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Biofilm Formation and Quinolone Resistance Determinants in Clinical *Klebsiella pneumoniae* Isolates from Babol, Northern Iran

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ABSTRACT

Background and Aim: *Klebsiella (K.) pneumoniae* is a major cause of hospital-acquired infections, where biofilm formation and quinolone resistance complicate the treatment outcomes. This research was aimed to examine the biofilm formation occurrence, plasmid-mediated quinolone resistance (PMQR) determinants, and chromosomal alterations among clinical *K. pneumoniae* isolates.

Materials and Methods: In this cross-sectional investigation, 120 unique *K. pneumoniae* isolates were obtained for evaluation. Antimicrobial susceptibility testing and biofilm production examination were conducted through disk diffusion technique and microtiter plate method, respectively. Minimum inhibitory concentration (MIC) was determined by the agar dilution approach. Polymerase chain reaction (PCR) and DNA sequencing were carried out to identify the resistance- and biofilm-related genes, along with mutations in the *gyrA* and *parC* genes. The statistical analysis was performed using SPSS 22.0, and the association between biofilm formation and fluoroquinolones (FQ) resistance was evaluated by Fisher's exact test.

Results: Bacteriocins from BSH1, BSH2, and BSH3 had molecular weights of 55.02 kDa, 51.15 kDa, and 50.12 kDa, respectively, classifying them as class III bacteriocins (>30 kDa). Amino acid analysis revealed the dominance of L-cystine (33–45%) and L-proline (9–20%), along with notable amounts of glutamic acid and leucine. These amino acids are essential for structural stability and antimicrobial activity.

Conclusion: The findings suggest that bacteriocins derived from cincalok not only broaden the understanding of bacteriocin diversity from local fermented foods but also demonstrate strong potential as natural antimicrobial agents for applications in food preservation and healthcare industries.

Keywords: Bacteriocins, Amino Acid Composition, Molecular Weight, Antimicrobial Peptides, Characterization

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1. Introduction

As an opportunistic pathogen, *Klebsiella (K.) pneumoniae* contributes to various infections in healthcare facilities as well as in the community **(1)**. The growing resistance of this microorganism to antimicrobial agents, particularly fluoroquinolones (FQs), has become a major therapeutic challenge **(2)**. FQs are widely prescribed broad-spectrum bactericidal agents derived from a 4-quinolone scaffold and are effective against various bacterial infections **(3)**. Fluoroquinolone resistance in *K. pneumoniae* arises through multiple mechanisms, including point mutations within the quinolone resistance–determining regions (QRDRs) of *gyrA*, *gyrB*, *parC*, and *parE* genes, modifications in membrane permeability, active efflux pumps, and plasmid-mediated pathways such as *qnr* genes (qnrA–S), *aac(6')-Ib-cr*, and efflux systems (*qepA*, *oqxAB*), which may spread via horizontal gene transfer (HGT) **(4–7)**.

The challenge becomes even greater when FQs resistance coexists with biofilm formation (BF), as both mechanisms synergistically enhance the bacterial survival and complicate infection management. BF further complicates this scenario by reducing antibiotic penetration and establishing a protective microenvironment that enhances the bacterial survival **(8)**. The association between biofilm production and resistance genes, particularly those conferring FQ resistance, contributes to therapeutic failure and infection persistence **(9)**. Beyond drug resistance, biofilms support bacterial adhesion to abiotic surfaces, help evade host immune defenses, and promote chronic colonization, especially in device-associated infections such as those involving urinary catheters and ventilators **(10)**. Several genetic determinants are involved in this process: *wzm* and *wbbM* are linked to capsular polysaccharide synthesis; *mrkA* encodes the structural component of type III fimbriae essential for the surface attachment; and *pgaA* is required for the biosynthesis and secretion of poly-β-1, 6-N-acetyl-D-glucosamine, a critical element of the biofilm matrix **(11)**. Collectively, these genes are supposed to enhance the biofilm stability and may contribute to both virulence and multidrug resistance.

The possible coexistence of biofilm-related determinants and quinolone resistance in *K. pneumoniae* represents a potential clinical concern by facilitating persistence and limiting therapeutic options. To address this knowledge gap, the current study aimed to investigate the prevalence of biofilm-related genes and their possible association with FQ resistance in clinical *K. pneumoniae* isolates from Babol, northern Iran, thereby providing insights into the molecular epidemiology of multidrug-resistant strains.

2. Materials and Methods

2.1 Sample Collection and Bacterial Identification

The required number of samples (n) was estimated using $n = z^2P\ (1–P)/d^2$, where *P* is the expected prevalence (0.45), *z* represents the standard normal value (0.975), and *d* is the permissible error margin (0.05) **(1)**. Consequently, over a one-year period (April 2022 to March 2023), a total of 120 no-repetitive *K. pneumoniae* were collected from different clinical specimens, including urine (28/120, 23.3%), wound exudates (25/120, 20.8%), bronchoalveolar lavage (25/120, 20.8%), blood (15/120, 12.5%), sputum (12/120, 10.0%), stool samples (12/120, 10.0%), and cerebrospinal fluid (CSF) (3/120, 2.5%) from two tertiary care teaching hospitals in Babol, northern Iran. The study included only pure cultures presumptively identified as *K. pneumoniae*. Exclusion criteria encompassed contaminated or mixed cultures, isolates confirmed as non-*K. pneumoniae* species, and specimens that failed to yield bacterial growth on culture media. The isolates were confirmed through standard biochemical and microbiological techniques, including Gram staining, SIM test (for motility, H2S, and indole production), lactose fermentation on TSI, Simmons' citrate utilization, urease activity, lysine decarboxylation, and methyl red-Voges-Proskauer (MR-VP) reactions. All confirmed *K. pneumoniae* strains were preserved in brain-heart infusion (BHI) broth (Becton Dickinson, Franklin Lakes, NJ) supplemented with 20% (v/v) glycerol (Merck, Germany) at –70°C for further experiments. *K. pneumoniae* ATCC 13883 served as control.

2.2 Antimicrobial Susceptibility Testing (AST)

Antimicrobial susceptibility testing was performed according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (M100-Ed33, 2023) using the Kirby-Bauer disk diffusion method on Mueller-Hinton agar (Merck, Darmstadt, Germany). Antibiotic disks included ciprofloxacin (CIP; 5 µg), ceftazidime (CAZ; 30 µg), cefotaxime (CTX; 30 µg), tetracycline (TET; 30 µg), imipenem (IPM; 10 µg), ampicillin (AMP; 10 µg), aztreonam (ATM; 30 µg), amikacin (AN; 30 µg), gentamicin (GM; 10 µg), and trimethoprim-sulfamethoxazole (SXT; 5 µg) (Padtan Teb Co., Iran) **(10)**. Bacterial isolates exhibiting resistance to three or more antimicrobial categories were labeled multidrug-resistant (MDR), whereas isolates resistant to all but two or fewer classes were categorized as extensively drug-resistant (XDR) **(11)**.

2.3 Determination of Minimum Inhibitory Concentration (MIC)

The MIC of CIP for *K. pneumoniae* isolates was determined using the agar plate dilution method strictly following CLSI M100 guidelines. CIP powder (≥98% purity; Guangzhou Nanxin Pharmaceutical, Guangzhou, China) was used to prepare two-fold serial dilutions ranging from 0.016 µg/mL to >512 µg/mL on Mueller-Hinton agar plates. Each plate was inoculated with approximately 10^6 CFU of bacterial suspension using a multipoint inoculator to ensure uniform deposition. After incubation at 35 ± 2°C for 18–24 hr, the MIC was recorded as the lowest concentration of CIP that completely inhibited visible bacterial growth **(8)**.

2.4 Biofilm Formation Evaluation by Microtiter Plate

A 96-well microtiter plate was used to assess the biofilm production. Briefly, 200 µL of a 24-hr BHI broth culture was added to each well. Following overnight incubation at 37°C, wells were washed twice with phosphate-buffered saline (PBS, pH 7.2) to remove planktonic cells, gently shaken, and air-dried to fix the adherent bacteria. The biofilms were then stained with 0.1% crystal violet (Sigma, St. Louis, USA) at 25°C for 5 min. Excess stain was rinsed off with tap water, and the plates were allowed to dry. The BF biomass was quantified by measuring the optical density at 570 nm ($OD570_{nm}$) using a microplate ELISA reader (BioTek, Bad Friedrichshall, Germany). All experiments were conducted in triplicate, and a cut-off value (ODc) was determined. BF was assessed using the crystal violet staining, and OD570 values were measured. The classification of BF was based on OD cut-offs relative to the mean OD of the negative control (ODc), as recommended in standard protocols: non-BF producer (OD570 ≤ ODc), weak (ODc < OD570 ≤ 2×ODc), moderate (2×ODc < OD570 ≤ 4×ODc), and strong (OD570 > 4×ODc) **(12)**. Sterile BHI was employed as the negative control, whereas *K. pneumoniae* ATCC 700603, a well-established strong biofilm-forming strain, was used as the positive control.

2.5 Cellular DNA Extraction

Pure microbial colonies were lysed by adding a full loop of bacteria to 25 µL of 0.25% sodium dodecyl sulfate (SDS) and 0.05 N NaOH solution, then heating for 15 min **(12)**. Subsequently, 200 µL of distilled water was added to the tube, and 5 µL of this diluted lysate was utilized as a template for PCR. The extracted DNA was evaluated for quality and quantity using 1.0% agarose gel electrophoresis. Additionally, DNA purity and concentration were determined by measuring absorbance ratios at 260/280 nm using a Thermo Scientific Nanodrop 2000 spectrophotometer. The extracted DNA samples were stored at -20°C for future use.

2.6 Molecular Detection of Quinolone Resistance Genes

As detailed in Table 1, specific primer sets were employed for PCR assays. The PCR was executed using a Techne TC-512 thermal cycler (Eppendorf, Hamburg, Germany) in a total volume of 25 µL, consisting of 1.0–1.8 µL genomic DNA, 12.5–13.6 µL Taq DNA Polymerase Master Mix RED (Ampliqon, Stenhuggervej, Odense M, Denmark), 0.8–1.5 µL of each primer (CinnaGen Co., Iran), and nuclease-free water to the final volume. The thermal cycling program included an initial denaturation at 94–95°C for 5–7 min, followed by 30–32 cycles of denaturation at 94–95°C for 35–60 sec, annealing at 55–58°C for 45–60 sec, and extension at 72°C for 60 sec, with a final extension at 72°C for 5–6 min. PCR amplicons were separated by electrophoresis on a 1.0% agarose gel prepared with 0.5X TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.0), run at 100 V for one hr. Following staining with DNA Safe Stain (SinaClon, Tehran, Iran), amplicons were visualized under UV light using a Bio-Rad transilluminator (Hercules, USA).

2.7. DNA Sequencing and Analysis

The PCR products of the *gyrA* and *parC* genes were sequenced in both forward and reverse directions using an ABI3730XL DNA sequencer (Applied Biosystems, Foster City, USA). The resulting nucleotide sequences were analyzed by performing similarity searches through the National Center for Biotechnology Information (NCBI) BLAST tool, accessible at http://www.ncbi.nlm.nih.gov/BLAST/.

2.8. Statistical Analysis

Statistical analyses were conducted using SPSS 22.0 (IBM Corp., Armonk, NY, USA), and the Fisher's exact test was applied to examine the relationship between biofilm formation and FQ resistance. Statistical significance was defined as a p-value less than 0.05.

Table 1. Primer sequences and PCR cycling conditions.

Set	Genes	Primer sequences (5'→3')	Amplicon size (bp)	Cycles of amplification	Reference
S1	*gyrA*	F;5′ -GGATAGCGGTTAGATGAGC-3′ R;5′ -CGTTCACCAGCAGGTTAGG-3′	521	30	Firouzjaei et al (13)
	parC	F;5′ -AATGAGCGATATGGCAGAGC-3′ R;5′ -TTGGCAGACGGGCAGGTAG-3′	488		
	qnrA	F;5′ -TCAGCAAGAGGATTTCTCA-3′ R;5′ -GGCAGCACTATTACTCCCA-3′	626		
	acc (6')-Ib-cr	F;5′ -TTGGAAGCGGGGACGGAM-3′ R;5′ -ACACGGCTGGACCATA-3′	260		
	qepA	F;5′ -GCAGGTCCAGCAGCGGGTAG-3′ R;5′ -CTTCCTGCCCGAGTATCGTG-3′	199		
S2	*qnrS*	F;5′ -ACGACATTCGTCAACTGCAA-3′ R;5′ -TAAATTGGCACCCTGTAGGC-3′	417		
	qnrB	F;5′ - GGMATHGAAATTCGCCACTG-3′ R;5′ -TTTGCYGYYCGCCAGTCGAA -3′	264		
	oqxA	F;5′ - CTCGGCGCGATGATGCT -3′ R;5′ - CCACTCTTCACGGGAGACGA -3′	392		
	oqxB	F;5′ - TTCTCCCCCGGCGGGAAGTAC -3′ R;5′ - CTCGGCCATTTTGGCGCGTA -3′	512		
S3	*wzm*	F:5'- TGCCAGTTCGGCCACTAAC -3′ R: 5'- GACAACAATAACCGGATGG -3′	148	32	Vuotto et al (14)
	mrkA	F:5'- CACCAAACAGGATGATGTGAG -3′ R: 5'- CGCATAGCCGACGTAGTAAG -3′	262		
S4	*wbbm*	F:5'- ATGCGGGTGAGAACAAACCA -3′ R: 5'- AGCCGCTAACGACATCTGAC -3′	120	33	Devanga Ragupathi et al (15)
	pgaC	F:5'- ATGCCTGTTCCACGCTGTGG -3′ R: 5'- CAGGCTTCCTTTTCCCCGGT -3′	125		

3. Results

In general, 120 *K. pneumoniae* isolates were collected from different clinical specimens as mentioned above. A total of 42 isolates were obtained from pediatric patients (aged 1 month to 18 years), while the remaining 78 isolates were collected from adults (aged 19 to 90 years). The patients' mean age was 32.2 ± 1.3 years. The distribution of isolates across different hospital wards was as follows: Intensive Care Unit (ICU) (30/120, 25.0%), Infectious Diseases Ward (27/120, 22.5%), Pediatric Intensive Care Unit (PICU) (22/120, 18.3%), Emergency Department (22/120, 18.3%), Internal Medicine (13/120, 10.8%), Surgical Ward (4/120, 3.3%), and Hematology-Oncology Ward (2/120, 1.7%).

The resistance rates among the isolates were as follows: AMP (77.5%, 93/120), TET (70.8%, 85/120), ATM (68.3%, 82/120), AN (62.5%, 75/120), CIP (60.0%, 72/120), SXT (58.3%, 70/120), GM (55.0%, 66/120), CAZ (52.5%, 63/120), CTX (44.2%, 53/120) and IPM (38.3%, 46/120) (Table 2). The rate of resistance to IPM (P=0.037), GM (P=0.045), AMP (P=0.000) and CAZ (P=0.003) was significantly different in adults and children. Furthermore, out of 120 isolates examined, 69.2% (83/120) were identified as BF producers, of which 16.8% (14), 27.7% (23), and 55.4% (46) were categorized as weak, moderate, and strong biofilm formers, respectively. The prevalence of BF-producing strains was higher among adult (53) than pediatric isolates (30). Fisher's exact test revealed that BF-producing isolates demonstrated significantly higher resistance to antimicrobials compared to non-producers, particularly against AMP, IPM, GM, and CAZ (P< 0.05) (Figure 1). MDR and XDR strains were detected in 64.2% (77/120) and 11.7% (14/120) of isolates, respectively. MIC results showed that 56.7% (68/120) of the isolates were

resistant to CIP and were consequently classified as FQR-Kp. BF-producing isolates exhibited higher resistance to CIP compared to non-BF producers. Among the total BF-producing isolates, 22.9% (19/83) were resistant to CIP, of which 15.8% (3/19), 36.8% (7/19) and 47.4% (9/19) strains exhibited weak, moderate, and strong BF formation capacity, respectively. Additionally, 31.5% (6/19) of CIP-resistant BF-forming isolates were obtained from pediatric patients, while 68.4% (13/19) were isolated from adults.

The results of PCR showed that the prevalence of *gyrA*, *parC*, *qnrA*, *qnrS*, *acc (6')-Ib-cr*, *qepA*, *qnrB*, *oqxA*, *oqxB*, *wzm*, *mrkA*, *wbbm* and *pgaC* genes were 42.6% (29/68), 45.5% (31/68), 7.4% (5/68), 57.4% (39/68), 75.0% (51/68), 85.3% (58/68), 45.5% (31/68), 72.1% (49/68), 85.3% (58/68), 19.1% (13/68), 89.7% (61/68), 27.9% (19/68) and 48.5% (33/68), respectively. The *qnrA* gene was detected only in the strong BF-producers. Notably, the prevalence of FQ resistance genes was markedly greater among BF-producing strains (P<0.05). The observed co-occurrence of resistance determinants among the isolates was as follows: *gyrA/ parC* (23.5%, 16/68), *qnrB/ qnrS/ acc (6')-Ib-cr* (27.9%, 19/68), *qnrA / acc (6')-Ib-cr* (2.9%, 2/68), *qnrB/ qnrS* (30.8%, 21/68), *qepA/ oqxA / oqxB* (39.7%, 27/68), and *gyrA/ parC/ qnrB/ qnrS/ acc (6')-Ib-cr, qepA/ oqxA / oqxB* (2.9%, 2/68) (Table 3). Genes *qepA* (P=0.014) and *oqxB* (P=0.044) were significantly more prevalent among BF-producing isolates. The S83I mutation in *gyrA* was the most dominant change in FQ-resistant isolates selected for direct sequencing. The second most substitution in *gyrA* was D87N. Prevalent mutations in *parC* were S80I and E84V. The *gyrA* D87N mutation was detected in 55.2% (16/29) of isolates. Thus, the *ParC* S80I and E84V substitutions were identified in 38.7% (12/31) and 29.0% (9/31), respectively. Notably, 18.7% (3/16) of *gyrA*-mutated isolates harbored at least two concurrent mutations in this gene.

Table 2. Resistance patterns to antibiotics among biofilm-forming isolates.

Resistant (R), n (%)	Intermediate (I), n (%)	Susceptible (S), n (%)	Total (n)	Group	P-value	Antimicrobial
34 (45.9)	2 (2.7)	38 (51.4)	74	Adults	0.037	IPM
12 (26.1)	0 (0.0)	34 (73.9)	46	Pediatric		
6 (28.5)	1 (4.7)	14 (66.7)	21	No-BF Adults		
4 (25.0)	0 (0.0)	12 (75.0)	16	No-BF Pediatric		
28 (52.8)	1 (1.9)	24 (45.3)	53	BF Adults		
8 (26.7)	0 (0.0)	22 (73.3)	30	BF Pediatric		
53 (71.6)	1 (1.3)	20 (27.0)	74	Adults	0.451	ATM
29 (63.0)	1 (2.2)	16 (34.7)	46	Pediatric		
15 (71.4)	0 (0.0)	6 (28.5)	21	No-BF Adults		
11 (68.7)	0 (0.0)	5 (31.3)	16	No-BF Pediatric		
38 (71.6)	1 (1.9)	14 (26.4)	53	BF Adults		
18 (60.0)	1 (3.3)	11 (36.7)	30	BF Pediatric		
59 (79.7)	0 (0.0)	15 (20.3)	74	Adults	0.108	AN
16 (34.7)	0 (0.0)	30 (65.2)	46	Pediatric		
13 (61.9)	0 (0.0)	8 (38.1)	21	No-BF Adults		
7 (43.7)	0 (0.0)	9 (56.3)	16	No-BF Pediatric		
46 (89.7)	0 (0.0)	7 (13.2)	53	BF Adults		
9 (30.0)	0 (0.0)	21 (70.0)	30	BF Pediatric		
49 (66.2)	2 (2.7)	23 (31.1)	74	Adults	0.279	SXT
21 (45.6)	3 (6.5)	22 (47.8)	46	Pediatric		
18 (85.7)	0 (0.0)	3 (14.3)	21	No-BF Adults		
5 (31.3)	1 (6.3)	10 (62.5)	16	No-BF Pediatric		
31 (58.5)	2 (3.7)	20 (37.7)	53	BF Adults		
16 (53.3)	2 (6.7)	12 (40.0)	30	BF Pediatric		

Resistant (R), n (%)	Intermediate (I), n (%)	Susceptible (S), n (%)	Total (n)	Group	P-value	Antimicrobial
49 (66.2)	2 (2.7)	23 (31.1)	74	Adults	0.045	GM
17 (36.9)	3 (6.5)	26 (56.5)	46	Pediatric		
16 (76.2)	1 (4.7)	4 (19.0)	21	No-BF Adults		
6 (37.5)	1 (6.3)	9 (56.3)	16	No-BF Pediatric		
33 (62.3)	1 (1.9)	19 (35.8)	53	BF Adults		
11 (36.7)	2 (6.7)	17 (56.7)	30	BF Pediatric		
62 (83.7)	4 (5.4)	8 (10.8)	74	Adults	<0.001	AMP
31 (67.4)	0 (0.0)	15 (32.6)	46	Pediatric		
16 (76.2)	1 (4.7)	4 (19.0)	21	No-BF Adults		
4 (25.0)	0 (0.0)	12 (75.0)	16	No-BF Pediatric		
46 (86.7)	3 (5.7)	4 (7.5)	53	BF Adults		
27 (90.0)	0 (0.0)	3 (10.0)	30	BF Pediatric		
52 (70.3)	0 (0.0)	22 (29.7)	74	Adults	0.419	TET
33 (71.7)	1 (2.2)	12 (26.1)	46	Pediatric		
14 (66.7)	0 (0.0)	7 (33.3)	21	No-BF Adults		
12 (75.0)	0 (0.0)	4 (25.0)	16	No-BF Pediatric		
38 (71.7)	0 (0.0)	15 (28.3)	53	BF Adults		
21 (70.0)	1 (3.3)	8 (26.7)	30	BF Pediatric		
42 (56.7)	2 (2.7)	30 (40.5)	74	Adults	0.212	CTX
11 (23.9)	1 (2.2)	34 (73.9)	46	Pediatric		
8 (38.1)	0 (0.0)	13 (61.9)	21	No-BF Adults		
4 (25.0)	0 (0.0)	12 (75.0)	16	No-BF Pediatric		
34 (64.2)	2 (3.8)	17 (32.1)	53	BF Adults		
7 (23.3)	1 (3.3)	22 (73.3)	30	BF Pediatric		
49 (66.2)	3 (4.1)	22 (29.7)	74	Adults	0.003	CAZ
14 (30.4)	2 (4.3)	30 (65.2)	46	Pediatric		
19 (90.5)	1 (4.7)	1 (4.7)	21	No-BF Adults		
4 (25.0)	1 (6.3)	11 (68.7)	16	No-BF Pediatric		
30 (56.6)	2 (3.8)	21 (39.6)	53	BF Adults		
10 (33.3)	1 (3.3)	19 (63.3)	30	BF Pediatric		
52 (70.3)	3 (4.1)	19 (25.6)	74	Adults	0.267	CIP
20 (43.5)	2 (4.3)	24 (52.2)	46	Pediatric		
12 (57.1)	2 (9.5)	7 (33.3)	21	No-BF Adults		
9 (56.3)	0 (0.0)	7 (43.7)	16	No-BF Pediatric		
40 (75.5)	1 (1.9)	12 (22.6)	53	BF Adults		
11 (36.7)	2 (6.7)	17 (56.7)	30	BF Pediatric		

Table 3. PRISMA flowchart demonstrating literature search and selection process 126.

Target Genes	BF-production (n=83)		No-BF production (n=37)		Total (n=120)		P-value
	Adults (n=53)	Pediatric (n=30)	Adults (n=21)	Pediatric (n=16)	Adults (n=74)	Pediatric (n=46)	
gyrA	12 (22.6%)	7 (23.3%)	8 (38.1%)	2 (12.5%)	20 (27.0%)	9 (19.5%)	0.849
parC	11 (20.7%)	8 (26.7%)	8 (38.1%)	4 (25.0%)	19 (25.7%)	12 (26.1%)	0.293
qnrA	3 (5.7%)	1 (3.3%)	1 (4.7%)	0 (0.0%)	4 (5.4%)	1 (2.2%)	1.000
qnrS	11 (20.7%)	12 (40.0%)	9 (42.8%)	7 (43.7%)	20 (27.0%)	19 (41.3%)	0.554
acc (6')-Ib-cr	19 (35.8%)	12 (40.0%)	10 (47.6%)	8 (50.0%)	29 (39.2%)	22 (47.8%)	0.628
qepA	26 (49.1%)	6 (20.0%)	16 (76.2%)	10 (62.5%)	42 (56.7%)	16 (34.7%)	0.014
qnrB	11 (20.7%)	8 (26.7%)	6 (28.5%)	6 (37.5%)	17 (22.9%)	14 (30.4%)	0.554
oqxA	16 (30.2%)	12 (40.0%)	12 (57.1%)	9 (56.3%)	28 (37.8%)	21 (45.6%)	0.628
oqxB	19 (35.8%)	15 (50.0%)	13 (61.9%)	11 (68.7%)	32 (43.2%)	26 (56.5%)	0.044
Wzm	5 (9.4%)	3 (10.0%)	4 (19.0%)	1 (6.3%)	9 (12.2%)	4 (8.6%)	1.000
mrkA	19 (35.8%)	18 (60.0%)	14 (66.7%)	10 (62.5%)	33 (44.5%)	28 (60.8%)	0.600
Wbbm	7 (13.2%)	5 (16.7%)	4 (19.0%)	3 (18.7%)	11 (14.8%)	8 (17.4%)	1.000
pgaC	11 (20.7%)	11 (36.7%)	5 (23.8%)	6 (37.5%)	16 (21.6%)	17 (36.9%)	0.838

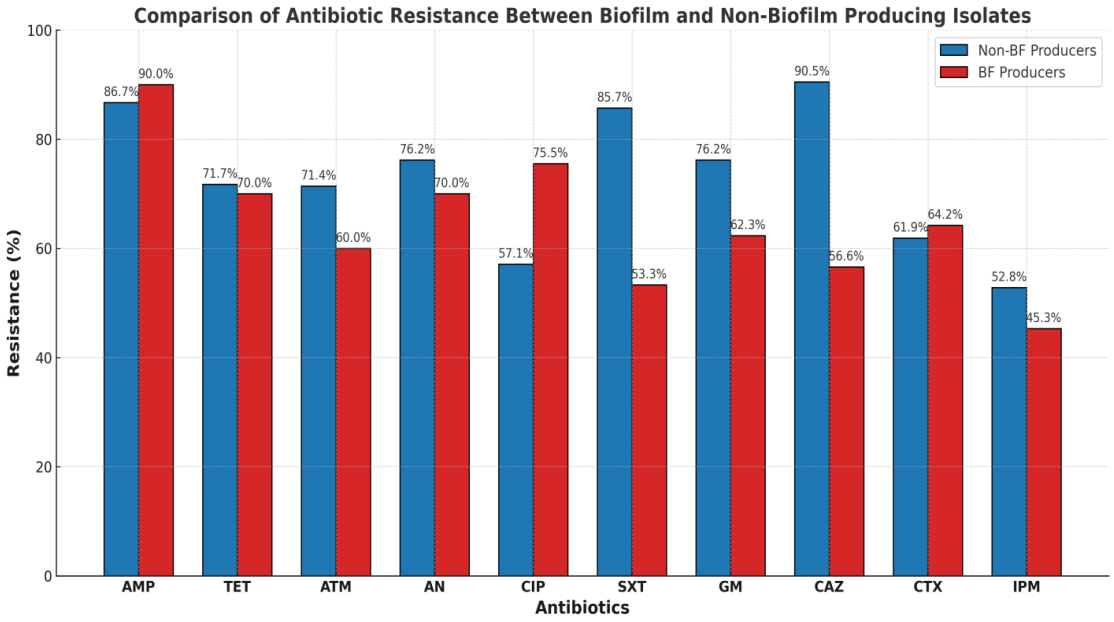

Figure 1. Antibiotic resistance among BF producers and non-BF producers **(Prepared by Authors, 2025)**.

4. Discussion

K. pneumoniae is a significant pathogenic bacterium related to a variety of illness in healthcare settings, especially among patients with weakened immunity. The formation of biofilms enables these bacteria to firmly attach to medical devices and host tissues while providing protection against antibiotics and immune responses, making infections harder to treat and more likely to persist **(16-18)**.

Our study is novel as it simultaneously evaluates biofilm formation, multiple resistance genes, and detailed *gyrA/parC* mutations in *K. pneumoniae* isolates from Babol, north of Iran. These integrated data, combined with comparisons to national and global reports, provide new insights into regional resistance patterns and their clinical implications.

In our study, high resistance was observed against AMP (77.5%), TET (70.8%), ATM (68.3%), AN (62.5%), and CIP (60.0%), which is consistent with Firouzjaei et **(13)** and Kashefieh et al **(19)**. Additionally, 38.3% of strains were classified as carbapenem-resistant *K. pneumoniae* (CRKp), consistent with Ferdosi-Shahandashti et al **(20)** but higher than the 14.6% reported by Bina et al **(21)**. Differences in resistance rates across studies likely reflect geographic, methodological, and clinical variations. BF-producing isolates displayed significantly higher antimicrobial resistance than non-producers (P<0.05), in line with Nirwati et al **(22)**, Shadkam et al **(23)**, Karimi et al **(24)**, Jomehzadeh et al **(25)**, and Pajohesh et al **(26)**. This enhanced resistance is attributed to the protective extracellular polymeric substances (EPS), reduced metabolic activity within the biofilm, and increased expression of resistance genes, which together support a mechanistic link between biofilm formation and multidrug resistance in *K. pneumoniae* **(27)**.

In our cohort, 69.2% of isolates formed biofilms, with 16.8% weak, 27.7% moderate, and 55.4% strong producers. Comparable studies reported slightly different distributions: Karimi et al **(24)** observed 75% biofilm formation with only 20% strong producers, while Shadkam et al **(23)** reported 75% overall with 25% strong, 19% moderate, and 31% weak producers. Variations may result from differences in isolate sources, environmental exposure, or genetic determinants. PCR analysis in our study revealed varying prevalence rates of multiple resistance and virulence-associated genes among *K. pneumoniae* isolates. Specifically, the *mrkA*, *qepA*, *oqxB*, *aac(6')-Ib-cr*, *oqxA*, *qnrS*, *pgaC*, *parC*, *qnrB*, *gyrA*, *wbbm*, *wzm*, and *qnrA* genes were detected in 89.7%, 85.3%, 85.3%, 75.0%, 72.1%, 57.4%, 48.5%, 45.5%, 45.5%, 42.6%, 27.9%, 19.1%, and 7.4% of isolates, respectively. In our study, no significant association was observed between the presence of genes *wzm*, *mrkA*, and *wbbm* and biofilm production. However, Vuotto et al **(14)** reported a direct correlation between the expression of these genes and biofilm formation. Such discrepancies may be attributed to differences in sample size as well as variations in the type of clinical specimens analyzed. These findings are comparable to reports from other regions. In India, Geetha et al **(5)** observed a high prevalence of resistance-associated genes among 110 *K. pneumoniae* isolates, with *aac(6')-Ib-cr* (89%), *gyrA* (85%), *parC* (80%), *gyrB* (77%), *parE* (58%), *qnrB* (12%), *oqxAB* (6.3%), and *qnrS* (4.5%). The discrepancy between our results and those of Geetha *et al.* may be explained by differences in geographical distribution of resistance determinants, antibiotic prescribing practices, and genetic diversity of circulating *K. pneumoniae* strains. Furthermore, variations in sample size, patient population, and methodological approaches could also contribute to the observed differences. Similarly, Kashefieh et al **(19)** in northwestern Iran reported detection rates of *qepA*, *oqxB*, and *oqxA* genes at 95%, 87.5%, and 70%, respectively in 100 isolates. These studies highlight the potential for HGT of plasmid-mediated quinolone resistance (PMQR) genes, which can accelerate the spread of FQ-resistance. Firouzjaei et al **(13)** analyzed 68 FQ-resistant strains and reported the following prevalence of resistance genes: *gyrA* (39.7%), *parC* (42.6%), *qnrA* (5.9%), *qnrS* (54.4%), *aac(6')-Ib-cr* (69.1%), *qepA* (94.1%), *qnrB* (41.2%), *oqxA* (69.1%), and *oqxB* (83.8%). In Iraq, Makhrmash et al **(28)** demonstrated that *fimH*, *mrkA*, and *mrkD* genes were present in 87.5% (49/56), 46.4% (26/56), and 53.6% (30/56) of isolates, respectively.

Collectively, these data underscore the widespread distribution of both FQ-resistance and virulence-related genes in *K. pneumoniae*, emphasizing the critical need for continuous molecular surveillance to prevent dissemination of MDR strains. Among FQ-resistant *K. pneumoniae* isolates, the most frequently observed mutation in the *gyrA* gene was S83I, while D87N was present in 55.2% of sequenced samples. In the *parC* gene, S80I and E84V were the predominant substitutions. The mutations identified in *gyrA* (S83I, D87N) and *parC* (S80I, E84V) are missense mutations, leading to single amino acid changes in the proteins. These substitutions occur in the QRDRs and are known to alter the local structure of the enzyme, reducing the FQ binding and contributing to resistance. No nonsense or frame-shift mutations were detected in our isolates. These observations were consistent with findings reported by Firouzjaei et al **(13)** and Azargun et al **(29)**. Huang et al **(30)** also noted that FQ-resistant *K. pneumoniae* commonly harbors S83L and D87N mutations in *gyrA*, along with S80I in *parC* **(30)**. Rezaei et al **(31)** analyzed 40 FQ-resistant isolates and found that codon 83 of *gyrA* and codon 80 of *parC* were the most frequently mutated positions. In *gyrA*, both single substitutions (Ser83→Ile, Asp87→Gly) and several double substitutions such as, Ser83→Phe/Asp87→Ala, Ser83→Tyr/Asp87→Ala, Ser83→Ile/Asp87→Tyr, Ser83→Phe/Asp87→Asn, and Ser83→Ile/Asp87→Gly were detected. In *parC*, single amino acid changes including Ser80→Ile and Glu84→Lys were identified **(31)**. Although we identified frequent single and double mutations in *gyrA* (e.g., S83I, D87N) and *parC* (S80I, E84V), their functional impact on FQ resistance was not directly assessed. Nevertheless, these substitutions are known to reduce drug binding and correlate with the resistance phenotypes observed in our isolates.

The present study has certain limitations, including the absence of genotyping analyses and functional assays to validate gene expression. In addition, phenotypic efflux pump activity was not evaluated,

and the limited number of pediatric isolates (n=42) reduces the reliability of subgroup inferences. As a consequence, high-level FQ resistance in our study was generally produced by cumulative point mutations in the QRDRs of *gyrA* and *parC*. Single substitutions, such as S83I or S80I, may confer low to moderate resistance, whereas combinations of mutations at key positions (e.g., S83I + D87N in *GyrA* along with S80I in *ParC*) synergistically reduce FQ binding to DNA gyrase and topoisomerase IV, resulting in high-level resistance. All these mutations are missense, causing single amino acid changes that alter the local conformation of the enzyme active site and decrease FQ affinity.

5. Conclusion

This study demonstrates that biofilm-producing *K. pneumoniae* isolates exhibit higher multidrug resistance, associated with resistance and virulence genes as well as cumulative point mutations in the QRDRs of *gyrA* and *parC* genes. High-level quinolone resistance appears to result from synergistic amino acid substitutions that reduce drug binding. Strong biofilm formation further enhances antibiotic tolerance, highlighting the importance of targeting biofilms and monitoring key missense mutations to improve treatment outcomes.

6. Declarations

6.1 Acknowledgment

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6.2 Ethical Considerations

This study was approved by the Ethics Committee of Babol University of Medical Sciences under the ethical approval code IR.MUBABOL.REC.1400.082.

6.3 Authors' Contributions

M.D. and M.K. contributed to the study design and laboratory experiments. M.Z. performed data analysis. A.Z.B. and M.H.T-A. Participated in data interpretation and manuscript revision. A.P. supervised the study and prepared the final manuscript. All authors read and approved the final version.

6.4 Conflict of Interests

The authors have no conflicts of interest to declare.

6.5 Financial Support and Sponsorship

This study was approved by Babol University of Medical Sciences and registered under the tracking code; 724133555.

6.6 Using Artificial Intelligence Tools (AI Tools)

All authors declare that there is no use of AI Tools in this study, including the writing of this manuscript.

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