and analyzing 64 strains from clinical samples and by conducting thorough biochemical and antimicrobial susceptibility testing. It revealed a solid understanding of the prevalence and resistance mechanisms associated with ESBL and AmpC beta-lactamase in clinical samples. These findings contribute significantly to the broader discourse on antibiotic resistance and underscore the importance of interdisciplinary approaches in addressing this global public health challenge. Ultimately, this study serves as a catalyst for continued exploration and innovation in the field of infectious diseases and microbiology, offering hope in the fight against antimicrobial resistance.

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- *Conflict of Interest*

There is no conflict of interest.

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