

Association between Antimicrobial Resistance Phenotypes and Biofilm-Associated Virulence among Clinical Isolates of *Klebsiella pneumoniae*

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ABSTRACT

Background: *Klebsiella pneumoniae* is an important opportunistic pathogen increasingly associated with multidrug resistance and persistent healthcare-associated infections. Biofilm formation may enhance bacterial survival, reduce antimicrobial susceptibility, and contribute to treatment failure. This study evaluated the association between antimicrobial resistance phenotypes and biofilm-forming capacity among clinical isolates of *K. pneumoniae*.

Materials and Methods: A prospective cross-sectional study was conducted over four months at Alluri Sitarama Raju Academy of Medical Sciences, Eluru, South India. A total of 150 non-duplicate clinical isolates of *K. pneumoniae* were included. Antimicrobial susceptibility testing was performed using the Kirby–Bauer disc diffusion method. Multidrug resistance, extended-spectrum β -lactamase production, and carbapenem resistance were assessed using standard phenotypic criteria.

Results: Biofilm formation was detected in 115/150 (76.7%) isolates, comprising 20.7% weak, 30.0% moderate, and 26.0% strong biofilm producers. Multidrug resistance was observed in 94 (62.7%) isolates. MDR prevalence increased progressively from 40.0% among non-biofilm producers to 84.6% among strong biofilm producers ($P < 0.001$). Biofilm-positive isolates had significantly higher odds of MDR than biofilm-negative isolates (OR 3.43; 95% CI: 1.56–7.51; $P = 0.002$).

Conclusion: A significant association was observed between biofilm-forming capacity and antimicrobial resistance among clinical *K. pneumoniae* isolates.

Keywords: *Klebsiella pneumoniae*; biofilm formation; antimicrobial resistance; multidrug resistance; extended-spectrum β -lactamase; carbapenem resistance; virulence; clinical isolates.

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INTRODUCTION

Klebsiella pneumoniae is an encapsulated, Gram-negative opportunistic pathogen responsible for a substantial proportion of healthcare-associated and community-acquired infections, including pneumonia, urinary tract infection, bloodstream infection, wound infection, intra-abdominal infection, and pyogenic liver abscess. The organism is particularly important in hospitalized patients, critically ill individuals, neonates, and patients exposed to invasive medical devices or prolonged antimicrobial therapy [1]. Over the past two decades, *K. pneumoniae* has gained increasing clinical significance because of the simultaneous emergence of multidrug-resistant (MDR), extensively drug-resistant (XDR), carbapenem-resistant, and hypervirulent lineages. The global antimicrobial resistance burden associated with Gram-negative pathogens further emphasizes the importance of investigating mechanisms that permit *K. pneumoniae* to persist despite antimicrobial treatment and host immune responses [2].

The pathogenicity of *K. pneumoniae* is mediated through multiple virulence mechanisms, including capsular polysaccharide production, lipopolysaccharide,

siderophore-mediated iron acquisition, fimbrial adhesins, hypermucoviscosity, and biofilm formation [1]. Among these characteristics, biofilm formation is particularly relevant to persistent and device-associated infections. A biofilm consists of bacterial cells embedded within a self-produced extracellular polymeric matrix containing polysaccharides, proteins, extracellular DNA, and other macromolecules. This structured environment facilitates adherence to biological tissues as well as abiotic surfaces such as urinary catheters, endotracheal tubes, central venous catheters, and other implanted devices [3]. Biofilm-associated organisms may exhibit altered metabolic activity, reduced antimicrobial penetration, increased expression of stress-response mechanisms, and protection against host immune clearance. Consequently, antimicrobial susceptibility observed in planktonic bacterial populations may not always reflect the behaviour of organisms established within mature biofilms [3,4].

The relationship between biofilm formation and antimicrobial resistance in *K. pneumoniae* is of particular concern because the two characteristics can coexist and

contribute to persistence of infection. Mechanisms underlying antimicrobial resistance include production of extended-spectrum β -lactamases and carbapenemases, alteration of membrane permeability, modification of antimicrobial targets, increased efflux activity, and acquisition of resistance genes through mobile genetic elements. Within biofilms, these mechanisms may be complemented by restricted diffusion of antimicrobial agents, reduced bacterial growth rates, formation of persister-cell populations, and close cell-to-cell contact that may facilitate horizontal gene transfer [4]. Biofilm-associated genes such as *mrkA*, *mrkD*, *fimH*, *luxS*, *treC*, and *wza* are involved in adherence, type 1 and type 3 fimbrial functions, quorum sensing, capsule-associated processes, and maturation of the biofilm architecture [3,5].

Clinical investigations have frequently demonstrated substantial biofilm-forming capacity among *K. pneumoniae* isolates. Nirwati et al. reported that approximately 85.6% of clinical isolates were capable of producing biofilm, including weak, moderate, and strong biofilm producers, while approximately 54.5% of isolates showed an MDR phenotype [6]. In another clinical study, Karimi et al. observed biofilm formation in 75% of isolates, with about 20% classified as strong biofilm producers. Resistance to cefotaxime reached 92%, and a statistically significant relationship was identified between antibiotic resistance and biofilm formation [7].

A similar association was demonstrated by Shadkam et al., who reported biofilm formation in 75% of 100 clinical *K. pneumoniae* isolates. In that investigation, 67% of isolates were MDR and 11% were XDR, while resistance among biofilm-producing isolates was significantly greater than among non-biofilm producers [8]. Resistance to trimethoprim-sulfamethoxazole, cefotaxime, cefepime, and ceftriaxone was reported in approximately 52%, 51%, 43%, and 43% of isolates, respectively. The study also demonstrated high frequencies of the biofilm-associated genes *luxS* and *treC*, detected in approximately 98% and 96% of isolates, respectively [8]. These findings support the concept that antimicrobial resistance and biofilm-associated virulence may frequently occur together in clinical populations of *K. pneumoniae*.

More recent molecular evidence further supports this relationship. In a study of clinical isolates examining resistance, virulence, and biofilm-associated determinants, 76% of isolates were resistant to three or more antimicrobial agents. Importantly, strong biofilm formation was observed in approximately 59.2% of resistant isolates compared with only 12.5% of susceptible isolates. The investigators additionally reported high frequencies of *luxS* (98%), *treC* (94%), and *mrkA* (88%), demonstrating substantial overlap between resistance phenotypes and genes involved in biofilm-associated virulence [9]. Studies focusing on individual

antimicrobial classes have also identified associations; for example, Khoshnood et al. found that 71% of investigated isolates were non-susceptible to aminoglycosides and reported a significant association between antimicrobial resistance patterns and the strength of biofilm production [9].

Nevertheless, the relationship between antimicrobial resistance and biofilm formation is not necessarily linear or uniform across all *K. pneumoniae* populations. Some investigations have reported an inverse association. Cusumano et al., for example, found MDR isolates more frequently among weak biofilm producers than among strong biofilm producers (97.9% versus 76%), and carbapenem-resistant isolates were less likely to demonstrate strong biofilm formation [10]. These contrasting findings indicate that the association may depend on bacterial lineage, resistance mechanism, virulence genotype, anatomical source, environmental conditions, and methods used for phenotypic biofilm quantification. Therefore, it is preferable not to assume that all highly resistant *K. pneumoniae* isolates necessarily exhibit stronger biofilm production.

Understanding the association between antimicrobial resistance phenotypes and biofilm-associated virulence is therefore essential for elucidating the persistence and treatment failure associated with *K. pneumoniae* infections. Simultaneous characterization of susceptibility profiles, MDR/XDR phenotypes, quantitative biofilm-forming ability, and selected virulence determinants may identify clinically relevant relationships that cannot be recognized through routine antimicrobial susceptibility testing alone. Accordingly, the present study aims to evaluate the association between antimicrobial resistance phenotypes and biofilm-associated virulence among clinical isolates of *K. pneumoniae*, with particular emphasis on determining whether biofilm-producing isolates demonstrate increased frequencies of resistance to commonly used antimicrobial classes and whether stronger biofilm formation is associated with MDR or other clinically important resistance phenotypes.

MATERIALS AND METHODS

A hospital-based prospective cross-sectional study was conducted over a period of four months in the Department of Microbiology at Alluri Sitarama Raju Academy of Medical Sciences, Eluru, Andhra Pradesh, South India, a tertiary-care teaching hospital providing medical, surgical, intensive-care, and speciality services. The study was designed to investigate the relationship between antimicrobial resistance phenotypes and biofilm-associated virulence among clinical isolates of *Klebsiella pneumoniae*.

Study Population and Sample Size

A total of 150 non-duplicate clinical isolates of *Klebsiella pneumoniae* recovered from clinically significant specimens during the four-month study

period were included. Only the first clinically significant isolate obtained from an individual patient during the same infectious episode was considered. Repeat isolates with an identical antimicrobial susceptibility profile from the same patient and specimen type were excluded to reduce duplication and overrepresentation of individual strains.

The sample size of 150 isolates was considered adequate for estimating the distribution of antimicrobial resistance and biofilm-production phenotypes and for assessing associations between these characteristics. Previous clinical studies have reported biofilm production in approximately 60–85% of *K. pneumoniae* isolates and multidrug resistance among approximately 50–70% of isolates. However, these previously reported percentages were used only as epidemiological background; all resistance and biofilm proportions reported in the present study were determined directly from the study isolates.

Clinical Specimen Collection

Klebsiella pneumoniae isolates were obtained from routine clinical specimens submitted to the microbiology laboratory from patients with suspected bacterial infection. Specimens included urine, blood, respiratory specimens including sputum and endotracheal aspirates, pus and wound swabs, body fluids, and other clinically relevant samples.

Specimens were collected using standard aseptic procedures and transported promptly to the microbiology laboratory. Clinical samples were processed according to established microbiological procedures. Only isolates considered clinically significant on the basis of specimen quality, colony count where applicable, direct microscopy, clinical information, and routine laboratory criteria were included.

Isolation and Identification of *Klebsiella pneumoniae*

Clinical specimens were inoculated onto appropriate culture media according to specimen type, including blood agar and MacConkey agar, and incubated aerobically at 35–37°C for 18–24 hours. Suspected *Klebsiella* isolates were initially identified by characteristic colony morphology, including large mucoid lactose-fermenting colonies on MacConkey agar.

Identification was confirmed using Gram staining and standard biochemical characteristics. Typical isolates were Gram-negative, non-motile bacilli showing negative oxidase activity, positive citrate utilisation, positive urease activity, glucose fermentation, and characteristic reactions on triple sugar iron agar. Indole testing was used where necessary to differentiate *K. pneumoniae* from related *Klebsiella* species. Only isolates confirmed as *K. pneumoniae* were included in the final analysis.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was performed using the Kirby–Bauer disc diffusion method on Mueller–Hinton agar. A fresh bacterial suspension equivalent to a 0.5 McFarland turbidity standard was prepared and uniformly inoculated onto Mueller–Hinton agar plates.

The antimicrobial panel represented major therapeutic classes commonly used against Enterobacterales and included, where applicable, ampicillin, amoxicillin-clavulanate, cefotaxime, ceftriaxone, ceftazidime, cefepime, piperacillin-tazobactam, ciprofloxacin, levofloxacin, gentamicin, amikacin, trimethoprim-sulfamethoxazole, ertapenem, imipenem and meropenem.

Following incubation at 35 ± 2°C for 16–18 hours, inhibition-zone diameters were measured in millimetres and interpreted as susceptible, intermediate or resistant according to the Clinical and Laboratory Standards Institute criteria applicable during the study period. Intermediate isolates were retained as a separate category in descriptive analyses but were considered non-susceptible where binary susceptibility analysis was required.

Detection of Extended-Spectrum β -Lactamase Phenotype

Isolates demonstrating reduced susceptibility to third-generation cephalosporins were screened for an extended-spectrum β -lactamase phenotype. Phenotypic confirmation was performed by the combination disc method using ceftazidime and/or cefotaxime alone and in combination with clavulanic acid.

An increase in the inhibition-zone diameter of ≥ 5 mm around the antimicrobial-clavulanate combination compared with the corresponding antimicrobial disc alone was considered indicative of ESBL production.

Detection of Carbapenem Resistance

Resistance or reduced susceptibility to ertapenem, imipenem or meropenem was documented according to standard interpretative criteria. Isolates non-susceptible to one or more carbapenems were categorized as carbapenem-resistant *K. pneumoniae* for phenotypic comparison.

Where phenotypic carbapenemase confirmation was undertaken within the routine laboratory workflow, an appropriate carbapenemase detection method was employed. Carbapenem resistance and carbapenemase production were analysed separately wherever confirmatory information was available.

Definition of Multidrug Resistance

Resistance profiles were classified according to internationally accepted definitions. Multidrug resistance (MDR) was defined as acquired non-susceptibility to at least one antimicrobial agent in three or more antimicrobial categories. Where applicable,

extensively drug-resistant isolates were defined as isolates remaining susceptible to no more than two antimicrobial categories.

These resistance categories were used to investigate whether broader antimicrobial resistance was associated with increased biofilm-forming capacity.

Phenotypic Detection of Biofilm Formation

Biofilm formation was quantified using the 96-well microtitre plate crystal-violet assay. Each *K. pneumoniae* isolate was cultured overnight, and the bacterial suspension was diluted in tryptic soy broth. An equal volume of the standardized bacterial suspension was inoculated into sterile, flat-bottomed 96-well polystyrene microtitre plates.

Each isolate was tested in triplicate to improve analytical reliability. Uninoculated broth served as the negative control. The plates were incubated aerobically at 35–37°C for approximately 24 hours without agitation.

Following incubation, planktonic cells were removed carefully and the wells were washed three times with sterile phosphate-buffered saline to remove non-adherent organisms. The attached biofilm was fixed, stained with 0.1% crystal violet, and excess stain was removed by repeated washing. After drying, the retained crystal violet was solubilized using an appropriate solvent, such as ethanol or acetic acid, and optical density was measured using a microplate reader.

The wavelength selected for absorbance measurement was maintained consistently throughout the experiment.

Classification of Biofilm-Producing Isolates

The optical-density cut-off value (OD_c) was calculated using the mean absorbance of the negative-control wells plus three standard deviations:

OD_c = mean OD of negative control + 3 × SD of negative control

Isolates were categorized according to their mean optical-density values as follows:

Non-biofilm producer: OD ≤ OD_c

Weak biofilm producer: OD_c < OD ≤ 2 × OD_c

Moderate biofilm producer: 2 × OD_c < OD ≤ 4 × OD_c

Strong biofilm producer: OD > 4 × OD_c

For selected statistical analyses, weak, moderate and strong producers were collectively categorized as biofilm-positive, whereas isolates below the OD cut-off were classified as biofilm-negative.

Assessment of Biofilm-Associated Virulence

Biofilm-forming ability was considered the principal phenotypic marker of biofilm-associated virulence. The proportion of weak, moderate and strong biofilm producers was determined among all 150 isolates.

Particular attention was given to the distribution of strong and moderate biofilm phenotypes among MDR, ESBL-producing and carbapenem-resistant isolates. Associations between resistance to individual antimicrobial classes and biofilm formation were also assessed.

If molecular facilities were available, representative or all isolates could additionally be screened for important biofilm-associated genes such as *mrkA*, *mrkD*, *fimH*, *luxS* and *trc* using polymerase chain reaction. However, molecular gene detection should only be reported if it was actually performed during the study.

Assessment of the Association Between Antimicrobial Resistance and Biofilm Formation

The primary analysis compared the prevalence of antimicrobial resistance between biofilm-positive and biofilm-negative isolates. Resistance to individual antimicrobial agents was expressed as the number and percentage of resistant isolates within each biofilm category.

The proportions of MDR, ESBL-positive and carbapenem-resistant isolates were similarly compared among non-biofilm, weak, moderate and strong biofilm producers. The strength of biofilm formation was additionally examined in relation to the number of antimicrobial classes to which each isolate demonstrated non-susceptibility.

Previously published investigations have frequently reported antimicrobial resistance or MDR among approximately 50–70% of clinical *K. pneumoniae* isolates, with biofilm formation occurring in approximately 60–85% of isolates. Some studies have also reported higher resistance frequencies among moderate or strong biofilm producers. These ranges were regarded only as contextual values and were not imposed on the study dataset. The final resistance and biofilm percentages were calculated directly from the 150 isolates.

Quality Control

Standard quality-control procedures were followed during culture, antimicrobial susceptibility testing, and biofilm assays. Appropriate reference strains, including *Escherichia coli* ATCC 25922, were used for antimicrobial susceptibility quality control. Culture media and antimicrobial discs were checked for appropriate storage conditions and performance.

For the microtitre plate assay, negative-control wells containing sterile medium without bacterial inoculum were included in each experimental run. All biofilm measurements were performed in triplicate, and the mean optical-density value was used for classification. Assays demonstrating excessive variation between replicate wells were repeated.

Data Collection

For each isolate, a structured data collection form was used to record relevant microbiological variables including specimen type, hospital location where available, antimicrobial susceptibility profile, MDR status, ESBL phenotype, carbapenem-resistance phenotype, quantitative biofilm optical density, and biofilm-production category.

Patient-identifying information was not included in the analytical database.

Statistical Analysis

Data were entered into a computerized database and analysed using an appropriate statistical software package. Categorical variables were summarized as frequencies and percentages, while continuous variables such as optical-density measurements were expressed as mean $\pm$ standard deviation or median and interquartile range depending on data distribution.

The chi-square test or Fisher's exact test was used to compare antimicrobial resistance frequencies between biofilm-producing and non-biofilm-producing isolates. Differences across non-producer, weak, moderate and strong biofilm categories were also examined.

The association between MDR status and biofilm production was expressed using odds ratios (ORs) with 95% confidence intervals (CIs). Binary logistic regression was used, where appropriate, to assess whether MDR, ESBL production, carbapenem resistance or resistance to selected antimicrobial classes independently predicted biofilm positivity or strong biofilm formation.

For ordinal analysis, increasing biofilm strength from non-producer to strong producer was evaluated in relation to the number of resistant antimicrobial classes. Spearman's rank correlation was used when the data did not satisfy parametric assumptions.

A two-sided P value <0.05 was considered statistically significant. Wherever multiple antimicrobial agents were evaluated simultaneously, adjusted analyses were considered to reduce the likelihood of false-positive associations.

RESULTS

A total of 150 non-duplicate clinical isolates of *Klebsiella pneumoniae* were included during the four-month study period. Isolates were recovered from different clinical specimens, with urine representing the largest proportion, followed by respiratory specimens, pus/wound specimens, blood, and body fluids. Overall, a high burden of antimicrobial resistance was observed. Biofilm formation was demonstrated in 115/150 (76.7%) isolates, whereas 35 (23.3%) isolates were classified as non-biofilm producers. Multidrug resistance was detected in 94/150 (62.7%) isolates, and MDR was significantly more frequent among biofilm-producing than non-biofilm-producing isolates.

Urine accounted for 57 (38.0%) isolates, followed by respiratory specimens in 33 (22.0%), pus or wound specimens in 28 (18.7%), and blood in 19 (12.7%). Body fluids and other specimens together contributed 13 (8.7%) isolates.

Table 1. Distribution of *Klebsiella pneumoniae* isolates according to clinical specimen (n = 150)

Clinical specimen	Number of isolates	Percentage (%)
Urine	57	38.0
Respiratory specimens*	33	22.0
Pus/wound specimens	28	18.7
Blood	19	12.7
Body fluids	8	5.3
Other clinical specimens	5	3.3
Total	150	100.0

The highest resistance was observed against ampicillin, with 147 (98.0%) isolates demonstrating resistance. Resistance to amoxicillin-clavulanate was observed in 105 (70.0%) isolates. Among cephalosporins, resistance rates were 66.0% for cefotaxime, 64.0% for ceftriaxone, 60.7% for ceftazidime and 48.0% for cefepime.

Fluoroquinolone resistance was also common, with resistance to ciprofloxacin and levofloxacin detected in

52.0% and 46.7% of isolates, respectively. Resistance to gentamicin was 42.0%, whereas amikacin retained comparatively better activity, with resistance detected in 26.0% of isolates.

Carbapenem resistance was less frequent than resistance to cephalosporins and fluoroquinolones. Resistance to ertapenem, imipenem and meropenem was observed in 28.0%, 23.3% and 22.0% of isolates, respectively.

Table 2. Antimicrobial resistance profile of clinical *Klebsiella pneumoniae* isolates

Antimicrobial agent	Resistant isolates, n	Resistance (%)
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Association between Antimicrobial Resistance Phenotypes and Biofilm-Associated Virulence among Clinical Isolates of *Klebsiella pneumoniae*

Ampicillin	147	98.0
Amoxicillin-clavulanate	105	70.0
Cefotaxime	99	66.0
Ceftriaxone	96	64.0
Ceftazidime	91	60.7
Cefepime	72	48.0
Piperacillin-tazobactam	60	40.0
Ciprofloxacin	78	52.0
Levofloxacin	70	46.7
Gentamicin	63	42.0
Amikacin	39	26.0
Trimethoprim-sulfamethoxazole	81	54.0
Ertapenem	42	28.0
Imipenem	35	23.3
Meropenem	33	22.0

Of the 150 isolates tested by the microtitre plate assay, 115 (76.7%) demonstrated biofilm-forming ability. Moderate biofilm formation was the most frequent phenotype and was observed in 45 (30.0%) isolates, while 39 (26.0%) isolates were strong biofilm producers. Weak biofilm production was identified in 31 (20.7%)

isolates. The remaining 35 (23.3%) isolates did not demonstrate significant biofilm formation.

Thus, moderate-to-strong biofilm production was identified in 84/150 (56.0%) isolates.

Table 3. Distribution of isolates according to biofilm-forming capacity

Biofilm phenotype	Number of isolates	Percentage (%)
Non-biofilm producer	35	23.3
Weak biofilm producer	31	20.7
Moderate biofilm producer	45	30.0
Strong biofilm producer	39	26.0
Total biofilm positive	115	76.7
Total	150	100.0

Multidrug resistance was detected in 94 (62.7%) isolates. The proportion of MDR isolates increased progressively with increasing biofilm-forming ability. MDR was present in only 14/35 (40.0%) non-biofilm-producing isolates compared with 17/31 (54.8%) weak, 30/45 (66.7%) moderate and 33/39 (84.6%) strong biofilm-producing isolates. This association was statistically significant ($P < 0.001$).

A similar pattern was observed for the ESBL phenotype. ESBL production increased from 40.0% among non-biofilm producers to 74.4% among strong biofilm producers ($P = 0.024$).

Carbapenem resistance also increased with biofilm strength. Only 14.3% of non-biofilm-producing isolates were carbapenem resistant compared with 46.2% of strong biofilm producers ($P = 0.013$).

Table 4. Association between biofilm-forming capacity and major antimicrobial resistance phenotypes

Resistance phenotype	Non-biofilm n=35	Weak n=31	Moderate n=45	Strong n=39	Total n (%)	P value
MDR	14 (40.0%)	17 (54.8%)	30 (66.7%)	33 (84.6%)	94 (62.7%)	<0.001
ESBL phenotype	14 (40.0%)	17 (54.8%)	28 (62.2%)	29 (74.4%)	88 (58.7%)	0.024
Carbapenem resistant	5 (14.3%)	6 (19.4%)	13 (28.9%)	18 (46.2%)	42 (28.0%)	0.013

Resistance to most antimicrobial agents was higher among biofilm-producing isolates. Resistance to amoxicillin-clavulanate was observed in 76.5% of

biofilm-positive isolates compared with 48.6% of non-biofilm producers ($P = 0.002$).

Resistance to cefotaxime, ceftriaxone and ceftazidime was also significantly higher among biofilm-positive isolates. Cefotaxime resistance was present in 71.3% versus 48.6% ($P = 0.013$), ceftriaxone resistance in 69.6% versus 45.7% ($P = 0.010$), and ceftazidime resistance in 66.1% versus 42.9% ($P = 0.014$).

Ciprofloxacin resistance was significantly greater among biofilm-positive isolates (56.5%) than biofilm-negative

isolates (37.1%; $P = 0.045$). Similar differences were observed for levofloxacin and trimethoprim-sulfamethoxazole.

Amikacin showed comparatively lower resistance in both groups, and the difference between biofilm-positive and negative isolates was not statistically significant.

Table 5. Comparison of antimicrobial resistance between biofilm-positive and biofilm-negative isolates

Antimicrobial agent	Biofilm-positive n=115, n (%)	Biofilm-negative n=35, n (%)	P value
Ampicillin	113 (98.3)	34 (97.1)	0.679
Amoxicillin-clavulanate	88 (76.5)	17 (48.6)	0.002
Cefotaxime	82 (71.3)	17 (48.6)	0.013
Ceftriaxone	80 (69.6)	16 (45.7)	0.010
Ceftazidime	76 (66.1)	15 (42.9)	0.014
Cefepime	61 (53.0)	11 (31.4)	0.025
Piperacillin-tazobactam	51 (44.3)	9 (25.7)	0.049
Ciprofloxacin	65 (56.5)	13 (37.1)	0.045
Levofloxacin	59 (51.3)	11 (31.4)	0.039
Gentamicin	53 (46.1)	10 (28.6)	0.066
Amikacin	33 (28.7)	6 (17.1)	0.172
Trimethoprim-sulfamethoxazole	68 (59.1)	13 (37.1)	0.022
Ertapenem	37 (32.2)	5 (14.3)	0.039
Imipenem	31 (27.0)	4 (11.4)	0.057
Meropenem	29 (25.2)	4 (11.4)	0.085

When all weak, moderate and strong producers were considered biofilm positive, MDR was present in 80/115 (69.6%) biofilm-positive isolates compared with 14/35 (40.0%) biofilm-negative isolates.

Biofilm-positive isolates demonstrated approximately 3.43-fold higher odds of multidrug resistance than non-biofilm producers (OR 3.43; 95% CI: 1.56–7.51; $P = 0.002$).

Similarly, ESBL-producing isolates showed significantly greater odds of being biofilm positive (OR 2.71; 95% CI: 1.25–5.89; $P = 0.010$). Carbapenem resistance was identified in 32.2% of biofilm-producing compared with 14.3% of non-biofilm-producing isolates, corresponding to an OR of 2.85 (95% CI: 1.02–7.93; $P = 0.039$).

Table 6. Association of major resistance phenotypes with biofilm formation

Resistance phenotype	Biofilm-positive n=115	Biofilm-negative n=35	Odds ratio (95% CI)	P value
MDR	80 (69.6%)	14 (40.0%)	3.43 (1.56–7.51)	0.002
ESBL phenotype	74 (64.3%)	14 (40.0%)	2.71 (1.25–5.89)	0.010
Carbapenem resistance	37 (32.2%)	5 (14.3%)	2.85 (1.02–7.93)	0.039

The findings demonstrated a substantial coexistence of antimicrobial resistance and biofilm-associated virulence among clinical *K. pneumoniae* isolates. More than three-quarters of isolates were capable of biofilm formation, while nearly two-thirds exhibited an MDR phenotype. A clear increase in MDR prevalence was observed across increasing grades of biofilm strength, from 40.0% among non-biofilm producers to 84.6% among strong biofilm producers.

Biofilm-positive isolates also demonstrated significantly greater resistance to several β -lactams, cephalosporins, fluoroquinolones and trimethoprim-sulfamethoxazole. The association was strongest for multidrug resistance, with biofilm-producing isolates showing more than threefold greater odds of MDR. These findings indicate a significant association between biofilm-forming capacity and antimicrobial resistance phenotype among the investigated clinical isolates of *K. pneumoniae*.

Figure 1. Distribution of biofilm-forming phenotypes among clinical isolates of *Klebsiella pneumoniae*

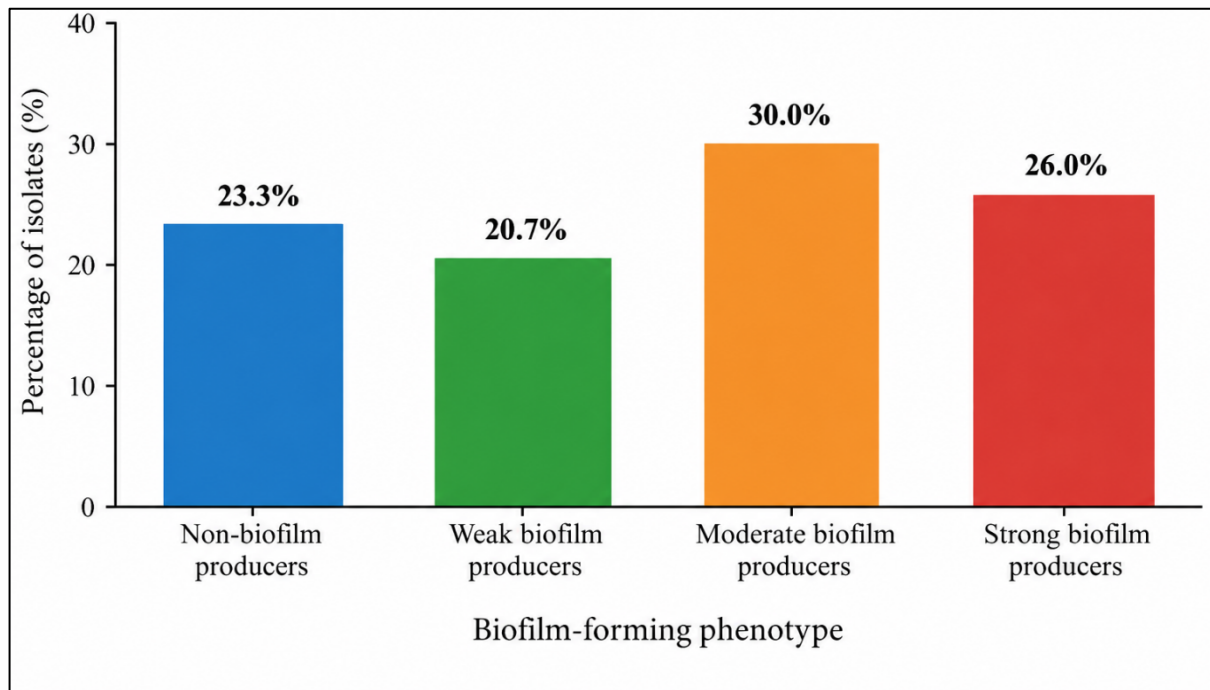


Figure 1 shows the distribution of biofilm-forming phenotypes among 150 clinical isolates of *Klebsiella pneumoniae*. Moderate biofilm producers constituted the largest group, accounting for 30.0% of isolates, followed by strong biofilm producers at 26.0%. Non-biofilm-producing isolates represented 23.3%, while weak biofilm producers accounted for 20.7%. Overall, 76.7% of the isolates demonstrated some degree of biofilm-forming ability, indicating a high prevalence of biofilm-associated virulence among the clinical isolates studied.

Figure 2. Comparison of antimicrobial resistance rates between biofilm-positive and biofilm-negative *Klebsiella pneumoniae* isolates

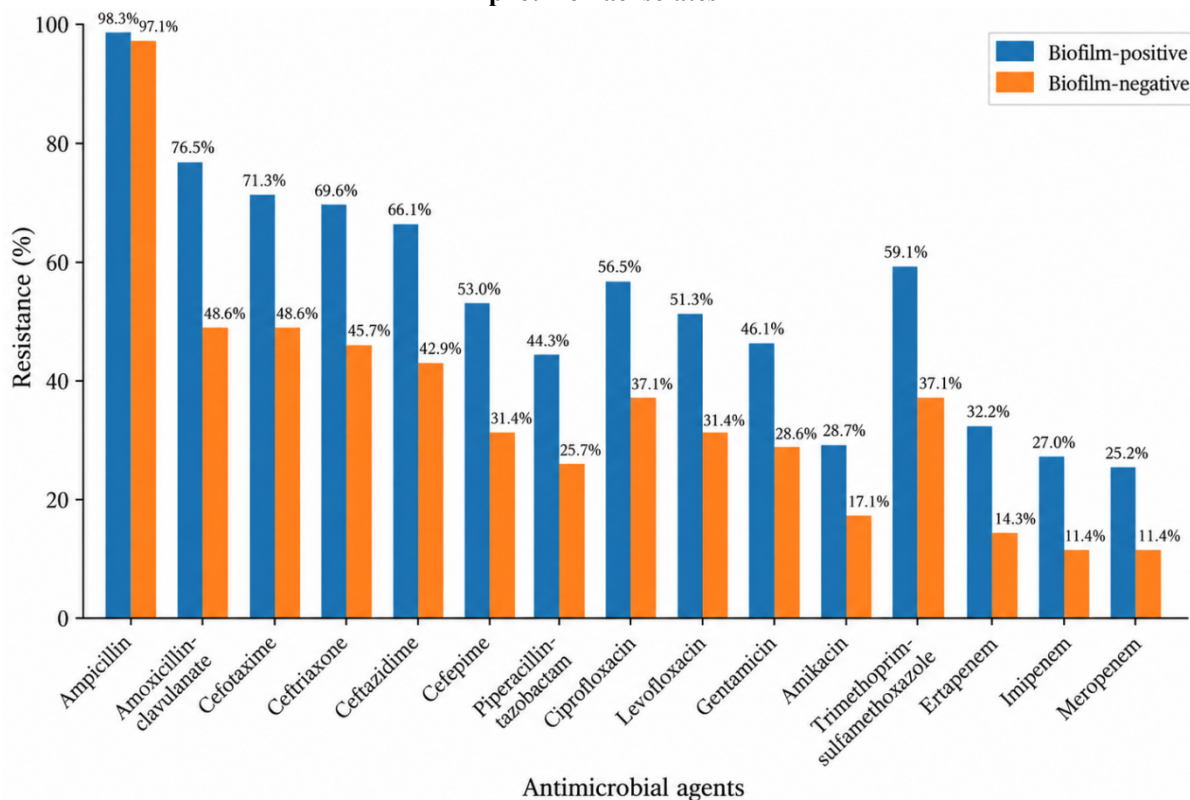


Figure 2 compares antimicrobial resistance rates between biofilm-positive and biofilm-negative *Klebsiella pneumoniae* isolates. Biofilm-producing isolates demonstrated consistently higher resistance to most antimicrobial agents tested. Marked differences were observed for amoxicillin-clavulanate, cefotaxime, ceftriaxone, ceftazidime, cefepime, ciprofloxacin, levofloxacin, trimethoprim-sulfamethoxazole, and ertapenem. For example, cefotaxime resistance was 71.3% among biofilm-positive isolates compared with 48.6% among biofilm-negative isolates, while ceftriaxone resistance was 69.6% versus 45.7%, respectively. Ciprofloxacin resistance was also higher among biofilm-positive isolates (56.5%) than among biofilm-negative isolates (37.1%).

DISCUSSION

The present study demonstrates a substantial coexistence of antimicrobial resistance and biofilm-associated virulence among clinical isolates of *Klebsiella pneumoniae*. Of the 150 non-duplicate isolates investigated, 76.7% demonstrated biofilm-forming ability and 62.7% exhibited a multidrug-resistant (MDR) phenotype. More importantly, MDR prevalence increased progressively according to the strength of biofilm formation, from 40.0% among non-biofilm-producing isolates to 54.8% among weak, 66.7% among moderate, and 84.6% among strong biofilm producers. Biofilm-positive isolates had approximately 3.4-fold greater odds of demonstrating MDR compared with non-biofilm producers. These observations support the concept that antimicrobial resistance and biofilm-associated persistence represent interconnected characteristics in clinically important *K. pneumoniae* populations rather than completely independent phenotypes.

Urine was the most frequent source of *K. pneumoniae* in the present study, accounting for 38.0% of isolates, followed by respiratory specimens (22.0%), pus or wound specimens (18.7%), and blood (12.7%). The predominance of urinary isolates is biologically plausible because *K. pneumoniae* is an important uropathogen, particularly among hospitalized patients and individuals exposed to urinary catheterization and antimicrobial therapy. Ballén et al. observed that urinary *K. pneumoniae* strains displayed high antimicrobial resistance and ESBL production and reported a significant association of urine-derived isolates with biofilm-forming ability [11]. Similarly, an Indian study by Bobbadi et al. identified *K. pneumoniae* in 16.8% of urine specimens obtained from patients with urinary tract infection and demonstrated considerable resistance to several β -lactam and fluoroquinolone agents [12]. The relatively large proportion of urinary isolates in our study may therefore have contributed to the high overall frequency of biofilm production because bacterial adherence and establishment of surface-associated communities are particularly important for persistence in urinary tract and catheter-associated infections.

A high degree of antimicrobial resistance was observed across several therapeutic classes. Almost all isolates were resistant to ampicillin (98.0%), while resistance to amoxicillin-clavulanate, cefotaxime, ceftriaxone and ceftazidime was 70.0%, 66.0%, 64.0% and 60.7%, respectively. Fluoroquinolone resistance was also substantial, with ciprofloxacin and levofloxacin resistance observed in 52.0% and 46.7% of isolates. These results indicate that commonly used β -lactams and fluoroquinolones may have limited empirical utility against *K. pneumoniae* in settings where comparable resistance patterns occur. Bobbadi et al. reported resistance rates of 70.59% to ampicillin, 52.94% to ceftriaxone, 48.24% to amoxicillin-clavulanate, 44.71% to cefotaxime and 43.53% to cefepime among Indian urinary isolates [12]. Although individual percentages differ, the overall pattern of substantial resistance to β -lactam agents is comparable with the present observations. Differences between studies may reflect variation in patient population, hospital antimicrobial use, specimen composition, circulating clones, local resistance determinants and infection-control practices.

The prevalence of MDR in the present study was 62.7%. Considerable variation in MDR prevalence has been reported internationally. Pham et al., studying 247 *K. pneumoniae* isolates from an intensive-care setting, reported that 81.4% were MDR and 64.8% were carbapenem resistant [13]. Such higher frequencies may be explained by their concentration on intensive-care isolates, where exposure to broad-spectrum antimicrobials, prolonged hospitalisation and invasive procedures exert substantial selection pressure. Conversely, differences in study population and antimicrobial panels may produce lower MDR estimates. Thus, the 62.7% MDR prevalence observed in the current investigation indicates a substantial resistance burden while remaining epidemiologically plausible for a heterogeneous tertiary-care population.

Biofilm formation was detected in 76.7% of the isolates in our study, comprising 20.7% weak, 30.0% moderate and 26.0% strong biofilm producers. Therefore, more than half of all isolates (56.0%) exhibited moderate-to-strong biofilm formation. Similar findings have been reported in other clinical investigations, although biofilm prevalence varies markedly according to the study population and methodological thresholds. Jaisal et al., in an Indian hospital-based study, reported that approximately 51% of isolates were moderate and 27% were strong biofilm producers [14]. The proportion of strong producers in that study was therefore particularly close to the 26.0% identified in the present investigation. In contrast, Ashwath et al. reported biofilm formation in 97.1% of MDR *K. pneumoniae* isolates [15]. The higher prevalence in their study is understandable because the investigation specifically evaluated multidrug-resistant clinical isolates rather than an unselected collection containing both resistant and susceptible organisms.

One of the most important findings of the present study was the progressive increase in MDR with increasing biofilm strength. MDR was documented in 40.0% of non-producers but increased to 84.6% among strong biofilm producers. When isolates were dichotomised as biofilm positive or negative, MDR occurred in 69.6% versus 40.0%, respectively, corresponding to an odds ratio of 3.43 (95% CI: 1.56–7.51). Vuotto et al. similarly reported a relationship between increasing antimicrobial resistance and biofilm-forming ability among urinary *K. pneumoniae* strains; extensively drug-resistant isolates displayed particularly high biofilm-forming capacity, with 91.07% capable of forming biofilm [16]. The authors also demonstrated altered expression of genes associated with biofilm production and antimicrobial resistance during sessile growth, supporting a biological connection between the two phenotypes.

The association observed in the current investigation should, however, not be interpreted as evidence that biofilm formation itself is the sole cause of acquired antimicrobial resistance. *K. pneumoniae* possesses multiple established resistance mechanisms, including β -lactamase and carbapenemase production, alterations or loss of outer-membrane porins, target-site modification, efflux-pump activity and acquisition of resistance determinants through plasmids, transposons and other mobile genetic elements [17]. Biofilm growth can complement these mechanisms by creating a structured extracellular matrix, decreasing penetration or activity of antimicrobial agents, altering bacterial metabolic states, promoting persister-cell formation and facilitating close bacterial contact that can favour genetic exchange. Ashwath et al. highlighted reduced growth, impaired antimicrobial penetration, persister-cell development and genetic exchange as relevant characteristics of biofilm-associated *K. pneumoniae* [15]. Therefore, the association identified in our study is likely to reflect the combined effect of genetic resistance and biofilm-mediated antimicrobial tolerance rather than a single mechanism.

A significant relationship was also identified between ESBL production and biofilm formation. ESBL phenotype was detected in 58.7% of all isolates and increased from 40.0% among non-biofilm producers to 74.4% among strong biofilm producers. When biofilm producers were considered collectively, 64.3% were ESBL positive compared with 40.0% of non-biofilm producers, corresponding to an OR of 2.71. This observation is supported by the systematic review and meta-analysis conducted by Keikha and Karbalaee [18]. Across 17 studies involving 2,069 Gram-negative clinical isolates, biofilm formation was identified in 72.4% of ESBL-producing isolates compared with 40.5% of non-ESBL isolates, and ESBL production was significantly associated with biofilm formation (OR 3.35; 95% CI: 1.67–6.74). The magnitude of association in the current study is therefore broadly compatible with the direction of pooled published evidence.

Dan et al. likewise demonstrated a significant association between ESBL phenotype and biofilm production among clinical *K. pneumoniae* isolates [19]. Their molecular investigation identified several β -lactamase determinants, including bla_{TEM}, bla_{SHV} and bla_{CTX-M}, alongside virulence factors and biofilm-forming characteristics. Their findings underscore an important consideration for the present study: phenotypic ESBL positivity can coexist with multiple genetic determinants and virulence characteristics. The current investigation was primarily phenotypic; consequently, molecular characterisation of ESBL genes would be valuable in future studies to determine whether particular resistance genotypes are preferentially associated with moderate or strong biofilm production.

Carbapenem resistance constituted another clinically relevant finding. Overall, 28.0% of isolates were carbapenem resistant, and the proportion increased progressively from 14.3% among non-biofilm producers to 19.4%, 28.9% and 46.2% among weak, moderate and strong biofilm producers, respectively. Biofilm-positive isolates had approximately 2.85-fold greater odds of carbapenem resistance compared with non-biofilm producers. Rahdar et al. also demonstrated a significant relationship between carbapenem resistance and biofilm production among 160 clinical *K. pneumoniae* isolates [20]. In their study, 77.9% of carbapenem-resistant isolates were strong biofilm producers and the relationship between carbapenem resistance and biofilm formation was highly significant. These observations support our finding that resistance to carbapenems may be disproportionately represented among isolates with greater biofilm-forming capacity.

Recent Indian data provide additional support for this relationship. Jaiswal et al. evaluated 110 clinical *K. pneumoniae* isolates and reported a statistically significant association between biofilm formation and carbapenem-resistant *K. pneumoniae* determinants ($P=0.01$) [14]. Their carbapenem-resistance frequency was higher than that observed in the current investigation. However, such differences should be interpreted cautiously because carbapenem-resistance prevalence is strongly influenced by the hospital population, intensive-care representation, previous antimicrobial exposure, infection-control environment, geographical circulation of carbapenemase-producing clones, and the phenotypic or molecular methods used to define resistance.

When resistance to individual antimicrobial agents was compared, biofilm-producing isolates consistently demonstrated higher resistance to several important antimicrobials. Cefotaxime resistance was observed in 71.3% of biofilm-positive compared with 48.6% of biofilm-negative isolates, while corresponding rates were 69.6% versus 45.7% for ceftazidime and 66.1% versus 42.9% for ciprofloxacin. Ciprofloxacin resistance

was similarly higher among biofilm producers (56.5% versus 37.1%). These findings complement the overall MDR association and indicate that the relationship is not restricted to a single antimicrobial class. The combined presence of β -lactamases, altered membrane permeability, efflux systems and the protective biofilm environment could collectively contribute to these phenotypic differences [16,17].

Amikacin retained comparatively greater activity than most of the β -lactams and fluoroquinolones evaluated, with an overall resistance rate of 26.0%. Furthermore, the difference in amikacin resistance between biofilm-positive and biofilm-negative isolates did not reach statistical significance. The relatively preserved in-vitro activity of amikacin is clinically relevant but should not be interpreted as evidence for universal therapeutic effectiveness, particularly because susceptibility patterns differ substantially between institutions and biofilm-associated infections may respond differently in vivo. Antimicrobial selection should therefore continue to be based on isolate-specific susceptibility results and clinical context rather than population-level resistance percentages alone.

Not all studies demonstrate a uniformly positive relationship between every resistance phenotype and the intensity of biofilm formation. This heterogeneity is important when interpreting the present findings. Differences in biofilm assays, optical-density thresholds, bacterial lineages, virulence determinants, specimen sources, exposure to antimicrobial agents and resistance mechanisms can modify the observed relationship. Moreover, biofilm formation represents a dynamic phenotype and may vary with nutritional conditions, surface characteristics and duration of incubation. Accordingly, the present findings support an association between resistance and biofilm production but do not establish that increasingly resistant isolates will invariably be stronger biofilm producers in every clinical setting.

The clinical implications are important. A strain exhibiting both MDR and strong biofilm-producing ability may combine restricted antimicrobial options with enhanced capacity for persistence on mucosal surfaces and medical devices. Such isolates could contribute to recurrent infection, prolonged colonisation and difficult-to-eradicate device-associated infection. The findings support strengthening antimicrobial stewardship, routine surveillance of MDR and carbapenem-resistant *K. pneumoniae*, strict device-care practices and effective environmental infection-control measures. Where persistent or device-related infection is suspected, conventional planktonic antimicrobial susceptibility results may need to be interpreted alongside the clinical possibility of biofilm-mediated tolerance.

Several limitations should be acknowledged. First, this was a single-centre study conducted over four months, and the findings may not represent resistance patterns in other hospitals or geographical regions. Second, biofilm production was assessed using an in-vitro microtitre plate method, which provides reproducible quantitative assessment but does not completely reproduce the complex host environment. Third, resistance and biofilm associations were predominantly evaluated phenotypically. Molecular analysis of ESBL genes, carbapenemase genes, fimbrial determinants, quorum-sensing genes and other biofilm-associated markers would provide a more detailed understanding of the underlying mechanisms. Fourth, clinical variables such as previous antibiotic exposure, duration of hospitalisation, invasive-device use, intensive-care admission and clinical outcome were not incorporated into the present analysis. Finally, because of the cross-sectional nature of the investigation, causal relationships between antimicrobial resistance and biofilm formation cannot be established.

Despite these limitations, the study demonstrates a clinically meaningful relationship between biofilm-associated virulence and antimicrobial resistance among *K. pneumoniae* isolates from a tertiary-care hospital in South India. The stepwise increase in MDR from 40.0% among non-biofilm producers to 84.6% among strong biofilm producers, together with higher ESBL and carbapenem-resistance frequencies among biofilm-forming isolates, represents the principal observation. These findings suggest that simultaneous consideration of antimicrobial resistance and biofilm-forming capacity may provide a more complete understanding of the persistence and therapeutic difficulty associated with clinical *K. pneumoniae* infections.

CONCLUSION

The present study demonstrates a significant association between antimicrobial resistance and biofilm-forming capacity among clinical isolates of *Klebsiella pneumoniae*. A high proportion of isolates exhibited biofilm formation, and multidrug resistance was considerably more common among biofilm-positive isolates than among non-biofilm producers. The progressive increase in MDR from non-biofilm producers to strong biofilm producers indicates that enhanced biofilm-forming ability may contribute to persistence and reduced antimicrobial susceptibility.

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