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Investigating Immune Evasion Genes Expression and Biofilm Formation of *Klebsiella pneumoniae* Isolates From Patients With Ventilator-associated Pneumonia

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ABSTRACT

Background: *Klebsiella pneumoniae*, a leading opportunistic pathogen, exhibits increasing multidrug resistance (MDR) and biofilm formation, posing significant challenges in hospital environments, especially in developing regions, such as Iran. This study aimed to characterize the antibiotic resistance profiles, biofilm-formation capacity, and expression levels of immune evasion genes (*fimH-1, mrkD, traT*) in *K. pneumoniae* isolates from patients with ventilator-associated pneumonia.

Materials and Methods: A total of *K. pneumoniae* isolates were obtained from sputum samples of patients with ventilator-associated pneumonia. Antibiotic susceptibility was determined using the Kirby-Bauer disk diffusion method. Biofilm formation was quantified by a crystal violet assay. The presence and expression of immune evasion genes were evaluated by polymerase chain reaction (PCR) and quantitative real-time PCR, respectively.

Results: High resistance rates were observed: 100% to ampicillin-sulbactam, 96.66% to ciprofloxacin, 93.33% to cefepime, and 83.33% to imipenem. About 50% of isolates were strong biofilm formers, 33.3% moderate, and 16.7% weak. *mrkD* and *fimH-1* genes were detected in 100% and 96.66% of isolates, respectively, while traT was present in 30%. Gene expression analysis revealed significant upregulation of *fimH-1* ($P=0.005$) and *mrkD* ($P<0.0001$), while *traT* expression showed no significant change ($P=0.2803$). No significant correlation was found between the prevalence of immune evasion genes and biofilm production ($P>0.05$).

Conclusion: This study highlights a high prevalence of multidrug-resistant (MDR) *K. pneumoniae* isolates with strong biofilm formation and upregulated immune evasion genes among patients with ventilator-associated pneumonia. The significant upregulation of *fimH-1* and *mrkD* suggests enhanced adaptation for persistence in the face of host defenses. These findings underscore the urgent need for targeted interventions to control *K. pneumoniae* infections amid the growing threat of antibiotic resistance.

Keywords:

Klebsiella pneumoniae, Antibiotic resistance, Biofilm, Virulence genes, Immune evasion, Ventilator-associated pneumonia

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Introduction

Klebsiella pneumoniae is a gram-negative, non-motile, encapsulated bacterium belonging to the family Enterobacteriaceae [1]. The bacterium is found everywhere, including on the surface of animal mucus, in the environment (such as water and soil), and in food [2, 3]. In humans, *K. pneumoniae* is present in the gastrointestinal and upper respiratory tract. Because it is an opportunistic pathogen, it can enter the bloodstream or other tissues, leading to infections, such as pneumonia, urinary tract infections (UTIs), and bacteremia [3, 4]. It is also one of the important causes of serious community-onset infections, such as necrotizing pneumonia, pyogenic liver abscesses, and endogenous endophthalmitis [5]. Individuals with underlying immunodeficiencies are at greater risk of *K. pneumoniae* infection than the general population [6]. *K. pneumoniae* is one of the most important pathogens involved in antibiotic resistance and is therefore classified as an ESKAPE organism (*Enterococcus faecium, Staphylococcus aureus*, *K. pneumoniae*, *Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* species) along with other very important multidrug-resistant (MDR) pathogens [5] and is considered one of the opportunistic pathogens frequently encountered in hospitals, especially in Iran [7, 8].

The increasing prevalence of MDR strains in recent years, driven by excessive and uncontrolled antibiotic use, has made infections caused by this bacterium a therapeutic challenge. *K. pneumoniae* employs various mechanisms to evade the immune system and resist antibiotics [7]. The most common mechanism is the production of bacterial biofilm, which reduces the effects of complement and phagocytosis by preventing the proximity of antimicrobial antibodies and peptides, thereby partially protecting the bacteria from the host's immune response [3]. Therefore, the study of genes associated with biofilm production and their susceptibility to antibiotics is of paramount importance for treatment and the control of hospital-acquired infections. Notable biofilm-related genes are *mrkD* (fimbria type 3) and *fimH-1* (fimbria type 1) [9, 10]. The *traT* gene, which encodes an outer membrane protein involved in bacterial conjugation, plays a crucial role in inhibiting the complement system [11]. These genes are also considered immune-related, as they are associated with biofilm production and complement resistance. Previous studies suggest that microbial immune evasive strategies contribute to the development and persistence of antimicrobial resistance. It is also accepted that antibiotic resistance most often imposes a cost in terms of reduced bacterial fitness and virulence [12]. Given the high prevalence of MDR *K. pneumoniae* isolates in Iran and the lack of studies on immune evasion gene expression, it is essential to characterize the properties of immune evasion genes and investigate antibiotic resistance profiles to inform the management and treatment of *K. pneumoniae* infections.

Although several studies have explored biofilm-associated and immune evasion genes in *K. pneumoniae* worldwide, there is a notable gap in understanding their expression dynamics in clinical isolates from ventilator-associated pneumonia in Iran. The unique antibiotic prescription patterns and high prevalence of MDR strains in Iranian hospitals may influence the regulation of these genes differently from global trends. Furthermore, few studies have simultaneously examined both biofilm phenotypes and real-time expression of key immune evasion genes (*fimH-1*, *mrkD*, and *traT*) in clinical isolates. Therefore, this study provides novel, region-specific insights into the molecular mechanisms underlying *K. pneumoniae* adaptation to local selective pressures.

Therefore, the primary objective of this study is to initially assess the antibiotic resistance profile of *K. pneumoniae* and biofilm formation and investigate the expression of genes related to immune evasion in isolates obtained from patients with pneumonia.

Materials and Methods

Bacterial isolation and identification

From July 2023 to March 2024, 30 respiratory *K. pneumoniae* samples were randomly collected from sputum cultures of patients with ventilator-associated pneumonia hospitalized in the special care units of Al-Zahra Hospital in Isfahan City, Iran. *K. pneumoniae* ATCC 13883 was also used as a control strain. For confirmation, all samples were cultured on McConkey agar and incubated at 37 ˚C for 24 h. The *K. pneumoniae* strains were identified using conventional microbiological tests [13]. Confirmed strains were stored in tryptic soy broth (TSB) containing 20% glycerol for further analysis.

Antimicrobial susceptibility test

After confirmation of *K. pneumoniae* samples, antibiotic susceptibility testing of these strains was performed using the Kirby-Bauer disk diffusion method according to Clinical and Laboratory Standards Institute (CLSI 2024) guidelines [14]. For this purpose, Mueller-Hinton agar (Merck, Germany) was used.

Then, the bacteria were cultivated on the plates with a turbidity concentration of 0.5 McFarland to measure the antimicrobial sensitivity to 7 different antibiotics using commercially available antibiotics (Rosco Diagnostica, Denmark), including gentamicin (10 µg), ciprofloxacin (5 µg), imipenem (10 µg), meropenem (10 µg), cefepime (30 µg), ampicillin/sulbactam (10 µg), and amikacin (15 µg). The plates were incubated aerobically at 37 ˚C for 18 h, and the inhibition zone diameter was subsequently measured in mm and interpreted according to CLSI guidelines. MDR isolates are defined as resistant to at least one drug from three or more antimicrobial classes.

Quantitative biofilm production assay

A quantitative biofilm test was performed in 96-well culture plates. Two hundred microliters of TSB culture medium supplemented with 1% glucose and containing 0.5 McFarland bacteria were added to each well. Three wells were considered for each sample. It was incubated at 37 ˚C for 24 hours; then, the medium and bacteria were discarded. Wells were washed 3 times with PBS. Then, 150 microliters of 96% ethanol were added to each well, incubated for 20 minutes, and discarded; the plate was then incubated at room temperature. After 24 hours, 150 µL of crystal violet 1% was added to the wells, and after 15 min, the dye was slowly drained using a pipette. The plates were washed using water until the additional dye was completely removed. Then, 150 µL of 96% ethanol was added, and the mixture was incubated at room temperature for 30 minutes. Finally, the optical density (OD) was read at 570 nm [15-17]. The OD cut-off (ODc) was defined as 3 standard deviations above the mean OD of the negative control. All the strains were classified based on the adherence capabilities into the following categories: non-biofilm producers (OD≤ODc), weak biofilm producers (ODc<OD≤2ODc), moderate biofilm producers (2ODc<OD≤4ODc), and strong biofilm producers (4ODc<OD) [18]. In addition, the standard strain *K. pneumoniae* ATCC 13833 and TSB with 1% glucose were used as positive and negative controls, respectively [19-21].

Polymerase chain reaction (PCR) detection of immune evasion-associated genes

Genomic DNA from each isolate was extracted using the boiling method [20, 21], and the immune evasion gene *traT* and biofilm-related genes *fimH-1* and *mrkD* were identified by PCR. The *rho* gene was used as a reference gene and internal control [22]. The primer sequence used in this research is listed in Table 1. The PCR program was as follows: Initial denaturation at 95 ˚C for 3 min, followed by 34 cycles, each consisting of 30 s at 95 °C for denaturation, 30 s at 60 ˚C for annealing, and 45 s at 72 ˚C for extension, and finally an additional extension phase at 72 ˚C for 3 min. Then the PCR products were electrophoresed in a 1.5% agarose gel in 1x TBE buffer and stained with SafeStain.

Immune evasion gene expression analysis

Gene expression analysis of immune evasion genes *traT*, *mrkD*, and *fimH-1* was performed using the quantitative real-time PCR (qRT-PCR) method in *K. pneumoniae* strains. Total RNA was extracted from each isolate using the RNA extraction kit (Sinaclon, Iran) according to the manufacturer's protocol. The concentration and purity of the extracted RNA were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). Complementary DNA (cDNA) was synthesized from 1 µg of total RNA using the cDNA synthesis kit (Add-Bio, Korea) following the manufacturer's instructions.

qRT-PCR was performed using SYBR Green PCR Master Mix (Sinaclon, Iran) on a Rotor-Gene Q, Qiagen. The primers used for *traT*, *mrkD*, *fimH-1*, and the reference gene *rho* are listed in Table 1. The qRT-PCR reaction consisted of an initial denaturation at 95 ˚C for 10 min, followed by 40 cycles of denaturation at 95 ˚C for 15 s, annealing at the specific annealing temperature for each primer (Table 1) for 30 s, and extension at 72 ˚C for 30 s. All reactions were performed in triplicate. The rho gene was used as an internal control for normalizing mRNA expression levels. The relative gene expression of *traT*, *mrkD*, and *fimH-1* was calculated using the $2^{-\Delta\Delta C_T}$ method. Briefly, the ΔC_T value was calculated by subtracting the C_T value of the rho gene from the C_T value of the target gene. The $\Delta\Delta C_T$ value was then calculated by subtracting the ΔC_T value of the control strain *K. pneumoniae* ATCC 13883 from the ΔC_T value of each isolate. The fold change in gene expression was calculated as $2^{-\Delta\Delta C_T}$.

Statistical analysis

All tests used in this study were done in triplicate. The t-test was used for statistical analysis, and $P<0.05$ was considered significant. Associations between $\log2(2^{-\Delta\Delta Ct})$ and biofilm OD were tested per gene with 2-tailed Spearman correlation (BH-FDR across genes). For MDR vs non-MDR, 2-tailed Mann-Whitney U compared log2 expression (Hodges-Lehmann median difference and 95% CI reported; BH-FDR across genes). When quantitative values were available only in MDR, the Fisher exact test (present/absent×MDR) was used with Haldane-Anscombe-corrected ORs (Analyses: GraphPad Prism v; FDR in Excel; α=0.05).

Results

Confirmation of bacterial strains

PCR and phenotypic characterization confirmed that all 30 isolates were *K. pneumoniae*. The *rho* gene, used as an internal control, was detected in all isolates.

Antimicrobial susceptibility test

Antimicrobial susceptibility testing revealed high resistance rates among the *K. pneumoniae* isolates. The percentages of resistant isolates were 100% to ampicillin-sulbactam, 96.6% to ciprofloxacin, 93.3% to cefepime, 90% to meropenem, 83.3% to imipenem, 73.3% to gentamicin, and 46.6% to amikacin (Table 2). So, 93.3% of the isolates exhibited MDR phenotypes.

Quantitative biofilm production assay

The quantitative biofilm assay using 1% crystal violet staining revealed varying biofilm-formation capacities among the isolates. Based on OD values, 50% (15 isolates) were classified as strong biofilm producers, 33.3% (10 isolates) as moderate, and 16.7% (5 isolates) as weak biofilm producers (Figure 1A). A significant increase in biofilm formation was observed in the isolates compared to the *K. pneumoniae* ATCC 13883 strain (P<0.0001) (Figure 1B). Among the MDR isolates, 46.4% were strong biofilm producers (Table 3). Table 4 shows the association between the prevalence of immune evasion genes (*traT*, *fimH-1*, and *mrkD*) and biofilm formation ability. According to the chi-square test, no significant relationship was found between the presence of *traT* (P>0.05), *fimH-1* (P>0.05), or *mrkD* (P>0.05) and the biofilm formation strength (strong, moderate, weak).

Investigating the prevalence of *fimH-1, mrkD,* and *traT* genes

The prevalence of biofilm-associated genes was observed among *K. pneumoniae* strains isolated from various sample types. The most commonly detected virulence genes amongst the *K. pneumoniae* strains were *mrkD* (100%) and *fimH-1* (96.6%). The prevalence of the outer membrane protein-coding gene *traT* (30%) was lower than that of other detected virulence genes.

The expression of *fimH-1, mrkD,* and *traT*

Analysis of the expression of *fimH-1* and *mrkD*, which are responsible for biofilm formation, and *traT*, which plays a crucial role in inhibiting the complement system and immune evasion, was performed using qRT-PCR in *K. pneumoniae* strains and compared with the *K. pneumoniae* ATCC 13883 strain. The *fimH-1* gene showed a statistically significant upregulation (P<0.05) with a mean fold change of approximately 3.2±0.6 (Figure 2A). At the same time, *mrkD* exhibited a stronger increase (P<0.0001) with a mean fold change of around 6.1±0.7 (Figure 2B). In contrast, the *traT* gene displayed no significant difference in expression (P=0.2803) with a fold change of about 0.8±0.2 relative to the *K. pneumoniae* ATCC 13883 strain (Figure 2C).

Table 1. The primers used for PCR and real-time PCR

Genes	Primers sequences (5′ to 3′)	Product Size (bp)	Annealing Temperature (℃)	Ref.
rho	F: AACTACGACAAGCCGGAAAA R: ACCGTTACCACGCTCCATAC	99	52	[23]
traT	F: GGTGTGGTGCGATGAGCACAG R: CACGGTTCAGCCATCCCTGAG	290	59	[24]
fimH-1	F: GCCAACGTCTACGTTAACCTG R: ATATTTCACGGTGCCTGAAAA	180	49	[11]
mrkD	F: CCACCAACTATTCCCTCGAA R: ATGGAACCCACATCGACATT	226	51	[11]

Table 2. Antibiotic resistance and sensitivity profiles of *K. pneumoniae* isolates

Antibiotic	Concentration (µg)	Resistant (%)	Intermediate (%)	Sensitive (%)
Imipenem	10	83.3	3.3	13.3
Meropenem	10	90	0	10
Gentamicin	10	73.3	0	26.6
Amikacin	15	46.6	10	43.3
Cefepime	30	93.3	3.3	3.3
Ampicillin/Sulbactam	10	100	0	0
Ciprofloxacin	5	96.6	0	3.3

Table 3. Comparing biofilm production between MDR and non-MDR *K. pneumoniae* isolates

Biofilm Formation	All Samples (%)	MDR (93.3) (%)	Non-MDR (6.6) (%)
Strong	50 [15]	46.4 [13]	100 [2]
Moderate	33.3 [10]	35.7 [10]	0
Weak	16.7 [5]	17.8 [5]	0
None	0	0	0

Table 4. The relationship between the prevalence of biofilm-related genes and biofilm formation

Biofilm-related Genes	No. (%)			P
	Biofilm Formation			
	Weak	Moderate	Strong	
+*traT*	3(10)	2(6.67)	4(13.3)	0.21
-*traT*	2(6.67)	10(33.3)	9(30)	
+*fimH-1*	5(16.7)	12(40)	12(40)	0.99
-*fimH-1*	0	0	1(3.3)	
+*mrkD*	5(16.7)	12(40)	13(43.3)	0.99
-*mrkD*	0	0	0	

Table 5. Spearman correlation between log2($2^{\Delta\Delta Ct}$) and biofilm OD (per gene)

Gene	No.	Spearman ρ	P	q (FDR)
fimH-1	30	0.05851	0.7588	0.7588
mrkD	27	0.2485	0.2114	0.5230
traT	10	-0.3333	0.3487	05230

Table 6. Comparing log2($2^{\Delta\Delta Ct}$) between MDR and non-MDR (2-tailed Mann–Whitney U)

Gene	Hodges–Lehmann Median Diff (MDR–non-MDR)	95% CI	Raw P (Two-tailed)	BH q Value
fimH-1	0.1	(−3.17, 4.47)	1.000	1.000
mrkD	1.05	(−13.43, 3.33)	0.522	1.000

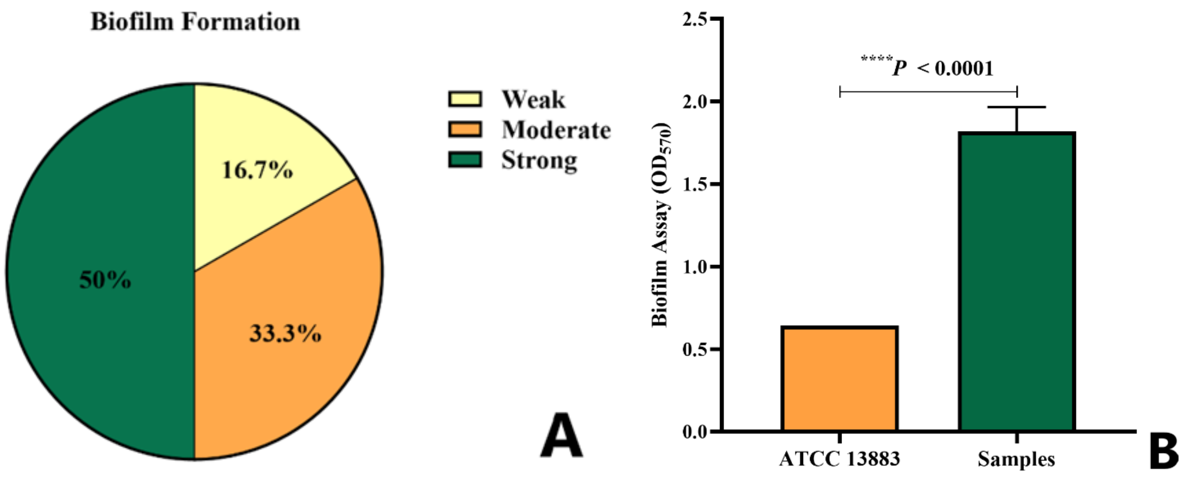

Figure 1. Biofilm production in *K. pneumoniae* isolates

Note: The pie chart displays the percentage distribution of isolates, visually showing biofilm production variability among the isolates (A). A significant increase in biofilm formation by the isolates compared to the standard strain has been observed (B).

Correlation between gene expression and biofilm

At the isolate level, log2($2^{-\Delta\Delta Ct}$) showed no statistically significant correlation with biofilm OD for any gene: *fimH-1* (ρ=0.05852, P=0.7588, q(FDR)=1.000, n=30), *mrkD* (ρ=0.2485, P=0.2114, q=0.855, n=27), and *traT* (ρ=-0.3333, P=0.3487, q=1.000, n=10). Thus, no gene exhibited a significant expression-biofilm association after FDR correction (Table 5).

Association between gene expression and MDR phenotype

When log2($2^{-\Delta\Delta Ct}$) was compared between MDR and non-MDR isolates using 2-tailed Mann–Whitney U tests, no gene showed a statistically significant difference after FDR correction (Table 6; given that only two isolates were non-MDR, statistical power was limited).

Special case

For *traT*, gene presence was detected only in MDR isolates. The Fisher exact test was not significant (P>0.05); therefore, detectable *traT* expression was not significantly associated with MDR status. The odds ratio is infinite due to a zero cell; applying the Haldane–Anscombe correction yielded OR=2.84 (95% CI, 0.12%, 64.88%).

Discussion

The present study focused on investigating the expression of immune-evasion genes in *K. pneumoniae* isolates from patients requiring mechanical ventilation. The *fimH-1*, *mrkD*, and *traT* genes were candidates as immune evasion genes. Moreover, rho is a reference gene, and 100% of the strains carry it. Based on previous studies, these genes showed variation in prevalence across isolates. In a study by Mirzaie et al. the frequency of the *traT* gene among *K. pneumoniae* isolates was 62%, and biofilm-related genes, such as *fimH-1* and *mrkD*, were detected in 88% of the tested isolates [7]. Samples obtained from patients with *K. pneumoniae* showed that *fimH-1* and *mrkD* genes are present in all isolated wounds and blood. Also, the *fimH-1* gene was common in sputum samples, the *mrkD* gene was common in urine samples, and serum resistance associated with the *traT* gene was detected in all blood isolates [11]. In a study by El Fertas-Aissani et al. 100% of samples carried *fimH-1*, 96.3% carried *mrkD*, and 1.8% carried *traT* [23]. In

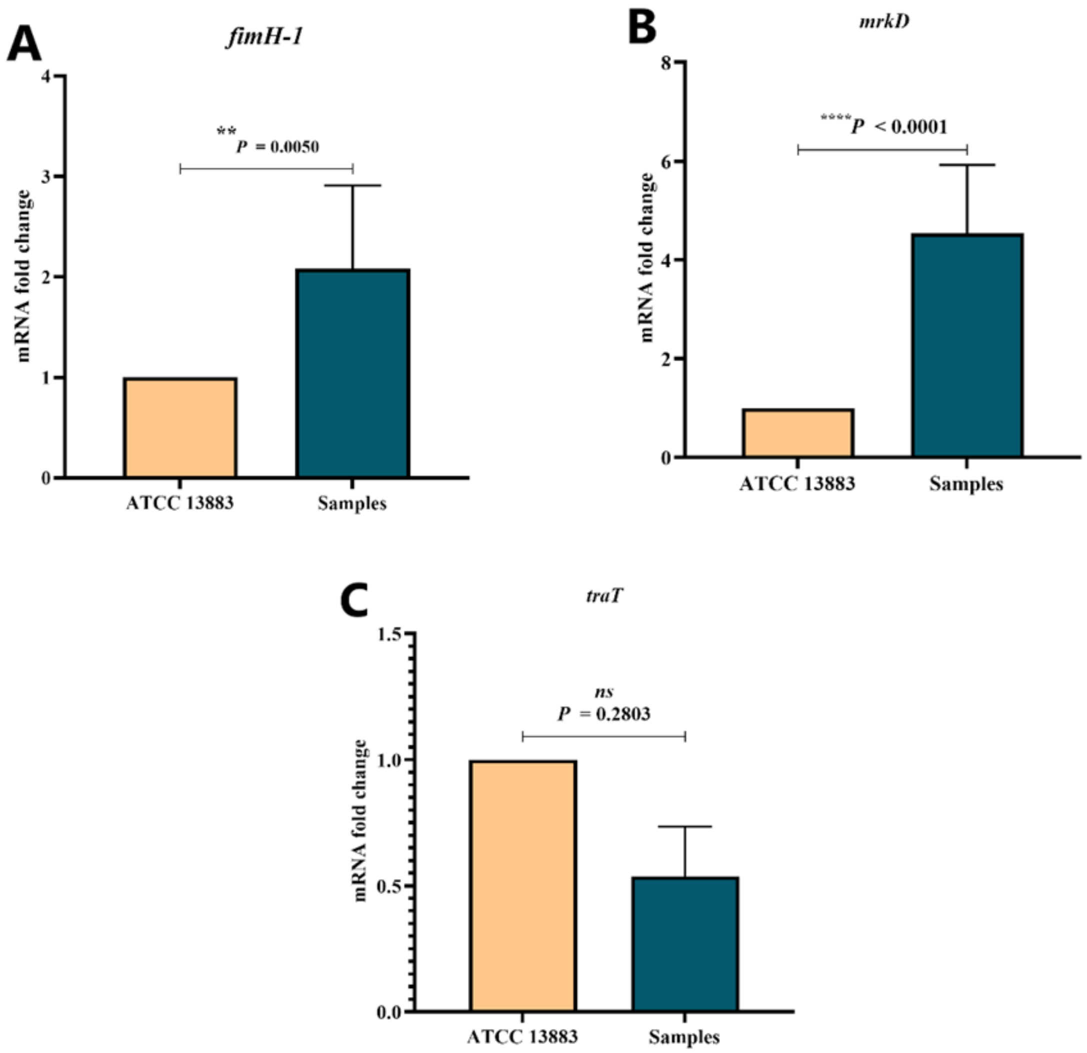

Figure 2. Fold change analysis of *fimH-1* (A), *mrkD* (B), and *traT* (C) genes in *K. pneumoniae*

Ns: Non-significant.

Note: *K. pneumoniae* ATCC 13883 was used as a control strain, and the *rho* gene served as an internal control. Increased expression levels were observed for the *fimH-1* and *mrkD* genes, while *traT* gene expression did not change significantly.

another study by Ranjbar et al. 93.04% of the samples carried *fimH-1*, 92.17% carried *traT*, and 84.34% carried mrkD [8]. In a study by Jabar et al. the *fimH* gene was detected in 82.5% of 40 *K. pneumoniae* isolates, whereas the *mrkD* gene was detected in 70.5% of the isolates by PCR amplification [24]. In a study by Makhrmash et al. the *fimH* gene was detected in 49 isolates (87.5%), while the *mrkD* gene was found in 30 isolates (53.6%) [25]. In one study by Ranjbar et al. 92.1% of the isolates had the *mrkD* gene [26], and in another study by María et al. 20% of them had this gene [27], and in the study by Tiria et al. the prevalence was 95.1% among 102 clinical isolates [28]. These results are consistent with our research, although in some studies, higher or lower percentages of genes are observed. Variability in the prevalence of these genes has been observed across studies, which may be due to differences in sample sources, geographic locations, and study methodologies.

The antibiotic sensitivity tests revealed a concerning level of resistance to multiple commonly used antibiotics, underscoring the challenge in managing infections caused by these isolates. In this study, the prevalence of MDR *K. pneumoniae* isolates was 93.3%. In another study 90.2% of *K. pneumoniae* isolates were MDR [29]. However, most isolates (60.6%) were extensively drug-resistant (XDR), while 30.3% were MDR, and only 9.2% were susceptible [30]. Also, in a study by Amirmozafari et al. 61.2% of *K. pneumoniae* isolates were drug-resistant, of which 20.4% were 100% resistant to all cephalosporins (cefixime, ceftriaxone, ceftazidime, etc.) [31]. In the present study, the rate of antibiotic resistance to imipenem and meropenem was 83.3% and 90%, respectively. Similar to this result, in a study by Bratu et al. the resistance rates to the 2 mentioned antibiotics were reported as 98% and 96% [32], and in another study by Seibert et al. they were 80% and 83% [33]. In another study by Al-Baz et al. with a slight difference, resistance to imipenem and meropenem was observed, including in one study, *K. pneumoniae* isolates were resistant 25.4% to meropenem and imipenem, 87.3% to cefepime, 77.5% to ampicillin/sulbactam, 47.9% to ciprofloxacin, 44.4% to gentamicin, and 43% to amikacin [30]. However, in a 2009 study by Ullah et al. 100% of isolates were resistant to ampicillin (similar to our research), whereas 93.48% and 86.96% were sensitive to meropenem and imipenem, respectively [34]. Also, in our study, the resistance levels to amikacin, gentamicin, cefepime, and ciprofloxacin were 46.6%, 73.3%, 93.3%, and 96.6%, respectively. In a study by Seibert et al., conducted on *K. pneumoniae* strains isolated from hospital infections, a high prevalence of resistance to amikacin (8.50%), gentamicin (42.60%), and cefepime (55%) was reported [33]. Also, in another study by Mekki et al. a higher prevalence of strains resistant to gentamicin (100%), ciprofloxacin (100%), and amikacin (39.47%) was reported [35]. In a study by Devanga Ragupathi et al. among 351 *K. pneumoniae* isolates from patients with bloodstream infections, 59% were susceptible to amikacin, while 31% were susceptible to ciprofloxacin [36].

Among all isolates tested, 50%, 33.3%, and 16.7% are strong, moderate, and weak biofilm formers, respectively. Previous research has shown that a significant percentage of MDR *K. pneumoniae* isolates are strong biofilm producers. For instance, one study by Vuotto et al. found that 67.5% of MDR *K. pneumoniae* samples are strong biofilm producers, 25% are moderate biofilm producers, and 7.5% are weak biofilm producers. Also, 70.8% of non-MDR isolates were strong biofilm producers [19]. In another study by Bellifa et al. 50% of the samples were strong biofilm producers, 41.6% were moderate, and 8.3% were not biofilm producers [37]. Also, in another study by Seifi et al. 33% of the samples were strong biofilm producers, 52.1% were moderate biofilm producers, 8.5% were weak biofilm producers, and 6.4% did not form biofilm [38]. In a study by Nirwati et al, 26.95% of the samples were strong biofilm producers, 28.74% were moderate biofilm producers, and 29.94% were weak biofilm producers [39]. In another study by Karimi et al. 20.4% of the samples were strong biofilm producers, 21.6% were moderate biofilm producers, and 32.5% were weak biofilm producers [40]. In a study by Makhrmash et al. 56 *K. pneumoniae* isolates were evaluated, and the results showed that 18% produced weak biofilms, 25% produced moderate biofilms, and 57% produced strong biofilms [25]. In the study by Tiria et al. 53.9% of the isolates were strong biofilm producers, 22.5% were moderate producers, 6.9% were weak producers, and 16.7% were non-biofilm producers, with the biofilm-forming phenotype showing a significant association with the presence of the *mrkD* gene [28]. The results of these studies are largely similar to those of the present research, and the differences observed may be due to various factors, including the year of the study and the number and type of samples. In 2012, an analysis showed that 80% of biofilm-producing strains collected from 100 urine samples from patients with symptoms of UTI over 6 months showed an MDR phenotype. Specifically, biofilm-positive isolates showed 93.3%, 83.3%, 73.3%, and 80% resistance to nalidixic acid, ampicillin, cefotaxime, and cotrimoxazole, respectively, compared to 70%, 60%, 35%, and 60% resistance by non-biofilm-producing strains. This result indicates the relationships between antibiotic resistance and the ability of *K. pneumoniae* strains to form biofilms [19].

Additionally, Ashwath et al. reported that *fimH-1* gene expression increased among strong biofilm formers, while mrkD expression decreased. Moderate biofilm formers showed variable expression of biofilm-related genes, and weak or non-biofilm formers had reduced expression of both fimbria genes [41]. These findings underscore the complexity of gene expression related to biofilm formation and its impact on virulence.

Conclusion

This study demonstrates a critical public health concern: *K. pneumoniae* isolates from patients with ventilator-associated pneumonia exhibit alarmingly high resistance to multiple antibiotics, coupled with a strong capacity for biofilm formation and significant upregulation of immune evasion genes (*mrkD* and *fimH-1*). These findings underscore the substantial challenges in treating *K. pneumoniae*-related pneumonia, particularly in hospitalized patients.

The observed significant upregulation of *fimH-1* and *mrkD* in clinical isolates compared to the control strain likely reflects adaptive responses to selective pressures within the host environment. This enhanced expression may facilitate robust biofilm formation, providing a crucial protective barrier against both host immune defenses and antibiotic therapies.

The observed upregulation of *fimH-1* and *mrkD* further indicates an enhanced capacity for adhesion and biofilm maturation. *fimH-1* encodes the type 1 fimbrial adhesin, which mediates initial attachment to epithelial and abiotic surfaces, whereas *mrkD* encodes the type 3 fimbrial adhesin, contributing to biofilm stability and resistance to shear stress [10]. This overexpression likely promotes bacterial persistence in hospital environments and may contribute to treatment failure by shielding biofilm-embedded cells from antibiotic penetration. Conversely, the lack of significant change in *traT* expression suggests that this gene may be constitutively expressed at optimal levels for immune evasion and that its regulation is tightly conserved. Given its relatively low prevalence (30% of isolates) and lack of transcriptional upregulation, *traT* may play a less prominent role in immune evasion in this clinical context compared to *fimH-1* and *mrkD*. While *traT* encodes an outer membrane protein that inhibits complement-mediated killing, its contribution may be more relevant during bloodstream infections rather than in respiratory tract colonization.

In contrast, *fimH-1* and *mrkD*, through their roles in adhesion and biofilm maturation, likely provide a more immediate advantage for persistence in the ventilator-associated pneumonia environment. Despite the critical roles of *fimH-1*, *mrkD*, and *traT* in adhesion and immune evasion, no statistically significant association was found between the presence of these genes and the strength of biofilm formation. Several factors can explain this apparent discrepancy. The mere presence of a gene does not guarantee its active transcription or translation; post-transcriptional and environmental regulatory mechanisms may suppress gene expression under certain conditions. In addition, biofilm development is a complex, multifactorial process influenced by a network of genes involved in quorum sensing, efflux pump activity, and exopolysaccharide production. Therefore, the lack of correlation likely reflects the multifaceted nature of biofilm regulation rather than the absence of functional importance of these genes [41].

The upregulation of *fimH-1* and *mrkD* may be driven by multiple regulatory and environmental factors. Exposure to subinhibitory levels of antibiotics in the hospital environment can exert selective pressure, inducing stress-response pathways that activate biofilm-associated genes. Moreover, quorum-sensing systems, such as LuxS/AI-2 signaling, and two-component regulatory systems (Rcs, CpxAR) are known to modulate the expression of adhesin and fimbrial genes under environmental stress. The presence of mobile genetic elements and plasmids carrying biofilm and resistance determinants also suggests that horizontal gene transfer may contribute to the co-selection of these traits. Together, these mechanisms could explain the concurrent increase in antibiotic resistance, biofilm formation, and expression of virulence-associated genes observed in clinical isolates.

Although this study was limited to 30 *K. pneumoniae* isolates, all samples were obtained from critically ill patients with ventilator-associated pneumonia during an extended sampling period, ensuring clinical relevance and methodological consistency. However, the relatively small sample size may limit the statistical power to detect subtle associations among resistance, gene expression, and biofilm formation. Furthermore, clinical outcome data, such as patient prognosis, hospitalization duration, or treatment response, were unavailable, preventing correlation between molecular findings and clinical severity. Another limitation is the restricted gene panel analyzed in this work, which focused on three key immune evasion and biofilm-associated genes (*fimH-1, mrkD, traT*). A broader genomic assessment could uncover additional virulence or regulatory genes involved in these phenotypes. Future research should therefore include larger, multicenter cohorts, comprehensive gene expression profiling, and whole-genome sequencing to identify novel resistance and virulence determinants. In addition, in vivo infection models or cell culture assays could be used to validate the biological roles of these genes under host-like conditions and to elucidate the molecular pathways linking antibiotic resistance, biofilm development, and immune evasion.

Ethical Considerations

Compliance with ethical guidelines

This study was approved by Isfahan University of Medical Sciences, Isfahan, Iran (Code: IR.ARI.MUI.REC.1402.151).

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Authors contribution's

Conceptualization, data collection, analysis, review, editing, and final approval: All authors; Writing the original draft: Marjan Gerami, Mahshid Azizi, and Hafez Mozayyan Esfahani; Supervision: Soodabeh Rostami.

Conflict of interest

The authors declared no conflict of interest.

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