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Phenotypic and Genotypic Detection of Biofilm Formation of *Klebsiella Pneumoniae* Clinical Isolates

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ABSTRACT

Background: This study aimed to investigate the biofilm formation ability of *K. pneumoniae* clinical isolates using phenotypic and genotypic methods. Additionally, the association of biofilm formation with antibiotic resistance and the presence of biofilm-related genes was investigated.
Materials & Methods: In this research, a total of 52 clinical isolates of *K. pneumoniae* were collected from educational hospitals affiliated with Babol University of Medical Sciences from March to October 2023. *K. pneumoniae* isolates were identified through standard microbiological and biochemical tests. Disk diffusion, microtiter plate, and polymerase chain reaction assays were also performed to evaluate the biofilm formation ability of these isolates.
Findings: *K. pneumoniae* isolates were obtained from various clinical specimens. The isolates showed the highest resistance to ceftazidime (54%) and the lowest resistance to amikacin (17%). More than 48% of the isolates were multidrug resistant. Of the 52 *K. pneumoniae* isolates, 43 (82.69%) isolates produced biofilm, whereas the remaining nine (17.3%) did not. *K. pneumoniae* isolates harbored biofilm formation genes, including *treC* (78.84%), *wcaG* (71.15%), *mrkD* (65.38%), *mrkA* (63.46%), *iutA* (40.38%), and *magA* (15.38%).
Conclusion: This study demonstrates that *K. pneumoniae* isolates are highly pathogenic because of antibiotic resistance and carrying biofilm genes. Given the biofilm formation propensity of these strains, it is imperative to elucidate the underlying mechanisms of biofilm formation in *K. pneumoniae*. Developing strategies to inhibit this process is paramount in the effective management of infections caused by this pathogen.

Keywords: *Klebsiella Pneumoniae,* Biofilms, Antibiotic, Infections

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Introduction

Klebsiella pneumoniae (*K. pneumoniae*) is a Gram-negative, non-motile, facultative anaerobic, usually encapsulated, rod-shaped bacterium that could cause various healthcare-associated diseases, including pneumonia, septicemia, soft tissue infections, and bloodstream infections [1, 2]. This bacterium grows on the mucosal surfaces of animals and in soil, plants, and water. Also, this bacterium often colonizes the oropharynx and gastrointestinal system of humans. It contributes to the increased incidence of opportunistic infections in individuals with weakened immune systems, such as those with bladder neuropathy or diabetes mellitus [3]. The main pathogenic characteristics of *K. pneumoniae* include the presence of a capsule, fimbriae, lipopolysaccharides , and the ability to form biofilms [4]. Biofilms represent structured consortia of bacteria encapsulated within a self-produced extracellular matrix. This matrix is a complex composite primarily composed of proteins, exopolysaccharides, DNA, and lipopeptides, which collectively contribute to biofilm robustness and function [5]. The ability of *K. pneumoniae* to form biofilm protects it against host immunological responses and antibiotics and increases its stability and survival on epithelial tissues and medical device surfaces [5, 6]. Various genetic components, including aerobactin (*iutA*), type III fimbriae (*mrkA* and *mrkD*), and capsular polysaccharide (CPS) (*treC*, *wcaG*, *magA*), are known to stimulate *K. pneumoniae* biofilm production [7]. Type III fimbriae are mainly composed of MrkA protein subunits, which contribute to the initiation of the biofilm formation process. MrkD, a protein at the end of fimbriae, imparts adhesive characteristics to these appendages and enables them to attach to polyethylene and polyvinyl chloride surfaces, enhancing the binding ability of fimbriae [8].

K. pneumoniae has recently developed antibiotic resistance due to biofilm production [9]. Bacteria enclosed in biofilm are protected from antibiotics, diminishing the efficacy of antibiotic therapy [10]. This is especially alarming considering the rapid dissemination of extensively drug-resistant and hypervirulent strains of *K. pneumoniae* worldwide [11].

Objectives: This study aimed to investigate the biofilm formation ability of *K. pneumoniae* clinical isolates using phenotypic and genotypic methods. Additionally, the association of biofilm formation with antibiotic resistance and the presence of biofilm-related genes was investigated.

Materials and Methods

Bacterial isolation and identification: In this cross-sectional study, a total of 52 non-duplicate *K. pneumoniae* isolates were collected from urine, blood, sputum, stool, wound swab, bronchoalveolar lavage (BAL), biopsy, and pus samples submitted to the Laboratories of hospitals affiliated to Babol University of Medical Sciences, Babol, Iran (Shahid Beheshti, Ayatollah Rouhani and Shahid Yahyanejad) from March to October 2023. Standard biochemical and microbiological tests such as gram staining, catalase, oxidase, reaction on SH2/indole/motility (SIM) medium, triple sugar iron agar (TSI), Voges-Proskaure (VP), citrate, and urease were used to confirm the isolates. After confirmation, the isolates were stored in brain heart infusion (BHI) broth (Merck, Germany) containing 15% glycerol at -70 °C [12].

Antimicrobial susceptibility: The antimicrobial susceptibility of the isolates was evaluated using the Kirby-Bauer disc diffusion method on Mueller–Hinton agar medium (Merck, Germany). The antibiotics (Padtan Teb Co, Iran) tested included ceftriaxone (30 µg), ceftazidime (30 µg), cefotaxime (30 µg), cefixime (5 µg), imipenem (10 µg), tete racycline (30 µg), trimethoprim-sulfamee thoxazole (1.25/23.75 µg), ciprofloxacin (5

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µg), gentamicin (10 µg), and amikacin (30 µg). Following incubation at 37 °C for 16-18 hours, the inhibition zone diameters were measured in millimeters. The results were subsequently analyzed in accordance with the 2023 criteria established by the Clinical and Laboratory Standards Institute (CLSI) [13]. *K. pneumoniae* isolates exhibiting resistance to three or more distinct categories of antimicrobials were categorized as multidrug-resistant (MDR) *K. pneumoniae* [14]. *Escherichia coli* (*E. coli*) ATCC25922 was employed as a control strain.

Microtiter plate biofilm formation assay: The ability of *K. pneumoniae* isolates to produce biofilm was assessed using the microplate technique [15]. Briefly, 200 µL of a *K. pneumoniae* suspension in BHI broth with a 0.5 McFarland standard turbidity was added to each microtiter plate (SPL, Korea) well and incubated overnight at 37 °C. Following three rounds of washing with normal saline, 200 µL of 96% methanol was added to each well to fix the cells. After 15 min, the contents were removed, and the plate was allowed to dry at room temperature. To initiate the staining process, 200 µL of 2% crystal violet was added to each well and left for five min. Then 200 µL of 33% acetic acid was added to each well after removing the dye and incubated at 37 °C for 15 min. Media-filled empty wells were used as a negative control. Using an ELISA reader (Bio Tek, USA), the absorbance of each well was measured at 570 nm.

The biofilm formation test was performed three times for each sample, and measurements were made. The average OD of the negative controls plus (3×) their standard deviation (SD) was used to determine the optical density cut-off value (ODc). The isolate with $OD \leq ODc$ was considered as a non-biofilm producer, $ODc < OD \leq 2ODc$ as a weak biofilm producer, $2ODc < OD \leq 4ODc$ as a moderate biofilm producer, and $OD > 4ODc$ as a strong biofilm producer [16]. *Acinetobacter baumannii* ATCC 19606 was used as a positive control in biofilm formation assay.

DNA extraction: The genomic DNA was extracted using the boiling method. To isolate DNA, *K. pneumoniae* colonies cultivated on MacConkey agar (Merck, Germany) were removed and added to 300 µL of Tris-EDTA buffer in an Eppendorf tube. The tube was heated at 100 °C for 15 min, frozen at -20 °C for 20 min, and then subjected to centrifugation at 8000 xg for 5 min. The resulting liquid (supernatant) was transferred to a new Eppendorf tube and store at -20 °C until used [17].

Detection of biofilm formation genes by

Table 1) Sequences of primers used in the study

Genes	Primer Sequence (5′→ 3′)	Annealing Temperature	Amplicon Size (bp)	Reference
iutA	5'- GGGAAAGGCTTCTCTGCCAT -3' 5'- TTATTCGCCACCACGCTCTT -3'	55° C	920	[29]
mrkA	5'- AATGTAGGCGGCGGTCAG -3' 5'- CTCTCCACCGATAACGCCA -3'	59° C	351	[27]
mrkD	5'- CTGAGTGAAACGGGATATGC -3' 5'- AGCGGTATGGTGATGTAGC -3'	58° C	224	[27]
treC	5'- CCGACAGCGGGCAGTATT -3' 5'- CGCCGGATTCTCCCAGTT -3'	53° C	71	[30]
wcaG	5'- AGCAACCGATTAGTGAGTCC -3' 5'- TCAACGCCAGTGCCTACG -3'	58° C	402	[27]
magA	5'- CATTGCCGCTACTACAGGAG -3' 5'- AGTGAACGAATTGATGCTTGG -3'	60° C	239	[27]

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polymerase chain reactions (PCRs): In the study, PCRs were performed using specific primers to identify genes associated with biofilm formation, including *iutA*, *mrkA*, *mrkD*, *treC*, *wcaG*, and *magA* (Table 1).

PCR amplification reactions were conducted in a total volume of 24 µL, consisting of 10 µL of Master Mix RED (Ampliqon, Denmark), one µL of each primer (synthesized by metabion, Germany), two µL of template DNA, and ten µL of sterile distilled water.

The PCR protocol involved an initial denaturation step at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 45 s, annealing at a gradient temperature ranging from 53 to 60 °C for 45 s, and extension at 72 °C for 45 s. Finally, a final extension step was performed at 72 °C for 5 min. PCR products were resolved by electrophoresis in 1% agarose gel (SinaClon, Iran) using 1× TBE (Tris/ borate/EDTA) buffer.

The gel was stained with safe stain load dye (SinaClon, Iran) and then visualized under UV (ultraviolet) light.

Statistical analysis: Statistical analyses were conducted using SPSS, Version 27.0. The chi-square test assessed the relationship between biofilm formation with specimen type and antibiotic susceptibility. A *p*-value of less than 0.05 was deemed statistically significant.

Findings

Distribution and identification of isolates: A total of 52 *K. pneumoniae* isolates were procured from various clinical specimens and hospital wards using conventional microbiological methods. Clinical specimens included urine (n=15, 28.84%), blood (n=10, 19.23%), sputum (n=8, 15.38%), stool (n=6, 11.53%), wound swab (n=5, 9.61%), BAL (n=5, 9.61%), biopsy (n=2, 3.84%), and pus (n=1, 1.92%) samples. The isolates were gathered from various hospital wards, including the intensive care unit (ICU) (n=22, 42.3%), internal medicine ward (n=10, 19.23%), infectious diseases ward (n=8, 15.38 %), urology ward (n=7, 13.46%), and emergency ward (n=5, 9.61%).

Antibiotic susceptibility profile: The results of antibiotic susceptibility tests demonstrated a pronounced resistance to ceftazidime, with 53.84% (n=28) of the isolates showing resistance. Conversely, the isolates showed the least resistance to amikacin, and only 17.3% (n=9) of the isolates were resistant (Figure 1). More than 48% of the isolates were MDR.

Biofilm formation: Of the 52 *K. pneumoniae* isolates evaluated, 43 (82.69%) isolates were found to be biofilm producers, whereas the remaining nine (17.3%) were not. Among the 43 isolates that produced biofilm, 12 (27.9%)

Table 2) Correlation between specimen type and biofilm formation

Sample type	Biofilm Formation				*P* -Value
	Non-Biofilm	Weak Biofilm	Moderate Biofilm	Strong Biofilm	
Urine No. (%)	3 (20)	4 (26.66)	1 (6.66)	7 (46.66)	.543
Blood No. (%)	0	3 (30)	5 (50)	2 (10)	.021
Sputum No. (%)	3 (37.5)	1 (12.5)	1 (12.5)	3 (37.5)	.403
Stool No. (%)	2 (33.33)	1 (16.66)	0	3 (50)	.460
Wound No. (%)	0	1 (20)	2 (40)	2 (40)	.528
BAL No. (%)	1 (20)	1 (20)	1 (20)	2 (40)	.997
Biopsy No. (%)	0	1 (50)	0	1 (50)	.691
Pus No. (%)	0	0	0	1 (100)	.681

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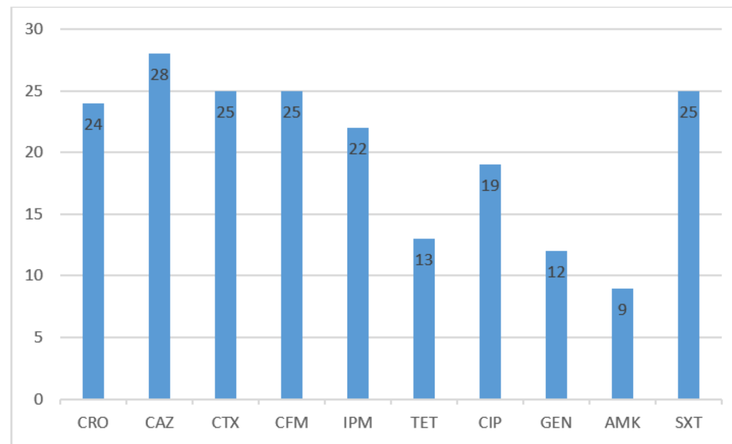

Figure 1) Resistance rate of *K. pneumoniae* isolates to antimicrobials: CRO: ceftriaxone, CAZ: ceftazidime, CTX: cefotaxime, CFM: cefixime, IPM: imipenem, TET: tetracycline, CIP: ciprofloxacin, GEN: gentamicin, AMK: amikacin, SXT: trimethoprim-sulfamethoxazole

Table 3) Association between biofilm formation ability and antimicrobial resistance

Antibiotics Non-Biofilm		Biofilm Formation				*P* -Value
		Non-Biofilm	Weak Biofilm	Moderate Biofilm	Strong Biofilm	
Ceftriaxone	R (No.)	4	4	8	8	.295
	I (No.)	0	0	0	1	
	S (No.)	5	8	2	12	
Ceftazidime	R (No.)	6	6	4	12	.552
	I (No.)	1	2	1	0	
	S (No.)	2	4	5	9	
Cefotaxime	R (No.)	3	5	6	11	.752
	I (No.)	0	0	0	1	
	S (No.)	6	7	4	9	
Cefixime	R (No.)	4	4	6	11	.611
	I (No.)	0	0	0	0	
	S (No.)	5	8	4	10	
Imipenem	R (No.)	4	5	5	8	.605
	I (No.)	1	0	1	0	
	S (No.)	4	7	4	13	
Tetracycline	R (No.)	2	2	5	4	.161
	I (No.)	0	0	1	0	
	S (No.)	7	10	4	17	
Ciprofloxacin	R (No.)	2	7	2	8	.396
	I (No.)	1	1	0	1	
	S (No.)	6	4	8	12	
Gentamicin	R (No.)	2	5	1	4	.554
	I (No.)	0	0	0	1	
	S (No.)	7	7	9	16	
Amikacin	R (No.)	1	5	0	3	.207
	I (No.)	1	0	1	1	
	S (No.)	7	7	9	17	
Trimethoprim-sulfamethoxazole	R (No.)	6	8	1	10	.079
	I (No.)	0	1	0	1	
	S (No.)	3	3	9	10	

R: resistant, I: intermediate, S: susceptible

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Table 4) Association between biofilm-related genes and biofilm formation

Biofilm Formation	Biofilm Formation No. (%)	Biofilm-Related Genes					
		Genes No. (%)					
		iutA	*mrkA*	*mrkD*	*treC*	*wcaG*	*magA*
Non-biofilm	9 (17.31)	3 (5.76)	3 (5.76)	5 (9.61)	7 (13.46)	6 (11.53)	0
Weak biofilm	12 (23.07)	5 (9.61)	8 (15.38)	8 (15.38)	10 (19.23)	10 (19.23)	1 (1.92)
Moderate biofilm	10 (19.23)	6 (11.53)	6 (11.53)	6 (11.53)	6 (11.53)	6 (11.53)	0
Strong biofilm	21 (40.38)	8 (15.38)	16 (30.76)	15 (28.84)	18 (34.61)	15 (28.84)	7 (13.46)

isolates were weak biofilm producers, 10 (23.25%) isolates were moderate biofilm producers, and 21 (48.83%) isolates were strong biofilm producers. Table 2 displays the correlation between sample type and biofilm production. No significant correlation was observed between the type of clinical sample and biofilm formation ability, except for blood samples. Statistical analysis showed no significant correlation between MDR and biofilm formation ability of *K. pneumoniae* isolates (P= .536). Also, no significant correlation was found between antimicrobial resistance and biofilm formation (Table 3).

Biofilm formation genes: The analysis of biofilm-encoding genes showed the presence of *treC* gene in 78.84% (n=41), *wcaG* in 71.15% (n=37), *mrkD* in 65.38% (n=34), *mrkA* in 63.46% (n=33), *iutA* in 40.38% (n=21), and *magA* in 15.38% (n=8) of *K. pneumoniae* isolates. Table 4 demonstrates that the isolates harboring *iutA*, *mrkA*, *mrkD*, *treC*, *wcaG*, and *magA* genes produce stronger biofilms than the isolates without these genes.

Discussion

This research examined the antibiotic resistance and biofilm formation ability of *K. pneumoniae* clinical isolates obtained from patients admitted to educational hospitals affiliated with Babol University of Medical Sciences. The findings indicated that most of the isolates exhibited resistance to multiple antibiotics, particularly ceftazidime, cefotaxime, and ceftriaxone. Furthermore, most of the isolates exhibited the ability to form biofilms of different types. In addition, this study findings showed no significant relationship between biofilm formation ability and antibiotic resistance of isolates. The widespread use of antimicrobial agents in the treatment of related infections has increased the antibiotic resistance of *K. pneumoniae* strains. Managing antimicrobial resistance of MDR *K. pneumoniae* strains is a major challenge for physicians due to limited access to effective medicines, leading to higher mortality rates, prolonged hospital stays, and high treatment costs [18]. The results of this investigation indicated that more than 48% of *K. pneumoniae* isolates were MDR. Another research conducted in Iran revealed that 58% of *K. pneumoniae* isolates were MDR [19]. A study conducted in Poland revealed that 68.8% of *K. pneumoniae* isolates were MDR [20]. In the current investigation, the highest and lowest resistance rates were related

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to ceftazidime (53.84%) and amikacin (17.3%), respectively. Farhadi et al. (2021) showed that the highest resistance rate was related to ampicillin/sulbactam (93%), and the lowest resistance rate was related to amikacin (8%) [19]. Heidary et al. (2018) indicated that the highest resistance rate among *K. pneumoniae* isolates was against ampicillin (82.2%) [21]. The present study identified biofilm formation in 82.69% of *K. pneumoniae* isolates. Furthermore, the majority of these isolates demonstrated strong biofilm production ability. In contrast, in a study conducted by Ochońska et al. (2021) on a set of 18 clinical isolates of *K. pneumoniae*, none of the isolates were strong biofilm producers; however, 22% of the isolates developed moderate biofilm, 22% created weak biofilm, and 56% formed no biofilm [8]. In another study conducted by Alcántar-Curiel et al. (2018) on 168 *K. pneumoniae* isolates, 69% of the isolates produced considerable biofilm, 20.3% produced weak biofilm, and 10.1% produced no biofilm [22].

In this study, biofilm-forming strains isolated from various sample types (urine, blood, sputum, stool, wound swab, BAL, biopsy, and pus) were comprehensively analyzed. Despite examining a diverse range of clinical samples, no statistically significant differences were observed in the frequency of biofilm formation except for blood samples. Similarly, in a study by Cepas et al. (2019), no significant difference was found between the strains isolated from different samples (blood, sputum, and urine) in terms of biofilm formation ability [23].

The analysis of biofilm-associated genes revealed that *treC* exhibited the highest frequency, while *magA* showed the lowest frequency among biofilm-encoding genes. In a study by Yadav and colleagues (2023), *mrkD* and *iutA* were detected in 31 (93.9%) and 28 (84.8%) *K. pneumoniae* isolates, respectively [24]. Goudarzi et al. (2023) reported that among 57 extended-spectrum beta-lactamase positive *K. pneumoniae* isolates, the occurrence of *mrkD* and *iutA* genes was 88 (80%) and 65 (59.1%), respectively [25]. In another study by Anis and colleagues (2021), biofilm formation was more prevalent in isolates that were positive for *wcaG* (86.7%) and *mrkD* (80%) [26]. Kelishomi et al. (2022) also showed that among a total of 74 *K. pneumoniae* isolates collected from wound site infections, the frequencies of *mrkA*, *mrkD*, *wcaG*, and *magA* genes were 75.7, 90.5, 55.4, and 9.5%, respectively [27]. Lastly, a study by Ghonaim et al. (2021) revealed that the frequency of *magA* genes among *K. pneumoniae* isolates was 3 out of 18 (16.7%) [28].

Overall, the discrepancies in study findings may be attributed to factors such as the geographic location, sample type and size, and the specific features of bacterial isolates, especially their antibiotic resistance patterns. It is important to note that some of the isolates used in this study might have originated from a single clone. This potential clonal origin could be affecting the generalizability of the findings. It is also suggested that the antibiotic resistance genes be investigated and compared with the biofilm formation ability of these isolates.

Conclusion

The present study results demonstrated a high prevalence of antibiotic-resistant *K. pneumoniae* strains. The highest resistance was found to be against ceftazidime, while the lowest resistance was against amikacin. A significant proportion of *K. pneumoniae* isolates exhibited the ability to form biofilms, and these strains often carried many genes associated with biofilm formation. Among the biofilm-related genes, the *treC* gene was found to have the highest frequency, while the *magA* gene had the

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lowest frequency. These findings underscore the substantial capacity of *K. pneumoniae* isolates for biofilm formation. However, no statistically significant relationship was observed between the ability of the isolates to form biofilm and resistance to different antibiotics. Given the biofilm formation propensity of these strains, it is imperative to elucidate the underlying mechanisms of biofilm formation in *K. pneumoniae*. Developing strategies to inhibit this process is paramount in the effective management of infections caused by this pathogen.

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Ethical permission: This study was approved by the Research Ethics Committee of Islamic Azad University, Ayatollah Amoli Branch, Amol, Iran (code number: IR.IAU.AMOL.REC.1401.087.

Authors' contributions: AF and EF conceived and designed the experiments. AF wrote the main manuscript text. AF, EF, FZ, and AP collected samples and performed the experiments. EF analyzed the data and prepared the figures. EF and AF reviewed and finalized the manuscript. All authors contributed to the article and approved the submitted version.

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Consent to participate: Patients were satisfied to participate in the study.

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