

Biofilm Positivity in Clinical *Klebsiella Pneumoniae* isolates and Its Associations with Carbapenem Resistance and mrkD, fimH and wcaG Carriage: A Cross-Sectional Study from Egypt

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Abstract

Background

Antimicrobial resistance and biofilm formation contribute to the persistence and treatment difficulty of healthcare-associated infections caused by *Klebsiella pneumoniae*. This study investigated the association between carbapenem resistance, biofilm-forming capacity, and selected biofilm-associated genes among clinical *Klebsiella pneumoniae* isolates from intensive care units.

Methods

This cross-sectional study included 87 non-duplicate clinical *Klebsiella pneumoniae* isolates collected from intensive care units (ICUs) at Tanta University Hospitals, Egypt, from January 2025 to June 2025. Antimicrobial susceptibility was assessed using disk diffusion, with colistin susceptibility determined by broth disk elution. Biofilm formation was quantified using a crystal-violet microtiter-plate assay and classified as none/weak, moderate, or strong. For binary analysis, moderate/strong producers were classified as biofilm positive. Polymerase chain reaction (PCR) was used to detect *mrkD*, *fimH*, and *wcaG*. Associations with biofilm positivity were evaluated using multivariable logistic regression.

Results

Among the 87 isolates, 57 (65.5%) were carbapenem-resistant, 51 (58.6%) met the study definition of multi-drug resistant, and 30 (34.5%) met the study definition of extensively drug-resistant. Biofilm positivity was observed in 54 isolates (62.1%), including 48 moderate and 6 strong producers. The prevalence of *wcaG*, *fimH*, and *mrkD* was 74.7%, 67.8%, and 62.1%, respectively. Biofilm positivity was more frequent among carbapenem-resistant isolates than non-carbapenem-resistant isolates [45/57 (78.9%) vs. 9/30 (30.0%), $P < 0.001$]. In the adjusted model, carbapenem resistance, *mrkD*, *fimH*, and *wcaG* carriage were each associated with higher odds of biofilm positivity.

Conclusion

Biofilm formation and carbapenem resistance were common among ICU-associated *Klebsiella pneumoniae* isolates in this single-center study. Carbapenem-resistant status and carriage of *mrkD*, *fimH*, and *wcaG* were independently associated with biofilm positivity after adjustment for the other prespecified predictors. Because of the cross-sectional design, modest sample size, targeted genetic analysis, and absence of clonal typing, these findings should be interpreted as associations rather than evidence of causality.

Background

Healthcare-associated infections (HAIs) remain an important challenge in clinical practice, particularly as antimicrobial resistance (AMR) increasingly limits the effectiveness of available therapies, representing an escalating threat to global public health. [1, 2] Among the pathogens of greatest concern is *Klebsiella pneumoniae* (*K. pneumoniae*), a member of the critical priority 'ESKAPE' group (*Enterococcus faecium*, *Staphylococcus aureus*, *K. pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.) that is considered a prominent cause of severe HAIs, including pneumonia, bloodstream infections, and sepsis. [3]

The clinical management of *K. pneumoniae* is complicated by its capability of acquiring resistance to multiple antimicrobial classes. The rising prevalence of extended-spectrum β -lactamases (ESBLs) and AmpC β -lactamases producing strains has extensively reduced the efficacy of many β -lactam antibiotics and forced reliance on carbapenems for infections caused by multidrug-resistant strains. [4, 5] Subsequently, carbapenem-resistant *K. pneumoniae* (CRKP) has evolved as an important clinical concern. Carbapenem resistance may arise through the acquisition of carbapenemases, including KPC, NDM, and OXA-48-type enzymes, or through non-enzymatic mechanisms such as reduced membrane permeability and increased efflux-pump activity. [6]

The ability of *K. pneumoniae* to form biofilms represents an additional challenge of its antimicrobial resistance. Biofilms consists of microbial communities where bacterial cells are embedded within a self-produced extracellular polymeric substance (EPS) matrix, which can increase bacterial persistence by limiting antimicrobial activity and enhancing tolerance to host defenses and environmental stress. [7] This phenotype involves various biological mechanisms is regulated by multiple genetic determinants, including fimbrial-mediated adhesion (*fimH* and *mrkD*), EPS production (*pga* operon, *wcaG*), and intercellular communication via quorum sensing (*luxS*). [7–10]

The relationship between antimicrobial resistance and biofilm formation in *K. pneumoniae* remains incompletely understood. Although several studies have reported greater biofilm-forming capacity among antimicrobial-resistant including ESBL-producing and extensively drug resistant (XDR) strains, or carbapenem-resistant isolates. [11–13] Nevertheless, other studies have found no association or even reduced biofilm formation in carbapenem-resistant isolates. [14, 15]

These inconsistent findings may reflect differences in resistance mechanisms, bacterial genetic backgrounds, clinical sources, and local epidemiological characteristics. [11, 13, 16] This highlights the urgent need for region-specific data to understand the unique interplay of these virulence factors in local pathogen populations. Therefore, understanding biofilm molecular mechanisms and its correlation with antimicrobial resistance is important to provide insight for designing new agents capable of controlling biofilm-related infections.

In Egypt, where antimicrobial resistance among healthcare-associated Gram-negative pathogens represents an important clinical concern, data examining the relationship between antimicrobial resistance, biofilm formation, and specific biofilm-associated genes in *K. pneumoniae* remain limited. Moreover, the extent to which resistance phenotypes are independently associated with biofilm formation after accounting for relevant genetic determinants has not been clearly established in clinical isolates from this setting. [3, 17] Characterizing these relationships may provide a better understanding of the factors associated with biofilm formation among circulating *K. pneumoniae* isolates and may help inform infection-control and antimicrobial-stewardship strategies.

Therefore, this study aimed to investigate the association between antimicrobial resistance phenotypes and biofilm formation among clinical *K. pneumoniae* isolates from intensive care units (ICUs) and to assess whether carriage of the biofilm-associated genes *mrkD*, *fimH*, and *wcaG* was independently associated with biofilm formation using multivariable logistic regression.

Methods

Study Design and Isolate Collection

This cross-sectional study was conducted from January 2025 to June 2025, at ICUs of Tanta University Hospitals, Egypt. A total of 87 non-duplicate clinical *K. pneumoniae* isolates were prospectively collected from patients diagnosed with HCAs. Healthcare-associated infections were defined according to applicable site-specific surveillance criteria adopted by Centers for Disease Control and Prevention / National Healthcare Safety Network (CDC/NHSN). In general, infection events occurring on or after the third calendar day of admission were classified as HCAs, subject to NHSN criteria and exceptions. [18]

Isolates were recovered from various clinical specimens, including blood, wound swabs, bronchoalveolar lavage, urine, and pus. Only the first isolate of *K. pneumoniae* per patient during the study period was included. Repeated isolates from the same patient were excluded.

Sample Size Calculation

Sample size was calculated using G*Power version 3.1.9.7 (Heinrich Heine University Düsseldorf, Germany) to detect an association between biofilm formation and antimicrobial resistance based on a chi-square test with one degree of freedom, a two-sided alpha level of 0.05. Assuming a medium effect size (Cohen's $w = 0.30$) and 80% statistical power, the required minimum sample size was 87 isolates.

Bacterial Identification and Preservation

Standard biochemical tests, including indole, motility, lactose fermentation, H_2S production, citrate utilization, urease, lysine decarboxylase, and methyl red–Voges–Proskauer (MR–VP) tests were initially used to identify *Klebsiella pneumoniae* isolated.

Species identification was subsequently confirmed using the API[®] 20E system (bioMérieux, Marcy-l'Étoile, France). Confirmed isolates were preserved for long-term storage in Brain Heart Infusion (BHI) broth (Oxoid, Basingstoke, UK) supplemented with 25% glycerol and stored at -80°C . [19]

Antimicrobial Susceptibility Testing & Phenotypic Detection of Resistance Mechanisms:

♣ Antimicrobial Susceptibility Testing (AST)

Antimicrobial susceptibility was determined for the 87 *K. pneumoniae* isolates using the Kirby–Bauer disk diffusion method on Mueller–Hinton agar (MHA) (Oxoid, Basingstoke, UK). Inhibition-zone diameters were measured and interpreted according to the Clinical and Laboratory Standards Institute (CLSI) M100 guidelines, 2025 edition. [20] The antimicrobial panel included amikacin (30 μg), imipenem (10 μg), meropenem (10 μg), ceftazidime (30 μg), ciprofloxacin (5 μg), ceftriaxone (30 μg), cefotaxime (30 μg), cefepime (30 μg), aztreonam (30 μg), amoxicillin–clavulanic acid (20/10 μg), trimethoprim–sulfamethoxazole (1.25/23.75 μg), ceftazidime (30 μg), and piperacillin–tazobactam (100/10 μg). Tigecycline (15 μg) susceptibility was interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakdown tables. Version 15.0 (2025). [21]

For colistin, susceptibility was performed using the broth disk elution method in accordance to the applicable CLSI recommendations. [20] Briefly, a 0.5 McFarland bacterial suspension was used to inoculate cation-adjusted Mueller–Hinton broth containing final colistin concentrations of 1, 2, and 4 $\mu\text{g}/\text{mL}$, prepared by eluting one, two, or four 10- μg colistin disks, respectively. Following incubation at 35°C for 16–20 hours, minimum inhibitory concentration (MIC) was determined as the lowest Colistin concentration that prevented visible bacterial growth. Colistin MICs were interpreted using EUCAST clinical breakpoints for Enterobacterales in Version 15.0 (2025). [21] *Escherichia coli* ATCC 25922 was used as the quality-control strain for AST producers.

♣ Phenotypic Detection of Resistance Mechanisms

Isolates exhibiting resistance to the relevant indicator antibiotics in the primary susceptibility screen were subjected to phenotypic confirmatory tests for selected resistance mechanisms according to CLSI guidelines (2025). [20]

1. **Extended-spectrum β -lactamase (ESBL) production:** Isolates resistant to third-generation cephalosporins were tested using the combined-disk method. A ≥ 5 mm increase in ceftazidime (30 μg) inhibition zone combined with clavulanic acid (10 μg), compared with ceftazidime alone, was considered indicative of ESBL production.
2. **AmpC β -lactamase production:** Isolates resistant to ceftazidime were evaluated using a double-disk synergy test. An expansion of the inhibition zones of ceftazidime and cefotaxime toward a cloxacillin (300 μg) disk placed 15 mm away was considered indicative of AmpC production.

3. **Carbapenemase production:** Modified carbapenem inactivation method (mCIM) and EDTA-modified carbapenem inactivation method (eCIM) were used to evaluate carbapenemase production among imipenem or meropenem resistant isolates. eCIM was interpreted only when the corresponding mCIM was positive (the zone of inhibition around the meropenem disk was 6–15 mm). An increase of ≥ 5 mm in the eCIM zone compared with the mCIM zone was considered indicative of metallo- β -lactamase (MBL) production.

Quantification of Biofilm Production

Biofilm formation was measured using the tissue culture plate method reformed from Stepanović et al. [23] An overnight bacterial culture was adjusted to a 0.5 McFarland standard and diluted 1:100 in fresh BHI broth. Triplicate 200 μ L volumes of the suspension were transferred into sterile 96-well flat-bottomed polystyrene microplates (Sigma-Aldrich, St. Louis, MO, USA), using uninoculated BHI broth as a negative control.

After a 24-hour incubation at 37°C, the wells were emptied and washed four times with sterile distilled water to eliminate non-adherent cells. The remaining biofilm was fixed using 200 μ L of 95% ethanol for 20 minutes, stained with 0.1% crystal violet for an additional 20 minutes, and the bound dye was ultimately solubilized in 200 μ L of 95% ethanol after rinsing away excess stain. Optical density (OD) was measured at 630 nm using a microplate reader. The final OD for each isolate was calculated as the mean OD of the three replicate wells minus the mean OD of the negative control wells. Based on the previously published OD thresholds, *K. pneumoniae* isolates were classified as non/weak biofilm producers (OD < 0.120), moderate biofilm producers (OD = 0.120–0.240), and strong biofilm producers (OD > 0.240). [24]

For the prespecified binary analysis, moderate and strong biofilm producers were grouped as biofilm-positive, whereas no/weak producers were grouped as biofilm-negative.

Molecular Detection of Biofilm Genes

♣ DNA Extraction

Using a commercial bacterial genomic DNA extraction kit (ABT002; Applied Biotechnology, Egypt), genomic DNA was extracted from overnight bacterial cultures following the manufacturer’s instructions. A NanoDrop spectrophotometer (Analytik Jena, Jena, Germany) was used to measure the A260/A280 ratio, thus assessing extracted DNA concentration and purity. Extracted DNA samples were stored at – 20°C until polymerase chain reaction (PCR) analysis.

♣ PCR Amplification of Target Genes

Uniplex PCR assays were performed to detect three biofilm-associated genes: *mrkD*, encoding a type 3 fimbrial adhesin; *fimH*, encoding a type 1 fimbrial adhesin; and *wcaG*, involved in capsular fucose biosynthesis. The primer sequences, target genes and expected amplicon sizes are presented in Table 1.

Table 1 Primers used for PCR amplification of biofilm-associated genes in <i>K. pneumoniae</i>		
Gene	Primer sequence (5'-3')	Product size (bp)
<i>mrkD</i> [25]	F: AAGCTATCGCTGTACTTCCGGCA	340 bp
	R: GGC GTTGGCGCTCAGATAGG	
<i>wcaG</i> [26]	F: GGT TGGKTCAGCAATCGTA	169 bp
	R: ACTATTCGCGCAACTTTTGC	
<i>fimH</i> [26]	F: TGCTGCTGGGCTGGTCGATG	550 bp
	R: GGGAGGGTGACGGTGACATC	

Each 25- μ L PCR reaction contained 12.5 μ L of 2 $\times$ Ready Mix (FastGene[®] Taq, Nippon Genetics, Tokyo, Japan), 0.5 μ L each of forward and reverse primers (10 pmol/ μ L), 2.0 μ L of template DNA, and nuclease-free water to obtain the final reaction volume. Amplification was performed using an Applied Biosystems 2720 Thermal Cycler (Thermo Fisher Scientific, Waltham, MA, USA).

For *mrkD*, PCR amplification consisted of an initial denaturation at 95°C for 15 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 90 seconds, and extension at 72°C for 60 seconds, followed by a final extension at 72°C for 10 minutes.

For *fimH* and *wcaG*, amplification consisted of an initial denaturation at 95°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 60 seconds, annealing at 58°C for 60 seconds, and extension at 72°C for 60 seconds, followed by a final extension at 72°C for 10 minutes.

♣ Agarose Gel Electrophoresis

PCR products were separated by electrophoresis on 1.5% agarose gels stained with ethidium bromide. A 100-bp DNA ladder (Enzynomics Co., Ltd., Daejeon, Korea) was run alongside the PCR products for amplicon-size estimation. Gels were documented using a gel documentation system (Biometra, Analytik Jena, Jena, Germany).

Data and Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics version 26.0 (IBM Corp., Armonk, NY, USA). Categorical variables were summarized as frequencies and percentages. Associations between categorical variables were assessed using the Pearson chi-square test or Fisher's exact.

Biofilm formation was analyzed as a binary outcome, with non/weak producers classified as biofilm-negative and moderate/strong producers as biofilm-positive. Multivariable binary logistic regression analysis was performed to assess associations with biofilm formation. The multivariable model included carbapenem resistance and the *mrkD*, *fimH*, and *wcaG* genes. Results were expressed as odds ratios (ORs) with 95% confidence intervals (CIs). A two-sided P value < 0.05 was considered statistically significant.

Results

Antimicrobial Susceptibility Profiles and Phenotypic Resistance Mechanisms:

A total of 87 clinical *K. pneumoniae* isolates were recovered from patients with HCAs at Tanta University Hospitals. As shown in Table 2, high resistance rates were observed for most tested β -lactam antibiotics, exceeding 90% for amoxicillin, cefotaxime, ceftriaxone, ceftazidime, and ceftazidime. All isolates were categorized as susceptible to colistin by the broth disk-elution method used, while aztreonam and tigecycline showed the highest susceptibility rates among the other tested agents (46.0% each).

Table 2
Antimicrobial susceptibility profile among the 87 *K. pneumoniae* isolates.

Antimicrobial agent	Susceptible	Intermediate	Resistant
Amoxicillin	6 (6.9%)	0 (0.0%)	81 (93.1%)
Aztreonam	40 (46.0%)	0 (0.0%)	47 (54.0%)
Cefotaxime	4 (4.6%)	0 (0.0%)	83 (95.4%)
Ceftriaxone	4 (4.6%)	0 (0.0%)	83 (95.4%)
Ceftazidime	6 (6.9%)	0 (0.0%)	81 (93.1%)
Ceftazidime	6 (6.9%)	0 (0.0%)	81 (93.1%)
Cefepime	11 (12.6%)	0 (0.0%)	76 (87.4%)
Piperacillin-tazobactam	11 (12.6%)	0 (0.0%)	76 (87.4%)
Amikacin	35 (40.2%)	10 (11.5%)	42 (48.3%)
Trimethoprim- Sulphamethoxazole	38 (43.7%)	1 (1.2%)	48 (55.2%)
Imipenem	30 (34.5%)	0 (0.0%)	57 (65.5%)
Meropenem	30 (34.5%)	0 (0.0%)	57 (65.5%)
Ciprofloxacin	25 (28.7%)	12 (13.8%)	50 (57.5%)
Tigecycline	40 (46.0%)	0 (0.0%)	47 (54.0%)
Colistin	87 (100.0%)	0 (0.0%)	0 (0.0%)

Overall, 51 isolates (58.6%) met the criteria for MDR, while 30 (34.5%) were classified as XDR. Carbapenem resistance was detected in 57 isolates (65.5%). Combined ESBL and AmpC production was identified in 18 isolates (20.7%). Among the 57 carbapenem-resistant isolates, 36 (63.1%) were positive for MBL production by the eCIM test.

Phenotypic Biofilm-Forming Capacity and Virulence Gene Distribution

Biofilm formation was assessed using the tissue culture plate assay. Of the 87 clinical *K. pneumoniae* isolates, 54 (62.1%) were classified as biofilm producers. Moderate biofilm formation was the most frequent phenotype, observed in 48 isolates (55.2%), whereas strong biofilm formation was observed in 6 isolates (6.9%) (Table 3).

Table 3
Association of carbapenem resistance with resistance pattern, biofilm formation, and biofilm-associated genes

Association of carbapenem resistance with resistance pattern, biofilm formation, and biofilm-associated genes				
Variable		Non-CRKP (n = 30, 34.5%)	CRKP (n = 57, 65.5%)	P
Resistance pattern	Not MDR (n = 6)	6 (20.0%)	0 (0.0%)	< 0.001*
	MDR, non-XDR (n = 51)	24 (80.0%)	27 (47.4%)	
	XDR (n = 30)	0 (0.0%)	30 (52.6%)	
Biofilm formation	No / Weak (n = 33)	21 (70.0%)	12 (21.1%)	< 0.001*
	Moderate (n = 48)	9 (30.0%)	39 (68.4%)	
	Strong (n = 6)	0 (0.0%)	6 (10.5%)	
mrkD Gene	Negative (n = 33)	16 (53.3%)	17 (29.8%)	0.04*
	Positive (n = 54)	14 (46.7%)	40 (70.2%)	
fimH Gene	Negative (n = 28)	16 (53.3%)	12 (21.1%)	0.004*
	Positive (n = 59)	14 (46.7%)	45 (78.9%)	
wcaG Gene	Negative (n = 22)	9 (30.0%)	13 (22.8%)	0.60
	Positive (n = 65)	21 (70.0%)	44 (77.2%)	
P value < 0.05 was considered statistically significant (*)				
CRKP: Carbapenem resistance <i>K. pneumoniae</i> , MDR: Multidrug resistance, XDR: Extensive drug resistance				

Among the three biofilm-associated genes investigated, *wcaG* was the most prevalent, detected in 65 isolates (74.7%), followed by *fimH* in 59 (67.8%) and *mrkD* in 54 (62.1%).

Associations with Carbapenem Resistance

Among the 57 carbapenem-resistant *K. pneumoniae* isolates, 45 (78.9%) were biofilm-positive (moderate or strong), compared with 9 of 30 (30.0%) non-carbapenem-resistant isolates (Table 3). Moderate biofilm formation was observed in 39 (68.4%) carbapenem-resistant isolates and 9 (30.0%) non-carbapenem-resistant isolates, while all six strong biofilm producers were carbapenem-resistant ($P < 0.001$). All 57 carbapenem-resistant isolates were classified as MDR, including 30 (52.6%) XDR and 27 (47.4%) MDR non-XDR isolates.

Carriage of *mrkD* and *fimH* was significantly more frequent among carbapenem-resistant isolates than among non-carbapenem-resistant isolates (*mrkD*: 70.2% vs. 46.7%, $P = 0.04$; *fimH*: 78.9% vs. 46.7%, $P = 0.004$). In contrast, *wcaG* prevalence did not differ significantly between the two groups (77.2% vs. 70.0%, $P = 0.60$).

Antimicrobial Susceptibility According to Biofilm-Formation Phenotype

Antimicrobial susceptibility profiles differed significantly between isolates with no/weak biofilm formation and those with moderate/strong biofilm formation (Table 4). The moderate/strong biofilm group showed higher resistance to several β -lactam antibiotics and carbapenems. Resistance to cefotaxime, ceftriaxone, ceftazidime, and ceftiofur was 100% in the moderate/strong biofilm group compared with 87.9%, 87.9%, 81.8%, and 81.8%, respectively, in the no/weak biofilm group ($P = 0.02, 0.02, 0.002, \text{ and } 0.002$, respectively).

Table 4
Multivariable logistic regression analysis of factors associated with biofilm positivity among *K. pneumoniae* isolates

Multivariable logistic regression analysis of factors associated with biofilm positivity among <i>K. pneumoniae</i> isolates						
Predictor [†]		Biofilm formation (frequency %)	OR	95% CI		P
				Lower	Upper	
Carbapenem resistance	Non-CRKP (n = 30)	9 (30.0%)	6.95	1.90	25.44	0.003*
	CRKP (n = 57)	45 (78.9%)				
<i>mrkD</i> Gene	Negative (n = 33)	12 (36.4%)	7.23	1.97	26.5	0.003*
	Positive (n = 54)	42 (77.8%)				
<i>fimH</i> Gene	Negative (n = 28)	9 (32.1%)	5.73	1.52	21.59	0.01*
	Positive (n = 59)	45 (76.3%)				
<i>wcaG</i> gene	Negative (n = 22)	8 (36.4%)	8.35	1.88	37.07	0.005*
	Positive (n = 65)	46 (70.8%)				
† Reference categories were non-CRKP, gene negative isolates and biofilm negative status. ORs are adjusted for all predictors shown in table						
P value < 0.05 was considered statistically significant (*)						
CR: Carbapenem resistance <i>K. pneumoniae</i> , CI: Confidence interval, OR: Odds ratio						

Resistance to cefepime and piperacillin-tazobactam was also higher among moderate/strong biofilm producers than among no/weak producers (98.1% vs. 69.7% for both agents; $P < 0.001$). Similarly, resistance to imipenem and meropenem was significantly higher in the moderate/strong biofilm group (83.3% vs. 36.4% for both agents; $P < 0.001$). In contrast, no significant differences were observed for amikacin, trimethoprim-sulfamethoxazole, ciprofloxacin, tigecycline, or colistin ($P > 0.05$).

Factors Independently Associated with Biofilm Formation

In the multivariable logistic regression model, carbapenem resistance remained associated with biofilm positivity after adjustment for *mrkD*, *fimH*, and was *wcaG* carriage (adjusted OR = 6.95, 95% CI: 1.90–25.44, $P = 0.003$). Carriage of *mrkD* (adjusted OR = 7.23, 95% CI: 1.97–26.50, $P = 0.003$), *fimH* (adjusted OR = 5.73, 95% CI: 1.52–21.59, $P = 0.010$), and *wcaG* (adjusted OR = 8.35, 95% CI: 1.88–37.07, $P = 0.005$) were also associated with higher odds of biofilm positivity. (Table 5).

These estimates represent adjusted associations and should not be interpreted as evidence that carbapenem resistance or gene carriage causes biofilm formation.

Table 5
Antimicrobial susceptibility profiles according to biofilm formation capacity among *K. pneumoniae* isolates

Biofilm Formation		AMC	AZT	CTX	CTR	CTZ	CFX	CFP	PIP	AMK	TRI	IMP	MRP	CIP
No / Weak	S	6 (18.2%)	14 (42.4%)	4 (12.1%)	4 (12.1%)	6 (18.2%)	6 (18.2%)	10 (30.3%)	10 (30.3%)	15 (45.5%)	13 (39.4%)	21 (63.6%)	21 (63.6%)	12 (36.4%)
33 (37.9%)	I	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	3 (9.1%)	0 (0%)	0 (0%)	0 (0%)	4 (12.1%)
	R	27 (81.8%)	19 (57.6%)	29 (87.9%)	29 (87.9%)	27 (81.8%)	27 (81.8%)	23 (69.7%)	23 (69.7%)	15 (45.5%)	20 (60.6%)	12 (36.4%)	12 (36.4%)	17 (51.5%)
Moderate / Strong	S	0 (0%)	26 (48.1%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (1.9%)	1 (1.9%)	20 (37.0%)	25 (46.3%)	9 (16.7%)	9 (16.7%)	13 (24.1%)
54 (62.1%)	I	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	7 (13.0%)	1 (1.9%)	0 (0%)	0 (0%)	8 (14.8%)
	R	54 (100%)	28 (51.9%)	54 (100%)	54 (100%)	54 (100%)	54 (100%)	53 (98.1%)	53 (98.1%)	27 (50.0%)	28 (51.9%)	45 (83.3%)	45 (83.3%)	33 (61.1%)
p [†]		0.002*	0.60	0.02*	0.02*	0.002*	0.002*	< 0.001*	< 0.001*	0.78	0.70	< 0.001*	< 0.001*	0.47

† P values compare distribution of susceptible, intermediate, and resistant categories between the two biofilm groups using pearson's χ^2 test or fisher's exact test. P value < 0.05 was considered statistically significant (*)

AMC: Amoxicillin, AMK, Amikacin, AZT: Aztreonam, CFP: Cefepime, CFX: Cefoxitin, CIP: Ciprofloxacin, CLS: Colistin, CTR: Ceftriaxone, CTX: Cefotaxime, CTZ: Intermediate, IMP: Imipenem, MDR: Multidrug resistance, MRP: Meropenem, PIP: Piperacillin-tazobactam, R: Resistant, TRI: Trimethoprim-sulphamethoxazole, XDR: Extensive drug resistance

Discussion

In the present study, 54 of 87 (62.1%) clinical *K. pneumoniae* isolates were biofilm producers. Moderate biofilm formation was the predominant phenotype (55.2%), whereas strong biofilm formation was observed in only 6.9% of isolates. Our observed biofilm positivity rate aligns closely with several clinical *K. pneumoniae* cohorts, though past literature highlights notable variation in biofilm-forming capacity. For instance, Shadkam et al. and Temel et al. documented biofilm positivity rates of 75% and 80.4%, respectively, while alternative studies noted lower overall rates or shifting distributions of biofilm intensity. [16, 27]

Similarly, the prevalence of moderate biofilm producers within our cohort mirrors findings from Seifi et al. and Jaisal et al., who both identified moderate biofilm activity in roughly half of their tested isolates. [28, 29] Such discrepancies across the literature likely stem from differences in clinical specimen sources, patient demographics, laboratory protocols, and the distinct genetic profiles of circulating *K. pneumoniae* strains.

We also performed molecular screening for three specific biofilm-related genes. Among these, *wcaG* emerged as the most prevalent target (74.7%), followed closely by *fimH* (67.8%) and *mrkD* (62.1%). *mrkD*, *fimH*, and *wcaG* were chosen because of their distinct biological roles in the multifactorial mechanism of *K. pneumoniae* biofilm formation. Far from being redundant markers, these genes represent separate functional components that involve both bacterial adherence and biofilm-associated extracellular environment.

The *mrkD* gene encodes the structural adhesin component of type 3 fimbriae, which serves as a primary surface appendage facilitating attachment to abiotic substrates and driving biofilm growth, particularly across catheter- and device-related environments. Experimental studies using isogenic mutants confirm that type 3 fimbriae heavily influence biofilm development, even if the precise impact of the individual MrkD adhesin varies slightly from the fimbrial structure as a whole. [10, 30] Meanwhile, *fimH* encodes the terminal adhesin of type 1 fimbriae, serving as an independent adhesion mechanism responsible for mannose-dependent binding to host surfaces and, under specific environmental cues, biofilm-mediated colonization. [31, 32] On the other hand, *wcaG* was targetting the capsular/exopolysaccharide part of the biofilm phenotype, given that capsule-associated polysaccharides actively influence surface attachment, biofilm maturation, and the structural properties of the bacterial community. [8]

Collectively, this three-gene panel offers a complementary look at both fimbrial attachment and extracellular polysaccharide-associated processes. It enables a clearer evaluation of whether biofilm phenotypes in clinical *K. pneumoniae* strains depend on multiple, potentially synergistic traits of the bacterial surface. and biofilm phenotype.

Notably, the distribution of these genes differed according to carbapenem resistance status. The prevalence of *mrkD* and *fimH* was significantly higher among carbapenem-resistant isolates than among non-carbapenem-resistant isolates (*mrkD*: 70.2% vs. 46.7%, $P = 0.04$; *fimH*: 78.9% vs. 46.7%, $P = 0.004$), whereas *wcaG* showed no significant difference between the two groups (77.2% vs. 70.0%, $P = 0.60$).

These findings suggest that the association between carbapenem resistance and the investigated biofilm-related genetic profile may be gene-specific rather than reflecting a uniform increase in all biofilm-associated determinants among resistant isolates. The higher prevalence of *mrkD* and *fimH* among

carbapenem-resistant isolates is consistent with observations from Abou-Dobara et al. who reported a significant association between *mrkD* carriage and carbapenemase producing *K. pneumoniae*, whereas a similar association was not observed for *fimH*. [33] Conversely, the absence of a significant association between *wcaG* and carbapenem resistance in our cohort suggests that the presence of this gene alone may not closely track the carbapenem-resistant phenotype.

The phenotypic comparison further demonstrated an association between biofilm-forming capacity and antimicrobial resistance. Isolates with moderate or strong biofilm formation showed significantly higher resistance to several β -lactam agents, including cefotaxime, ceftriaxone, ceftazidime, cefoxitin, cefepime, and piperacillin-tazobactam, compared with isolates showing no or weak biofilm formation. Resistance to imipenem and meropenem was also substantially higher among moderate/strong biofilm producers than among no/weak producers (83.3% vs. 36.4%, respectively; $P < 0.001$).

These findings are consistent with previous reports describing a positive association between biofilm formation and carbapenem resistance or broader multidrug-resistant phenotypes in *K. pneumoniae*. Jaisal et al. and Abou-Dobara et al. reported associations between biofilm formation and carbapenem-resistant isolates. [29, 33] Similarly, Rahdar et al. reported that all carbapenem-resistant isolates in their study exhibited at least medium-strength biofilm formation, with 77.9% classified as strong biofilm producers. [34] Furthermore, Khodadadian et al. observed a significant link between strong biofilm production and the presence of the metallo- β -lactamase genes *VIM-1* and *IMP-1* in clinical *K. pneumoniae* isolates. [35]

Even so, the interaction between antimicrobial drug resistance and biofilm capacity remains inconsistent throughout the literature. Some researchers describe an inverse relationship or no association at all, noting that carbapenem-resistant or β -lactamase-producing *K. pneumoniae* strains sometimes form weaker biofilms than their susceptible counterparts. [14, 15] For instance, Sabeça et al. found that most ESBL- and KPC-producing strains were weak biofilm formers, suggesting that antimicrobial resistance mechanisms do not necessarily guarantee enhanced biofilm capacity. [14] Similarly, Cusumano et al. reported that carbapenem-resistant isolates were less prone to strong biofilm phenotypes. [15]

These conflicting results likely stem from variations in the genetic backgrounds of the cohorts, the specific resistance mechanisms involved, clinical isolation sites, or diverse methodological protocols. Consequently, while our data point to a clear link between resistance phenotypes and biofilm formation in this specific cohort, they do not prove that resistance directly drives biofilm development.

An important finding of the present study was that carbapenem resistance remained independently associated with biofilm positivity after adjustment for the three investigated genes. In the multivariable logistic regression model, carbapenem-resistant isolates had approximately sevenfold higher odds of biofilm formation (OR = 6.95, 95% CI: 1.90–25.44, $P = 0.003$). Similarly, carriage of *mrkD* (OR = 7.23, 95% CI: 1.97–26.50, $P = 0.003$), *fimH* (OR = 5.73, 95% CI: 1.52–21.59, $P = 0.010$), and *wcaG* (OR = 8.35, 95% CI: 1.88–37.07, $P = 0.005$) was independently associated with higher odds of biofilm positivity.

This finding is particularly relevant because the relationship between carbapenem resistance and biofilm formation in *K. pneumoniae* has been inconsistent across clinical studies. Several investigations have reported a positive association between carbapenem resistance or broader multidrug resistance and stronger biofilm formation, whereas others have found weak or inverse associations among carbapenem-resistant isolates. [15, 16, 33]

The persistence of the association in our multivariable model, after adjustment for *mrkD*, *fimH*, and *wcaG*, suggests that the observed relationship between carbapenem resistance and biofilm positivity was not fully explained by carriage of the three investigated biofilm-associated genes. However, this finding should not be interpreted as evidence that carbapenem resistance itself promotes biofilm formation. Carbapenem resistance is a composite phenotype that may reflect diverse underlying mechanisms, including carbapenemase production, alterations in outer-membrane permeability, efflux activity, plasmid-associated resistance determinants, and strain-specific genetic backgrounds. These factors may coexist with, or be genetically linked to, determinants of adhesion and biofilm formation. The coexistence of resistance and biofilm-associated phenotypes may therefore reflect shared genetic backgrounds or linked biological traits rather than a direct causal effect of carbapenem resistance on biofilm formation. [11, 15, 33]

Because the model included only three targeted genes and did not assess clonal relatedness, carbapenemase genes, plasmid content, gene expression, or biofilm-regulatory pathways, residual confounding and shared genetic background remain possible explanations for the observed associations. Furthermore, the cross-sectional design and relatively small sample size limit causal inference and contribute to the relatively wide confidence intervals around several of the estimated effects.

The present study adds to the existing literature by integrating phenotypic biofilm assessment with targeted analysis of three functionally distinct biofilm-related genes in a clinically derived collection of *K. pneumoniae* isolates from an ICU setting. Rather than examining biofilm formation, antimicrobial resistance, or individual genetic markers in isolation, our analysis evaluated the association of the carbapenem-resistant phenotype with biofilm positivity while simultaneously accounting for *mrkD*, *fimH*, and *wcaG* carriage. Consequently, this comprehensive microbiological approach provides a foundation for future studies investigating the wider genetic and phenotypic drivers behind concurrent antimicrobial resistance and biofilm traits in clinical *K. pneumoniae* populations.

Limitations

The present study has several limitations to consider when interpreting the findings. The cross-sectional design precludes determination of temporal or causal links among drug resistance, biofilm production, and the carriage of biofilm-related genes. Furthermore, the moderate sample size originating from a single university center could constrain the generalizability of our observations and skew the precision of identified correlations, as indicated by the broad confidence intervals within the multivariable regression model. Additionally, our genetic screening targeted only three specific loci (*mrkD*, *fimH*, and *wcaG*), leaving out alternative genetic drivers involved in adhesion, capsule production, and biofilm regulation. Finally, while phenotypic assays successfully identified carbapenem resistance and carbapenemase expression, we did not perform molecular sequencing to confirm the specific carbapenemase genotypes involved.

Conclusions

In this single-center cohort of ICU-derived *Klebsiella pneumoniae* strains, a high prevalence of carbapenem resistance and biofilm production was observed, with CRKP isolates demonstrating a greater likelihood of biofilm positivity compared to non-CRKP isolates. Following adjustment for other investigated markers, carbapenem resistance and the carriage of *mrkD*, *fimH*, and *wcaG* remained independently associated with biofilm positivity. These data support an association between the carbapenem-resistant phenotype, selected biofilm-related genes, and the actual biofilm phenotype within this study cohort; however, they do not establish a causal relationship or show an effect on patient outcomes. These observations should thus be viewed as microbiological findings that require confirmation in larger, multicenter investigations using broader genomic profiling and clinical endpoints.

List of Abbreviations

- AMR Antimicrobial resistance
- BHI Brain Heart Infusion
- CDC Centers for Disease Control and Prevention
- CIs Confidence intervals
- CLSI Clinical and Laboratory Standards Institute
- CRKP Carbapenem-resistant *Klebsiella pneumoniae*
- eCIM EDTA-modified carbapenem inactivation method
- EDTA Ethylenediaminetetraacetic acid
- EPS Extracellular polymeric substance
- ESBL Extended-spectrum β -lactamase
- HCAIs Healthcare-associated infections
- ICU Intensive care unit
- MBL Metallo- β -lactamase
- mCIM Modified carbapenem inactivation method
- MDR Multidrug-resistant
- MHA Mueller–Hinton agar
- MIC Minimum inhibitory concentration
- MR-VP Methyl red–Voges–Proskauer
- NHSN National Healthcare Safety Network
- OD Optical density
- OR Odds ratio
- PCR Polymerase chain reaction
- XDR Extensively drug resistant

Declarations

Ethics Approval and Consent to Participate

The study protocol was reviewed and approved by the Research Ethics Committee of the Faculty of Medicine, Tanta University (Approval code: 36264PR1111/2/25). Written informed consent was obtained from all study participants or their legal guardians before sample collection.

Consent for Publication

Not applicable

Competing Interests

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Author Contribution

All authors meet the International Committee of Medical Journal Editors (ICMJE) criteria for authorship and approved the final version of the manuscript. MM, MR, and AR contributed to study conception and design. MM, MG, and AR contributed to sample collection and microbiological investigations, while MM, MG, MR, and AR performed molecular investigations. AN and MR contributed to statistical analysis. MM, AN, and AR drafted the manuscript. All authors contributed to interpretation and critical revision, approved the final manuscript, and accepted accountability for the work.

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Availability of Data and Materials

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

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