Upregulation of *pmrA, pmrB, pmrC, phoQ, phoP*, and *arnT* genes contributing to resistance to colistin in superbug *Klebsiella pneumoniae* isolates from human clinical samples in Tehran, Iran

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ABSTRACT

Background: Antibiotic resistance in *Klebsiella pneumoniae* isolates, particularly resistance to colistin, has become a growing concern. This study seeks to investigate the upregulation of specific genes (*pmrA, pmrB, pmrC, phoQ, phoP*, and *arnT*) that contribute to colistin resistance in *K. pneumoniae* isolates collected from human clinical samples in Tehran, Iran.
Methods: Thirty eight *K. pneumoniae* isolates were obtained and subjected to antibiotic susceptibility testing, as well as evaluation for phenotypic AmpC and ESBL production according to CLSI guidelines. The investigation of antibiotic resistance genes was conducted using polymerase chain reaction (PCR), whereas the quantification of colistin resistance related genes expressions was performed via Real-Time PCR.
Results: The highest and lowest antibiotics resistance were observed for cefotaxime 33 (86.8%) and minocycline 8 (21.1%), respectively. Twenty-four (63.2%) and 31 (81.6%) isolates carried AmpC and ESBLs, respectively. Also, antibiotic resistance genes containing bla_{NDM}, bla_{IMP}, bla_{VIM}, bla_{SHV}, bla_{TEM}, bla_{CTXM}, *qnrA, qnrB, qnrS*, and *aac (6')-Ib* were detected in *K. pneumoniae* isolates. Only 5 (13.1%) isolates were resistant to colistin and the MIC range of these isolates was between 4 and 64 µg ml^{-1}. Upregulation of the *pmrA, pmrB, pmrC, phoQ, phoP*, and *arnT* genes was observed in colistin-resistant isolates. The colistin-resistant isolates were found to possess a simultaneous presence of ESBLs, AmpC, fluoroquinolone, aminoglycoside, and carbapenem resistant genes.
Conclusions: This study reveals escalating antibiotic resistance in *K. pneumoniae*, with notable coexistence of various resistance traits, emphasizing the need for vigilant surveillance and innovative interventions.

1. Introduction

The irreversible increase in antibiotic resistance in countries around the world has led the World Health Organization (WHO) to declare antibiotic resistance as one of the three substantial menaces to public health [1–3]. Meanwhile, resistance to β-lactams such as cephalosporins, carbapenems, fluoroquinolones, aminoglycosides and other antibiotics, especially last resort antibiotics in Enterobacteriaceae, is a global challenge [4–6]. Today, the development of multidrug-resistant (MDR) and extensively drug-resistant (XDR) as well as

colistin-resistant isolates has been reported [7]. Based on CDC guidelines any *Klebsiella pneumoniae* (*K. pneumoniae*) isolate demonstrating either intermediate or resistant susceptibility to at least one antimicrobial agent in a minimum of three specified categories was classified as MDR. These categories included: Extended-spectrum cephalosporins (ceftriaxone, ceftazidime, cefepime, cefotaxime, ceftolozane/tazobactam, ceftazidime/avibactam), Fluoroquinolones (ciprofloxacin, levofloxacin, moxifloxacin), Aminoglycosides (amikacin, gentamicin, tobramycin), Carbapenems (the isolate must have exhibited resistance to imipenem, meropenem, doripenem, ertapenem,

meropenem/vaborbactam, or imipenem/relebactam), and Piper-acillin/tazobactam (http://www.cdc.gov/hai/surveillance). Various factors cause antibiotic resistance, which over the past few decades, their overuse has gradually spread resistance in bacteria, so that in some infections, resistance to last-line antibiotics such as colistin has been reported [8–10]. Most studies have considered resistance against colistin in *K. pneumoniae* is due to clinical use of colistin and the multiclonal transmission of hospitalized patients to another. Colistin-resistant *K. pneumoniae in vivo* is probably correlated with previous colistin exposure in patients but various reports declare colistin-resistant *K. pneumoniae* in humans without any prior use of colistin. The prevalence of colistin-resistant *K. pneumoniae* is thus not known among healthy individuals. The emergence of colistin-resistant in major researches have emphasized on patients infected with MDR *K. pneumoniae* which treated with colistin and conclude colistin-resistant development occur after previous exposure, as most of the isolated *K. pneumoniae* also harbors the carbapenemase genes [11]. The substantial mechanism involved to colistin resistance is the upregulating of a two-component system which called PhoQ/PhoP consequently activates the *pmrHFIJKLM* operon. Mutations in CrrAB as a signaling system is detected in colistin resistant bacteria [12]. Recently, a remarkable mechanism has been introduced in colistin-resistant *K. pneumoniae* which is the inactivation of *mgrB* by mutation. The *mgrB* gene encodes a 47–amino acid transmembrane protein which applies a negative feedback effect on PhoQ/PhoP, consequently upregulates the PhoQ/PhoP, then develops resistance to colistin [11]. In addition to the chromosomal mutations that are responsible for colistin resistance, various mechanisms contribute to resistance to this antibiotic that can be spread by horizontal gene transmission between bacteria [13]. However, colistin-resistant isolates as superbug bacteria usually have the ability to resist most of the routine antibiotics for treatment by different mechanisms such as ESBLs, AmpC, fluoroquinolone, aminoglycoside, and carbapenem resistant genes [14]. Hence, the aim of current study was to investigate different transmissible antibiotic resistant mechanisms with upregulation of different operons contributing in resistance to colistin in superbug *K. pneumoniae* isolates.

2. Material and methods

The study was carried out between October 2020 and January 2021 at the Iran University of Medical Sciences, Tehran, Iran, with approval from the local Ethical Committee. *K. pneumoniae* samples described in this study were part of a set of pathogens in the hospital microbiology laboratory that are routinely isolated in the hospital. No further clinical samples were collected for purposes of this study; therefore, there was no need for ethical permission or patient consent to use these samples.

2.1. Sample collection

A total of 38 clinical *K. pneumoniae* samples were obtained from blood, sputum, and urine of individuals who had not taken antibiotics in the last one or two months. The samples were collected from five hospitals in Tehran (A, B, C, D, and E) over a period of 4 months. *K. pneumoniae* isolates re-grown on the culture medium and were identified with classic bacteriologic methods such as gram staining and biochemical tests [15]. All of *K. pneumoniae* isolates were transferred to TSB medium containing 10% glycerol and stored in −20 °C for subsequent tests.

2.2. Antibiotics susceptibility test and phenotypic detection of AmpC and ESBLs

The level of antibiotics resistance of *K. pneumoniae* isolates was checked using the disk diffusion method that disks including trimethoprim + sulfamethoxazole, imipenem, ceftazidime, cefotaxime nor-floxacin, ciprofloxacin, kanamycin, chloramphenicol, ceftriaxone,

gentamicin, fosfomycin, and minocycline from Mast Company (Liverpool, England) were used for this purpose following the instructions of the Clinical and Laboratory Standards Institute. Subsequently, the *K. pneumoniae* isolates were not susceptible to at least one antibiotic in three or more groups of antibiotics were considered MDR (http://www.cdc.gov/hai/surveillance). AmpC-β-lactamase-producing *K. pneumoniae* isolates were distinguished by AmpC disk test as described previously. Ceftazidime and cefotaxime discs were applied on Mueller Hinton agar 1.5 cm away from the center of two these discs combined with clavulanic acid and incubated at 37 °C for 24 h. Positive results were interpreted in accordance with previous studies, where *Escherichia coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 were considered as negative and positive controls, respectively [16–18].

2.3. Minimum inhibitory concentration

In this study, all the isolates that were confirmed as MDR were checked for resistance to colistin by microbroth dilution method. A final concentration (1024 μg/ml) of powder of colistin sulfate salt (Sigma-Aldrich, Merck, Germany) were stored at −80 °C. Consequently, different working concentrations of colistin range from 0.5 to 16 mg/L were prepared in the tubes of Mueller-Hinton broth according to the dilution recommendations by European Committee on Antimicrobial Susceptibility Testing guidelines. The MICs of colistin for each *K. pneumoniae* isolates were defined by microbroth dilution in triplicate. Colistin-resistant *K. pneumoniae* isolates were defined as a MIC ≥ 4 μg mL^{-1} [19].

2.4. DNA extraction and detection resistant genes

The *K. pneumoniae* isolates overnight culture at 37 °C in Luria–Bertani media were used to extract of bacterial genomic DNAs by Qiagen DNA Extraction Kit (QIAGEN, Crawley, UK). The presence of the antibiotic resistance genes including bla_{NDM}, bla_{IMP}, bla_{VIM}, bla_{KPC}, bla_{SHV}, bla_{TEM}, bla_{CTXM}, *qnrA*, *qnrB*, *qnrS*, and *aac(6')-Ib* in the *K. pneumoniae* isolates were studied with PCR analysis. *K. pneumoniae* ATCC7881 served as the positive control for detecting bla_{SHV}, bla_{TEM}, bla_{CTXM} genes. *Enterobacter* spp. strain KEJ-1, with bla_{NDM} (GenBank accession no. KP347135), and *K. pneumoniae* ATCC BAA-1705, which carries bla_{VIM}, bla_{IMP}, and bla_{KPC} genes, were utilized as positive controls for detecting Metallo-β-lactamase (MBL) genes. Additionally, *K. pneumoniae* UAB1 Strain containing the PMG252 plasmid (*qnrA*) or PMG298 plasmid (*qnrB*), and *Salmonella enterica* serotype *Bovis morbificans* strain AM12888 (*qnrS*) were employed as positive controls for the presence of the *qnr* gene. *Enterobacter cloacae* strain HM 477 was used as the positive control for detecting the *aac(6')-Ib* gene. Water was utilized as the negative control in the experimental setup. The primers sequences used in this study are shown in Table 1. The PCR was carried out with a 25 μl of final volume including 12.5 μL of Master Mix 2x (Qiagen HotStar Taq polymerase), 7.5 μL nuclease-free water, 1 μL of 10 pmol of reverse and forward primers, 3 μL of genomic DNA (50 ng/μL). The thermal profile of PCR on a thermal cycler included initial denaturation, 94 °C for 5 min; 30 cycles of denaturation, 94 °C for 1 min; annealing variable temperature (Table 1) for 1 min; elongation, 72 °C for 1 min; and final extension, 72 °C for 5 min. Amplified products were detected on 1.5% agarose gel by electrophoresis and visualized by safe stain under a UV transilluminator. The sizes of amplifications were compared with a 1 kb DNA ladder.

2.5. Expression of colistin resistant genes

Expressions of colistin resistance related genes including *pmrA, pmrB, pmrC, phoQ, phoP,* and *arnT* were quantified by Real-Time PCR (Rotor-Gene, Qiagen Corbett, Hilden, Germany) via specific primers and probes (Table 1). The RNA of colistin resistant *K. pneumoniae* isolates were harvested from the log-phase growth of bacteria using a bacterial

Table 1
Details of primers and probes used to perform PCR and Real Time PCR.

Gene name	Oligonucleotide Sequence (5′–3′)		Annealing temperature (°C)	Reference
		Real Time PCR		
pmrA	F	5′-CGCAGGATAATCTGTTCTCCA-3′	61	[37]
	R	5′-GGTCCAGGTTTCAGTTGCAA-3′		
pmrB	F	5′-GCGAAAAGATTGGCAAATCG-3′	61	[37]
	R	5′-ATCAATGGGTGCTGACGTT-3′		
pmrC	F	5′-CTCTCGCCTCGTTCCTGAA-3′	61	[37]
	R	5′-CGGAGTGGTGTCGAGGATA-3′		
phoQ	F	5′-CCACAGGACGTCATCACCA-3′	59	[37]
	R	5′-GCAGGTGTCTGACAGGGATT-3′		
phoP	F	5′-GAGCGTCAGACTACTATCGA-3′	59	[37]
	R	5′GTTTTCCCATCTCGCCAGCA-3′		
arnT	F	5′-ATGGCGCATTACGCTTTGCT-3′	60	[38]
	R	5′-CCAGGGGGAGACCACAAATG-3′		
Conventional PCR			**Product size (bp)**	
qnrA	F	5′-GATCGGCAAAGGTTAGGT-3′	53	627	[4]
	R	5′-ATTTCTCACGCCAGGATT-3′		
qnrB	F	5′-GATCGTGAAAGCCAGAAA-3′	53	562	[4]
	R	5′-ACGATGCCTGGTAGTTGT-3′		
qnrS	F	5′-GCAGAATTCAGGGGTGTGAT-3′	57	118	[27]
	R	5′-AACTGCTCCAAAAGCTGCTC-3′		
aac(6′)-Ib	F	5′-TTGCGATGCTCTATGAGTGGC-3′	55	472	[4]
	R	5′-CTCGAATGCCTGGCGTG-3′		
*bla*NDM	F	5′-GCAGCTTGTCGGCCATGCGGGC-3′	52	621	[40]
	R	5′-GGTCGCGAAGCTGAGCACCGCAT-3′		
*bla*IMP	F	5′-GGAATAGAGTGGCTTAAYTCTC-3′	58	232	[17]
	R	5′-GGTTTAAYAAAACAACCACC-3′		
*bla*VIM	F	5′-GATGGTGTTTGGTCGCATA-3′	53	390	[17]
	R	5′-CGAATGCGCAGCACCAG-3′		
*bla*KPC	F	5′- CGTCTAGTTCTGCTGTCTTG -3′	48	798	[40]
	R	5′- CTTGTCATCCTTGTTAGGCG-3′		
*bla*SHV	F	5′- GGTTATGCGTTATATTCGCC-3′	58	867	[40]
	R	5′- TTAGCGTTGCCAGTGCTC-3′		
*bla*TEM	F	5′- ATGAGTATTCAACATTTCCG-3′	44	931	[40]
	R	5′- CCAATGCTTAATCAGTGAGC-3′		
*bla*CTXM	F	5′-TTTGCGATGTGCAGTACCAG-3′	58	544	[40]
	R	5′-CGATATCGTTGGTGGTGCCATA-3′		

RNeasy mini kit (Qiagen). Then, the RNA from each isolate was synthesized to cDNA via Quantiscript Reverse Transcription kit (Qiagen). The Real-Time PCR reactions were in a volume of 20 µl including of 0.4 ml of TaqMan probe, 10 µL Power Sybr Green Master Mix (Takara Bio, Shiga, Japan), 0.4 ml of primer (forward and reverse), 5.8 ml water, and 1 ml of first-strand cDNA. The amplification conditions in thermocycler were in two stages as follows: a first denaturation of 1 cycle for 4 min at 94 °C, followed by 40 cycles at 94 °C for 5 min (annealing) and at 60 °C for 30s (extension). The expression of *rpoB* as a housekeeping gene was used for normalizing transcript levels. All experiments for studied genes were performed in triplicate. Finally, fold changes of expression of each colistin resistance gene were measured based on the threshold cycle (2-ΔΔCt) method.

2.6. Statistical analysis

All the results from *K. pneumoniae* isolates were collected and entered into SPSS version 22 after conducting the experiments of this research. Although the demographic information of the isolates was explained using descriptive statistics, the relationship between different variables such as sample type, gender, and hospital with the rate of antibiotic resistance, etc., were analyzed using the chi-square test.

3. Result

The frequency of *K. pneumoniae* infection according to the gender of the patients showed that 8 (21.2%) and 30 (78.9%) isolates were isolated from men and women, respectively. It was also found that 4 (10.5%) isolates were isolated from blood, 19 (50%) isolates from urine, and 15 (39.5%) isolates from sputum of patients. As mentioned, the samples were collected from 5 hospitals including A to E, that 1 (2.6%),

12 (31.6%), 15 (39.5%), 7 (18.4%), and 3 (7.9%) *K. pneumoniae* isolates were isolated from hospitals A, B, C, D, and E, respectively. According to the results of the antibiotic sensitivity test the highest and lowest resistance among *K. pneumoniae* isolates were to cefotaxime 33 (86.8%) and minocycline 8 (21.1%), respectively. Also, the most intermediate resistance of the isolates was obtained to minocycline 18 (47.4%). Nineteen (50%) of *K. pneumoniae* isolates were sensitive to fosfomycin. The details of the antibiogram results of clinical isolates can be seen in Table 2. The analysis of the antibiotic resistance pattern of isolates revealed that among 38 *K. pneumoniae* isolates, 35 (92.1%) were identified as MDR isolates. Examining the frequency of ESBL revealed that 31 (81.6%) of the isolates had the ability to produce these antibiotic resistance enzymes phenotypically. Statistical analysis showed that

Table 2
Antibiotics resistance pattern of *K. pneumoniae* clinical isolates from five hospitals.

Antibiotics	Total samples N (%)		
	Resistance	Intermediate	Sensitive
Ceftazidime	29 (76.3)	3 (7.9)	6 (15.8)
Cefotaxime	33 (86.8)	2 (5.3)	3 (7.9)
Ceftriaxone	25 (65.8)	3 (7.9)	10 (26.3)
Cefoxitin	27 (71.0)	7 (18.4)	4 (10.5)
Imipenem	31 (81.6)	2 (5.3)	5 (13.2)
Chloramphenicol	15 (39.5)	17 (44.7)	6 (15.8)
Norfloxacin	26 (68.4)	4 (10.5)	8 (21.1)
Fosfomycin	12 (31.6)	7 (18.4)	19 (50.0)
Gentamicin	26 (68.4)	2 (5.3)	10 (26.3)
Ciprofloxacin	29 (76.3)	4 (10.5)	5 (13.2)
Minocycline	8 (21.1)	18 (47.4)	12 (31.6)
Kanamycin	18 (47.4)	14 (36.8)	6 (15.8)
Trimethoprim + sulfamethoxazole	31 (81.6)	1 (2.6)	6 (15.8)

Table 3
The correlation of different investigated variables in this study.

		MDR		ESBL				Abundance (N)								Abundance (N)					
								bla_{VIM}		bla_{TEM}		qnrS				bla_{NDM}		bla_{IMP}		qnrS	
		No	Yes	No	Yes			No	Yes	No	Yes	No	Yes			No	Yes	No	Yes	No	Yes
AmpC	No	3	11	5	9	**TOF**	**B**	0	4	1	3	0	4	**H**	**A**	1	0	1	0	1	0
	Yes	0	24	2	22		**U**	15	4	16	3	12	7		**B**	11	1	10	2	7	5
p-value		0.04		0.04			**S**	13	2	14	1	9	5		**C**	14	1	6	9	10	5
						p-value		0.04		0.01		0.04			**D**	2	5	3	4	2	5
															E	3	0	2	1	1	2
														p-value		0.04		0.03		0.04	

****TOF:** Type of sample; **B:** blood, **U:** urine, **S:** sputum; **H:** Hospital.

there was a significant relationship between ESBL-producing isolates and MDR resistance (P<0.05). Phenotypic test to identify AmpC-producing isolates showed that 24 (63.2%) isolates carried these antibiotic resistance enzymes. The statistical analysis revealed a significant relationship between AmpC-producing and MDR isolates (P = 0.04), as well as ESBL-producing isolates (P=0.04). Also, there was no significant relationship between hospitals, type of sample and gender of patients with the level of antibiotic resistance (P>0.05). The results of micro-broth dilution on MDR isolates showed that among 38 isolates, only 5 (13.1%) isolates were resistant to colistin and the MIC range of these isolates was between 4 and 64 µg ml^{-1} (Table 4). All 5 colistin-resistant isolates were resistant to all other antibiotics that were investigated in this study. The abundance of antibiotic resistance genes using PCR technique revealed that 16 isolates (42.1%) carried bla_{NDM}, 17 isolates (44.7%) carried bla_{IMP}, 10 isolates (26.3%) carried bla_{VIM}, 14 isolates (36.8%) carried bla_{SHV}, 16 isolates (42.1%) carried bla_{TEM}, 26 isolates (68.4%) carried bla_{CTXM}, 12 isolates (31.6%) carried *qnrA*, 17 isolates (44.7%) carried *qnrB*, 7 isolates (18.4%) carried *qnrS*, and 15 isolates (39.5%) carried *aac(6')-Ib*. On the other hand, none of the isolates had the bla_{KPC} gene. On one side, a significant relationship was observed between the type of sample and the abundance of bla_{VIM}, bla_{TEM}, and *qnrS* genes, on the other hand, there was a significant relationship between hospitals and the abundance of bla_{NDM} (P=0.04), bla_{IMP} (P=0.03), and *qnrS* (P=0.04) genes (Table 3).

Investigating the upregulation of genes involved in colistin resistance by Real Time PCR determined that the expression level of *pmrA* gene enhanced between 3.91 and 288.00-fold in all colistin resistant isolates compared to the susceptible isolate. Besides that, 36.00 to 274-fold of the *pmrB*, 5.09 to 57.28-fold of *pmrC*, 6.58 to 196.00-fold of *phoQ*, 3.73 to 82.71-fold of *phoP*, and 1.03 to 135.00-fold of *arnT* genes overexpression were showed in colistin resistant isolates in comparison to the susceptible isolate (Table 4). An interesting observation from our data analysis was the co-existence of ESBLs, AmpC, fluoroquinolone, aminoglycoside, and carbapenem resistant genes in colistin-resistant isolates, which also exhibited overexpression of colistin-resistant related genes (*pmrA, pmrB, pmrC, phoQ, phoP,* and *arnT*).

4. Discussion

The presence of antibiotic resistance mechanisms such as ESBLs, AmpC, fluoroquinolone, aminoglycoside, and carbapenem resistant genes in MDR Gram-negative bacteria especially colistin-resistant bacteria are a warning sign for increasing antibiotic resistance [20,21]. Therefore, resistance to colistin as one of the last-line antibiotics is a challenging issue in treatment, so identifying the colistin-resistant mechanisms is very vital, because it causes an appropriate and accurate response, especially for native isolates of each geographical region [22]. In this study, the highest and lowest resistance among *K. pneumoniae* isolates were to cefotaxime (86.8 %) and minocycline (21.1 %), respectively. Studies have been conducted in line with our study, which determined that minocycline was one of the most effective antibiotics and reported high resistance to cephalosporins against *K. pneumoniae* clinical isolates [4,17,23]. Recently, activity of minocycline against MDR Gram negative isolates has been documented in some studies as a resulting minocycline has been suggested as an alternative therapy for Gram negative isolates [24]. Also, high resistance to cephalosporins was reported in *K. pneumoniae* isolates that has been increasing during the last few years, which confirms our results in the field of reducing the effectiveness of these antibiotics [25–28]. In our analysis of antibiotic susceptibility patterns, we identified a high number of MDR (92.1%) *K. pneumoniae* isolates. Similar to our results, in recent years studies conducted on *K. pneumoniae* clinical isolates have reported a high rate of MDR. Abossedgh et al. reported the high rate of MDR (60.8%) in *K. pneumoniae* isolates [27]. In another study, Kiaei et al. reported that 63.3% of 30 *K. pneumoniae* clinical isolates collected from patients hospitalized in the burn ward were MDR [17]. In 2020, it was reported that 100% colistin-resistant isolates *K. pneumoniae* were MDR [23]. In our research, we found that a significant number of *K. pneumoniae* isolates tested positive for AmpC and ESBL enzymes, with 63.2% and 81.6% of the isolates carrying AmpC and ESBL, respectively. In 2019, a study found that 63.3% and 16.7% of *K. pneumoniae* isolates carried ESBL and AmpC, respectively [17]. In line with our results in 2020, it was found in the study that *K. pneumoniae* isolates collected from urinary infections had a high frequency of ESBL (94.1%) [27]. In consistent with

Table 4
Characteristics and expression level of resistance mechanisms in colistin-resistant isolates.

Isolates number	hospital	Source of sample	MIC µg ml^{-1}	Upregulation of genes						Co-existence of other resistance mechanisms
				pmrA	pmrB	pmrC	phoQ	phoP	arnT	
K7	C	Suptum	64	35.01± 1.03	36.00± 0.34	57.28± 0.42	11.87± 0.87	12.81± 0.72	32.75± 0.76	ESBL, AmpC, bla_{NDM}, *qnrA, qnrB, qnrS,* and *aac(6')-Ib*
K9	D	Blood	16	93.05± 0.73	93.05± 0.52	5.81± 2.1	6.58± 2.01	3.73± 0.59	80.44± 1.05	ESBL, AmpC, bla_{NDM}, bla_{IMP}, bla_{VIM}, *qnrA, qnrB, qnrS,* and *aac(6')-Ib*
K10	C	Suptum	4	288.0± 0.88	52.70± 1.01	56.00± 0.35	96.00± 0.56	49.00± 1.2	135.0± 2.4	ESBL, AmpC, bla_{NDM}, bla_{IMP}, *qnrA, qnrB, qnrS,* and *aac(6')-Ib*
K15	B	Blood	32	39.39± 1.45	136.0± 0.78	25.81± 0.67	55.71± 0.91	47.50± 0.66	1.03± 0.27	ESBL, AmpC, bla_{NDM}, bla_{IMP}, bla_{VIM}, *qnrA, qnrB, qnrS,* and *aac(6')-Ib*
K17	D	Blood	16	3.91± 2.22	274.0± 0.88	5.09± 1.2	196.00± 2.8	82.71± 0.46	29.65± 0.84	ESBL, AmpC, bla_{NDM}, bla_{IMP}, *qnrA, qnrB, qnrS,* and *qnrS*

our study, Khalifa et al. reported a high frequency of ESBL and AmpC enzymes in *K. pneumoniae* isolates, and the frequency of both enzymes in these isolates was 82.9% [29]. According to our PCR result, 36.8%, 42.1%, and 68.4% of *K. pneumoniae* isolates harbored bla_{SHV}, bla_{TEM}, and bla_{CTXM} genes, respectively. In recent years, various studies have reported the high frequency of bla_{SHV}, bla_{TEM}, and bla_{CTXM} genes in clinical isolates, which confirms the results obtained from our study. Moradi et al. found 76.7% of *K. pneumoniae* isolates have the bla_{TEM} gene, 92.8% of the isolates have the bla_{CTXM} gene, and 96.4% of the isolates carry the bla_{SHV} gene [30]. In 2021, high rates of bla_{SHV} (66%), bla_{TEM} (74%), and bla_{CTXM} (86%) genes were carried in *K. pneumoniae* isolates which is in agreement with our results [29]. In another study, it was found that the prevalence of ESBL genes varied, with bla_{SHV} exhibiting the highest prevalence at 94.2% and bla_{TEM} showing the lowest at 74.2%. Notably, 60% of the isolates in the study were identified as carrying all three ESBL genes: bla_{SHV}, bla_{TEM}, and bla_{CTXM} [31]. Based on our result, the prevalence of carbapenmase genes including bla_{NDM}, bla_{IMP}, and bla_{VIM} in *K. pneumoniae* isolates were 42.1%, 44.7%, 26.3%, respectively. In line with our results, Alizadeh et al. reported a high amount of carbapenemase genes including bla_{VIM} (75%), bla_{IMP} (17.5%), and bla_{NDM} (17.5%) in *K. pneumoniae* isolates [32]. Urmi et al. reported the frequency of carbapenemase genes in *K. pneumoniae* isolates including bla_{IMP}, bla_{NDM}, and bla_{VIM} were 10.3%, 22.4%, and 19%, respectively [33]. Based on our results, the frequency of plasmid-borne fluoroquinolone resistant genes including *qnrA*, *qnrB*, and *qnrS* was reported 31.6%, 44.7%, and 18.4%, respectively. Several studies have been conducted by researchers on the frequency of *qnr* genes, which show the increasing number of these genes in *K. pneumoniae* isolates. In this regard, the frequency of *qnrA*, *qnrB*, and *qnrS* genes were reported 5.6%, 32.9%, and 26.1% of *K. pneumoniae* isolates, respectively [4]. In 2020, the high frequency of *qnrA*, *qnrB*, and *qnrS* genes were detected in 13.7%, 29.4%, and 33.3 % of *K. pneumoniae* isolates, respectively [27]. In 2022, Roshani et al. reported the high frequency of *qnr* genes, that 22.5% of *K. pneumoniae* isolates carrying *qnrA*, 26.75% isolates had *qnrB*, and 46.25% of isolates horbored *qnrS* gene. These results confirm the high frequency of *qnr* genes in our study [34]. Also, in our study, it was shown that 39.5% isolates were expressing the *aac(6')-Ib* gene. Similar to the results of our study, Moghadam et al. reported the frequency of *aac(6')-Ib* antibiotic resistance gene was 31.8% in *K. pneumoniae* isolates [4]. Jomehzadeh et al. reported a high amount of *acc(6')-Ib-cr* (82.7%) from *K. pneumoniae* clinical isolates [35]. Analysis of the MIC results from this study revealed that a mere 13.1% of the isolates exhibited resistance to colistin. The MIC values for these resistant isolates ranged from 4 to 64 µg ml^{-1}. The colistin-resistant isolates exhibited up-regulation of *pmrA*, *pmrB*, *pmrC*, *phoQ*, *phoP*, and *arnT* genes in comparison to the susceptible isolates. The emergence of colistin-resistant Gram-negative bacteria poses a substantial threat to public health, as colistin serves as the last line antibiotic against carbapenem-resistant Gram-negative bacteria. Colistin resistant Gram negative bacteria has previously been documented in different regions of the world. Although various mechanisms had been reported for resistance to colistin, increasing the expression of some chromosomal operons was one of the most important mechanisms in Gram-negative bacteria [36]. In their study, Khoshbayan et al. identified a notable association between colistin-resistant *K. pneumoniae* isolates and the upregulation of the *pmrHFIJKLM* and *pmrCAB* operons. The research reported that the expression levels of these operons were markedly elevated in colistin-resistant isolates compared to colistin-sensitive ones [37]. In another study, Sato et al. indicated that expression of *arnT* gene was increased in four colistin-resistant isolates compared to colistin-sensitive isolates [38]. Similar to our results, Cannatelli et al. reported overexpression of *phoP*, *phoQ*, and *pmr* operon in colistin-resistant *K. pneumoniae* isolates [39]. In addition to high antibiotic resistance, our results showed the simultaneous presence of different transmissible mechanisms including ESBLs, AmpC, fluoroquinolone, aminoglycoside, and carbapenem resistant genes in colistin-resistant *K. pneumoniae* isolates. Up-regulating of *pmrA*, *pmrB*,

pmrC, *phoQ*, *phoP* and *arnT* genes in colistin-resistant isolates compared to colistin-sensitive isolates was considered one of the mechanisms of colistin resistance. Although ongoing studies have focused more on elucidating the mechanisms involved in colistin resistance, infection control and the application of antimicrobial stewardship strategies are urgently needed to prevent the spread of untreatable pathogens. One limitation of the study is the relatively small sample size, which may impact the generalizability of the results to a larger population. Additionally, the study focused on a specific set of genes and mechanisms related to colistin resistance, and other potential factors contributing to resistance may not have been fully explored. Furthermore, the study did not investigate the impact of other potential resistance mechanisms or genetic mutations that could also play a role in colistin resistance. Future research with larger sample sizes and a more comprehensive analysis of resistance mechanisms could provide a more nuanced understanding of colistin resistance in Gram-negative bacteria.

5. Conclusion

This study highlights the concerning rise in antibiotic resistance among *K. pneumoniae* isolates in Tehran, Iran. Our findings demonstrate a high prevalence of resistance to multiple antibiotics, with a significant proportion of isolates showing resistance to colistin. The detection of various antibiotic resistance genes, including ESBLs, AmpC, and carbapenemase genes, underscores the complex mechanisms driving resistance in these isolates. Furthermore, the upregulation of specific genes associated with colistin resistance, such as pmrA, *pmrB*, *pmrC*, *phoQ*, *phoP*, and *arnT*, in colistin-resistant isolates emphasizes the importance of understanding the molecular mechanisms underlying resistance development. The simultaneous presence of multiple resistance traits in colistin-resistant isolates highlights the urgent need for comprehensive surveillance and effective interventions to combat multidrug-resistant *K. pneumoniae* strains. Based on our findings, we recommend enhanced monitoring of antibiotic resistance patterns, implementation of strict infection control measures, and promotion of antibiotic stewardship programs in healthcare settings. Additionally, the development of novel treatment strategies and continued research into alternative therapies are crucial to address the escalating threat of antibiotic resistance in *K. pneumoniae*.

Ethics approval

Not applicable.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

All data supporting the conclusions of this article are included within the article.

Code availability

Not applicable.

CRediT authorship contribution statement

Maryam Mirshekar: Investigation, Methodology, Writing – original draft. **Rezvan Golmoradi Zadeh:** Investigation. **Majid Taati Moghadam:** Software, Writing – original draft. **Shahla Shahbazi:** Investigation, Software, Writing – review & editing. **Faramarz Masjedian Jazi:** Project administration, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

None.

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