ORIGINAL ARTICLE

Genetic characterization of CTX-M-15, AmpC, and metallo-β-lactamase genes among clinical *Klebsiella pneumoniae* isolates in a tertiary-care hospital

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Abstract

Background: The co-existence of Extended-Spectrum β-Lactamases (ESBL), AmpC β-lactamases and Metallo-β-Lactamases (MBL) in *Klebsiella pneumoniae* limits therapeutic options and complicates infection control in tertiary-care settings. *Aim and Objectives:* To determine the phenotypic and molecular profile of β-lactamase-mediated resistance and associated co-resistance to aminoglycosides and fluoroquinolones among clinical *K. pneumoniae* isolates. *Material and Methods:* In this laboratory-based cross-sectional study, 254 non-duplicate clinical isolates were subjected to antimicrobial susceptibility testing and phenotypic detection of ESBL, AmpC and MBL. Multiplex polymerase chain reaction was performed for blaTEM, blaSHV, blaCTX-M-15, blaDHA and blaNDM genes in multidrug-resistant isolates. *Results:* Resistance to third-generation cephalosporins and fluoroquinolones exceeded 70%, while carbapenem resistance ranged from 20.9% to 25.6%. ESBL, AmpC and MBL production was detected in 68.1%, 22.0% and 9.8% of isolates, respectively. Among the genotyped isolates, blaTEM and blaSHV were present in 98.0% each and blaCTX-M-15 in 88.0%, with blaNDM co-detected in 64.0%. Frequent co-resistance to aminoglycosides and fluoroquinolones was observed. *Conclusion:* The convergence of ESBL, AmpC and blaNDM-mediated resistance in K. pneumoniae highlights the need for routine molecular surveillance and targeted antimicrobial-stewardship interventions in tertiary-care hospitals.

Keywords: *Klebsiella pneumoniae*, AmpC beta-Lactamases, Metallo-beta-Lactamases, Carbapenem Resistance

Introduction

Klebsiella pneumoniae (K. pneumoniae) is a major cause of healthcare-associated infections worldwide and is recognised for its remarkable capacity to acquire, accumulate, and disseminate Antimicrobial Resistance (AMR) determinants. The World Health Organization (WHO) classifies carbapenem -resistant *K. pneumoniae* as a Priority 1: Critical pathogen, underscoring its global clinical significance and the urgent need for enhanced diagnostic and surveillance strategies [1]. Infections caused by Multidrug-Resistant (MDR) *K. pneumoniae* are associated with prolonged hospitalisation, increased morbidity and mortality, and severely restricted therapeutic options, particularly in resourcecons-trained settings [2, 3].

Among β-lactam resistance mechanisms, Extended -Spectrum β-Lactamases (ESBLs), AmpC β-lactamases, and Metallo-β-Lactamases (MBLs) represent some of the most clinically consequential determinants. CTX-M-15 has emerged as the predominant ESBL type globally and is strongly linked to resistance to third-generation cephalo-sporins [4, 5]. Plasmid-mediated AmpC enzymes further compromise the efficacy of broad-spectrum

cephalosporins and are often challenging to detect reliably using routine phenotypic assays [6]. MBLs such as New Delhi Metallo-β-lactamase (NDM), Verona Integron-encoded Metallo-β-lactamase (VIM), and Imipenemase (IMP) hydrolyse nearly all β-lactams, including carbapenems, leaving extremely limited treatment options [7, 8].

The co-harbouring of CTX-M-15, AmpC, and MBL genes in *K. pneumoniae* is frequently associated with co-resistance to aminoglycosides and fluoroquinolones, driven largely by plasmid-mediated resistance determinants, efflux mechanisms, and selective pressure from empirical broad-spectrum antibiotic use [9–11]. This MDR phenotype considerably narrows viable therapeutic options, complicates empirical treatment decision-making, and increases the likelihood of clinical failure. The simultaneous presence of ESBL, AmpC, and MBL genes combined with resistance to non-β-lactam agents poses substantial challenges for formulating evidence-based Antimicrobial Stewardship (AMS) strategies, strengthening Infection Prevention and Control (IPC) programmes, and developing rational, hospital-specific antibiotic policies in tertiary-care settings.

The present study aimed to characterise the phenotypic and genotypic profiles of clinical K. pneumoniae isolates co-harbouring CTX-M-15, AmpC, and MBL genes in a tertiary-care hospital. By correlating antimicrobial susceptibility profiles with Polymerase Chain Reaction (PCR) based gene detection and integrating relevant clinical data including treatment compliance we seek to identify co-resistance trends, particularly resistance to aminoglycosides and fluoroquinolones, which commonly accompany β-lactamase-mediated resistance. Additionally, the study aimed to determine associated clinical risk factors and to evaluate the implications of these MDR profiles for therapeutic decision-making, AMS planning, and hospital antibiotic policy development.

Although ESBL, AmpC and MBL genes have been reported individually from India, data on their simultaneous coexistence with non-β-lactam co-resistance and associated clinical risk factors from this region remain limited. Furthermore, genotype –phenotype correlation for CTX-M-15-dominant ESBL backgrounds co-harbouring blaNDM and blaDHA in a single institutional setting has not been adequately explored. The present study therefore provides integrated phenotypic, molecular and clinical correlation to support AMS decision-making.

Material and Methods

Study design and bacterial isolates

This prospective laboratory-based cross-sectional study conducted in the Department of Microbiology, Symbiosis Medical College for Women and Symbiosis University Hospital and Research Centre, Symbiosis International (Deemed University), Pune, India, between May 2022 and December 2022. Consecutive non-duplicate clinical isolates were included using a consecutive sampling technique.

The minimum sample size was calculated using the formula

$n = Z^2P(1-P)/d^2$ taking ESBL prevalence 55%, 95% confidence level and 6% precision → required sample ≈ 250. Two hundred and fifty-four non-duplicate *K. pneumoniae* isolates were recovered from diverse clinical specimens, including urine, blood, respiratory secretions, pus, and indwelling-catheter tips, obtained from hospitalised patients.

Patient demographic and clinical data including age, sex, immune status, underlying comorbidities, hospitalisation history, antimicrobial exposure, mechanical ventilation, indwelling catheterisation, focal site of infection, and indicators of disease severity were collected from hospital records.

This study was approved by the Institutional Ethics Committee of Symbiosis International (Deemed University), Pune (Ref: SMCW/IEC/2022/034). All data were anonymised; no patient identifiers were recorded.

Isolation and identification

Clinical specimens were inoculated onto Mac Conkey agar and blood agar (HiMedia Laboratories, Mumbai, India), whereas urine samples were cultured on Cystine Lactose Electrolyte Deficient (CLED) agar. All plates were incubated aerobically at 37 °C for 24 hours. The isolation and preliminary identification of *K. pneumoniae* were performed using standard conventional microbiological procedures [12]. Confirmatory Identification and antimicrobial susceptibility testing, including Minimum Inhibitory Concentration (MIC) determination, were conducted using the VITEK-2 Compact automated system (bioMérieux, France) in accordance with the manufacturer's instructions. Phenotypic confirmation of ESBL, AmpC and MBL production was undertaken using established phenotypic methods [7, 11, 13].

Phenotypic detection of β-lactamase production

ESBL detection

Screening for ESBL production was undertaken using cefotaxime (30 µg) and ceftazidime (30 µg) discs alone, and in combination with clavulanic acid (10 µg). An increase of ≥ 5 mm in the inhibition-zone diameter for either cephalosporin–clavulanate disc compared with the corresponding cephalosporin disc alone was interpreted as indicative of ESBL production, in accordance with the Clinical & Laboratory Standards Insitute (CLSI) recommendations [7, 11, 14, 15].

AmpC β-lactamase detection

Isolates exhibiting cefoxitin resistance (zone diameter < 18 mm) were screened for AmpC β-lactamase production using an inhibitor-based method employing Phenyl-Boronic Acid (PBA). PBA (95% benzeneboronic acid; Sigma-Aldrich, India) was dissolved at 120 mg in 3 mL dimethyl sulfoxide and 3 mL sterile distilled water; 20 µL of this solution was dispensed onto a cefoxitin (30 µg) disc. An increase of ≥ 4 mm in the inhibition-zone diameter for the cefoxitin–PBA disc relative to cefoxitin alone was interpreted as AmpC positive [12]. Additionally, MICs for cefoxitin alone and in combination with cloxacillin were evaluated, with a cefoxitin/cefotetan–cloxacillin MIC ratio ≥ 8 confirming AmpC β-lactamase production [2, 3, 14, 15].

MBL detection

MBL production was assessed using the imipenem –EDTA combined disc test as described by Yong et al. [14]. Two imipenem (10 µg) discs (HiMedia, India) were placed onto Mueller–Hinton agar plates inoculated with a 0.5 McFarland suspension of the test isolate. Ten microlitres of EDTA solution (750 µg/disc) was applied to one disc. Plates were incubated at 35 °C for 16–18 hours, and an increase of ≥ 7 mm in the inhibition-zone diameter around the imipenem–EDTA disc compared with the imipenem disc alone indicated MBL production [2, 3, 14, 15]

Carbapenemase screening: Modified Hodge test (MHT)

Carbapenemase activity was further evaluated using the MHT following CLSI recommendations [11]. A lawn culture of *Escherichia coli* ATCC 25922 was prepared on Mueller–Hinton agar. After drying, the test strain was streaked from the central imipenem (10 µg) disc toward the periphery. Following overnight incubation at 35°C, the presence of a characteristic clover-leaf-shaped indentation in the *E. coli* lawn along the test-strain streak was interpreted as indicative of carbapenemase production [14, 15].

Quality control

All phenotypic β-lactamase detection assays were Quality-Controlled (QC) using CLSI recommended reference strains. *Escherichia coli* ATCC 25922 served as the negative control for ESBL, AmpC, MBL, and carbapenemase testing; *Klebsiella pneumoniae* ATCC 700603 as the ESBL-positive control; *Enterobacter cloacae* ATCC 13047 as the AmpC-positive control; *Klebsiella pneumoniae* ATCC BAA-1705 and ATCC BAA-1706 as carbapenemase-positive and -negative controls, respectively. All control strains produced expected results, and only QC validated runs were included in the analysis.

Genotyping of antimicrobial resistance genes

Molecular detection of β-lactamase genes

Plasmid-encoded β-lactamase genes conferring resistance to β-lactams, including extended-spectrum β-lactamases (ESBLs; *SHV, TEM, CTX-M-15*), AmpC β-lactamases (*DHA*), and carbapenemases (*KPC-2, NDM-1*), were detected in 50 MDR *K. pneumoniae* isolates using conventional multiplex PCR. Previously sequenced gene templates were used as positive controls (*SHV* KY115-613, *TEM* KY115614, *CTX-M-15* KY115615, *DHA* KY115612, *KPC-2* KY364013, *NDM-1*) while nuclease-free water functioned as the negative control.

Primer sequences and expected amplicon sizes were used as previously described. Each primer set was designed to amplify clinically relevant β-lactamase determinants associated with ESBL, AmpC and carbapenemase-mediated resistance as described by Jacob *et al.,* Nordman *et al.*, Ranjan *et al.* [2,3,8].

PCR amplification conditions

PCR amplification was performed in 25 µL reaction mixtures containing 100–200 ng genomic DNA, 1× PCR buffer, 2.5 mM $MgCl_2$, 50 µM dNTPs, 0.2 µM of each primer, 1 U Taq DNA polymerase, 5 % (v/v) dimethyl sulfoxide and nuclease-free water. Amplification was undertaken in a 2720 Thermal Cycler (Thermo Fisher Scientific, USA), using an annealing temperature of 55 °C for all primer sets, consistent with published multiplex PCR protocols.

Agarose gel electrophoresis

Amplified PCR products were resolved on 2 % (w/v) agarose gels prepared in 0.5× TBE buffer containing LabSafe nucleic-acid stain (G-Biosciences, USA). Each reaction (5 µL) was mixed with 1 µL of 6× loading dye and electrophoresed alongside a molecular weight ladder at 5 V cm^{-1} until adequate band separation was achieved. Bands were visualised under UV illumination using a BIO-RAD GelDoc XR system, and amplicon sizes were verified against expected sizes. All target genes produced amplicons of the anticipated lengths.

Results

Demographic distribution and clinical profile

A total of 254 non-duplicate clinical isolates of K. pneumoniae were obtained from various specimens collected between May 2022 and December 2022.

Table 1 summarises the demographic profile, clinical characteristics, hospital distribution, specimen sources, and predisposing factors of patients from whom *Klebsiella pneumoniae* was isolated, and analyses their association with carbapenem resistance. Of these, 149 (58.7 %) originated from male patients and 105 (41.3 %) from female patients. The mean patient age was 49.6 ± 18.3 years, ranging from 1 month to 87 years. Most isolates were derived from patients admitted to Intensive Care Units (ICUs) (87 isolates; 34.2 %), followed by the

medical wards (67 isolates; 26.3 %), surgical wards (55 isolates; 21.6 %), and outpatient clinics (45 isolates; 17.9 %). The predominant specimen type was urine (96 isolates; 37.8 %), followed by respiratory secretions (62 isolates; 24.4 %), pus/wound swabs (48 isolates; 18.9 %), blood (36 isolates; 14.1 %), and catheter-related samples (12 isolates; 4.7 %) (Table 1, Figure 1). Most isolates were recovered from patients with underlying conditions such as diabetes mellitus (70 patients; 27.5 %), chronic kidney disease (46 patients; 18.1 %), or prolonged hospitalisation (> 10 days) (91 patients; 35.8 %) (Table 1). Of the 254 isolates included in the study, 118 (46.5%) were Carbapenem-Resistant (CRKP) and 136 (53.5%) were Carbapenem-Susceptible (CSKP). There was no statistically significant difference in sex distribution between the two groups (p = 0.21). However, patients with CRKP infection were significantly older than those with CSKP (mean age 52.4 ± 17.6 vs 47.2 ± 18.7 years; p = 0.02). A significantly higher proportion of CRKP isolates originated from ICUs compared with CSKP isolates (47.5% vs 22.8%), and ICU stay emerged as a strong risk factor for carbapenem resistance (OR = 3.06, 95% CI 1.78–5.26; p < 0.001). With regard to specimen type, respiratory samples and blood cultures were significantly associated with carbapenem resistance. Respiratory isolates showed more than three-fold higher odds of being carbapenem-resistant (OR = 3.17, p = 0.001), while bloodstream isolates demonstrated a 2.5-fold increased risk (p = 0.03).

Among the healthcare-associated risk factors, prolonged hospitalisation, prior exposure to broad-spectrum antibiotics, presence of indwelling medical devices, and mechanical ventilation were all strongly associated with CRKP (p < 0.001 for each). Mechanical ventilation showed the highest risk (OR = 6.68, 95% CI = 3.14–14.2). Regarding comorbid conditions, diabetes mellitus (OR = 1.80, p = 0.04) and chronic kidney disease (OR = 2.54, p = 0.006) were significantly associated with carbapenem resistance, whereas hypertension, chronic lung disease, malignancy, chronic liver disease, and immunosuppression did not show statistically significant associations.

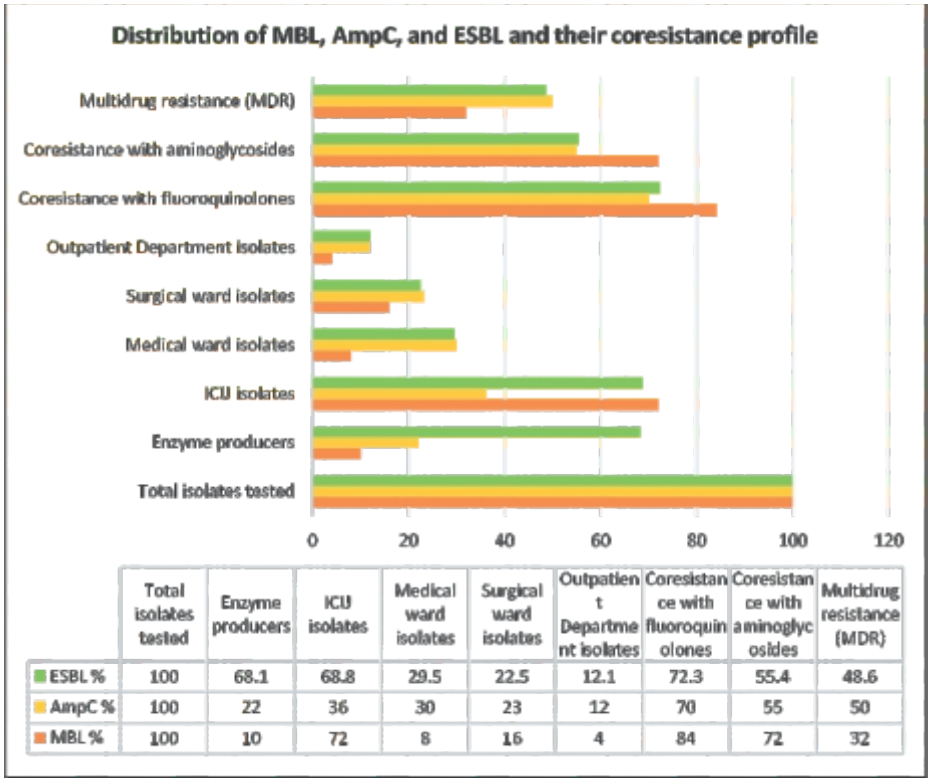
Table 1: Association of clinical and demographic factors with carbapenem resistance among *K. pneumoniae* isolates (n = 254)

Variable	Category	CRKP (n = 118) n (%)	CSKP (n = 136) n (%)	Total n (%)	Odds Ratio (95% CI)	*p*
Sex	**Male**	74 (62.7)	75 (55.1)	149 (58.7)	1.36 (0.83–2.21)	0.21
	Female	44 (37.3)	61 (44.9)	105 (41.3)		
Age (years)	**Mean ± SD**	52.4 ± 17.6	47.2 ± 18.7	49.6 ± 18.3		0.02†
Hospital location	**ICU**	56 (47.5)	31 (22.8)	87 (34.2)	3.06 (1.78–5.26)	<0.001
	Wards/OPD	62 (52.5)	105 (77.2)	167 (65.8)		

Continued...

Specimen type	**Urine**	32 (27.1)	64 (47.1)	96 (37.8)		
	Respiratory	38 (32.2)	24 (17.6)	62 (24.4)	3.17 (1.63–6.17)	0.001
	Pus / wound	22 (18.6)	26 (19.1)	48 (18.9)	1.69 (0.79–3.59)	0.17
	Blood	20 (16.9)	16 (11.8)	36 (14.1)	2.50 (1.08–5.77)	0.03
	Catheter	6 (5.1)	6 (4.4)	12 (4.7)	2.00 (0.55–7.21)	0.29
Prolonged hospitalisation	**>10 days**	62 (52.5)	29 (21.3)	91 (35.8)	4.10 (2.34–7.17)	<0.001
Prior antibiotic exposure	**Yes**	78 (66.1)	36 (26.5)	114 (45.0)	5.40 (3.09–9.44)	<0.001
Indwelling medical device	**Yes**	55 (46.6)	21 (15.4)	76 (30.0)	4.76 (2.63–8.61)	<0.001
Mechanical ventilation	**Yes**	41 (34.7)	10 (7.4)	51 (20.0)	6.68 (3.14–14.2)	<0.001
Diabetes mellitus	**Yes**	40 (33.9)	30 (22.1)	70 (27.5)	1.80 (1.01–3.21)	0.04
Hypertension	**Yes**	31 (26.3)	29 (21.3)	60 (23.7)	1.32 (0.72–2.43)	0.36
COPD / CLD	**Yes**	33 (28.0)	29 (21.3)	62 (24.4)	1.43 (0.79–2.59)	0.23
CKD	**Yes**	30 (25.4)	16 (11.8)	46 (18.1)	2.54 (1.29–5.01)	0.006
Malignancy	**Yes**	18 (15.3)	13 (9.6)	31 (12.2)	1.71 (0.78–3.76)	0.17
Chronic liver disease	**Yes**	11 (9.3)	9 (6.6)	20 (7.9)	1.44 (0.57–3.66)	0.43
Immunosuppression	**Yes**	10 (8.5)	5 (3.7)	15 (5.9)	2.42 (0.79–7.41)	0.11

CRKP: carbapenem-resistant Klebsiella pneumoniae; CSKP:carbapenem-susceptible Klebsiella pneumoniae; ICU: intensive-care unit; COPD: chronic obstructive pulmonary disease; CKD: chronic kidney disease; OR: odds ratio; CI: confidence interval.



	Total isolates tested	Enzyme producers	ICU isolates	Medical ward isolates	Surgical ward isolates	Outpatient Department isolates	Coresistance with fluoroquinolones	Coresistance with aminoglycosides	Multidrug resistance (MDR)
ESBL %	100	68.1	68.8	29.5	22.5	12.1	72.3	55.4	48.6
AmpC %	100	22	36	30	23	12	70	55	50
MBL %	100	10	72	8	16	4	84	72	32

Figure 1: Distribution of MBL, AmpC, and ESBL Producers and Their Coresistance Profiles

Detection of CTX-M-15

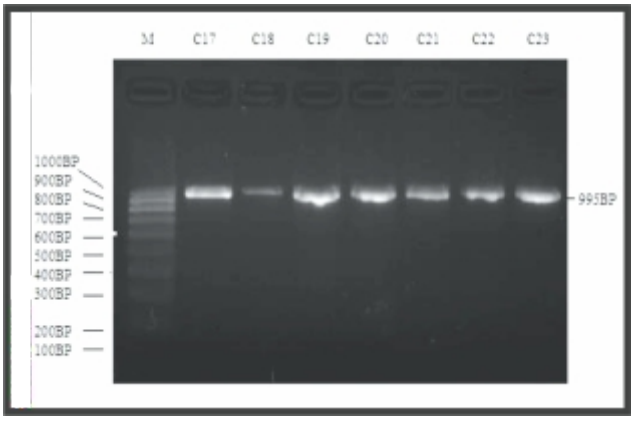


Figure 2: Agarose (2% w/v) gel electrophoresis of amplification products from CTXM gene

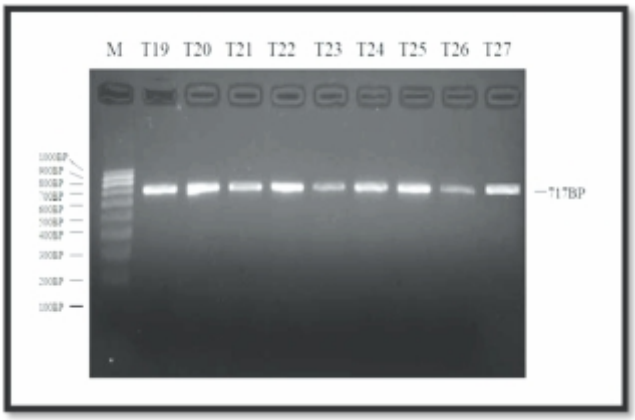


Figure 3: Agarose (2% w/v) gel electrophoresis of amplification products from TEM gene

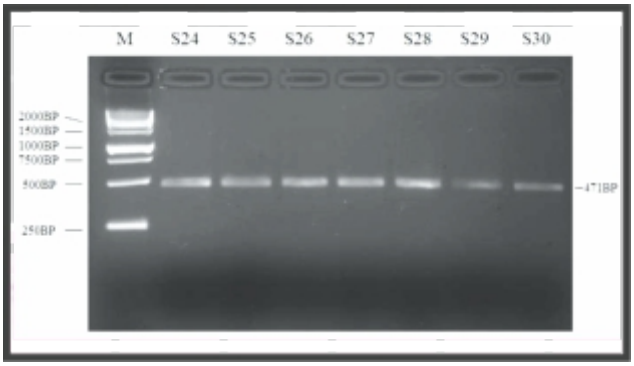


Figure 4: Agarose (2% w/v) gel electrophoresis of amplification products from SHV gene

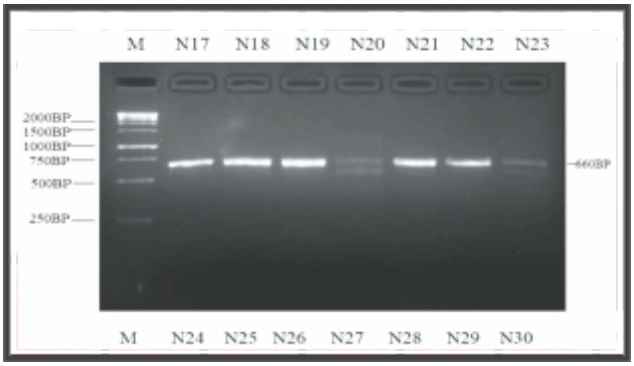


Figure 5: Agarose (2% w/v) gel electrophoresis of amplification products from NDM gene

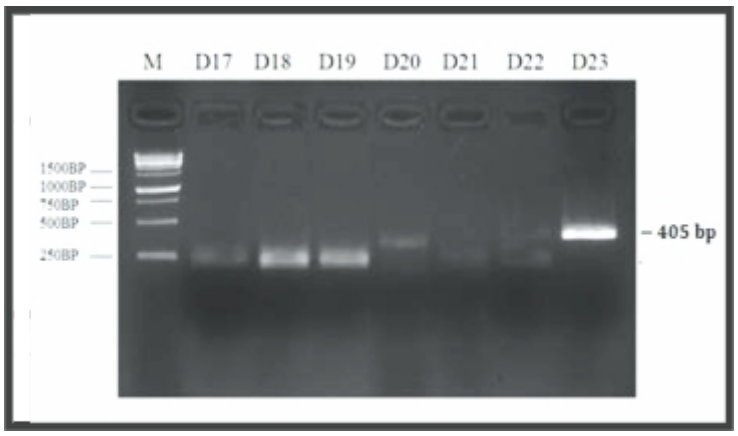


Figure 6: Agarose (2% w/v) gel electrophoresis of amplification products from BLA-DHA gene

Antimicrobial susceptibility profiles

Of the total 254 samples tested; high level of resistance was observed against third-generation cephalosporins, with 182 isolates (71.7%) resistant to cefotaxime, 176 (69.3%), ceftazidime, and 172 (67.8%) to ceftriaxone. Resistance to cefoxitin was seen in 142 isolates (55.9%), indicating possible AmpC β-lactamase activity. Among β-lactam/β-lactamase inhibitor combinations, 148 isolates (58.3%) were resistant to piperacillin–tazobactam.

Carbapenem resistance was detected in 53 isolates (20.9%) for imipenem and 65 (25.6%) for meropenem, suggestive of MBL production. For aminoglycosides, 107 isolates (42.1%) were resistant to gentamicin and 88 (34.6%) to amikacin, reflecting moderate sensitivity retention. Resistance to ciprofloxacin was widespread, affecting 179 isolates (70.5%), while cotrimoxazole resistance was noted in 162 isolates (63.8%). In contrast, last-resort agents colistin and tigecycline showed low resistance rates 10 isolates (3.9%) and 14 isolates (5.5%), respectively indicating retained efficacy. Phenotypic β-lactamase detection confirmed 173 isolates (68.1%) as Extended-Spectrum β-Lactamase (ESBL) producers, 56 isolates (22.0%) as AmpC β-

lactamase producers, and 25 isolates (9.8%) as MBL, positive.

Overall, 115 isolates (45.3%) demonstrated MDR, defined as non-susceptibility to ≥ 3 antimicrobial classes. These findings collectively indicate a high prevalence of ESBL-mediated resistance with emerging carbapenem resistance, underscoring the urgent need for reinforced infection control and AMS interventions in tertiary-care hospital settings (Table 2).

Table 2: Antimicrobial susceptibility pattern and phenotypic β-lactamase resistance among *K. pneumoniae* isolates (n = 254)

Antimicrobial class	Antimicrobial agent	Resistant isolates (n)	Resistance (%)
β-lactams / Cephalosporins	**Cefotaxime**	182	71.7
	Ceftazidime	176	69.3
	Ceftriaxone	172	67.8
	Cefoxitin	142	55.9
β-lactam / β-lactamase inhibitor	**Piperacillin–tazobactam**	148	58.3
Carbapenems	**Imipenem**	53	20.9
	Meropenem	65	25.6
Aminoglycosides	**Gentamicin**	107	42.1
	Amikacin	88	34.6
Fluoroquinolones	**Ciprofloxacin**	179	70.5
Sulphonamides	**Cotrimoxazole**	162	63.8
Polymyxins / Others	**Colistin**	10	3.9
	Tigecycline	14	5.5
Phenotypic β-lactamase detection	**Extended-spectrum β-lactamase positive**	173	68.1
	AmpC β-lactamase positive	56	22.0
	Metallo-β-lactamase positive	25	9.8

Phenotypic detection of β-lactamase production

ESBL

Of the 254 isolates, 173 (68.1%) demonstrated a ≥ 5 mm increase in inhibition-zone diameter with clavulanic acid, confirming ESBL production. The majority of ESBL-producing isolates were recovered from ICUs (62/173, 35.8%), followed by medical wards (51/173, 29.5%), surgical wards (39/173, 22.5%), and outpatient departments (21/173, 12.1%). Co-resistance to fluoroquinolones and aminoglycosides was observed in 125 (72.3%) and 96 (55.4%) isolates, respectively. MDR was detected in 84 (48.6%) ESBL producers (Table 2)

AmpC β-lactamase

AmpC production was confirmed in 56/254 isolates (22.0%). Ward-wise distribution showed a predominance in ICUs (20/56, 35.8%), followed by medical wards (17/56, 29.5%), surgical wards (13/56, 22.5%), and outpatient settings (7/56, 12.1%). Wound specimens constituted the most common source (22/56, 39.3%), followed by respiratory samples (14/56, 25.0%), urine (13/56, 23.2%), and blood cultures (7/56, 12.5%). Prior β-lactam exposure was documented in 38 cases (67.8%), and indwelling devices were present in 18 (32.1%). Co-resistance to fluoroquinolones and aminoglycosides occurred in 39 (70.0%) and 31 (55.0%) isolates, respectively, while MDR was observed in 28 (50.0%) (Table 2).

MBL

MBL production was detected in 25/254 isolates (9.8%) using the imipenem–EDTA combined disc test; of these, 22 (88.0%) were positive by the MHT. Most MBL producers were from ICUs (18/25, 72.0%), followed by surgical wards (4/25, 16.0%), medical wards (2/25, 8.0%), and outpatient settings (1/25, 4.0%). A prolonged hospital stay (> 10 days) and the presence of indwelling devices were noted in 19 (76.0%) and 16 (64.0%) cases, respectively. High rates of co-resistance were observed for fluoroquinolones (21/25, 84.0%) and aminoglycosides (18/25, 72.0%), whereas MDR was present in 8 isolates (32.0%) (Table 2).

Molecular genotyping of K. pneumoniae for the production of blaCTX-M, blaTEM. blaSHV, blaKPC, blaNDM and blaDHA genes to identify the resistance mechanism

Among the 50 K. pneumoniae isolates analysed, ESBL genes were highly prevalent. blaTEM and blaSHV were detected in 98% of isolates each (95% CI 89.4–99.9), while blaCTX-M was identified in 88% (95% CI 75.7–95.5). The prevalence of blaTEM and blaSHV was significantly higher than that expected by chance ($p < 0.001$) (Table 3). Among carbapenemase genes, blaNDM was detected in 64% of isolates (95% CI 49.2–77.1), whereas blaKPC was not detected in any isolate (0%; 95% CI 0.0–7.1), and this difference was statistically significant ($p < 0.001$). The AmpC gene blaDHA was present in 14% of isolates (95% CI 5.8–26.7), which was significantly lower than the ESBL gene prevalence ($p < 0.001$).

The present analysis demonstrates that carbapenem resistance in K. pneumoniae is predominantly driven by healthcare-associated factors rather than patient sex or most underlying comorbidities. ICU stay emerged as a major determinant, reflecting the selective antimicrobial pressure, high device utilisation, and increased severity of illness in critically ill patients. The strong association with prior broad-spectrum antibiotic exposure underscores the pivotal role of AMS in limiting the emergence and dissemination of carbapenem-resistant strains.

Table 3: Distribution of β-lactamase genes among *Klebsiella pneumoniae* isolates (n = 50)

Gene category	Gene	Amplicon size (bp)	Positive isolates n (%)	95% CI for proportion	*p*
ESBL	**blaCTX-M**	995	44 (88.0)	75.7–95.5	0.07
	blaTEM	717	49 (98.0)	89.4–99.9	<0.001
	blaSHV	471	49 (98.0)	89.4–99.9	<0.001
Carbapenemase	**blaKPC**	863	0 (0.0)	0.0–7.1	<0.001
	blaNDM	660	32 (64.0)	49.2–77.1	—
AmpC	**blaDHA**	406	7 (14.0)	5.8–26.7	<0.001

95% Confidence Intervals (CI) for proportions were calculated using the Wilson method. The one-sample proportion test was used to assess the significance of gene prevalence. A two-tailed p value < 0.05 was considered statistically significant

Mechanical ventilation and indwelling devices showed the highest effect sizes, highlighting the importance of biofilm-mediated persistence and cross-transmission in hospital settings. The significant association with respiratory and bloodstream infections suggests that invasive infections are more likely to be caused by resistant strains, possibly due to prolonged hospital stay and repeated antibiotic exposure. Among comorbidities, diabetes mellitus and chronic kidney disease were independent clinical correlates, which may be explained by frequent healthcare contact, immune dysregulation, and repeated antimicrobial therapy in these patient groups. Overall, these findings reinforce that carbapenem resistance in K. pneumoniae is a marker of healthcare exposure and invasive supportive care, and they provide clinically actionable targets for infection-control interventions and AMS programmes.

Discussion

This study reveals a substantial burden of antimicrobial-resistant K. pneumoniae across diverse clinical specimens, with infections disproportionately affecting critically ill patients, those with prolonged hospitalisation, and individuals exposed to broad-spectrum antimicrobials. The predominance of isolates from ICUs reflects established risk factors for healthcare-associated K. pneumoniae infection, including invasive procedures, indwelling devices, and sustained antimicrobial pressure. Similar demographic clustering has been reported by Diekema et al. (2019) who documented increased incidence among hospitalised adults with multiple comorbidities [18]. The high proportion of urinary isolates further supports earlier observations identifying K. pneumoniae as a key uropathogen, particularly among patients with diabetes mellitus or long-term catheterisation [19].

The antimicrobial resistance profile observed is concerning, with resistance exceeding 70% to third-generation cephalosporins and fluoroquinolones. These patterns are consistent with the widespread dissemination of ESBL-producing K. pneumoniae, particularly across South and South-East Asia, where

antimicrobial consumption remains high [20]. Carbapenem resistance rates ranging from 20.9% to 25.6% are comparable to those reported in East and South Asian settings, indicating sustained regional transmission of carbapenem-resistant lineages [21]. In contrast, relatively preserved susceptibility to colistin and tigecycline mirrors global reports suggesting that last-resort agents retain activity, although their continued effectiveness depends on strict antimicrobial stewardship [1]. At the mechanistic level, ESBL production was the dominant resistance determinant, identified in 68.1% of isolates. This prevalence aligns with regional and global data documenting the expansion of CTX-M-type enzymes within healthcare environments [5, 20, 22]. The concentration of ESBL producers in ICUs highlights the role of ecological pressures, including prolonged antibiotic exposure and frequent patient-to-patient transmission, in amplifying resistance. The frequent co-resistance to fluoroquinolones and aminoglycosides further supports plasmid-mediated co-selection of resistance determinants associated with ESBL genes, with nearly half of the isolates exhibiting multidrug-resistant phenotypes [1, 14, 18, 23].

AmpC β-lactamases were detected in 22% of isolates, predominantly from high-risk hospital settings. Their distribution across wound and respiratory specimens is consistent with nosocomial acquisition pathways and reflects the selective impact of repeated β-lactam exposure and device-associated interventions [24]. The observed co-resistance patterns parallel global trends in which AmpC-encoding plasmids frequently harbour additional resistance loci, contributing to complex multidrug-resistant profiles. The detection of MBL production in 9.8% of isolates represents the most clinically significant component of the resistance landscape. MBLs, predominantly encoded by blaNDM variants, are among the most critical antimicrobial resistance threats due to their ability to hydrolyse nearly all β-lactams and their efficient plasmid-mediated dissemination [24]. Their predominance in ICU patients with prolonged hospitalisation and indwelling devices aligns with recognised risk factors for colonisation by carbapenemase-producing organisms. The high levels of fluoroquinolone and aminoglycoside co-resistance reflect the dense genetic linkage of MBL genes with multiple resistance modules. Concordance between EDTA-based synergy testing and the MHT supports the reliability of accessible phenotypic detection strategies in high-burden settings [12, 14, 24, 25].

Genotypic analysis demonstrated extensive co-harbouring of β-lactamase genes, underscoring the remarkable genomic plasticity of K. pneumoniae in tertiary-care hospitals. The near-universal presence of blaTEM and blaSHV (98.0% each), together with a high prevalence of blaCTX-M-15 (88.0%), indicates that ESBL-mediated resistance is firmly entrenched within dominant hospital-adapted clones. Frequent intra-isolate coexistence of these ESBL determinants suggests dissemination through multi-replicon plasmids and composite mobile genetic elements, facilitating both horizontal transfer and long-term stability under sustained antimicrobial pressure [26]. Such clustering may synergistically enhance hydrolytic activity against third-generation cephalosporins, further narrowing therapeutic options [27].

Of particular concern is the high rate of blaNDM co-detection (64.0%) among ESBL-producing isolates. This convergence of ESBL and MBL reflects the successful mobilisation of carbapenemase-encoding platforms into pre-existing ESBL backgrounds, likely mediated by conjugative

plasmids, insertion sequences, and integron-associated gene cassettes [3, 20, 26-28]. ESBL–NDM co-harbouring strains exhibit resistance to nearly all β-lactam agents, including carbapenems, thereby severely constraining treatment options and complicating infection-control efforts [5, 7, 28].

Although blaDHA was detected at a lower frequency (14.0%), its coexistence with ESBL and blaNDM genes is clinically relevant. AmpC β-lactamases can compromise the activity of β-lactam/β-lactamase inhibitor combinations and may remain undetected by routine phenotypic methods, facilitating silent dissemination within healthcare environments. The absence of blaKPC in this cohort highlights region-specific carbapenemase epidemiology and reaffirms NDM as the dominant mechanism of carbapenem resistance in the Indian subcontinent.

Importantly, the β-lactamase genes identified in this study are highly mobile and readily disseminate across genera within the Enterobacteriaceae and to other clinically important Gram-negative pathogens, including Escherichia coli and Acinetobacter species. This interspecies transfer has driven the widespread emergence of multidrug-resistant and extensively drug-resistant phenotypes across health-care and community settings. The largely silent dissemination of these resistance determinants underscores antimicrobial resistance as an evolving global "silent pandemic", necessitating coordinated surveillance and robust AMS to curb further spread.

Conclusion

Clinical K. pneumoniae isolates in this study exhibited extensive multidrug resistance driven by the co-occurrence of ESBL, AmpC, and metallo-β-lactamase genes, frequently accompanied by resistance to aminoglycosides and fluoroquinolones. The convergence of plasmid-mediated resistance mechanisms within hospital-adapted clones severely constrains therapeutic options and facilitates long-term persistence in high-risk healthcare environments. These findings underscore the urgent need for routine molecular surveillance, targeted infection-control interventions, and robust AMS strategies to limit the dissemination of high-risk K. pneumoniae lineages in tertiary-care settings.

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