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RESEARCH ARTICLE

Mutation in *mgrB* is the major colistin resistance mechanism in *Klebsiella pneumoniae* clinical isolates in Tehran, Iran

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ABSTRACT

Colistin is considered as one of a last resort antimicrobial agent against multidrug-resistant Gram-negative bacteria including *Escherichia coli* and *Klebsiella pneumoniae*. However, the recent emergence of colistin resistance (ColR) worldwide that severely restricts therapeutic options is a serious threat to global public health. In this study we have investigated the molecular determinants in ColR *K. pneumoniae* isolates collected from clinical specimens.

A total of 98 *E. coli* and 195 *K. pneumoniae* clinical isolates were collected from two hospitals from August 2018 to December 2019 in Tehran, Iran. Colistin susceptibility and minimum inhibitory concentrations (MIC) were determined according to the Clinical and Laboratory Standards Institute by disk diffusion method, and microdilution method, respectively. For isolates with colistin MIC $\geq$4 μg mL^{-1}, PCR was performed for the detection of *mcr-1* to *mcr-4* genes. Moreover, nucleotide sequences of *mgrB, phoP, phoQ, pmrA*, and *pmrB* genes were determined by sequencing. Finally, the transcriptional level of *pmrK* and *pmrC* genes was evaluated by quantitative reverse transcription PCR (RT-qPCR).

None of the *E. coli* isolates were resistant to colistin while 21 out 195 *K. pneumoniae* isolates were identified as resistant, 19 of which carried mutation in the *mgrB* gene. Three different mutations were observed in the *pmrB* gene in 3 *K. pneumoniae* isolates. None of the ColR isolates showed alternations in *pmrA, phoP,* and *phoQ* genes. Furthermore, none of the plasmid-encoding genes were detected. Transcriptional level of the *pmrK* gene increased in all ColR isolates meanwhile, *pmrC* overexpression was detected in 16 out 21 (76.19%) isolates. Eventually, all ColR isolates were susceptible to tigecycline. Our results demonstrated that the alternation of *mgrB* gene is the main mechanism related to colistin resistance among ColR *K. pneumoniae* isolates in this study.

KEYWORDS

colistin resistance, colistin, *Klebsiella pneumoniae*, *mgrB*

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INTRODUCTION

The spread of various antimicrobial resistance mechanisms among different kinds of pathogens has led to the emergence of multi-drug resistance (MDR) bacteria. In *Enterobacteriaceae* family, the main resistance pathogens including *Escherichia coli* and *Klebsiella pneumoniae* are raising concerns because the available therapeutic options are narrowing [1,

2]. The emergence of carbapenemase-producing isolates around the world has recently resulted in a highly limited therapeutic choice, which makes polymyxin antibiotics as one of the last options for the treatment of infections caused by MDR Gram-negative bacteria (GNB) [3]. The polymyxin family such as polymyxin B and colistin are cationic antimicrobial peptides affecting the bacterial lipopolysaccharide (LPS) component in the outer membrane [4, 5]. In recent years, many studies reported the resistance to colistin among *K. pneumoniae* and *E. coli* around the world [3, 6–8].

The most common mechanism associated with colistin resistance among *Enterobacteriaceae* such as *K. pneumoniae* and *E. coli* is LPS modifications via positively charged groups. This phenomenon neutralized the negative charge of LPS and led to the decreased affinity of cationic colistin. Moreover, the modification of LPS has known to be mostly mediated by genetic mutations in two-component regulatory systems including PhoPQ, PmrAB, and CrrAB [3, 9–11]. Additionally, two-component regulatory systems regulate the downstream genes located in *pmrCAB* and *pmrHFIJKLM* operons. The occurrence of mutations in these two-component regulatory systems caused the continuous expression of the mentioned operons and led to the modification of LPS [12, 13]. In addition, the inactivation of the negative regulator of the PhoPQ signaling system, which is encoded by the *mgrB* gene, reported as the key and common mechanism of resistance to colistin among *K. pneumoniae* isolates [10, 14, 15]. Finally, plasmid-encoded colistin resistance (*mcr*-type genes) has been reported as another colistin resistance mechanism in *Enterobacteriaceae* in addition to chromosomal mutations [16–18]. In this regard, the current study aimed to investigate the presence of colistin resistance among *E. coli* and *K. pneumoniae* clinical isolates collected from two hospitals in Tehran and to determine the molecular mechanisms of colistin resistance in such isolates.

MATERIALS AND METHODS

Bacterial isolates

A total of 293 isolates, including 98 *E. coli* and 195 *K. pneumoniae* isolates were collected from August 2018 to December 2019 from individual patients in two hospitals in Tehran, Iran. The isolates were recovered from various clinical specimens including, bronchoalveolar lavage (BAL), tracheal aspirate, wound, urine, abdominal fluid, and blood. The isolates were cultured on MacConkey agar for ensuring of pure culture. After the incubation for 18–24 h at 37 °C, the isolates were identified using gram-staining and biochemical tests such as triple-sugar iron (TSI), urea, Simmon's citrate, Methyl Red/Voges-Proskauer (MR/VP) and sulfide indole motility (SIM). The confirmed isolates were selected for further investigations [19, 20]. Moreover, *E. coli* isolates were confirmed by the amplification of the 16S rDNA gene by using ECO primers (Table 1) [21].

Antimicrobial susceptibility testing

The antibiotic disks were used to determine susceptibility profiles using the disk diffusion method in accordance with

Table 1. Nucleotide sequences of primers used in this study

Primer name	Sequence (5′ to 3′)	Size of product (bp)	Reference
ECO-1	GACCTCGGTTTAGTTCACAGA	585	[21]
ECO-2	CACACGCTGACGCTGACCA		
pmrA-F	CGCAGGATAATCTGTTCTCCA	808	[12]
pmrA-R	GGTCCAGGTTTCAGTTGCAA		
pmrB-F	GCGAAAAGATTGGCAAATCG	1113	[12]
pmrB-R	ATCAATGGGTGCTGACGTT		
mgrB-F	ACCACCTCAAAGAGAAGGCGTT	347	[12]
mgrB-R	GGCGTGATTTTGACACGAACAC		
phoP-F	GAGCGTCAGACTACTATCGA	912	[12]
phoP-R	GTTTTCCCATCTCGCCAGCA		
phoQ-F	CCACAGGACGTCATCACCA	1594	[12]
phoQ-R	GCAGGTGTCTGACAGGGATT		
mcr-1-F	CGGTCAGTCCGTTTGTTC	309	[7]
mcr-1-R	CTTGGTCGGTCTGTAGGG		
mcr-2-F	TGTTGCTTGTGCCGATTGGA	567	[17]
mcr-2-R	AGATGGTATTGTTGGTTGCTG		
mcr-3-F	TTGGCACTGTATTTTGCATTT	542	[16]
mcr-3-R	TTAACGAAATTGGCTGGAACA		
mcr-4-F	ATTGGGATAGTCGCCTTTTT	478	[18]
mcr-4-R	TTACAGCCAGAATCATTATCA		
pmrC-F	CTCTCGCCTCGTTCCTGAA	170	[12]
pmrC-R	CGGAGTGGTGTCGAGGATA		
pmrK-F	GGTGTATGCGATTGGCACCTA	179	[12]
pmrK-R	AGCAGCACGTAGCCCAGTAT		
rpsl-F	CCGTGGCGGTCGTGTTAAAGA	106	[30]
rpsl-R	GCCGTACTTGGAGCGAGCCTG		

the Clinical and Laboratory Standards Institute (CLSI) guidelines [22]. In the first step, screening for colistin resistance and MDR *E. coli* and *K. pneumoniae* isolates was performed by using the following disks: colistin (10 µg), tigecycline (15 µg), imipenem (10 µg), cefepime (30 µg), and piperacillin/tazobactam (100/10 µg). Furthermore, the phenotype was defined as MDR based on the International Expert proposal for Interim Standards Guidelines. For colistin-resistant (ColR) isolates further susceptibility testing was performed using the disk diffusion method for ciprofloxacin (5 µg), nitrofurantoin (300 µg), gentamicin (10 µg), norfloxacin (10 µg), trimethoprim/sulfamethoxazole (25 µg), chloramphenicol (30 µg), cefixime (5 µg), tetracycline (30 µg), and ampicillin (10 µg) [19, 23, 24].

Minimum inhibitory concentration (MIC) of colistin

Colistin MIC was determined by the microdilution method using colistin sulfate using two-fold dilutions ranging from 0.5 to 16 µg mL^{-1} according to Wiegand et al. and CLSI guidelines [22, 25]. The isolates with MIC ≥4 µg mL^{-1} were considered as resistant in accordance with the CLSI guidelines [12, 22]. Notably, *K. pneumoniae* ATCC 700603 was used as standard control for colistin susceptibility testing.

Molecular characterization of ColR isolates by Polymerase Chain Reaction (PCR)

DNA was extracted by the boiling method as previously described [26]. To detect chromosomal mutations in ColR isolates, *mgrB*, *phoP*, *phoQ*, *pmrA*, and *pmrB* genes were amplified by the PCR, and amplicons were sent for DNA sequencing. In addition, for detection of plasmid mediated ColR the *mcr-1*, *mcr-2*, *mcr-3*, and *mcr-4* were subjected to PCR testing as described previously [12]. Furthermore, PCR product visualization was performed by electrophoresis on 1% agarose gels containing safe stain. The set of primers used in this study are listed in Table 1.

Transcriptional analysis of *pmrK* and *pmrC* genes by Quantitative reverse transcription PCR(RT-qPCR)

The *pmrCAB* and *pmrHFIJKLM* operons could be upregulated in ColR isolates [13]. The expression level of *pmrC* and *pmrK* genes encoding the LPS modification enzyme was evaluated to investigate this phenomenon. All ColR isolates along with *K. pneumoniae* ATCC 700603 as a colistin-susceptible isolate had grown in the tryptic soy broth medium for the logarithmic phase of growth. Then, the total RNA was extracted using the SinaPure TM RNA extraction kit. Contamination with genomic DNA was removed using the DNaseI, RNase-free enzyme and the purification was performed based on the manufacturer's instructions. In the next step, the synthesis of the first-strand cDNA happened by using the PrimeScript 1st strand cDNA Synthesis Kit. Moreover, the real-time PCR amplification was performed using a SYBR Green Master Mix with a Rotor-Gene Q as 1 cycle of 95 °C for 12 min, 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 25 s. Furthermore, after each run, melting curve analyses were performed to assure single amplicon production [12, 27]. Eventually, the relative gene expression level was evaluated with the *rpsL* gene as the internal control using the $2^{-\Delta\Delta Ct}$ formula [28, 29]. Table 1 provides the list of the applied primers for the RT-qPCR.

Genbank accession number

The nucleotide sequences of the altered *mgrB* and *pmrB* genes were deposited in the GenBank database. The accession number of *mgrB* is MT947805 and that of *pmrB* are MW238772, MW321545 and MW308132.

RESULTS

Molecular profiles of the gene determinants of the colistin resistance

All ColR isolates were *K. pneumoniae* (21 out of 195 isolates) (10.8%), and all *E. coli* isolates were susceptible to colistin. The MIC for *K. pneumoniae* ATCC 700603 was 0.125 µg mL^{-1} and MIC range for ColR isolates were between 4 and 32 µg mL^{-1}. All ColR isolates were resistant to ciprofloxacin, nitrofurantoin, gentamicin, norfloxacin, cefixime, trimethoprim/sulfamethoxazole, chloramphenicol, imipenem, cefepime, piperacillin/tazobactam, and ampicillin. On the other hand, all isolates were susceptible to tigecycline. All isolates except for one were susceptible to tetracycline. Additionally, in 19 out of 21 (90.47%) of ColR isolates alternation in the *mgrB* gene was detected by Sanger sequencing, which was the insertion of insertion sequence (IS) elements belonged to the *IS*1-like family (768 bp). The insertion site was located at -60 upstream of the *mgrB* gene, which corresponds to the putative promoter region and protein start codon. Further, in 3 *K. pneumoniae* isolates three different nucleotide changes were found in the *pmrB* gene as follows: P95L (CCG > CTG), A246T (GCC > ACC), and D150Y (GAC > TAC). Noteworthy, A246T substitution was observed in 2 *K. pneumoniae* isolates (i.e., Y2 and Y3) and one of the isolates (Y2) had two further mutations of D150Y and A246T. In addition, one isolate (W16) had P95L alternations in the *pmrB* gene in addition to *IS*1 insertion in *mgrB*. The remaining isolates with mutations in *mgrB* represented no alternation in the *pmrB* gene (Table 2). Likewise, all ColR isolates demonstrated wild types of *phoQ*, *phoP*, and *pmrA* genes. Based on our results, none of the plasmid-encoded resistance genes (*mcr-1* to *mcr*-4 genes) were detectable. The phenotypic and genotypic characteristics of ColR isolates are shown in Table 2.

Overexpression of related operons

To investigate the possible connection between the upregulation of *pmrCAB* and *pmrHFIJKLM* operons and colistin resistance, the expression of *pmrC* and *pmrK* genes were evaluated by the RT-qPCR. The *pmrK* transcription level increased between 1.31 and 293.74-fold in all ColR isolates compared to the susceptible isolate. Furthermore, 1.82 to

Table 2. Phenotypic and genotypic characteristics of the ColR *K. pneumoniae* isolates

Isolate	Hospital and year	Ward, and source of isolation	Colistin MIC (mg ml^{-1})	*mgrB* sequence	*pmrB* sequence	Relative expression level	
						pmrK	*pmrC*
W1	Hospital A/2018	BAL/ICU	16	Inactivation by Insertional IS*1*-like at promoter region (-60)	WT	7.24 ± 2.22	22.47 ± 0.1
W2	Hospital A/2018	BAL/ICU	16	Inactivation by Insertional IS*1*-like at promoter region (-60)	WT	9.975 ± 0.72	3.97 ± 1.86
W4	Hospital A/2018	Tracheal aspirate/ICU	16	Inactivation by Insertional IS*1*-like at promoter region (-60)	WT	10.22 ± 0.78	62.24 ± 1.42
W5	Hospital A/2018	BAL/ICU	16	Inactivation by Insertional IS*1*-like at promoter region (-60)	WT	8.12 ± 0.45	-
W6	Hospital A/2018	Tracheal aspirate/ICU	8	Inactivation by Insertional IS*1*-like at promoter region (-60)	WT	3.74 ± 0.88	-
W8	Hospital A/2018	Blood/ICU	16	Inactivation by Insertional IS*1*-like at promoter region (-60)	WT	4.20 ± 1.97	-
W11	Hospital A/2019	Abdominal fluid/Surgical	16	Inactivation by Insertional IS*1*-like at promoter region (-60)	WT	2.01 ± 0.01	-
W12	Hospital A/2019	Wound/ICU	32	Inactivation by Insertional IS*1*-like at promoter region (-60)	WT	7.78 ± 1.02	21.45 ± 0.3
W14	Hospital A/2019	BAL/ICU	16	Inactivation by Insertional IS*1*-like at promoter region (-60)	WT	4.97 ± 0.08	35.01 ± 4.12
W15	Hospital A/2019	Blood/ICU	16	Inactivation by Insertional IS*1*-like at promoter region (-60)	WT	54.38 ± 0.94	8.05 ± 0.9
W16	Hospital A/2019	Tracheal aspirate/ICU	32	Inactivation by Insertional IS*1*-like at promoter region (-60)	P95L	293.74 ± 21.27	20.82 ± 2.1
W17	Hospital B/2018	Urine/ICU	32	Inactivation by Insertional IS*1*-like at promoter region (-60)	WT	29.35 ± 0.71	9.15 ± 1.50
CR1	Hospital A/2019	Wound/Surgical	32	Inactivation by Insertional IS*1*-like at promoter region (-60)	WT	1.31 ± 0.24	49.86 ± 1.52
W19	Hospital B/2018	Tracheal aspirate/ICU	16	Inactivation by Insertional IS*1*-like at promoter region (-60)	WT	9.31 ± 0.13	-
CR11	Hospital A/2019	BAL/ICU	32	Inactivation by Insertional IS*1*-like at promoter region (-60)	WT	13.73 ± 0.19	14.12 ± 2.1
W22	Hospital B/2018	Tracheal aspirate/ICU	32	Inactivation by Insertional IS*1*-like at promoter region (-60)	WT	260.4 ± 3.53	9.2 ± 2.0
W23	Hospital A/2019	Wound/ICU	16	Inactivation by Insertional IS*1*-like at promoter region (-60)	WT	18.12 ± 1.02	1.82 ± 0.24
W24	Hospital A/2019	BAL/ICU	32	Inactivation by Insertional IS*1*-like at promoter region (-60)	WT	188.70 ± 4.53	3.4 ± 0.07
W27	Hospital A/2019	Tracheal aspirate/ICU	16	Inactivation by Insertional IS*1*-like at promoter region (-60)	WT	80.5 ± 8.53	64 ± 3.04
Y2	Hospital B/2018	Pleural fluid/ICU	32	WT	D150Y- A246T	19.42 ± 2.06	15.77 ± 1.19
Y3	Hospital B/2018	Blood/ICU	4	WT	A246T	9 ± 2.63	7.26 ± 2.1

ICU: Intensive care unit, BAL: Bronchoalveolar lavage, WT: Wild type.

64-fold overexpression of the *pmrC* gene was observed in 76.19% (16 of 21) of ColR isolates in comparison to the susceptible isolate.

DISCUSSION

Alarming rates of antimicrobial resistance is recognized as a great threat to public health worldwide. The infection caused by MDR-GNB has been increasingly reported around the world. Colistin is known as one of the last available drugs against highly resistant *Enterobacteriaceae* species [31–33]. Despite the lack of any reports about colistin resistance for many decades (1960–1990), the frequent utilization of colistin in clinical settings has led to emergence of resistance to this drug [12, 15]. In current study, no *mcr* family genes were detected, indicating that colistin resistance in these ColR isolates was mediated via chromosomally encoded mechanisms.

Based on our findings, 21 *K. pneumoniae* isolates were resistant to colistin while none of the *E. coli* isolates showed resistance to this antibacterial drug. Additionally, an alternation was detected in the *mgrB* genes in 19 out of 21 ColR isolates by detection of an *IS*1-like element in the -60 upstream of the promoter region of *mgrB* gene. Noteworthy, these results are in line with previous study indicating insertional inactivation of the *mgrB* gene, encoding a negative-feedback regulator of the PhoQ-PhoP signaling system, can be responsible for colistin resistance in *K. pneumoniae* clinical isolates [14]. Besides, in two individual studies by Poirel et al. and Cannatelli et al., the insertion of *IS*1-like, *IS*5-like, *ISKpn*13, and *ISKpn*14 elements caused the alternation of the *mgrB* gene, and subsequently, involved with colistin resistance in *K. pneumoniae* isolates [10, 14]. Interestingly, the result of our study has been correlated with other reports from Iran about the mechanism of colistin resistance in *K. pneumoniae* and it seems that *mgrB* is the main cause of resistance among *K. pneumoniae* isolates in Iran [9, 12, 34–36]. In addition, three isolates had mutations in the *pmrB* gene, including A246T, D150Y, and P95L. It seems that these point mutations in the *pmrB* gene are not related to the induction of colistin resistance in mutated isolates. Similarly, this phenomenon was confirmed in the study by Haeili et al. reporting L213M, A246T, and R256G mutations in the *pmrB* gene, and the complementation assay test with the wild-type *pmrB* gene could not restore susceptibility to colistin in mutated isolates [12]. Furthermore, in another study also reported, the R256G mutation in ColR isolates was not related to colistin resistance using the site-directed mutagenesis method [37]. Further, Aires et al. reported that the A246T mutation is not contributed to resistance to colistin as it has been found in both susceptible and resistant isolates to colistin [38]. Thus, the mechanism of the colistin resistance of two isolates (i.e., Y2 and Y3) has not been unraveled completely.

Based on our results, there was a remarkable correlation between resistance to colistin and the overexpression of *pmrHFIJKLM* and *pmrCAB* operons. Moreover, all isolates demonstrated overexpressed *pmrK* while 76.19% of them represented overexpression in the *pmrC* gene. Our results showed that some isolates with same mutations and MIC values had different expression level of *pmrK* and *pmrC* genes. Moreover, this phenomenon could be related to other genes that regulate these operons such as CrrAB and CrrC and could be in associated with other unknown genetic alternation and other mechanisms that are related to colistin resistance such as efflux pumps or capsular polysaccharide [11–13].

In a study on ColR carbapenemase-producing *K. pneumoniae*, the results demonstrated that insertion of the *ISKpn*26 element in *mgrB* and the alternation of A41Y, G256R, and L213M in *pmrB* caused the overexpression of *pmrK* about 20-fold in one isolate. In addition, the truncation of MgrB at 29th amine acid and G256R and E57G alternations in *pmrB* caused to 40-fold increase in expression of *pmrK* [6]. Furthermore, the observation of different MIC values and the distinct expressions level of *pmrK* and *pmrC* genes in isolates with the same mutation profile could be related to the involvement of other resistance mechanisms such as the CrrAB two-component regulatory system. Additionally, other resistance mechanisms including efflux pumps or an increase in the capsular polysaccharide mass could be contributed to different MIC values [13].

Moreover, our results showed that tigecycline was the most effective drug and inhibited the growth of all isolates. Tigecycline is one of the therapeutic choices against ColR-GNB, and some studies reported that tigecycline susceptibility was maintained in ColR isolates [39–41]. Moreover, combination therapy with tigecycline with promising results had been used in several studies [41–43]. Humphries et al. suggested that a high dosage of tigecycline in combination with colistin seems to be effective in treating pan-drug resistant *K. Pneumoniae*, and that tigecycline plays a major role in successful treatment [41].

However, evaluation of the role of the CrrAB system in colistin resistance isolates and performing of cluster analysis of the ColR isolates using multi locus sequence typing and/or whole genome sequencing in these ColR isolates could be next step in near future.

In summary, the mutation of the *mgrB* gene was the key mechanism of acquired colistin resistance in *K. pneumoniae* isolates in this study. Additionally, the overexpression of *pmrHFIJKLM* and *pmrCAB* operons, which cause LPS modifications, had a notable contribution part to the procedure leading to colistin resistance.

DECLARATION SECTION

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in comprehensive research and sample collection. A.K. and A.S. performed the study. A.K., A.S. and D.D. wrote the paper and participated in manuscript editing and A.K. prepared tables. Notably, all authors have review and approved the manuscript.

Ethics approval and consent to participate: It was obtained from the ethics committee of Iran University of medical science. Reference number: IR. IUMS.FMD.REC.1398.160. Tehran, Iran. Collection of bacterial isolates occurred as part of routine patients care investigation in the studied hospitals and informed consent was not necessary.

Consent for publication: Not applicable.

Availability of data and materials: All data were included and the nucleotide sequences of the altered *mgrB* and *pmrB* genes were deposited in the NCBI GenBank database under following accession numbers MT947805, MW238772, MW321545 and MW308132, respectively.

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Not applicable.

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