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ORIGINAL ARTICLE

Phenotypic and genotypic ınvestigation of virulence factors in *Klebsiella pneumoniae* strains ısolated from ventilator-associated pneumonia (VAP) cases

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Abstract

Klebsiella pneumoniae is an opportunistic pathogen that normally resides in the gastrointestinal tract of humans and animals, as well as on water and plant surfaces. It can cause a variety of infections, particularly in immunocompromised individuals. *K. pneumoniae* possesses numerous virulence factors including capsular polysaccharides, hemolysin production, erythrocyte agglutination, hypermucoviscosity (HV), biofilm formation, fimbriae, toxins, and siderophores. This study aimed to determine the virulence factors of *K. pneumoniae* strains isolated from cases of ventilator-associated pneumonia (VAP). A total of 19 K. *pneumoniae* isolates identified using the VITEK system and obtained from tracheal aspirate cultures of patients on mechanical ventilation in the ıntensive care unit of Kırşehir Ahi Evran Training and Research Hospital between 2022 and 2023 were included in the study. The presence of capsule, hemagglutination, and biofilm formation was detected in 100%, 100%, and 52.63% of the isolates, respectively. The virulence genes *uge*, *wab*G, *ycf*M, *fim*H, *mrk*D, *ure*A, *iut*A, *ybt*A, *ent*B, and *fyu*A were present in 100% of the isolates; *kpn* was detected in 52.63%, and *kfuBC* in 94.73%. In contrast, the *rmp*A, *wca*G, *iro*N, *iro*D, *all*s, and *cnf*-1 genes were not detected in any of the isolates. The findings of this study may serve as a guide for pathogen-targeted therapy and the development of preventive strategies against VAP.

Keywords Ventilator-associated pneumonia (VAP) · *Klebsiella pneumoniae* · Virulence factors · Biofilm

Introduction

Klebsiella pneumoniae is a non-motile, rod-shaped, non-spore-forming, lactose-fermenting, oxidase-negative, facultative anaerobic, encapsulated Gram-negative bacterium that typically produces mucoid colonies. *K. pneumoniae* exists as a normal flora member in the gastrointestinal tracts of humans and animals, as well as in environmental sources such as water and plant surfaces. It is considered an opportunistic pathogen, frequently associated with various infections in immunocompromised individuals, particularly in the presence of risk factors such as urinary catheterization, mechanical ventilation, surgical interventions, and prolonged hospitalization in intensive care units (Mba et al. 2025; Paczosa and Mecsaz 2016).

K. pneumoniae can produce a wide range of virulence factors that facilitate colonization of the human body, adherence to host cells, evasion and suppression of the immune response, and subsequent progression of infection. Key virulence determinants include the presence of lipopolysaccharides, capsule production, fimbriae and siderophore synthesis, the ability to form biofilms, α-hemolysin production, hemagglutination, and the HV phenotype (Kuş et al. 2017).

The capsule surrounding the surface of *K. pneumoniae*, composed of polysaccharides, is one of its primary virulence factors and is closely associated with the hyperviscous phenotype. The bacteria capsule contains strain-specific polysaccharides. It makes a dense stringy colony (Mohammadi et al. 2023). These capsular polysaccharides are also referred to as K antigens. To date, 79 different capsular serotypes of *K. pneumoniae* have been identified, based on the diversity

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and structure of these polysaccharides. The capsule plays a crucial role in enabling the bacteria to evade phagocytosis. The genes responsible for capsular polysaccharide (CPS) biosynthesis are located within the *cps* locus on the chromosome and are composed of multiple genes. The *cps* gene cluster, which spans from *gal*F to *ugd*, contains more than 20 genes and is primarily regulated by three promoters situated upstream of the *gal*F, *wzi*, and *man*C genes, respectively. The translocation and assembly of CPS on the bacterial surface are regulated by proteins encoded by conserved genes located at the 5′ end of the *cps* locus, including *gal*F, *orf*2, *wzi*, *wza*, *wzb*, and *wzc*. Variations in *K. pneumoniae* capsule types are primarily attributed to differences in nucleotide sequences and the number of genes present within the *cps* cluster (Yoshida et al. 2000). The regulatory genes *rmp*A and *rmp*A2, which encode the regulators of the mucoid phenotype A and A2, are notable virulence determinants and may be located on either plasmids or the chromosome. Strains lacking *rmp*A exhibit reduced CPS expression and tend to display a non-hypermucoviscous phenotype. The *wzi* gene encodes an outer membrane protein responsible for anchoring capsular polysaccharides to the outer membrane, thereby facilitating capsule formation. In the absence of *wzi*, an acapsular phenotype is typically observed. The *wca*G gene encodes enzymes involved in fucose biosynthesis, which contributes to capsule structure and enhances the bacterium's ability to evade phagocytosis by macrophages (Zhu et al. 2021; Bushell et al. 2013).

In *K. pneumoniae*, siderophore systems are categorized into four main types: enterobactin (*ent*), yersiniabactin (*ybt*), salmochelin (*iro*), and aerobactin (*iuc*). These siderophore systems enable the bacterium to scavenge iron from the extracellular environment, thereby supporting its survival and enhancing its growth potential. The contribution of each siderophore to virulence and its expression levels may vary. The transport of enterobactin into the cell is mediated by specific transport proteins and receptors encoded by the *fep* locus (*fep*ABCDG) [8]. Enterobactin can be neutralized by lipocalin-2, a host-derived protein that binds and sequesters siderophores. To evade this immune response, certain *K. pneumoniae* strains have evolved "stealth" siderophores, such as salmochelin, a highly glycosylated derivative of enterobactin, and alternative systems like yersiniabactin, which are not recognized by lipocalin-2 (Bachman et al. 2012; Holden et al. 2014). *Iro*N and *iro*D function as specific receptors for salmochelin, facilitating its transport across the bacterial cell membrane. The synthesis of yersiniabactin requires proteins encoded by the *irp* genes, while its uptake is mediated by specific receptors encoded by the *ybt* and *fyu* genes (Paczosa and Mecsaz 2016). The *iuc* gene locus (*iuc*ABCD), which is essential for aerobactin biosynthesis, and the *iut*A gene, encoding the specific receptor protein for aerobactin, are typically located on a large virulence plasmid. In addition to these four major siderophore systems, an alternative iron acquisition system known as *kfu* has been identified, which is more commonly found in hypervirulent *K. pneumoniae* (hvKP) strains compared to classical *K. pneumoniae* (cKP). Experimental studies using animal models have demonstrated that *kfu*ABC mutants exhibit significantly reduced virulence compared to their wild-type counterparts (Zhu et al. 2021).

Lipopolysaccharide (LPS) is composed of three distinct regions: the highly conserved and hydrophobic lipid A moiety anchored to the outer membrane; the core polysaccharide, which connects lipid A to the O-antigen; and the O-antigen itself, which constitutes the highly variable outermost domain of the LPS structure (Karakamış 2022). LPS is also referred to as the endotoxin of *K. pneumoniae*. Endotoxin-associated genes, such as *wab*G, *uge* (which encodes a capsular lipoprotein), and *ycf*M (which promotes outer membrane protein expression), contribute significantly to virulence by enhancing resistance to phagocytosis (Remya et al. 2019).

Fimbriae are key mediators of adhesion in *K. pneumoniae*. At least four types of fimbrial adhesins have been experimentally characterized in this species: type 1, type 3, Kpc, and KPF-28 (Karakamış 2022). Type 1 fimbriae mediate bacterial adhesion to mannose-containing structures on host cells or extracellular matrices via the *fim*H adhesin. Type 3 fimbriae possess an adhesive subunit encoded by *mrk*D, which enables binding to collagen molecules and is located at the tip of the fimbriae. Type 3 fimbriae also play a major role in *K. pneumoniae* biofilm formation. Kpc fimbriae are strongly associated with the hypermucoviscous phenotype of *K. pneumoniae*. The *kpn* gene, a *fim*H-like adhesin, is highly prevalent among virulent *K. pneumoniae* strains (Paczosa and Mecsaz 2016).

Hypermucoviscosity is associated with invasive syndromes caused by *K. pneumoniae* and is believed to contribute to increased bacterial virulence. In *K. pneumoniae* strains, the mucoviscosity-associated gene (*mag*A) and the regulator of the mucoid phenotype (*rmp*A) have been found to be closely linked to the HV phenotype (Hartman et al. 2019; Wiskur et al. 2008).

Hemagglutinins responsible for hemagglutination are localized on bacterial fimbriae. Type 1 fimbriae, which mediate mannose-sensitive hemagglutination (MS-HA), have the ability to agglutinate guinea pig erythrocytes an effect that is inhibited in the presence of mannose. The expression of these fimbriae is regulated by the *fim* gene cluster. In contrast, mannose-resistant hemagglutination (MR-HA) is mediated by type 3 fimbriae, which are under the control of the *mrk* gene cluster (Podschun and Sahly 1991).

Biofilm is a structured community of bacterial cells enclosed in a self-produced polymeric matrix that adheres to

surfaces or host tissues. This matrix provides protection to bacteria against environmental stressors such as fluctuations in temperature, humidity, and pH. Within the biofilm, bacteria are more resistant to phagocytosis and can more effectively evade the host immune response (Høiby 2017).

Alpha-hemolysin is one of several bacterial hemolysins, which are extracellular cytotoxic polypeptides. A defining characteristic of alpha-hemolysin is its ability to lyse erythrocytes. It exhibits toxicity toward mammalian cells, particularly rabbit erythrocytes, and also possesses dermonecrotic and neurotoxic properties (Müştak & Esendal 2008).

Many enteric pathogens, including *K. pneumoniae*, are capable of producing cytoplasmic urease to hydrolyze urea into ammonia and carbon dioxide, using it as a nitrogen source for growth. The *ure*A gene encodes the urease enzyme responsible for this process. Additionally, allantoin metabolism represents another virulence factor observed in hypervirulent *K. pneumoniae* isolates. Allantoin is a degradation product derived from nucleic acids and can serve as a nitrogen source for bacterial growth. The chromosomally encoded *alls* gene acts as an activator of allantoin metabolism (Al-Kubaisi 2022).

In recent years, there has been a significant increase in hospital-acquired infections caused by *K. pneumoniae* (Liu et al. 2020). Numerous studies have been conducted to elucidate the antibiotic resistance genes and virulence characteristics of *K. pneumoniae* strains responsible for these infections (Juan et al. 2019; Le et al. 2021). However, to date, comprehensive gene-level investigations specific to our country remain limited. The aim of this study is to determine the phenotypic and genotypic virulence factors of *K. pneumoniae* isolates obtained from VAP patients in the intensive care unit of Kırşehir Training and Research Hospital.

Methods

Clinical samples

A total of 19 *Klebsiella pneumoniae* isolates obtained from tracheal aspirate cultures of mechanically ventilated patients in the intensive care unit of Kırşehir Training and Research Hospital between 2022 and 2023 were included in this study. Identification of the isolates and determination of their antibiotic resistance profiles were performed using the VITEK automated system. Colistin susceptibility was assessed using the broth microdilution method.

Phenotypic characterization of virulence

Hemolytic activity

K. pneumoniae isolates were incubated overnight at 37 °C on 5% sheep blood agar. Hemolytic activity was determined by the presence of hemolysis zones surrounding the bacterial colonies (Kuş et al. 2017).

String test

To identify hypermucoviscous *K. pneumoniae* colonies, the isolates were inoculated onto 5% sheep blood agar and incubated overnight at 37 °C. A standard inoculation loop was gently touched to the colony surface and lifted vertically. If a viscous string of ≥ 5 mm formed, the isolate was considered hypermucoviscous (Lin et al. 2014).

Detection of hemagglutination activity

The presence of type 1 and type 3 fimbriae in *K. pneumoniae* isolates was phenotypically evaluated using hemagglutination assays. For type 1 fimbriae, hemagglutination was assessed using a 3% suspension of human O group erythrocytes in 96-well microtiter plates. Bacterial suspensions were prepared at a turbidity equivalent to 0.5 McFarland standard. In each well, 20 µL of the bacterial suspension was added to 180 µL of 5% erythrocyte suspension, with and without 50 mM mannose. The plates were incubated overnight at room temperature. Hemagglutination was evaluated based on the appearance of uniform or irregular sedimentation patterns of erythrocytes at the bottom of the wells (Mishra et al. 2001).

For type 3 fimbriae, hemagglutination was assessed using 5% human O group erythrocyte suspensions pretreated with 0.03% tannic acid in 96-well microtiter plates. Bacterial suspensions were adjusted to a turbidity equivalent to 0.5 McFarland standard. In each well, 20 µL of the bacterial suspension was added to 180 µL of the tannic acid–treated 5% erythrocyte suspension, both in the presence and absence of 50 mM mannose. The plates were incubated overnight at room temperature. Hemagglutination was evaluated based on the presence of uniform or irregular sedimentation patterns of erythrocytes at the bottom of the wells (Mishra et al. 2001; Struve et al. 2009).

Investigation of biofilm formation

Biofilm formation by the isolates was assessed using the crystal violet staining method in 96-well flat-bottom microtiter plates (Christensen et al. 1985). Isolates were incubated overnight at 37 °C in 3 mL of tryptic soy broth (TSB) supplemented with 2% glucose. Then, 180 µL of fresh TSB (2% glucose) was added to each well of a 96-well plate, followed by 20 µL of the overnight culture. The plates were incubated at 37 °C for 48 h under static conditions. After incubation, the contents of the wells were discarded, and the wells were washed three times with sterilized distilled water. Subsequently, 200 µL of 0.2% crystal violet solution

was added to each well and allowed to stain for 45 min at room temperature. The dye was then discarded, and the wells were washed three times with 200 µL of distilled water and air-dried. To solubilize the stained biofilm, 200 µL of 96% ethanol was added to each well. From this, 100 µL of the solubilized solution was transferred to a new plate, and the absorbance was measured at 590 nm using a spectrophotometer. All measurements were performed in triplicate.

Biofilm formation was evaluated based on the classification criteria described by (Stepanović et al. 2007). According to this method, the cut-off optical density (ODc) was calculated using the following formula:

OD_c = average OD of negative control + (3 × SD of negative control).

Based on the optical density (OD) values, the *K. pneumoniae* isolates from VAP cases were classified into four groups: non-biofilm producers (OD ≤ ODc), weak biofilm producers (ODc < OD ≤ 2 × ODc), moderate biofilm producers (2 × ODc < OD ≤ 4 × ODc), and strong biofilm producers (OD > 4 × ODc).

Genotypic characterization of virulence

Total DNA extraction from *K. pneumoniae* isolates was performed using the boiling method as described by Ausubel et al. (1995). A loopful of bacterial growth from LB agar plates was suspended in 500 µL of sterile distilled water. The suspensions were then boiled in a water bath at 100 °C for 10 min. Following boiling, the tubes were centrifuged at 14,800 rpm for 10 min. The supernatant was transferred to a new tube and used as template DNA for PCR reactions (Ausubel et al. 1995).

In *K. pneumoniae* isolates, virulence genes associated with capsule formation (*rmp*A, *wca*G, *wzi*, *uge*, *wab*G, *ycf*M), fimbriae (*fim*H, *mrk*D, *kpn*), nitrogen metabolism (*alls*, *ure*A), siderophores (*iut*A, *ybt*A, *ent*B, *kfuB*C, *fyu*A, *iro*D, *iro*N), and cytotoxic necrotizing factor-1 (*cnf*-1) were amplified using PCR. The primers, reaction conditions, and thermocycler parameters used for gene amplification are presented in Table 1. Following PCR, the amplified products were subjected to electrophoresis on 1% agarose gels containing ethidium bromide, run at 100 V for approximately 45 min. The PCR products were visualized under ultraviolet (UV) light.

The *wzi* gene, which is used for capsule serotype determination, was amplified by PCR and subsequently sent for DNA sequencing (BMLabosis, Turkey). The resulting DNA sequences were processed using the BioEdit software, and capsule serotypes were identified by comparing the *wzi* sequences to those available in the Pasteur Institute database (https://bigsdb.pasteur.fr/klebsiella/). The wzi gene sequences of each *K. pneumoniae* isolate were deposited in the GenBank database under the accession number of PV617745-PV617763.

Results

Phenotypic analysis of virulence in *K. pneumoniae* isolates from VAP cases revealed that all isolates possessed a capsule; however, none of the isolates exhibited a hypermucoviscous capsule phenotype. Regarding hemolytic activity, none of the isolates demonstrated α-hemolysis. Hemagglutination assays indicated that all isolates carried both type 1 and type 3 fimbriae (Table 2).

Evaluation of biofilm formation among the isolates revealed a cut-off optical density (ODc) value of 0.2. Based on this threshold and classification criteria, 16% of the isolates ($n = 3$; Kp3, Kp12, Kp13) were classified as non-biofilm producers, 21% ($n = 4$; Kp6, Kp9, Kp14, Kp15) as weak biofilm producers, 26% ($n = 5$; Kp2, Kp5, Kp8, Kp17, Kp18) as moderate biofilm producers, and 37% ($n = 7$; Kp1, Kp4, Kp7, Kp10, Kp11, Kp16, Kp19) as strong biofilm producers (Table 2).

Genotypic analysis of virulence factors in *K. pneumoniae* isolates from VAP cases revealed that the capsule-associated genes *rmp*A and *wca*G were negative in all isolates, whereas *uge*, *wab*G, and *ycf*M were positive in all isolates, as determined by PCR (Fig. 1, Table 2). Analysis of the *wzi* gene sequences showed that a majority of the isolates (79%, $n = 15$) belonged to capsule type KL64. The remaining isolates were identified as follows: two isolates (11%) were classified as KL15/KL17/KL51/KL52, one isolate (5%) as KL2/KL30, and one isolate (5%) as KL36.

The fimbrial genes *fim*H and *mrk*D, responsible for fimbriae production, were found to be positive in all isolates, whereas the *kpn* gene was detected in the following isolates: Kp1, Kp2, Kp4, Kp7, Kp10, Kp11, Kp16, Kp17, Kp18, and Kp19. Among the genes associated with nitrogen utilization, *alls* was negative in all isolates, while *ure*A was positive in all isolates.

The presence of siderophore-related genes was also evaluated: *ent*B for enterobactin, *iro*D and *iro*N for salmochelin, *ybt*A and *fyu*A for yersiniabactin, and *iut*A for aerobactin. PCR analysis showed that all isolates carried *ent*B, *ybt*A, *fyu*A, and *iut*A. However, *iro*D and *iro*N, which are required for salmochelin production, were not detected in any of the isolates. The *kfu*BC gene was positive in all isolates except Kp1 (Fig. 1).

Additionally, the *cnf*-1 gene, which encodes cytotoxic necrotizing factor-1, was negative in all isolates (Fig. 1, Table 2).

Table 1 Primers and PCR reactions used in the study

Gene	Primer	Sequences (5′−3′)	bp	Tm °C	PCR conditions			References
					Cycle	°C	sn	
Capsule	*wzi*	F:GTGCCGCGAGCGCTTTCTATC TTGGTATTCC R:GAGAGCCACTGGTTCCAGAAT TACCGC	550	58	1 35 1	95 95 Tm 72 72	180 50 50 60 300	(Mishra et al. 2001)
	*rmp*A	F: ACTGGGCTACCTCTGCTTCA R: CTTGCATGAGCCATCTTTCA	535	56				(Le et al. 2021)
	*wca*G	F: GGTTGGKTCAGCAATCGTA R: ACTATTCCGCCAACTTTTGC	169	56				
	uge	F: GATCATCCGGTCTCCCTGTA R: TCTTCACGCCTTCCTTCACT	534	58				
	*wab*G	F: CGGACTGGCAGATCCATATC R: ACCATCGGCCATTTGATAGA	683	56				
	*ycf*M	F:ATCAGCAGTCGGGTCAGC R: CTTCTCCAGCATTCAGCG	160	55				(Lin et al. 2014)
Fimbria	*fim*H	F:ATGAACGCCTGGTCCTTTGC R:GCTGAACGCCTATCCCCTGC	688	55				(Le et al. 2021)
	*mrk*D	F:CCACCAACTATTCCCTCGAA R:ATGGAACCCACATCGACATT	240	52				
	kpn	F:GTATGACTCGGGGAAGATTA R:CAGAAGCAGCCACCACACG	626	55				(Lin et al. 2014)
Nitrogen	*alls*	F: CATTACGCACCTTTGTCAGC R:GAATGTGTCGGCGATCAGCTT	764	56				(Le et al. 2021)
	*ure*A	F:CAAGCTGTTGCTGTTTACCG R:ATCGGGTTGTGAACGGTGAC	270	58				
Siderofor	*iut*A	F-GGGAAAGGCTTCTCTGCCAT R-TTATTCGCCACCACGCTCTT	920	58				
	*ybt*A	F: ATGACGGAGTCACCGCAAAC R: TTACATCACGCGTTTAAAGG	960	55				
	*ent*B	F:ATTTCCTCAACTTCTGGGGC R:AGCATCGGTGGCGGTGGTCA	371	57				
	*kfu*BC	F-GGCCTTTGTCCAGAGCTACG R-GGGTCTGGCGCAGAGTATGC	638	58				
	*fyu*A	F:GCGACGGGAAGCGATGATTTA R:TAAATGCCAGGTCAGGTCACT	547	58				
	*iro*D	F:GCATAGGCGGATACGAACAT R:CACAGGGCAATTGCTTACCT	556	58				
	*iro*N	F:AAGTCAAAGCAGGGGTTG CCCG R:GACGCCGACATTAAGACGCAG	665	63				
Toxin	*cnf*-1	F:AAGATGGAGTTTCCTATGCAG GAG R:CATTCAGAGTCCTGCCCTCAT TATT	498	56				(Lin et al. 2014)

Discussion

Klebsiella pneumoniae is frequently responsible for a significant proportion of nosocomial infections in intensive care units, including ventilator-associated pneumonia (VAP) and urinary tract infections (UTIs). These infections are associated with increased morbidity and mortality rates, prolonged hospital stays, and elevated healthcare costs (Kalil et al. 2016).

VAP is a clinically significant healthcare-associated infection due to its high prevalence and elevated mortality rate (Sikora & Zahra 2025). Recent studies have demonstrated that the presence of biofilms on the surface of endotracheal tubes plays a critical role in the pathogenesis

Table 2 Genotypic and phenotypic characteristics of *Klebsiella pneumoniae* isolates caused VAP

K. pneumoniae isolates	Genotypic characteristics																			Phenotypic characteristics				
	Capsule					Fimbria			Urea		Siderofor							Toxin	KL type (*wzi*)	HV	Type 1 fimbria	Type 3 fimbria	Hemolysis	Biofilm
	*rmp*A	*wca*G	*uge*	*wab*G	*ycfm*	*fim*H	*mrk*D	*kpn*	*alls*	*ure*A	*ent*B	*iro*D	*iro*N	*ybt*A	*fyu*A	*iut*A	*kfu*BC	*cnf*-1						
Kp 1	−	−	+	+	+	+	+	+	−	+	+	−	−	+	+	+	−	−	KL15K-L17KL51	−	+	+	−	Strong
Kp 2	−	−	+	+	+	+	+	+	−	+	+	−	−	+	+	+	+	−	KL64	−	+	+	−	Moderate
Kp 3	−	−	+	+	+	+	+	−	−	+	+	−	−	+	+	+	+	−	KL64	−	+	+	−	Negative
Kp 4	−	−	+	+	+	+	+	+	−	+	+	−	−	+	+	+	+	−	KL64	−	+	+	−	Strong
Kp 5	−	−	+	+	+	+	+	−	−	+	+	−	−	+	+	+	+	−	KL64	−	+	+	−	Moderate
Kp 6	−	−	+	+	+	+	+	−	−	+	+	−	−	+	+	+	+	−	KL64	−	+	+	−	Weak
Kp 7	−	−	+	+	+	+	+	+	−	+	+	−	−	+	+	+	+	−	KL64	−	+	+	−	Strong
Kp 8	−	−	+	+	+	+	+	−	−	+	+	−	−	+	+	+	+	−	KL64	−	+	+	−	Moderate
Kp 9	−	−	+	+	+	+	+	−	−	+	+	−	−	+	+	+	+	−	KL64	−	+	+	−	Weak
Kp 10	−	−	+	+	+	+	+	+	−	+	+	−	−	+	+	+	+	−	KL64	−	+	+	−	Strong
Kp 11	−	−	+	+	+	+	+	+	−	+	+	−	−	+	+	+	+	−	KL64	−	+	+	−	Strong
Kp 12	−	−	+	+	+	+	+	+	−	+	+	−	−	+	+	+	+	−	KL64	−	+	+	−	Negative
Kp 13	−	−	+	+	+	+	+	+	−	+	+	−	−	+	+	+	+	−	KL64	−	+	+	−	Negative
Kp 14	−	−	+	+	+	+	+	−	−	+	+	−	−	+	+	+	+	−	KL64	−	+	+	−	Weak
Kp 15	−	−	+	+	+	+	+	−	−	+	+	−	−	+	+	+	+	−	KL64	−	+	+	−	Weak
Kp 16	−	−	+	+	+	+	+	−	−	+	+	−	−	+	+	+	+	−	KL2KL30	−	+	+	−	Strong
Kp 17	−	−	+	+	+	+	+	−	−	+	+	−	−	+	+	+	+	−	KL36	−	+	+	−	Moderate
Kp 18	−	−	+	+	+	+	+	+	−	+	+	−	−	+	+	+	+	−	KL64	−	+	+	−	Moderate
Kp19	−	−	+	+	+	+	+	+	−	+	+	−	−	+	+	+	+	−	KL15K-L17KL51	−	+	+	−	Strong

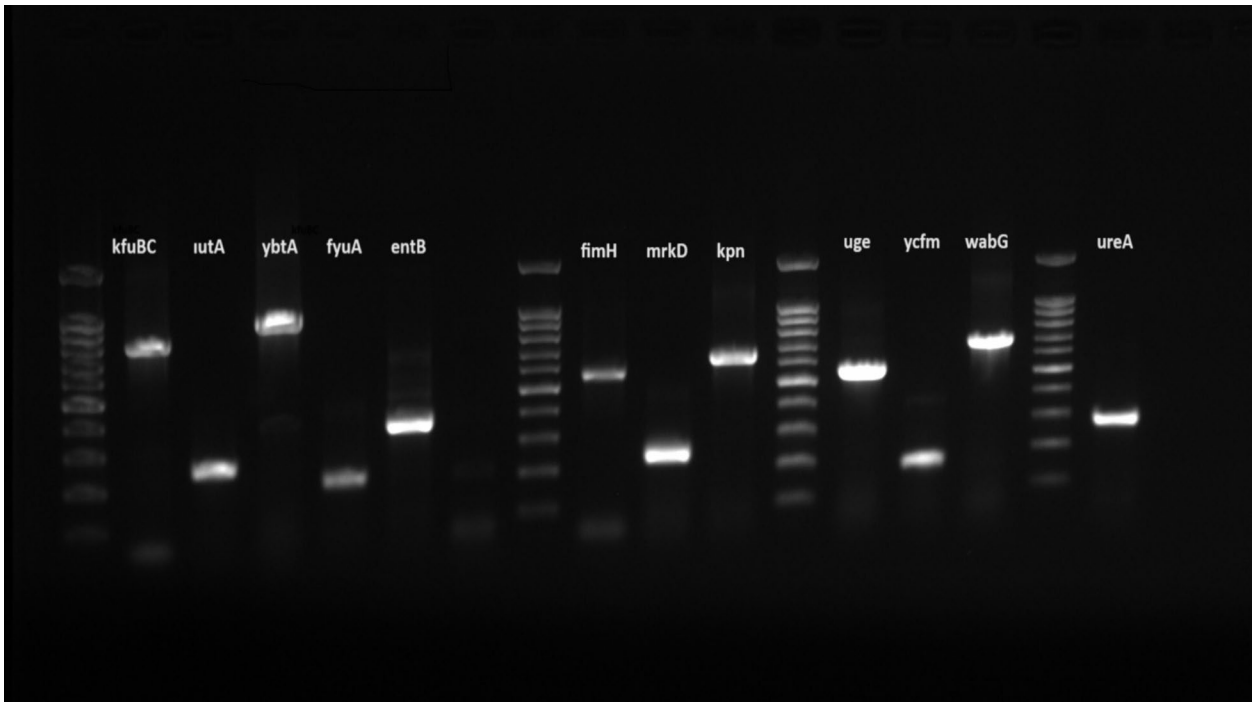

Fig. 1 Agarose gel electrophoresis image of PCR products for virulence genes in *K. pneumoniae* isolates

of VAP (Diaconu et al. 2018). In our study, an evaluation of biofilm-forming capacity in *K. pneumoniae* strains isolated from VAP cases revealed that 84% of the isolates were capable of forming biofilms at varying levels (weak, moderate, or strong). The *mrk*D gene encodes the adhesin protein of type 3 fimbriae, which facilitates bacterial adherence to extracellular matrices (Jagnow & Clegg 2003). The *fim*H gene encodes the adhesin subunit of type 1 fimbriae. While type 1 fimbriae primarily play a significant role in urinary tract infections, type 3 fimbriae facilitate *K. pneumoniae* adherence to various tissues such as the kidneys, lungs, and bladder. In addition, type 3 fimbriae contribute substantially to a strong biofilm formation on both biotic and abiotic surfaces, which increases the risk of infection, particularly in immunocompromised and catheterized patients. In the present study, all isolates were found to be positive for both *mrk*D and *fim*H genes (Table 2). The presence of these genes is thought to play a key role in the biofilm-forming ability of the majority of the isolates. These findings are consistent with previous studies in the literature indicating that type 1 and type 3 fimbriae are commonly found in *K. pneumoniae* strains and play an important role in both virulence and biofilm formation (Struve et al. 2009; Schroll et al. 2010). A review of the literature also reveals that a large proportion of *K. pneumoniae* isolates associated with VAP have biofilm-forming capacity (Yan et al. 2016; El Fertas-Aissani et al. 2013). In our study, 84% of *K. pneumoniae* isolates were found to produce biofilm at varying levels (weak, moderate, or strong) (Table 2), which aligns well with previously published data.

The capsule is a major virulence factor that enables bacteria to evade two critical host defense mechanisms: phagocytosis and direct inhibition by the host immune system. In *K. pneumoniae*, 79 different capsular serotypes have been identified, eight of which (K1, K2, K5, K16, K20, K54, K57, and KN1) are associated with hvKP. Among them, K1 and K2 are the most commonly encountered serotypes. Certain capsule types, particularly K1, K2, K54, K57, K20, and K5, are frequently linked to a community-acquired invasive pyogenic liver abscess syndrome. The K64 serotype, on the other hand, has been associated with multidrug-resistant hypervirulent *K. pneumoniae* (MDR-hvKP) strains (Yang et al. 2021). K15 and K36 capsular serotypes have also been reported in MDR-*K. pneumoniae* strains, with K15 being more prevalent in South America and K36 primarily detected in Brazil (Cerdeira et al. 2018). Recently, the K64 serotype has emerged as a predominant pathogen among carbapenem-resistant *K. pneumoniae* (CRKP) isolates reported from Brazil, Singapore, Taiwan, the USA, and several European countries. According to previous epidemiological studies, K64 is among the most frequently identified serotypes in CRKP infections in China (Li et al. 2021). In the study conducted by Li et al. (2021), based on Wzi genotyping, a total of 67 *Klebsiella pneumoniae* isolates were classified into nine distinct capsular types: KL64 (48 isolates),

KL19 (4), KL47 (3), KL60 (2), KL61 (2), KL54 (2), KL1 (2), KL2 (2), and KL24 (2). In another study, among 43 K. *pneumoniae* isolates obtained from blood, urine, sputum, and wound cultures, four capsular serotypes were identified: K1 (5 isolates), K2 (25), K5 (4), and K54 (2). Additionally, the genes responsible for capsule phenotypes were reported as follows: *wca*G in 48.83% (21/43) and *rmp*A in 32.55% (14/43) of the isolates (Li et al. 2021). In the study by Turton et al. (2010), the *rmp*A gene was reported to be associated with K1 and K2 capsular serotypes, while *wcaG* was found in K1, K54, and, to a lesser extent, K16 and K58 serotypes. In our study, four different capsular serotypes were identified among 19 isolates: KL64 (15), KL15KL17KL51 (2), KL2 (1), and KL36 (1) (Table 2). While these findings were consistent with some previous studies in the literature, they differed from others. These discrepancies were attributed to regional variations, the limited number of isolates, and the diversity of clinical sample sources. Notably, *rmp*A and *wca*G genes were not detected in any of the isolates in our study. This absence is considered consistent with the literature, as hypervirulent capsular serotypes such as K1, K2, K54, K16, and K58 were not identified among the *K. pneumoniae* isolates (Table 2).

Hypermucoviscosity is an important virulence factor for *K. pneumoniae*; yet, it remains a challenging phenotype to detect. In a study investigating 70 K. *pneumoniae* isolates from ventilator-associated pneumonia (VAP) patients, 20% of the strains were reported as HV-positive. These isolates were found to possess K1 and K2 capsular types and tested positive for the *rmp*A and aerobactin genes. Notably, the HV phenotype was particularly associated with the presence of the *rmp*A gene (Guo et al. 2016). In another study, 50.7% of *K. pneumoniae* isolates from VAP patients were identified as HV-positive. In that study, the K1 capsular type as well as *rmp*A and *mag*A genes were detected (Liu & Guo 2018). Another study confirmed the regulatory role of the *rmp*A gene in the expression of the HV phenotype (Cheng et al. 2010). In our study, the HV phenotype was not observed in any of the isolates. While the *mag*A gene was not examined, the *rmp*A gene was found to be negative in all isolates. This result supports the established positive correlation between *rmp*A and the HV phenotype reported in the literature.

Bacterial hemolysins are extracellular cytotoxic polypeptides that exhibit in vitro toxic effects not only on erythrocytes but also on polymorphonuclear leukocytes, monocytes, and fibroblasts. Studies have shown that isolates obtained from patients with bacteremia and pyelonephritis produce higher levels of alpha-hemolysin compared to those isolated from stool samples or patients with asymptomatic bacteriuria (Kuş et al. 2017). Although hemolytic activity in VAP-associated *K. pneumoniae* isolates has not been widely investigated, some studies have examined this feature in isolates obtained from other clinical specimens. These studies reported no alpha-hemolysin production and failed to detect the associated *hly*A and *cnf*-1 genes (Kuş et al. 2017; El Fertas-Aissani et al. 2013). Similarly, in our study, no hemolytic activity was observed, and the *cnf*-1 gene was found to be negative in all isolates, consistent with findings reported in the literature.

Hemagglutination refers to the agglutination of erythrocytes caused by the binding of bacterial fimbriae to specific receptors on the erythrocyte surface. Most *K. pneumoniae* strains express two types of fimbrial adhesins (hemagglutinins): type 1 (*Fim*H), which is mannose-sensitive, and type 3 (*Mrk*D), which is mannose-resistant (Klemm & Schembri 2000). Type 1 fimbriae, particularly in *Klebsiella* and *Escherichia coli*, have been shown to play a significant role in urinary tract infections (Maayan et al. 1985; Mulvey et al. 1998). Type 3 fimbriae, on the other hand, facilitate the adhesion of *Klebsiella* species to various human cells, including epithelial and endothelial cells in the respiratory and urinary tracts (Hornick et al. 1991). Moreover, type 3 fimbriae are essential for *K. pneumoniae* to form biofilms on abiotic surfaces such as plastics and extracellular matrices of human tissues. This enables the formation of treatment-resistant biofilms on indwelling medical devices, such as catheters and endotracheal tubes. In the study by El Fertas-Aissani et al. (2013), among 54 K. *pneumoniae* isolates, mannose-sensitive hemagglutination (MSHA), specific to type 1 fimbriae, was observed in 94.5% of isolates, while mannose-resistant hemagglutination (MRHA), specific to type 3 fimbriae, was found in 68.5%. In our study, since all isolates tested positive for both *fim*H and *mrk*D genes, MSHA and MRHA were also found to be positive in all isolates (Table 2).

The lipopolysaccharide (LPS) structure, also known as the endotoxin of *K. pneumoniae*, plays a critical role in virulence by conferring resistance to phagocytosis. In addition to protecting the bacterium from complement-mediated killing, LPS also triggers a strong immune response in the host (Pruss et al. 2023). Among the genes responsible for normal LPS synthesis, *uge* has been frequently detected in *K. pneumoniae* isolates, as reported in several studies. The absence of the *uge* gene has been associated with the inability of *K. pneumoniae* to cause infections such as urinary tract infections, septicemia, and pneumonia. Similarly, the *wab*G gene has been detected in approximately 88–100% of isolates across multiple studies (Paczosa & Mecsaz 2016; Pruss et al. 2023).

In a study investigating *K. pneumoniae* isolates from various clinical specimens, the *ycf*M, *wab*G, and *uge* genes were detected at frequencies of 88%, 84%, and 72%, respectively. These genes are associated with the synthesis of capsule components, capsule-associated lipoproteins, and outer membrane proteins. The same study reported that carbapenem-susceptible strains lacked at least one of these

genes, whereas all three genes were present in resistant strains. These findings are consistent with previous studies suggesting a relationship between the LPS structure in pathogenic bacteria and antibiotic resistance (Sora et al. 2021; Mohammed 2024). Other studies in the literature have similarly reported the *uge*, *ycf*M, and *wab*G genes to be present in 87–100% of isolates (Karakamış 2022; Aljanaby & Alhasani 2016). In our study, consistent with the literature, all isolates tested 100% positive for the *uge*, *wab*G, and *ycf*M genes. Considering that all of our isolates were multidrug-resistant (MDR), these results support the hypothesis that LPS-related genes may contribute to antimicrobial resistance.

The production of multiple siderophores by *K. pneumoniae* provides an alternative strategy for successful colonization of various tissues and/or evasion of host-mediated neutralization of a single siderophore. Among the four main siderophores, enterobactin is highly conserved across strains and contains three catechol rings, giving it the highest binding affinity for Fe^{3+}. This makes enterobactin essential and the primary iron acquisition system in both cKP and hvKP strains (Bachman et al. 2012; Holden et al. 2014). Salmochelin, in contrast, is reported to be significantly more prevalent in hvKP strains, with studies detecting its presence in up to 90% of hvKP isolates (Paczosa & Mecsas 2016). Notably, yersiniabactin is expressed during pulmonary infections and remains active during early stages of lung infection, as it is not inhibited in vivo by lipocalin-2. This enables *K. pneumoniae* to reach high bacterial loads in the lungs during infection. In hvKP, approximately 90% of the total siderophore production consists of aerobactin. Analysis of the best-characterized virulence plasmids in *K. pneumoniae*, namely pK2044 and Kp52.145pII, has revealed that they carry gene loci encoding both aerobactin (*iuc*ABCD, *iut*A) and salmochelin (*iro*BDCE) siderophores, as well as *rmp*A genes responsible for the hypermucoid phenotype considered a hallmark of hvKP strains (Zhu et al. 2021; Kochan et al. 2022). In light of the above information, a comparison of our findings with the literature reveals that the *ent*B gene, which encodes the primary siderophore enterobactin, as well as the yersiniabactin-associated genes *ybt*A and *fyu*A, were detected in all isolates likely due to the fact that our isolates were derived from VAP cases (Table 2). In contrast, salmochelin genes, typically associated with hypervirulent strains, were not detected in any of our isolates, supporting the conclusion that these were not hvKP strains. Among the aerobactin-related genes, only *iut*A was investigated, and it was found to be present in all isolates. While the literature frequently reports the co-occurrence of aerobactin genes with *rmp*A and salmochelin genes in hypervirulent strains, the isolated presence of *iut*A in our study further supports the notion that our isolates do not belong to the hypervirulent *K. pneumoniae* lineage.

Pathogens such as *K. pneumoniae* can produce urease to utilize urea as a nitrogen source for growth. The *ure*DABCEFG operon encodes both the structural subunits of the metalloenzyme urease (*ure*A, *ure*B, and *ure*C) and the accessory nickel-binding proteins (*ure*D, *ure*E, *ure*F, and *ure*G), which are responsible for incorporating nickel ions into the enzyme's active site (Li et al. 2014). Additionally, allantoin metabolism represents a degradation pathway of nucleic acids that yields products some bacteria can utilize as a nitrogen source (Choby et al. 2020). The *all*S gene locus, which is responsible for allantoin metabolism, is highly associated with hypervirulent *K. pneumoniae* and is emphasized to play a crucial role in the pathogenesis of liver abscesses caused by *K. pneumoniae* (Paczosa & Mecsas 2016; Li et al. 2014; Ku et al. 2008). According to the literature, *K. pneumoniae* isolates obtained from various clinical samples were reported to be positive for the *all*S gene at a rate of 8% and for the *ure*A gene at 87.5% (Al-Kubaisi 2022). In our study, the *all*S gene was negative in all isolates, while the *ure*A gene was found to be positive in all of them. Our findings are consistent with the literature, and the low presence of the *all*S gene supports the fact that the isolates were not obtained from liver abscesses.

Conclusion

As is well known, the identification of the causative agent, its specific virulence factors, and its antimicrobial susceptibility profile is critical for the diagnosis of infectious diseases and for understanding disease pathogenesis. Numerous studies have demonstrated that virulence factors contribute to the development of antibiotic resistance in pathogenic bacteria (El Fertas-Aissani et al. 2013; Arısoy et al. 2008). The detection of virulence factors in *K. pneumoniae* isolates is particularly important when evaluated alongside antibiotic resistance, as it allows for the assessment of the potential course of infection in the human body and facilitates the development of appropriate treatment strategies. The strains in our study are multidrug-resistant (MDR), and these pathogens have the potential to acquire virulence plasmids such as pLVPK, which could lead to their transformation into hypervirulent strains. This phenomenon is increasingly observed in *K. pneumoniae* (Riwu et al. 2022). The treatment of hvKp strains that also exhibit extensive drug resistance (XDR) is particularly challenging and has been associated with increased mortality. In our study, all isolates tested positive for key virulence genes associated with biofilm formation *fim*H, *mrk*D, *ent*B, and *iut*A. The presence of these genes suggests that these strains are capable of forming biofilms on the surface of endotracheal tubes in VAP patients, contributing significantly to infection development. These findings highlight a critical issue that must be taken

into account when managing VAP, particularly in terms of treatment planning and the implementation of preventive strategies. Considering these virulence traits may improve clinical outcomes.

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Data availability No datasets were generated or analyzed during the current study.

Declarations

Ethics approval This study was approved by the Clinic Research Ethics Committee of Kırsehir Ahi Evran University, Kırsehir, Turkey (Number: 2022/22–190).

Competing interests The authors declare no competing interests.

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