Association Between Antibiotic Resistance, Biofilm Formation, and Virulence Gene Profiles in Clinical Isolates of *Klebsiella pneumoniae*

Abstract

Klebsiella pneumoniae is a leading cause of healthcare-associated infections, increasingly characterized by multidrug resistance and enhanced virulence. This study aimed to investigate the interplay between antibiotic resistance, biofilm formation, and virulence gene profiles among clinical isolates of *Klebsiella pneumoniae* in Tehran, Iran. Clinical confirmed isolates were recovered from urine, wound, blood, catheter, and respiratory specimens. Antimicrobial susceptibility testing revealed alarming resistance rates: 100% to imipenem, ~80% to ampicillin and cefotaxime, and 69.23% were ESBL producers. Notably, a significant association was found between the hypervirulent *Klebsiella pneumoniae* (hvKp) phenotype and ESBL production ($P = 0.003$), indicating the emergence of convergent strains combining resistance and virulence. Phenotypic assays showed that 76% of isolates were capable of biofilm formation. Molecular analysis revealed high prevalence of key virulence genes: *mrkA* (53.84%), *wzm* (46.15%), *pgaA* (34.61%), and *wbbM* (23.07%). Strong positive correlations were observed between *wbbM* and both *wzm* ($r_s = 0.734$) and pgaA ($r_s = 0.549$), as well as between *mrkA* and *wbbM* ($r_s = 0.458$), suggesting synergistic roles in biofilm development. hvKp isolates were predominantly recovered from respiratory samples (66.66%), whereas classical *Klebsiella pneumoniae* (cKp) dominated urinary and bloodstream sources. These findings underscore a concerning convergence of carbapenem resistance, hypervirulence, and robust biofilm-forming capacity in clinical *Klebsiella pneumoniae* isolates in Iran. Such strains pose a serious therapeutic challenge and highlight the urgent need for enhanced surveillance, rapid diagnostics, and alternative treatment strategies targeting virulence and biofilm mechanisms.

Keywords: *Klebsiella pneumoniae*; antibiotic resistance; biofilm formation; virulence genes

Introduction

Klebsiella pneumoniae is a Gram-negative, rod-shaped, lactose-fermenting member of the Enterobacteriaceae family(1). It is oxidase-negative, facultatively anaerobic, and closely related genetically to other clinically relevant genera such as *Escherichia, Salmonella, Shigella,* and *Yersinia*. While commonly found in soil, water, and environmental surfaces, *K. pneumoniae* also colonizes the human nasopharynx and gastrointestinal tract asymptomatically in approximately one-third of the population(2). However, in immunocompromised individuals such as those with diabetes, on glucocorticoid therapy, or post-organ transplantation it can cause severe opportunistic infections, including pneumonia, urinary tract infections, septicemia, meningitis, and liver abscesses, often with high mortality rates(3). A major challenge in managing *K. pneumoniae* infections stems from its ability to form biofilms and develop multidrug resistance. Biofilms are structured microbial communities encased in a protective extracellular matrix that impedes antibiotic penetration and shields bacteria from host immune defenses. Within these biofilms, horizontal gene transfer facilitates the dissemination of resistance determinants among bacterial species(4). Notably, *K. pneumoniae* frequently acquires extended-spectrum beta-lactamase (ESBL) genes, which confer resistance to a broad range of beta-lactam antibiotics, including penicillins and cephalosporins, thereby severely limiting therapeutic options(5). Given the interplay between biofilm-associated virulence and antibiotic resistance, this study aimed to investigate the association between antimicrobial resistance profiles and the prevalence of virulence genes involved in biofilm formation among clinical isolates of *K. pneumoniae*.

Materials and Methods

Sample Collection, Bacterial Isolation, and Identification

A total of 203 clinical specimens were collected from patients with suspected urinary tract infections (UTIs) attending medical centers in Tehran, Iran, between June and October 2023. All samples were processed for bacterial isolation by inoculation onto standard culture media, including blood agar (as a non-selective medium) and eosin methylene blue (EMB) agar (as a differential medium). Presumptive colonies exhibiting typical morphological characteristics of *Klebsiella pneumoniae* were subjected to a battery of standard biochemical tests for definitive identification. These tests included Gram staining, catalase, oxidase, indole production, citrate *utilization, urease activity, and lactose fermentation. Isolates were confirmed as K*. pneumoniae based on their Gram-negative rod morphology, oxidase-negative and catalase-positive reactions, and characteristic biochemical profile (e.g., indole-negative, citrate-positive, urease-positive, and lactose-fermenting)(6).

Antimicrobial Susceptibility Testing

A bacterial suspension was prepared by adjusting the turbidity of isolated *Klebsiella pneumoniae* colonies in 5 mL of sterile physiological saline to match that of a 0.5 McFarland standard. The optical density (OD) of the suspension was verified spectrophotometrically at 625 nm, with an acceptable OD range of 0.08–0.13. This turbidity corresponds to approximately 1.5×10^8 colony-forming units (CFU)/mL, as specified in CLSI document 2023 for broth microdilution and disk diffusion methods(7). Antimicrobial susceptibility testing was performed using the Kirby–Bauer disk diffusion method according to CLSI guidelines (M100, 2023). The following antibiotic disks were used: ampicillin (AMP, 10 µg), gentamicin (GN, 10 μg), cefotaxime (CTX, 30 μg), ceftazidime (CAZ, 30 μg), imipenem (IMI, 10 μg), piperacillin (PIP, 100 μg), ciprofloxacin (CIP, 5 μg), amoxicillin/clavulanate (AML+CL, 20/10 μg), and cotrimoxazole (STX, 1.25/23.75 μg)(8). *Escherichia coli* ATCC 25922 was included as a quality control strain to ensure the accuracy and reproducibility of the testing procedure.

Biofilm Formation Assay

The biofilm-forming capacity of *K. pneumoniae* isolates was evaluated using a microtiter plate assay. Briefly, isolates were grown in tryptic soy broth (TSB) supplemented with 1% glucose at 37 °C for 18–20 h. Cultures were adjusted to a 0.5 McFarland standard, and 100 μL of each suspension was inoculated into individual wells of a sterile 96-well polystyrene microplate. After 24 h of static incubation at 37 °C, non-adherent cells were removed by washing the wells three times with phosphate-buffered saline (PBS). Adherent biofilms were fixed with 250 μL of 96% ethanol for 15 min, stained with 0.2% crystal violet for 5 min, and gently rinsed with distilled water to remove unbound dye. Following air-drying, biofilm-bound crystal violet was solubilized with 200 μL of 33% glacial acetic acid, and absorbance was measured at 492 nm using an ELISA reader. Each assay was performed in triplicate on three separate occasions to ensure reproducibility. Biofilm production was classified based on optical density (OD_{492}) as follows: non-producer ($OD < 1.0$), weak producer ($1.0 \leq OD < 2.0$), moderate producer ($2.0 \leq OD < 3.0$), and strong producer ($OD \geq 3.0$)(9).

Molecular Detection of Virulence Genes by PCR

Genomic DNA was extracted from *K. pneumoniae* isolates using the Karmania Pars Gene commercial DNA extraction kit, and eluted DNA was stored at –20 °C for downstream applications. Prior to PCR, isolates were cultured in Tryptic Soy Broth (TSB) for 24 h at 37 °C. The presence of virulence-associated genes (*wzm, mrkA, pagA,* and *wbbM*) was assessed by conventional PCR using gene-specific primers. Each 25 μL reaction mixture contained: 2.6 μL of 5× PCR master mix (including 0.05 U/μL Taq DNA polymerase and 3 mM $MgCl_2$), 4.0 μL of dNTPs (10 mM), 8.0 μL of each primer (0.8 μM final concentration), 7.0 μL of genomic DNA (10 ng/μL), and 6.4 μL of nuclease-free water. Amplification was carried out in a gradient thermal cycler under the following conditions: initial denaturation at 95 °C for 5 min, followed by 32 cycles of denaturation at 95 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 2 min, with a final extension at 72 °C for 7 min. PCR products were resolved by electrophoresis on a 1% (w/v) agarose gel in 1× TAE buffer at 90 V for 60 min. Gels were visualized under UV light using a Gel Doc imaging system, and the presence of target genes was confirmed by comparing band sizes with a DNA ladder. Each gene was amplified in separate reactions to ensure specificity.

Table 1. Primer sequences of genes

Genes	Sequence (3-5)	Product size
wzm	5'- TGCCAGTTCGGCCACTAAC -3'	129
	5'- GACAACAATAACCGGGATGG -3	
markA	5'- ACGTCTCTAACTGCCAGGC -3'	114
	5'- TAGCCCTGTTGTTTGCTGGT -3'	
pgaA	5'- GCAGACGCTCTCCTATGTC -3'	156
	5'- GCCGAGAGCAGGGGAATC -3'	
wbbm	5'- ATGCGGGTGAGAACAAACCA -3'	121
	5'- AGCCGCTAACGACATCTGAC -3'	

Results

In this study, 26 clinical isolates were identified as *K. pneumoniae* based on phenotypic characteristics, including glucose and lactose fermentation, absence of hydrogen sulfide production and motility, negative indole test, positive citrate utilization, and a positive Voges–Proskauer (VP) reaction. Of these, 18 isolates (69.23%) were confirmed as extended-spectrum β-lactamase (ESBL) producers. The isolates were further classified into classical *K. pneumoniae* (cKp) and hypervirulent *K. pneumoniae* (hvKp) based on the presence of specific virulence markers. Among the 26 isolates, 14 (53.85%) were recovered from urine cultures, of which 1 (7.15%) belonged to the hvKp lineage and 13 (92.86%) were cKp. 6 (23.08%) originated from wound, blood, or catheter samples, all of which (100%) were classified as cKp. 6 (23.08%) were isolated from otorhinolaryngological specimens and sputum, with 4 (66.66%) identified as hvKp and 2 (33.34%) as cKp.

Statistical analysis revealed no significant association between sample source and ESBL production (P = 0.109) or between sample source and hvKp prevalence (P = 0.215). However, a statistically significant correlation was observed between hvKp status and ESBL production (P = 0.003), suggesting a strong link between hypervirulence and antibiotic resistance in these clinical isolates. This finding highlights the emerging convergence of multidrug resistance and enhanced pathogenic potential in *K. pneumoniae*, posing a serious public health concern.

Results of microbial sensitivity determination K. pneumoniae

Table 2: Antibiotic resistance pattern of the isolates

Resistance Status / Antibiotics	resistant		sensitive		Total	
	N	**%**	**N**	**%**	**N**	**%**
Ampicillin	18	23.69	8	77.30	26	100
Cefotaxime	21	77.80	5	23.19	26	100
Cotrimoxazole	15	69.57	11	31.42	26	100
Ceftazidime	10	46.38	16	54.61	26	100
Gentamicin	10	46.38	16	54.61	26	100
Amoxicillin/ Clavulanate	18	23.69	8	77.30	26	100
Ciprofloxacin	20	93.76	6	07.23	26	100
Piperacillin	22	62.84	4	38.15	26	100
imipenem	26	100	0	0	26	100
Total	123	57.52	111	43.47	26	100

The results related to antibiotic sensitivity on 26 clinical samples of *K. pneumoniae* showed that 2.97% of the isolates were resistant to at least one of the 10 antibiotics tested. The highest resistance rates to antibiotics were observed with Imipenem at 100%, followed by Penicillin and Cefotaxime at approximately 80%. A significant association between antibiotic resistance and the type of antibiotic was obtained using the Chi-Square test (P-Value=0.000). These results demonstrated that the highest sensitivity rates, at 54.61%, were observed for Gentamicin and Cefazidime in the samples.

Phenotypic investigation of biofilm formation

In total, 76% of the isolates (20 bacteria) were capable of forming biofilms, while 24% of the isolates (6 bacteria) failed to produce biofilms. Figure 1 illustrates the reactions of isolates with strong, moderate, and weak biofilm formation on the polystyrene microplate. The observed purple color indicates the level of bacteria adhered to the surface or bacterial biofilm. This color intensity can be quantified using a microplate reader at a specific wavelength, converting it into quantitative data.

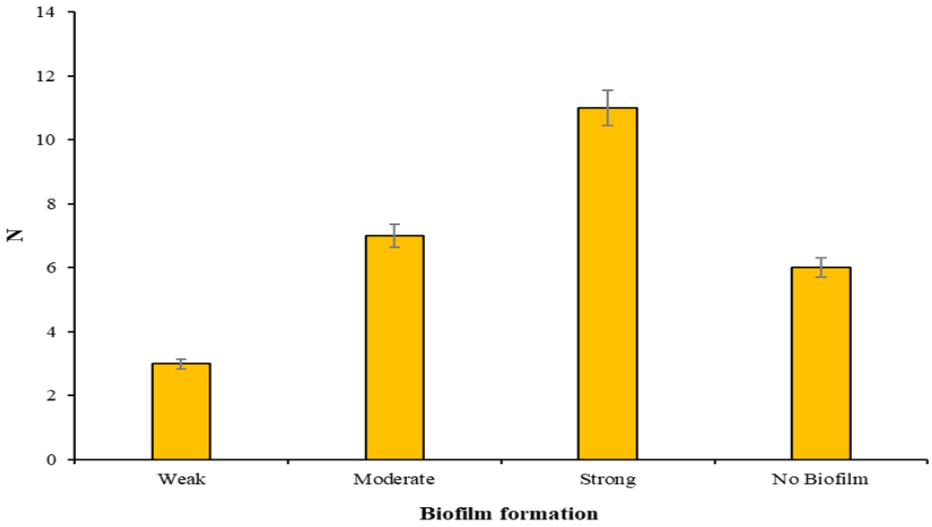


Figure 1: Phenotypic investigation of biofilm production ability in identified isolates

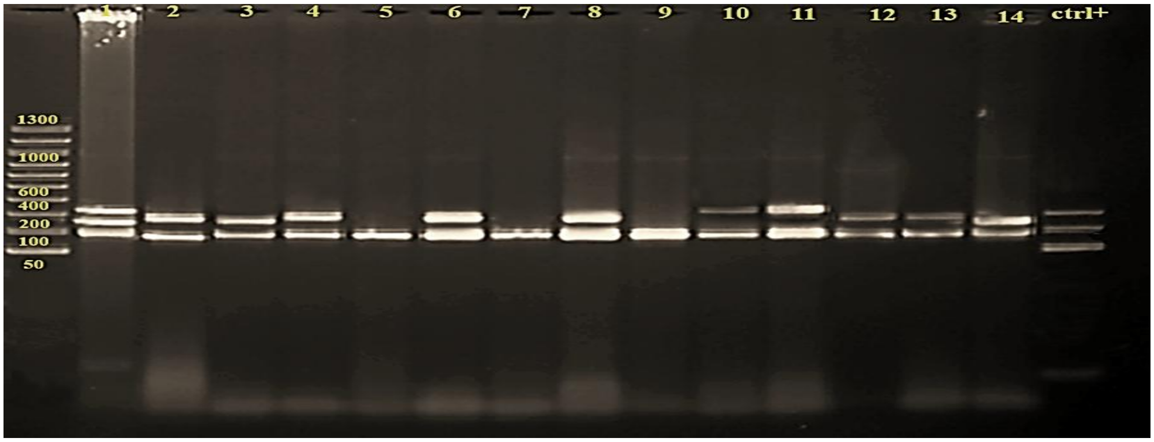
Virulence Gene Prevalence and Association with Biofilm Formation

The prevalence of virulence genes among the 26 *K. pneumoniae* isolates was as follows: *wzm* in 12 isolates (46.15%), *wbbM* in 6 (23.07%), *pgaA* in 9 (34.61%), and *mrkA* in 14 (53.84%). A strong positive correlation was observed between *wbbM* and both *pgaA* (Spearman's $r_s = 0.549$, $P < 0.05$) and *wzm* ($r_s = 0.734$, $P < 0.05$). Additionally, *mrkA* showed a significant positive association with *wbbM* ($r_s = 0.458$, Fisher's exact test $P = 0.0106$), suggesting potential co-regulation or functional synergy among these genes in biofilm formation and virulence.

Table 3: Investigating biofilm production in *K. pneumoniae* isolates containing virulence genes.

Biofilm formation	*markA*	*pgaA*	*wbbm*	*wzm*	Isolates
Strong	+	+	-	+	**1**
Strong	+	+	-	-	**2**
Weak	+	-	-	-	**3**
Strong	+	-	-	-	**4**
No biofilm	-	-	-	+	**5**
Weak	+	-	-	+	**6**
No biofilm	+	-	-	-	**7**
Moderate	+	-	-	-	**8**
Moderate	+	-	-	-	**9**
Moderate	+	+	-	-	**10**
Strong	+	+	-	+	**11**
Strong	+	+	-	+	**12**
Strong	-	-	+	+	**13**
Strong	-	+	-	+	**14**
Strong	+	-	+	+	**15**
Moderate	-	+	-	+	**16**
No biofilm	-	+	-	-	**17**
No biofilm	-	-	+	-	**18**
Weak	+	-	-	-	**19**
Moderate	-	-	+	-	**20**
Moderate	-	-	-	-	**21**
Moderate	-	-	+	-	**22**
Strong	-	+	+	+	**23**
No biofilm	-	-	-	+	**24**
Strong	+	-	-	+	**25**
No biofilm	-	-	-	-	**26**

Molecular results



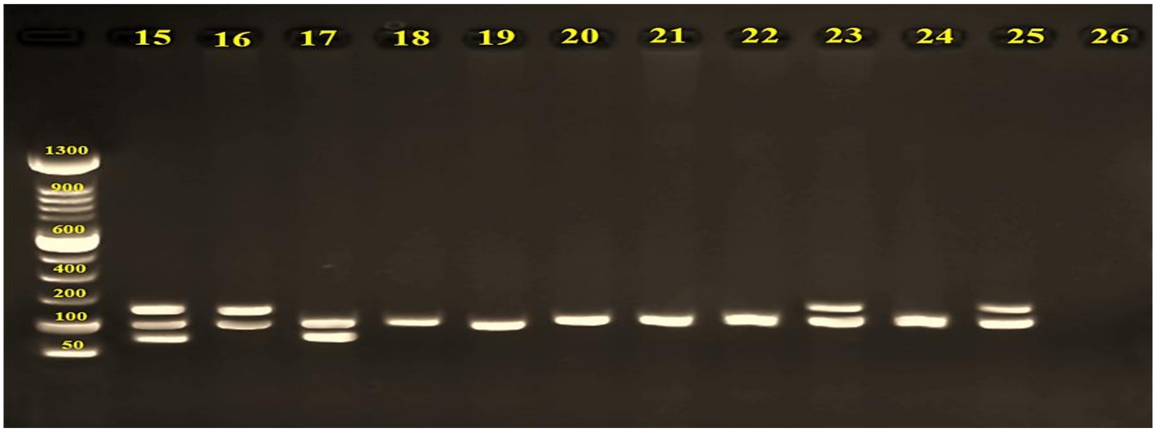


Figure 2: The electrophoresis product of DNA marker 100 bp pulse, strain sample number 1-26, the target genes bands were *wzm* 129 bp, *markA* 114 bp, *pgaA* 156 bp, *wbbm* 121 bp K. pneumonia strains isolated from clinical samples.

According to Figure 3, the relationship between the genes involved in biofilm formation and the ability to produce biofilm has been shown phenotypically, experimentally, the presence of some target genes in the production of strong, medium and weak biofilm is mandatory and the absence of It also caused weak biofilm formation or no biofilm formation was observed. *wzm* and *markA* genes play a specific role in biofilm formation.

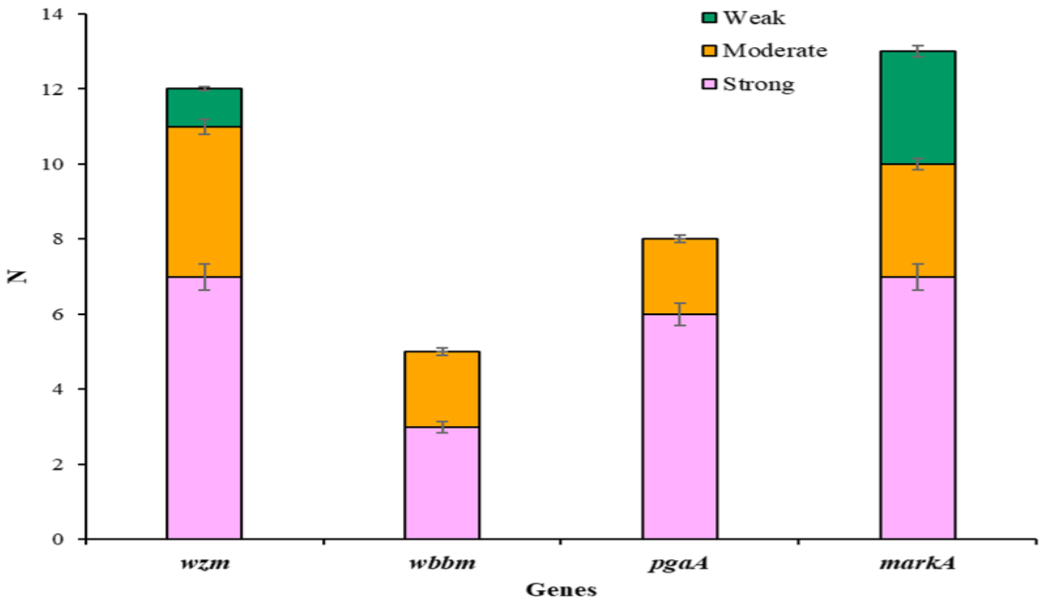


Figure 3: The relationship between the ability to form biofilm and the presence of genes involved in it among *K. pneumonia* strains isolated from clinical samples

Discussion

Klebsiella pneumoniae is one of the infectious agents and causes respiratory damage in humans. In this research study, various isolates of *K. pneumoniae* were obtained from clinical human samples to investigate their antibiotic resistance and the presence of biofilm-producing genes(10). The clinical human samples that demonstrated resistance to different antibiotics were isolated. The isolates were then subjected to antibiotic susceptibility testing to determine their resistance levels. Additionally, molecular techniques were used to identify the presence of biofilm-producing genes in the isolates(١١). The findings of this study highlight the concerning convergence of hypervirulence and multidrug resistance in clinical isolates of *K. pneumoniae* from Tehran, Iran. Among 26 confirmed isolates, a striking 69.23% were ESBL producers, underscoring the high prevalence of β-lactam resistance in this setting, all isolates exhibited 100% resistance to imipenem a carbapenem typically reserved for severe Gram-negative infections, suggesting possible carbapenemase production or other resistance mechanisms beyond ESBLs, which warrants further molecular investigation. Notably, a statistically significant association was observed between the hypervirulent *K. pneumoniae* (hvKp) phenotype and ESBL production ($P = 0.003$), reinforcing growing global evidence of the emergence of "convergent" strains that combine enhanced virulence with multidrug resistance. While hvKp has traditionally been associated with community-acquired, invasive infections in immunocompetent hosts, and classical *K. pneumoniae* (cKp) with nosocomial, multidrug-resistant infections, our dataparticularly the high proportion of hvKp among respiratory specimens (66.66%) suggest a blurring of these historical boundaries. This phenotypic convergence poses a serious therapeutic and epidemiological challenge, as such strains may cause severe, difficult-to-treat infections in both hospital and community settings(12). The high capacity for biofilm formation (76% of isolates) further exacerbates the clinical threat. Biofilms not only protect bacteria from host immune responses but also significantly reduce antibiotic penetration, contributing to chronic and recurrent infections particularly in catheter- or device-associated UTIs, which constituted over half of our isolates(13). The strong phenotypic link between the presence of key virulence genes (*wzm, mrkA, pgaA, wbbM*) and biofilm intensity supports their functional role in adhesion and extracellular matrix production(14). Specifically, *mrkA* (encoding the major subunit of type 3 fimbriae) and *wzm* (involved in capsular polysaccharide export) were among the most prevalent genes and showed significant co-occurrence with robust biofilm phenotypes, consistent with prior studies implicating fimbriae and capsule in surface attachment and immune evasion(15). The significant positive correlations between *wbbM* and both *wzm* ($r_s = 0.734$) and *pgaA* ($r_s = 0.549$), as well as between *mrkA* and *wbbM*, suggest coordinated genetic regulation of capsule synthesis, fimbrial assembly, and polysaccharide intercellular adhesin (PIA) production key components of the biofilm architecture. This genetic synergy may enhance the structural integrity and resilience of *K. pneumoniae* biofilms, thereby promoting persistence in clinical environments(16). Despite the lack of a statistically significant association between sample source and either ESBL status or hvKp classification ($P > 0.05$), the concentration of hvKp in respiratory samples and cKp in urinary and bloodstream isolates aligns with known tropisms: hvKp is increasingly linked to pulmonary and invasive syndromes, while cKp dominates in healthcare-associated UTIs and bacteremia. In conclusion, our results reveal a worrisome trend: the emergence of *K. pneumoniae* strains in Iran that simultaneously exhibit hypervirulence traits, extensive biofilm-forming capacity, and high-level resistance to critically important antibiotics including carbapenems. This triad of pathogenic features demands enhanced surveillance, rapid molecular diagnostics to distinguish hvKp from cKp, and the development of novel anti-biofilm or virulence-targeted therapies. Without such interventions, these convergent strains may fuel untreatable, life-threatening infections with significant public health implications.

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