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ORIGINAL ARTICLE

Molecular evaluation of colistin resistance in *Klebsiella pneumoniae* strains isolated from patients in hospitals of Qom province

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ABSTRACT

Background and Objectives: Colistin is considered as one of the last antibiotic choices for addressing infections resulting from multidrug-resistant *Klebsiella pneumoniae*. Nevertheless, the rising resistance to colistin is emerging as a growing threat to public health. The aim of the present study was to explore the molecular mechanisms underlying resistance to colistin in a clinical isolate of *K. pneumoniae.*

Materials and Methods: Colistin resistance was confirmed through antimicrobial susceptibility testing, and the sequence type was identified using Multilocus sequence typing (MLST). The molecular mechanism of colistin resistance was investigated by sequence analysis of resistance-associated loci, including *mgrB*, *pmrAB*, *phoPQ*, *crrAB*, and PCR detection of plasmid-mediated *mcr* genes.

Results: Among the studied 38 clinical *K. pneumoniae* isolates, one strain was colistin-resistant, which belonged to sequence type ST377. PCR results showed that the colistin resistance genes carried by plasmid (*mcr-1* to *mcr-4*) were not present. While gene sequencing revealed wild-type *pmrAB* and *phoPQ*, the *mgrB* and *crrA* genes were found to be disrupted by insertion of IS elements in the promoter (position-45) and coding regions (position +365/+366), respectively. Moreover, a Q296L amino acid substitution was detected in CrrB.

Conclusion: This study demonstrates that resistance to colistin in the *K. pneumoniae* ST377 isolate was mainly mediated by inactivation of MgrB and CrrA without the involvement of *mcr* plasmid genes or *pmrAB* or *phoPQ* genetic alterations. To our knowledge, no previous study has reported insertion sequence-mediated disruption of the *crrA* coding region in *K. pneumoniae.* The results highlighted the complexity of chromosomal resistance mechanisms and the importance of molecular surveillance in managing colistin-resistant infections.

Keywords: *Klebsiella pneumoniae*; Colistin resistance; Antimicrobial susceptibility testing; Multilocus sequence typing; Insertion sequences; *mgrB* gene; CrrAB two-component systems

INTRODUCTION

Infections resulting from Gram-negative bacteria resistant to multiple drugs threaten the public health globally. Over the past several decades, the widespread dissemination of antibiotic-resistant Enterobacteriaceae has markedly accelerated the emergence of antimicrobial resistance, resulting in increased morbidity and mortality worldwide (1). *Klebsiella pneumoniae* is a significant human patho-

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gen known for its ability to acquire and spread various antimicrobial resistance genes. It is responsible for many diseases, such as pneumoniae, urinary tract infections, and bacteremia with local lesions in disabled people, and septicemia associated with hospital infections. *K. pneumoniae* infection is common in neonatal units, especially in intensive care units and premature infants (2).

Initially, *K. pneumoniae* infections were effectively managed using the third- and fourth-generation of cephalosporins. However, the emergence of extended-spectrum β-lactamases (ESBLs) significantly diminished the efficacy of these β-lactam agents (3). As antimicrobial resistance progressively escalated, carbapenems were adopted as the treatment of choice. Unfortunately, *K. pneumoniae* subsequently developed resistance to carbapenems by producing carbapenemases, further compromising the therapeutic effectiveness of these antibiotics (4). In response to this growing resistance, polymyxins, particularly colistin, were reintroduced into clinical practice, despite their established nephrotoxic potential. This reintroduction was endorsed by the World Health Organization (WHO) as part of a global strategy aimed at combating multidrug-resistant Gram-negative pathogens (5, 6).

Polymyxins, including colistin (polymyxin E), exert their antimicrobial effects through interaction with the lipid A component of lipopolysaccharides (LPS) in the cell wall of Gram-negative bacteria. This interaction leads to the disruption of membrane integrity, elevated permeability, and eventual cell lysis and death (7). Despite their potency, resistance to colistin has emerged, posing a significant clinical challenge. One of the primary resistance mechanisms involves modifications of lipid A by incorporating groups with positive charge like phosphoethanolamine (pEtN) or 4-amino-4-deoxy-L-arabinose (L-Ara4N), which decreases the colistin's binding affinity for its anionic target. These modifications can be mediated by chromosomal mutations, particularly in regulatory systems such as *pmrAB*, *phoPQ*, *crrAB*, and the small transmembrane regulator gene *mgrB* or by acquisition of plasmid-associated resistance genes including *mcr-1* (mobilized colistin resistance-1) to 10 (8, 9). Considering the pivotal role of colistin as a final defense strategy against infections due to multidrug-resistant Gram-negative bacteria, this research aimed to evaluate the colistin susceptibility and identify molecular determinants of resistance to colistin in *K. pneumoniae* isolates obtained from intensive care units (ICUs) patients in three hospitals of Qom, a central city in Iran.

MATERIALS AND METHODS

Isolation and identification of bacteria. In this study, 38 *K. pneumoniae* isolates were gathered over a 3-month period between 2019 and 2020 from various clinical specimens of three hospitals in Qom province, Iran. The isolates were aseptically transferred to the microbiology laboratory of Kashan University for identification and further analysis. The clinical samples collected from patients were initially cultured on EMB agar medium and incubated at 37°C for 18-24 hours. Colonies exhibiting morphological characteristics consistent with *K. pneumoniae* were subjected to a series of biochemical and differential tests. For long-term preservation of the isolates, Tryptic Soy Broth (TSB) supplemented with 15% glycerol was used, and the cultures were stored at -20°C. All the culture media and materials utilized in this study were sourced from Merck, Germany.

Antimicrobial susceptibility testing. In order to identify colistin-resistant bacteria, susceptibility of 38 *K. pneumoniae* isolates to colistin was determined by broth dilution assay and inoculating the isolates in to Mueller Hinton broth media containing different concentration of colistin sulfate (Glentham, UK) (0, 1, 2 and 4 mg/L). Moreover, the sensitivity of colistin-resistant isolates to other agents was evaluated using disk diffusion assay with ampicillin (10 µg), tetracycline (30 µg), cefepime (30 µg), cefoxitin (30 µg), ceftriaxone (30 µg), ciprofloxacin (5 µg), doxycycline (30 µg), imipenem (10 µg), fosfomycin (200 µg), gentamicin (10 µg), levofloxacin (5 µg), nalidixic acid (30 µg), nitrofurantoin (300 µg), and tigecycline (15 µg) disks. Antimicrobial susceptibility findings were analyzed in accordance with the breakpoints outlined by the Clinical and Laboratory Standards Institute (CLSI) guidelines. For tigecycline and colistin, however, interpretation was based on the Enterobacteriaceae-specific breakpoints established by the European Committee on Antimicrobial Susceptibility Testing (EUCAST). According to this standard, strains with colistin MICs of ≥4 µg/mL were considered resistant.

Genotyping of colistin-resistant isolates by MLST assay. Multilocus sequence typing (MLST) was conducted using seven conserved housekeeping genes, including *rpoB, gapA, mdh, pgi, phoE, infB,* and *tonB,* following the protocol described by Diancourt et al. (10). The obtained allele profiles and corresponding sequence types (STs) were interpreted using the *K. pneumoniae* MLST database hosted by the Institute Pasteur in Paris, France (https://pubmlst.org/bigsdb?db=pubmlst_mlst_seqdef&page=profiles).

Genetic surveys of colistin resistance in isolated strains. DNA extraction of confirmed colistin-resistant strains was done by the boiling method. The chromosomal and plasmid genes associated with colistin resistance were amplified and investigated using specific primers (Table 1). To amplify the chromosomal resistance genes, including *pmrA, pmrB, mgrB, phoP, phoQ, crrA,* and *crrB,* a reaction mixture with a total volume of 50 µL containing 25 µl of 2x Master mix PCR (Amplicon), 1.4 µL of primer F, 1.4 µL of primer R, 20.2 µL of sterile distilled water, and 2 µL of extracted DNA was added to separated microtubes on ice. To amplify the plasmid resistance genes, including *mcr-1, mcr-2, mcr-3,* and *mcr-4,* a reaction mixture with a total volume of 25 µL, containing 12.5 µl of 2x Master mix PCR (Amplicon), 0.7 µL of primer F, 0.7 µL of primer R, 10.1 µL of sterile distilled water, and 1 µL of extracted DNA was prepared. In the negative control tubes, distilled water was added instead of the genome. The chromosomal and plasmid genes for colistin resistance were amplified with a relatively similar program as follows: initial denaturation at 96ºC for 10 min, repeating cycles of denaturation at 96ºC for 30 s, annealing temperatures as indicated in Table 1 for 40 S, extension at 72ºC for 45s (28 cycles for *pmrA* and *pmrB,* 25 cycles for *mgrB,* and 30 cycles for other genes), and final extension at 72ºC for 10 min. The PCR amplicons were separated using electrophoresis on a 1% agarose gel with Safe DNA Gel Stain, followed by visualization under UV light.

The amplified PCR fragments were subjected to sequencing, and both nucleotide and predicted amino acid sequences were examined using tools available on the NCBI website. Insertion sequences were identified and characterized through the database of IS Finder (https://www-is.biotoul.fr/). Additionally, any novel amino acid changes detected were further assessed using the PROVEAN tool (http://provean.jcvi.org/index.php) to evaluate their potential impact on protein function (17).

RESULTS

Bacterial isolates and antimicrobial susceptibility testing. In this research, 38 clinical isolates of *K. pneumoniae* were screened for colistin resistance using broth dilution assay and strains with MIC greater than 4 µg/mL were considered resistant according to breakpoints defined by EUCAST. Out of the 38 screened *K. pneumoniae* strains, only one strain was found as resistant to colistin with MIC>4 mg/L. Testing susceptibility of this strain to other antibiotics revealed resistance to ampicillin, cefoxitin, ceftriaxone, cefepime, imipenem, nalidixic acid, ciprofloxacin, levofloxacin, and nitrofurantoin, but susceptibility to, tetracycline, doxycycline, gentamicin, fosfomycin and tigecycline (Table 2). Based on current definitions, this profile was consistent with a multidrug-resistant (MDR) phenotype with resistance to both carbapenems and colistin as important last-resort agents, having an important clinical significance.

Genotyping of the strain obtained by MLST assay. The internal fragments of seven housekeeping genes of the colistin-resistant *K. pneumoniae* strain were amplified by the PCR method. The obtained sequences were assigned to a specific MLST sequence type (ST) based on the alleles at each locus by PubMLST. Based on the analyzed sequences, the colistin-resistant strain belonged to ST377.

The results of tracking plasmid genes. To evaluate the role of plasmid genes in creating resistance to colistin, the detection of *mcr*-1, *mcr-2, mcr-3,* and *mcr-4* genes was done using genomic DNA extracted from the colistin-resistant isolate by the PCR method. The results showed that plasmid genes were not present in the studied isolate. This finding indicated that colistin resistance in the studied isolate was probably caused by the alterations of chromosomal genes.

Nucleotide sequence analysis of chromosomal genes. The sequence analysis of *pmrB, pmrA, phoP,* and *phoQ* genes, revealed no mutation indicating that these genes do not contribute in colistin resistance development in the studied isolate. While the nucleotide

Table 1. Characteristics of the primers and amplification conditions used in this study

Primer name	Sequence (5'-3')	Product size (base pair)	Annealing temperature (°C)	Reference
pmrA-F	CGCAGGATAATCTGTTCTCCA	808	60	(11)
pmrA-R	GGTCCAGGTTTCAGTTGCAA			
pmrB-F	GCGAAAAGATTGGCAAATCG	1182	60	(11)
pmrB-R	ATCAATGGGTGCTGACGTT			
mgrB-F	ACCACCTCAAAGAGAAGGCGTT	347	61	(11)
mgrB-R	GGCGTGATTTTGACACGAACAC			
phoP-F	GAGCGTCAGACTACTATCGA	912	58	(11)
phoP-R	GTTTTCCCATCTCGCCAGCA			
phoQ-F	CCACAGGACGTCATCACCA	1594	58	(11)
phoQ-R	GCAGGTGTCTGACAGGGATT			
crrA-F	GCATGTTGTCATCAGCACTGTG	821	57	(12)
crrA-R	GGAACCGAGTATTGCAATGG			
crrB-F	GGATTGAAGGGCATTCCGGA	1182	57	(12)
crrB-R	GCAGTATGTGGGATCTGTCT			
mcr-1-F	CGGTCAGTCCGTTTGTTC	309	55	(13)
mcr-1-R	CTTGGTCGGTCTGTAGGG			
mcr-2-F	TGTTGCTTGTGCCGATTGGA	567	56	(14)
mcr-2-R	AGATGGTATTGTTGGTTGCTG			
mcr-3-F	TTGGCACTGTATTTTGCATTT	542	52	(15)
mcr-3-R	TTAACGAAATTGGCTGGAACA			
mcr-4-F	ATTGGGATAGTCGCCTTTTT	487	52	(16)
mcr-4-R	TTACAGCCAGAATCATTATCA			

Table 2. Antimicrobial susceptibility profile of the studied isolate

Antibiotic Class	Antibiotic	Sensitivity
B-lactams	Ampicillin, Cefoxitin, Ceftriaxone, Cefepime, Imipenem	Resistant
Quinolones	Nalidixic acid, Ciprofloxacin, Levofloxacin	Resistant
Tetracyclines	Tetracycline, Doxycycline	Susceptible
Aminoglycosides	Gentamicin	Susceptible
Phosphonates	Fosfomycin	Susceptible
Glycylcyclines	Tigecycline	Susceptible
Nitrofurans	Nitrofurantoin	Resistant

sequence analysis of coding region of *mgrB* gene revealed no change, the promoter region of *mgrB* was disrupted by insertion of the IS1 family with a length of 768 bp at position -45 (Fig. 1). Moreover, the analysis of *crrAB* system revealed disruption of coding region of *crrA* gene by insertion of *IS5* family, (length of 1057 bp) at positions between +365 and +366 (Fig. 1) and amino acid substitution Gln296Lys (CAG>CTG) in *crrB* gene. The nucleotide sequences of the studied seven chromosomal genes associated with colistin resistance have been submitted to the GenBank nucleotide sequence database under the accession numbers PX373520 to PX373526.

DISCUSSION

In this study, a colistin-resistant *K. pneumoniae* strain was analyzed for its resistance mechanisms. Although the strain typed as ST377 by MLST had

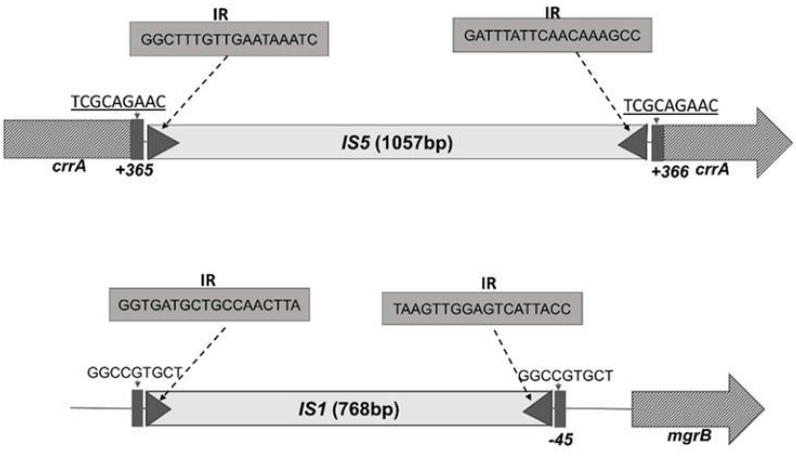

Fig. 1. Schematic representation of the *crrA* coding region and *mgrB* promoter disruption by insertion of IS elements. IR: inverted repeat

the criteria for MDR rather than XDR, the co-resistance to carbapenems and colistin is alarming as these agents are often considered as last-line therapeutic options. According to Andre Shelenkov et al.'s 2020 research, ST377 represents one of the rarest cases of multidrug resistance (MDR) to colistin, indicating the emergence of a new and potentially dangerous source of infection (18). This typing sequence has been observed with resistance to carbapenems. While colistin remains a final treatment option for multidrug-resistant (MDR) *K. pneumoniae* infections, increasing resistance especially in high-risk clones poses a serious therapeutic threat (9).

Molecular analysis revealed the absence of plasmid-associated *mcr* genes (*mcr-1* to *mcr-4*) in the resistant isolate. This finding indicates that resistance was not horizontally acquired but likely developed via chromosomal alterations, consistent with earlier studies in which mcr genes were not identified in colistin-resistant strains (11, 19).

Among chromosomal determinants, sequencing showed no mutations in *pmrA*, *pmrB*, *phoP*, or *phoQ*, suggesting that these commonly implicated regulatory systems did not contribute to resistance in this case. This is noteworthy since *pmrAB* and *phoPQ* are frequently reported to undergo adaptive mutations under colistin selection pressure (20-22). Similarly, in a study conducted by Haeili et al. (2017), no changes were reported in these two genes among the studied colistin-resistant isolates (11).

A critical finding was the insertion of an *IS1* family element into the *mgrB* gene, disrupting its promoter and likely silencing gene expression. Inactivation of the *mgrB* gene has been widely recognized as a significant cause of colistin resistance, particularly in clinical isolates lacking *mcr* genes (23-25). Cheng et al. (2015) showed that inactivation of the *mgrB* gene leads to increased expression of the operon involved in LPS changes in *K. pneumoniae* (26). Also, in a similar study, Haeili et al. (2017) investigated colistin resistance in *K. pneumoniae* strains and showed that inactivation of the MgrB protein is the main factor in resistance (11). Poirel et al. (2015) reported the introduction of additional IS fragments resulting in creation of a premature termination codon in the *mgrB* gene among colistin resistance *K. pneumoniae* strains. MgrB is a small protein that normally regulates the PhoPQ two-component system in *K. pneumoniae* negatively. Once activated, PhoP induces the expression of genes that chemically modify lipid A to reduce the negative charge on the bacterial surface. When MgrB is inactivated (by mutation or disruption), it can no longer suppress PhoPQ. This leads to constitutive activation of PhoPQ, which in turn continuously activates the lipid A modification pathways, reducing colistin binding (23).

Additionally, analysis of the *crrAB* locus revealed two key features. First, an *IS5* insertion within the coding region of *crrA* resulting in inactivation of this protein with a response regulatory function. Second, a CrrB Q296L substitution, which was categorized as neutral change by PROVEAN prediction tool (PROVEAN score: -0.33). The CrrB Q296L mutation has been previously reported among colistin-re-

sistant *K. pneumoniae* from Brazil (27). Although the biological impact of this specific mutation on CrrB and its association with colistin resistance is not fully understood, prior studies have shown that *crrB* mutations can activate *pmrC*, promoting lipid A modifications and enhancing colistin resistance (28). Cheng et al. (2016) reported that *crrAB* is involved in resistance to colistin through changes in LPS by controlling the expression of genes in the *pmrHFIJKLM* operon. Therefore, it can be assumed that CrrAB changes trigger CrrC expression, thereby stimulating the overexpression of the *pmrHFIJKLM* operon and *pmrC* locus, leading to increased colistin resistance (29).

Collectively, these findings highlight the multifactorial nature of resistance to colistin in *K. pneumoniae*. The coexistence of IS-mediated *mgrB* expression disruption and *crrB* point mutation supports a model of cumulative chromosomal evolution under antibiotic pressure. Notably, the absence of plasmid-borne *mcr* genes suggests that colistin resistance in this isolate is not easily horizontally transferable, but could become so if IS elements mobilize adjacent resistance determinants.

The identification of ST377 as the sequence type of the resistant strain is also of interest. Although not as globally prevalent as ST258 or ST11, this ST has been increasingly reported in MDR and XDR backgrounds and warrants further surveillance (18, 30).

Although plasmid-borne *mcr* genes have drawn significant attention due to their potential for horizontal transmission, a growing body of evidence suggests that chromosomal mutations remain a dominant resistance mechanism in clinical settings, especially in regions where *mcr* prevalence is low (31).

There are several limitations in this study that should be considered. The analysis was performed on a limited number of isolates from a single hospital, which may restrict the broader applicability of the results. Only key resistance-associated genes were examined, and functional validation of the *mgrB* and *crrA* disruptions was not performed. Furthermore, screening was limited to *mcr-1* to *mcr-4*, leaving the possibility that other emerging resistance mechanisms may have been missed.

CONCLUSION

In conclusion, understanding the molecular bases of colistin resistance is vital for the advancement of effective diagnostic, therapeutic, and epidemiological strategies. While previous studies have focused on globally dominant clones such as ST258 or ST11, less is known about resistance mechanisms in other sequence types. The colistin resistance observed in this study is most likely driven by IS-mediated inactivation of *mgrB* or *crrA* genes rather than plasmid-mediated mechanisms or classical mutations in *pmrAB* or *phoPQ*. These results contribute to a growing understanding of the diversity and complexity of resistance pathways in *K. pneumoniae*, emphasizing the need for integrated molecular diagnostics and strict antibiotic stewardship.

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