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Investigation of Virulence Factors by Phenotypic and Genotypic Methods and Evaluation of Their Relationship with Antibiotic Resistance in *Klebsiella pneumoniae* Strains Isolated from Clinical Specimens

Klinik Örneklerden İzole Edilen *Klebsiella pneumoniae* Suşlarında Virülans Faktörlerinin Fenotipik ve Genotipik Yöntemlerle Araştırılması ve Antibiyotik Direnci ile İlişkilerinin İncelenmesi

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ABSTRACT

Background: Infections caused by *Klebsiella pneumoniae (K. pneumoniae)*, have become a challenging health issue, especially with the emergence of extensively drug-resistant (XDR) strains. The widespread use of antibiotics to treat infections caused by *K. pneumoniae* has led to the development and spread of resistance to these drugs. This study aims to identify the virulence factors of *K. pneumoniae* isolates obtained from clinical specimens using phenotypic and genotypic methods and to examine their relationship with antimicrobial resistance.

Materials and Methods: A total of 100 *K. pneumoniae* isolates were included in the study, and their identification and antimicrobial susceptibility tests were performed using the VITEK-2 automated system. The production of capsule and alpha-hemolysin, biofilm formation ability, and hypermucoviscosity phenotype were examined by phenotypic methods; the presence of adhesin (*ycfM, mrkD, kpn*), invasin (*traT*), siderophore (*entB*, *iutA, fyuA, iroN*), and toxin (*hlyA*) genes were investigated using genotypic methods.

Results: The production of alpha-hemolysin and the hypermucoviscosity phenotype were only detected in eight strains (8%). Among the adhesin genes, *ycfM* was positive in 99%, *mrkD* in 97%, and *kpn* in 46% of the isolates; among the invasin genes, *traT* was positive in 2%; among the siderophore genes, *entB* was positive in 96%, *iutA* in 79%, *fyuA* in 71%, and *iroN* in 3%; and among the toxin genes, *hlyA* was positive in 2% of the isolates.

Conclusion: In evaluating the virulence factors of the isolates categorized as XDR, multidrug-resistant (MDR), and susceptible based on antibiotic susceptibility results, it was found that the aerobactin siderophore receptor gene *iutA* was significantly more prevalent in the XDR and MDR groups, that the two isolates with the *traT* virulence gene were in the MDR group, and that the *kpn* and *iroN* genes were more frequently observed in isolates from the MDR and susceptible groups, suggesting a possible negative correlation with antibiotic resistance.

Keywords: *Klebsiella pneumoniae*, antibiotic resistance, virulence factors

ÖZ

Amaç: Enterobactericeae ailesinin en önemli fırsatçı patojenlerinden biri olan *Klebsiella pneumoniae (K. pneumoniae)*'nın neden olduğu enfeksiyonlar, özellikle çok ilaca dirençli (ÇİD) suşların ortaya çıkmasıyla birlikte tedavi edilmesi güç bir sağlık sorunu haline gelmiştir. *K. pneumoniae*'nın neden olduğu enfeksiyonları tedavi etmek için antibiyotiklerin yaygın olarak kullanılmaya başlanması, bu ilaçlara karşı direncin ortaya çıkmasına ve yayılmasına sebep olmuştur. Bu çalışmada, klinik örneklerden izole edilen *K. pneumoniae*

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ÖZ

izolatlarının virülans faktörlerinin fenotipik ve genotipik yöntemlerle belirlenmesi ve antimikrobiyal ilaçlara karşı direnç durumuyla ilişkisinin incelenmesi hedeflenmiştir.

Gereç ve Yöntemler: Çalışmaya 100 *K. pneumoniae* izolatı dahil edilmiş, izolatların tanımlama ve antimikrobiyal duyarlılık testleri VITEK-2 otomatize sistemi ile yapılmıştır. Kapsül ve alfa hemolizin üretimi, biyofilm oluşturma yeteneği ve hipermukoviskozite fenotipi özelliği fenotipik yöntemlerle incelenmiştir.

Bulgular: Yüz izolat, yaygın ilaca dirençli (YİD), ÇİD ve duyarlı olmak üzere üç grupta incelenmiştir. Tüm izolatlarımızda kapsüle sahip olduğu ve biyofilm oluşturduğu bulunmuştur. Alfa hemolizin üretimi ve hipermukoviskozite fenotipi özelliği ise yalnızca sekiz suşta (%8) tespit edilmiştir. Adezin genlerinden *ycfM* %99, *mrkD* %97 ve *kpn* %46; invazin genlerinden *traT* %2; siderofor genlerinden *entB* %96, *iutA* %79, *fyuA* %71, *iroN* %3 ve toksin genlerinden *hlyA* ise %2 oranında pozitif bulunmuştur.

Sonuç: Çalışmamızda antibiyotik duyarlılık sonuçlarına göre YİD, ÇİD ve duyarlı olmak üzere üç gruba ayrılan izolatların virülans faktörleri açısından değerlendirilmesinde; aerobaktin siderofor reseptör geni *iutA*'nın YİD ve ÇİD grupta anlamlı derecede fazla bulunduğu, *traT* virülans genine sahip iki izolatın da ÇİD grupta yer aldığı, *kpn* ve *iroN* geninin ÇİD ve duyarlı grupta bulunan izolatlarda daha fazla görüldüğü, bu nedenle antibiyotik direnci ile arasında negatif bir kolerasyon olabileceği sonucuna varılmıştır.

Anahtar Kelimeler: *Klebsiella pneumoniae*, antibiyotik direnci, virulans faktörleri

Introduction

Klebsiella pneumoniae (K. pneumoniae) is a significant opportunistic pathogen, particularly causing various infections in immunocompromised patients, often associated with risk factors such as urinary catheterization, mechanical ventilation, surgical procedures, and prolonged stays in intensive care units (ICUs) (1). It is commonly linked to urinary tract infections (UTIs), pneumonia, sepsis, and wound infections (2). Hospital isolates of *K. pneumoniae* frequently exhibit multidrug-resistant (MDR) phenotypes due to the presence of extended-spectrum beta-lactamases (ESBLs) or carbapenemases, complicating the selection of appropriate antibiotics for treatment (3). The bacterium's ability to reproduce rapidly enhances its ability to develop mutations, which in turn increases its ability to develop antibiotic resistance. In addition to its high prevalence, *K. pneumoniae* is a significant factor in the spread of antibiotic resistance. Clinical isolates of *K. pneumoniae* often display MDR phenotypes due to the presence of ESBLs or carbapenemases, making it challenging to choose appropriate antibiotics for treatment (4). MDR strains of *K. pneumoniae* are frequently isolated as causative agents of hospital infections.

In recent years, strains of *K. pneumoniae* with MDR have posed serious problems in many countries, including Türkiye, and virulence factors significantly contribute to the infections caused by the bacteria (5). Virulence is defined as the ability of a microorganism to infect the host and cause disease. Virulence factors are molecules which assist the bacterium in colonizing the host. These factors can be secretory, membrane-associated, or cytosolic by nature. Cytosolic factors facilitate the bacterium's rapid, adaptive, metabolic, physiological, and morphological changes. Membrane-associated virulence factors aid the bacterium in adhering to and evading host cells. The secretory virulence factors possessed by the bacterium are crucial components that help it evade the host's innate and adaptive immune responses. In extracellular pathogens, secretory virulence factors act synergistically to kill host cells (6). The differences in the clinical features of *K. pneumoniae* infections are associated with the characteristics and number of virulence factors expressed (7). Although *K. pneumoniae* is considered one of the most important Gram-negative opportunistic pathogens, the mechanisms by which this bacterium causes different diseases remain unclear, and many studies have limitations because of the limited number of investigated virulence factors (8). The pathogenicity of *K. pneumoniae* is associated with the expression of various virulence factors, including capsular polysaccharide, lipopolysaccharide, iron acquisition systems (siderophores), adhesins, hypermucoviscosity, and outer membrane lipoprotein (6).

The primary virulence factors involved in the pathogenicity of *K. pneumoniae* include capsule formation, biofilm formation, alpha-hemolysin production, hypermucoviscosity, fimbriae, siderophores, and toxins (9). *K. pneumoniae* possesses several virulence genes encoding factors such as *mrkD*, *kpn*, *ycfM* (adhesins), *traT* (invasin), *entB* (enterobactin siderophore), *iutA* (aerobactin siderophore), *fyuA* (yersiniabactin siderophore), *iroN* (catechols receptor), and *hlyA* (toxin) (5). These virulence factors enable the bacterium to survive under adverse conditions and manage the pathogenesis of infection through critical mechanisms such as biofilm formation, capsule formation, adhesin, and invasion capabilities, siderophores, and toxin production (10). Although there have been some studies focused solely on the virulence factors of *K. pneumoniae*, there are a limited number of studies examining the relationship between

virulence factors in extensively or MDR strains and those in susceptible strains (8).

The aim of this study was to determine the presence of capsules, biofilm formation ability, hypermucoviscosity, and alpha-hemolysin production in *K. pneumoniae* strains isolated from clinical specimens using phenotypic methods, to investigate the presence of adhesin, invasion, siderophore, and toxin genes using genotypic methods, and to evaluate the potential virulence factors together with their relationship to antimicrobial resistance.

Material and Methods

This study was conducted with the approval of the University of Health Sciences Türkiye, Hamidiye Scientific Research Ethics Committee Presidency (approval number: 35/13, dated: 19.11.2021). Following ethical approvals, informed consent was obtained from participants included in this study. Our study included 100 *K. pneumoniae* isolates which were obtained as causative pathogens from various clinical specimens submitted by inpatients to the Medical Microbiology Laboratory of the University of Health Sciences Türkiye, Sultan 2. Abdülhamid Han Training and Research Hospital between January 2021 and December 2021. In cases where *K. pneumoniae* strains were isolated from different clinical specimens of the same patient, only the strain isolated from the first clinical specimen was included in the study. The isolates were stored in stock medium at -80 °C until the study was conducted. The bacterial identification and determination of antibiotic susceptibility were performed using the automated VITEK-2 system (bioMérieux, France). Colistin susceptibility was determined using the broth microdilution method according to the recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST). The antibiotic susceptibilities of the isolates were evaluated according to EUCAST criteria (11). The presence of a capsule in *K. pneumoniae* isolates was investigated using the India ink staining method. For this method, the isolates were first sub-cultured onto 5% sheep blood agar medium and incubated at 37 °C for 24 hours. The following day, a loopful of fresh colonies from this medium was mixed with a drop of India ink on a slide. The mixture was spread as a thin smear using another slide and left to dry at room temperature. After drying, the slides were stained with crystal violet for one minute; then they were washed and left to dry again at room temperature. The presence of a capsule was investigated under a microscope using a 100x objective with immersion oil. At the end of the method, the bacteria appeared purple, the background black, and the capsule around the bacteria was seen as a white halo.

Investigation of Biofilm Formation Ability

The biofilm formation capacity of *K. pneumoniae* isolates was investigated using the microplate method. For this process, the isolates were subcultured daily for three days in sterile glass tubes containing Luria-Bertani (LB) medium (Thermo Scientific, USA). After the third day, the bacterial density in each glass tube was adjusted to 0.56-0.64 using a McFarland densitometer. Two hundred microliters of the adjusted culture samples were transferred into a 96-well microplate. Two wells containing only LB medium were used as controls. The microplate was incubated at room temperature for 24 hours. After incubation, 25 µL of crystal violet stain was added to each well. The microplate was shaken in an HTX Multi-Mode Microplate Reader (Synergy, Germany) for one minute and then left at room temperature for 15 minutes. Subsequently, the medium and dye in the wells were carefully discarded into a disinfectant-containing container by inverting the microplate. The microplate was then thoroughly washed three times with phosphate-buffered saline, and 200 µL of 96% ethanol was distributed into each well. The microplate was then placed back into device for measurement, with the device set to a wavelength of 590 nm. The absorbance values of the wells containing bacteria had their absorbance value subtracted. Based on the new value obtained, the isolates were classified as high-level biofilm producers [optical density (OD >0.500)], moderate-level biofilm producers (OD between 0.100 and 0.500), and weak-level biofilm producers (OD <0.100).

Investigation of Hypermucoviscosity Presence

To detect the presence of hypermucoviscosity, *K. pneumoniae* isolates were inoculated onto brain heart infusion agar (Merck, Germany) plates using the dilution streaking method and incubated at 37 °C for 24 hours. Afterward, a standard disposable loop was touched to the colonies on the medium and slowly lifted. If the bacterial structure extended upward by more than 5 mm, hypermucoviscosity was considered positive.

Investigation of Alpha-Hemolysin Production

To investigate alpha-hemolysin production, *K. pneumoniae* isolates were inoculated onto 5% sheep blood agar medium and incubated at 37 °C for 18-24 hours. At the end of the incubation period, if a greenish zone formed around the bacterial colonies due to incomplete lysis of erythrocytes, the isolate was considered positive for alpha-hemolysin.

Investigation of Virulence Genes by Polymerase Chain Reaction (PCR)

DNA extraction from *K. pneumoniae* isolates was performed using the boiling method. The presence of virulence genes was investigated using the PCR method. The virulence genes included *ycfM* (outer membrane lipoprotein), *mrkD* (type 3 adhesin), *kpn* (*FimH*-like adhesin), *traT* (outer membrane lipoprotein associated with serum resistance), *entB* (enterobactin biosynthesis), *iutA* (aerobactin receptor), *iroN* (salmochelin catecholate siderophore receptor), *fyuA* (yersiniabactin receptor), and *hlyA* (hemolysin A) (Table 1). The following markers were used as reported in previous articles (12-14).

The PCR reaction mixture was prepared in PCR tubes with a total volume of 25 µL. Amplification of the gene regions encoding virulence factors was performed under reaction conditions suitable for the primers, utilizing a T100 Thermal Cycler (Bio-Rad, USA). The amplified PCR products were visualized using gel electrophoresis. The agarose gel was prepared with 1X TAE buffer at a 2% agar concentration, and the samples were run at 110V for 1 hour. The amplification products were visualized using the GBox Chemi XX6 (Syngene, UK) gel imaging system, and the images were analyzed using the GeneSys software.

Statistical Analysis

Statistical analysis was performed using the SPSS software. The chi-square test was used to determine the statistical relationship between categorical data (phenotypic tests, virulence genes, and antibiotic susceptibility profiles) and a p-value of <0.05 was considered statistically significant.

Results

Distribution of Clinical Samples

Of the studied samples, tracheal aspirate fluid was the sample from which *K. pneumoniae* was most frequently isolated, accounting for 50% (n=50) of all samples. at 50% (n=50). This was followed by blood at 16% (n=16), urine at 11% (n=11), wound swabs at 9% (n=9), sputum at 8% (n=8), bronchoalveolar lavage fluid at 4% (n=4), and catheter tip samples at 2% (n=2). Of these clinical samples, 70% were collected from patients in ICUs, while 30% were from patients in clinical wards.

Antibiotic Susceptibility of *K. pneumoniae* Isolates

The antibiotic susceptibilities of the 100 *K. pneumoniae* isolates included in the study were determined using the VITEK-2 (bioMérieux, France) automated system. Colistin susceptibility was assessed by the broth microdilution method, and the susceptibility results were evaluated according to EUCAST criteria. Based on these results, the isolates were categorized into three distinct groups.

Group 1: consisted of 67 extensively drug-resistant (XDR) isolates that were susceptible to two or fewer antibiotic classes. Group 2: Comprising 17 MDR isolates that were resistant to three or more antibiotic classes. Group 3: Included 16 isolates that were resistant to fewer than three antibiotic classes.

Antibiotic Resistance Profiles

Group 1 (XDR): The isolates in the XDR group showed 100% resistance to amikacin, gentamicin, amoxicillin/clavulanic acid, ertapenem, meropenem, piperacillin/tazobactam, cefepime, cefoxitin, ceftazidime, ceftriaxone, ciprofloxacin, and trimethoprim/sulfamethoxazole. They exhibited 70% resistance to colistin.

Group 2 (MDR): The isolates in the MDR group were 100% susceptible to colistin. The susceptibility to other antibiotics was as follows: 88% to amikacin, 82% to ertapenem and meropenem, 65% to cefoxitin, 59% to gentamicin, 35% to piperacillin/tazobactam, 24% to cefepime, 18% to amoxicillin/clavulanic acid and trimethoprim/sulfamethoxazole, and 6% to ceftazidime, ceftriaxone, and ciprofloxacin.

Group 3 (Susceptible): The isolates in group 3 were 100% susceptible to amikacin, gentamicin, ertapenem, meropenem, colistin, and ciprofloxacin. The susceptibility rates were 94% to cefoxitin, 87% to piperacillin/tazobactam and cefepime, 81% to ceftazidime, ceftriaxone, and trimethoprim/sulfamethoxazole, and 75% to amoxicillin/clavulanic acid (Table 2).

Microscopic Analysis of Capsule Presence and Biofilm Formation in *K. pneumoniae* Isolates

Using the Chinese ink staining technique, all 100 *K. pneumoniae* isolates examined under a microscope displayed the presence of capsules. Since capsules were observed in all isolates, no statistically significant difference was found among the groups (p>0.05).

All isolates in our study were observed to possess biofilm-forming ability. The number of isolates forming strong biofilms was 37, forming moderate biofilms was 42, forming weak biofilms was 21. The distribution of strong biofilm formers was as follows: 32 (47.8%) in the XDR group, 3 (17.7%) in the MDR group, and 2 (12.5%) in Group 3. Moderate biofilm formers numbered 24 (35.8%) in the XDR group, 5 (29.4%) in the MDR group, and 13 (81.3%) in Group 3. Weak biofilm formers included 11 (16.4%) in the XDR group, 9 (52.9%) in the MDR group, and 1 (6.2%) in Group 3. Statistical evaluation revealed a significant difference in biofilm formation among the groups (p<0.05).

Table 1. List of primers used in this study

Primer	Nucleotide sequence (5'-3')	TA (°C)	Amplicon size (bp)	Reference
ycfM-F	ATCAGCAGTCGGGTCAGC	55	160	8
ycfM-R	CTTCTCCAGCATTCAGCG			
mrkD-F	CCACCAACTATTCCCTCGAA	52	240	12
mrkD-R	ATGGAACCCACATCGACATT			
kpn-F	GTATGACTCGGGGAAGATTA	55	626	8
kpn-R	CAGAAGCAGCCACCACACG			
traT-F	GGTGTGGTGCGATGAGCACAG	63	290	13
traT-R	CACGGTTCAGCCATCCCTGAG			
entB-F	ATTTCCTCAACTTCTGGGGC	57	371	8
entB-R	AGCATCGGTGGCGGTGGTCA			
iutA-F	GGCTGGACATCATGGGAACTGG	63	300	13
iutA-R	CGTCGGGAACGGGTAGAATCG			
iroN-F	AAGTCAAAGCAGGGGTTGCCCG	63	665	13
iroN-R	GACGCCGACATTAAGACGCAG			
fyuA-F	GCGACGGGAAGCGATGATTTA	56	547	14
fyuA-R	TAAATGCCAGGTCAGGTCACT			
hlyA-F	AACAAGGATAAGCACTGTTCTGGCT	63	1177	13
hlyA-R	ACCATATAAGCGGTCATTCCCGTCA			

TA: Annealing temperature

Table 2. Resistance status of isolates to tested antibiotics

Antibiotics	XDR group		MDR group		Susceptible group		All isolates	
	Susceptible	Resistant	Susceptible	Resistant	Susceptible	Resistant	Susceptible	Resistant
Amikacin	0 (0%)	67 (100%)	15 (88%)	2 (12%)	16 (100%)	0 (0%)	31 (31%)	69 (69%)
Gentamicin	0 (0%)	67 (100%)	10 (59%)	7 (41%)	16 (100%)	0 (0%)	26 (26%)	74 (74%)
Amoxicillin/Clavulanic acid	0 (0%)	67 (100%)	3 (18%)	14 (82%)	12 (75%)	4 (25%)	15 (15%)	85 (85%)
Ertapenem	0 (0%)	67 (100%)	14 (82%)	3 (18%)	16 (100%)	0 (0%)	30 (30%)	70 (70%)
Meropenem	0 (0%)	67 (100%)	14 (82%)	3 (18%)	16 (100%)	0 (0%)	30 (30%)	70 (70%)
Piperacillin/Tazobactam	0 (0%)	67 (100%)	6 (35%)	11 (65%)	14 (87%)	2 (13%)	20 (20%)	80 (80%)
Colistin	20 (30%)	47 (70%)	17 (100%)	0 (0%)	16 (100%)	0 (0%)	53 (53%)	47 (47%)
Cefepime	0 (0%)	67 (100%)	4 (24%)	13 (76%)	14 (87%)	2 (13%)	18 (18%)	82 (82%)
Cefoxitin	0 (0%)	67 (100%)	11 (65%)	6 (35%)	15 (94%)	1 (6%)	26 (26%)	74 (74%)
Ceftazidime	0 (0%)	67 (100%)	1 (6%)	16 (94%)	13 (81%)	3 (19%)	14 (14%)	86 (86%)
Ceftriaxone	0 (0%)	67 (100%)	1 (6%)	16 (94%)	13 (81%)	3 (19%)	14 (14%)	86 (86%)
Ciprofloxacin	0 (0%)	67 (100%)	1 (6%)	16 (94%)	16 (100%)	0 (0%)	17 (17%)	83 (83%)
Trimethoprim/Sulfamethoxazole	0 (0%)	67 (100%)	3 (18%)	14 (82%)	13 (81%)	3 (19%)	16 (16%)	84 (84%)

XDR: Extensively drug-resistant, MDR: Multidrug-resistant

For the detection of hypermucoviscosity, 100 *K. pneumoniae* isolates were cultured on Brain Heart Infusion agar,with eight isolates (8%) showing the hypermucoviscosity phenotype. The remaining 92 isolates (92%) were negative for hypermucoviscosity. Statistical analysis showed no significant difference in hypermucoviscosity presence among the groups (p>0.05).

When testing for alpha-hemolysis on 5% sheep blood agar, alpha-hemolysin production was observed in eight isolates (8%). Statistical analysis did not reveal a significant difference in alpha-hemolysis among the groups (p>0.05).

The presence of the *ycfM* gene was investigated using PCR. This virulence gene was found in 99 out of 100 isolates, with only one isolate not exhibiting this gene. *ycfM* was the most frequently detected virulence gene in our study. Statistical analysis did not reveal a significant difference in the presence of the *ycfM* gene among the groups (p>0.05). The presence of the *mrkD* gene was detected in 97 out of 100 *K. pneumoniae* isolates, (97%), making it the second most frequent virulence gene in our study. Statistical analysis showed no significant difference in the presence of the *mrkD* gene among the groups (p>0.05). The *kpn* gene was detected in 46 out of 100 *K. pneumoniae* isolates (46%). This virulence gene was more prevalent in isolates from Group 1 and 2. Statistical analysis revealed a significant difference in the presence of the *kpn* gene among the groups (p<0.05). The *traT* gene was detected in only two out of 100 *K. pneumoniae* isolates (2%), both of which were in the MDR group. Statistical analysis indicated a significant difference in the presence of the *traT* gene among the groups (p<0.05). The *entB* gene was found in 96 out of 100 *K. pneumoniae* isolates. No significant difference was detected in the presence of the *entB* gene among the groups (p>0.05). The *iutA* gene was positive in 79 out of 100 *K. pneumoniae* isolates (79%). This virulence gene was predominantly found in the XDR and MDR groups, with statistical analysis revealing a significant difference among the groups (p<0.05). The *fyuA* gene was detected in 71 out of 100 *K. pneumoniae* isolates (71%). Statistical analysis did not reveal a significant difference in the presence of the *fyuA* gene among the groups (p>0.05). The *iroN* gene was found in three out of 100 *K. pneumoniae* isolates (3%). Of these, two were in group 2 and one in Group 3. Statistical analysis indicated a significant difference in the presence of the *iroN* gene among the groups (p<0.05).

The presence of the *hlyA* gene was observed in only two out of 100 *K. pneumoniae* isolates (2%). Statistical analysis did not show a significant difference in the presence of the *hlyA* gene among the groups (p>0.05). The amplicon sizes for the investigated virulence genes were as follows: *ycfM* (160 bp), *traT* (290 bp), *mrkD* (240 bp), *kpn* (626 bp), *entB* (371 bp), *iutA* (300 bp), *fyuA* (547 bp), *iroN* (665 bp), and *hlyA* (1177 bp) (Table 3).

Table 3. Genotypic and phenotypic resistance status of the tested isolates

	Phenotypic methods				Hypermucoviscosity	Alpha Hemolysis	Genotypic resistance markers										
	Capsul	Biofilm					Adhesin			Invasin	Siderophore					Toxin	
		Strong	Medium	Weak			*ycfM*	*mrkD*	*kpn*	*traT*	*iutA*	*entB*	*fyuA*	*iroN*		*hlyA*	
XDR group (n=67,%67)	67 (100%)	32 (47.8%)	24 (35.8%)	11 (16.4%)	6 (8.9%)	7 (10.4%)	66 (98%)	65 (97%)	22 (33%)	0 (0%)	62 (92%)	63 (94%)	46 (69%)	0 (0%)		1 (1.4%)	
MDR group (n=17,%17)	17 (100%)	3 (%17.7%)	5 (29.4%)	9 (52.9%)	0 (0%)	0 (0%)	17 (100%)	16 (94%)	12 (70%)	2 (11.8%)	12 (70%)	17 (100%)	13 (76%)	2 (12%)		0 (0%)	
Susceptible grup (n=16,%16)	16 (100%)	2 (125%)	13 (81.3%)	1 (6.2%)	2 (12.5%)	1 (6.2%)	16 (100%)	16 (100%)	12 (75%)	0 (0%)	5 (31%)	16 (100%)	12 (75%)	1 (6%)		1 (6%)	
Total		37 (37%)	42 (42%)	21 (21%)	8 (8%)	8 (8%)	99 (99%)	97 (98%)	46 (46%)	2 (2%)	79 (79%)	96 (96%)	71 (71%)	3 (3%)		2 (2%)	
p	p>0.05	**p<0.05**			p>0.05	p>0.05	p>0.05	p>0.05	**p<0.05**	**p<0.05**	**p<0.05**	p>0.05	p>0.05	**p<0.05**		p>0.05	

XDR: Extensively drug-resistant, MDR: Multidrug-resistant

Discussion

The widespread use of antibiotics for treating infections caused by *K. pneumoniae* has led to the emergence and spread of resistance to these drugs (15). According to data from a 2019 surveillance study by the European Centre for Disease Prevention and Control, approximately 40% of *K. pneumoniae* isolates in Europe exhibit resistance to at least one class of antibiotics used in treatments, including fluoroquinolones, third-generation cephalosporins, aminoglycosides, and carbapenems (16). Identifying the virulence factors that play a crucial role in the pathogenesis of *K. pneumoniae* is essential for addressing this resistance issue and developing alternative treatment strategies. In our study aimed at contributing to anti-virulence therapeutic strategies, we investigated various virulence factors in *K. pneumoniae* isolates categorized into three groups based on antibiotic susceptibility. The presence of factors such as capsule formation, biofilm production, hypermucoviscosity phenotype, and alpha-hemolysin production was examined using phenotypic methods, while the presence of adhesin, invasion, siderophore, and toxin genes was assessed using genotypic methods. The relationship between these virulence factors and antibiotic resistance was analyzed.

One of the most important factors in the pathogenesis of *K. pneumoniae* is its capsule. In this study, capsule presence was examined using the Chinese ink staining method, and all isolates were found to produce capsules. Kuş et al. (5) investigated capsule presence in 53 *K. pneumoniae* isolates using the Chinese ink staining technique in Konya in 2015, detecting capsule formation in all isolates. Although the capsule is considered a significant virulence factor, no association was found among the groups, and there was no statistically significant difference ($p>0.05$).

For bacteria to colonize and cause disease, they must first adhere to the host cells. Bacteria use surface extensions such as pili, fimbriae, and flagella to adhere to surfaces (17). Clinical *K. pneumoniae* strains have two types of fimbrial adhesins: type 1 and type 3 fimbriae (18). The type 1 fimbrial adhesin encoded by the *FimH* virulence gene plays a significant role in UTIs caused by these strains, while the type 3 fimbrial adhesin encoded by the *mrkD* virulence gene promotes biofilm development (19). El Fertas-Aissani et al. (8) reported positivity rates of 96.3% for *mrkD*, 96.3% for *ycfM*, and 63% for *kpn* genes in their study with 54 *K. pneumoniae* isolates. In our study, the *mrkD* gene was found positive in 97% of *K. pneumoniae* isolates. The other adhesin genes, *ycfM* and *kpn*, were positive in 99% and 46% of isolates, respectively. Statistical analysis revealed no significant difference among the three groups for the *mrkD* and *ycfM* genes ($p>0.05$); however, a significant difference was found for the *kpn* gene. The positivity rates for the *kpn* gene were 70% in the Group 2, 75% in the Group 3, and 33% in Group 1. These results suggest a potential negative correlation of antibiotic resistance with the presence of the *kpn* gene. Further studies are needed to support this hypothesis. The *traT* gene was detected in only 2% of the 100 isolates in our study. Both isolates with the *traT* invasion gene were in Group 2. Statistical analysis detected a significant difference among the groups ($p<0.05$), but more isolates are needed for clearer results.

Bacteria have developed siderophores, which are iron acquisition tools, to compete with the host. *K. pneumoniae* expresses four types of siderophores: enterobactin, yersiniabactin, salmochelin, and aerobactin (20,21). The *iutA* gene was found in 79% of our isolates. Candan and Aksöz (1) reported a 26% positivity rate for the *iutA* gene. Statistical analysis showed a significant difference among the groups ($p<0.05$). The *iutA* gene was detected in 96% of isolates in Group 3 and 70% in Group 2, suggesting a positive correlation between antibiotic resistance and the *iutA* gene. The *entB* gene was detected in 96% of the isolates, and the *fyuA* gene was found in 71%. Eghbalpoor et al. (22) reported 100% positivity for the *entB* gene in their study in Iran. No significant differences were observed between the groups for *entB* and *fyuA* genes ($p>0.05$). The *iroN* gene was detected in 3% of isolates. Two positive isolates were in Group 2, and one was in Group 3. Statistical analysis revealed a significant difference among the groups ($p<0.05$), suggesting a possible negative correlation between *IroN* and antibiotic resistance. However, the low number of positive isolates should be considered when interpreting these results. Further research on salmochelin siderophore genes is needed. In studies examining the correlation between virulence factors and antibiotic resistance, Eghbalpoor et al. (22) reported that the *traT* and *fyuA* genes correlated with antibiotic resistance. In our study, we observed a correlation between the presence of *kpn, traT, iutA,* and *iroN* virulence genes and antibiotic resistance.

Hemolysins are toxins that make certain nutrients, such as iron ions in hemoglobin, available and also form pores (23). In our study, alpha-hemolysin production was examined both phenotypically and genotypically. Alpha-hemolysin formation was observed in 8% of the 100 *K. pneumoniae* isolates on 5% sheep blood agar. The *hlyA* gene was detected in only 2% of isolates. Among these, one isolate exhibited alpha-hemolysis, while the other did not. Statistical analysis found no significant differences among the groups for alpha-hemolysis and the *hlyA* gene ($p>0.05$). Pereira and Vanetti (24) did not detect alpha-hemolysin production in any of the 21 *K. pneumoniae* isolates studied in Brazil in 2015.

The hypermucoviscosity phenotype has long been associated with invasive infections in healthy individuals caused by *K. pneumoniae*. In our study, 8% of the 100 *K. pneumoniae* isolates exhibited the hypermucoviscosity phenotype. When evaluating the relationship between this virulence factor and antibiotic resistance, it was observed that six of the eight hypermucoviscous isolates were in Group 1, while two were in Group 3. No statistically significant difference was found among the groups for the hypermucoviscosity phenotype ($p>0.05$), indicating that more isolates should be included for further evaluation.

The relationship between biofilm formation and antibiotic resistance has not yet been fully elucidated (25). Some studies have reported a positive correlation between biofilm formation and antibiotic resistance, while others have indicated a negative correlation (26,27). Our results showed that strong biofilm formation was observed in 47.8% of isolates in Group 1, compared to 17.7% and 12.5% in Group 2 and 3, respectively. A significant difference was found between strong and medium/weak biofilm formation in Group 1 ($p<0.05$). In Group 2, 52.9% of isolates formed weak biofilms, and in Group 3, medium-level biofilm formation was high (81.3%), with significant differences detected ($p<0.05$). Given the conflicting results, attributing antibiotic resistance solely to biofilm formation would be a bold claim.

Study Limitations

This study has several limitations that should be acknowledged. First, it was conducted in a single center, which may limit the generalizability of the findings to broader populations or different geographic regions. Second, although only the initial isolate from each patient was included to avoid duplication, genotypic analysis of these isolates could not be performed. The absence of molecular characterization restricts deeper insights into the genetic diversity and potential transmission dynamics of the isolates.

Conclusion

Our study on the virulence factors of *K. pneumoniae* revealed potential associations between antibiotic resistance and the presence of the *kpn* gene, *traT* gene, *iutA* gene, and *iroN* gene. Comparison of our data with other studies suggests that the high antimicrobial resistance potential of *K. pneumoniae* is not solely related to virulence factors but involves more complex mechanisms. Research into anti-virulence therapeutics underscores the importance of understanding bacterial pathogens and their interactions with host-environment factors. If virulence can be controlled, it is believed that the host immune system can overcome any non-therapeutic infection. In the absence of such control, anti-virulence therapies could be used synergistically with traditional antimicrobials to reduce antibiotic consumption.

Ethics

Ethics Committee Approval: University of Health Sciences Türkiye, Hamidiye Scientific Research Ethics Committee Presidency (approval number: 35/13, dated: 19.11.2021).

Informed Consent: Following ethical approvals, informed consent was obtained from participants included in this study.

Footnotes

Authorship Contributions

Concept: İ.S., O.B., M.K., E.S.B., Design: İ.S., O.B., Data Collection or Processing: İ.S., N.B., Analysis or Interpretation: İ.S., M.K., E.S.B., N.B., Literature Search: İ.S., O.B., M.K., N.B., Writing: İ.S., M.K.

Conflict of Interest: No conflict of interest was declared by the authors.

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