Original Article

Plasmid-Mediated Colistin and Fosfomycin Resistance among Clinical Isolates of ESBL- and Carbapenemase-Producing *Klebsiella Pneumoniae* in Northern Iran

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ABSTRACT

The emergence of extensively-resistant strains of *Klebsiella pneumoniae* (*K. pneumoniae*) in healthcare settings is linked to prolonged hospitalization and uncontrolled use of antibiotics. There is a paucity of data regarding the prevalence and mechanisms of colistin and fosfomycin resistance encoding genes rate and mechanisms in Iran. The objective of this study was to determine the prevalence of biofilm formation and fosfomycin and colistin resistance among *K. pneumoniae strains* producing ESBL and carbapenemases by detecting the *mcr-1*, *mcr-2*, and *fosA* genes in Tehran, Iran, during the 2020-2021 period. After collecting 73 samples, the isolates were identified using biochemical tests. Antibiotic susceptibility test was performed using the disk diffusion method. The phenotypic determination of extended-spectrum beta-lactamases (ESBLs) and carbapenemase enzymes was conducted using combined disk and CARBA-NP tests, respectively. The biofilm formation was conducted using a microtiter tissue plate assay. Polymerase chain reaction (PCR) was employed to detect the *mcr-1*, mcr-2 and *fosA* genes,which are associated with colistin and fosfomycin resistance, respectively. The highest resistance rate was observed against ampicillin (97%), chloramphenicol (90%), and ciprofloxacin (87%), respectively.In contrast, the lowest resistance rate was noted against gentamicin (4%), amikacin (10%), and cotrimoxazole (18%). Moreover, 44 and 23 isolates were identified as ESBL and carbapenemase -producing *K. pneumonia*), respectively. Of the fortyeight isolates that formed strong biofilms,one was a non-biofilm producer. The PCR test revealed the amplification of the *fosA2* gene in four isolates and the *mcr-2* genes in one isolate. However, no amplification of the *fosA3* or *mcr-1* genes was observed. The present study demonstrated that the frequency of *K. pneumoniae* isolates producing ESBL and carbapenemase, as well as *mcr-1*, *mcr-2* and *fosA* genes, was relatively low.However,given the potential for these genes to be disseminated more widely, it is imperative to implement effective isolation and control measures. Moreover, these strains demonstrated the capacity to form biofilms *in vitro*, which can lead to persistent infections in the hospital settings.

Keywords: *Klebsiella Pneumoniae*, Antibiotic resistance, Colistin;Fosfomycin, ESBL, Carbapenemase

1. Introduction

Klebsiella species,including *Klebsiella pneumoniae* (*K. pneumoniae*),are a significant cause of nosocomial bacterial infections,exhibiting a high level of drug non-susceptibility in recent years (1-5). *Klebsiella* belongs to the family *Enterobacteriaceae*. These bacteria can causes a variety of infections in infants, including septicemia, urinary tract infections (UTIs), infections of thecentral nervous system (CNS), lungs, skin and soft tissue infections in infants (4,5). *K. pneumoniae* infections are of particular significancein infants, the elderly, and immunocompromised individuals within healthcare settings.Furthermore, the organism is also responsible for a significant number of communicable infections in communities around the world.These infections are distinguished by their ability to disseminate to different tissues (metastasis) and their considerable impact on morbidity and mortality. The emergence of Carbapenem-resistant *K. pneumoniae* (CR-Kp) may be attributed to selective pressure exerted by the treatment of infections caused by extended-spectrum β-lactamase (ESBL)-producing strains with carbapenem (6). In 2013, the United States designated CRE as an immediate public health threat. Of the 9,000 CRE infections, 80% were caused by Klebsiella species. Resistance can occur as a result of increased expression of efflux pumps and altered expression of outer membrane purines in the core genome,as well as overproduction of carbapenemase enzymes. It is also possible that theplasmid encoding carbapenem resistance enzymes may also encode virulence factors. Some CR-Kp isolates encode an important virulence factor in Ybt and can obtain highly pathogenic plasmids (7). Colistin is a polycationic antibiotic that is effective against Gram-negative bacteria by affecting the outer membrane milieu. The emergence of carbapenem-resistant *Enterobacteriaceae* (CRE) has rendered the colistin as one of the most efficacious pharmaceutical agents in the treatment of infections caused by these organisms. However, mutations in the genes responsible for bacterial lipid A are the primary cause of non-susceptibility.The bacterial lipid A is the target of polymyxin antibiotics and its mutation results in a reduction in the efficacy of polymyxin. Another resistance mechanism is plasmid-mediated resistance, which is exerted via various *mcr* transmissible genes .Theprevalence of the *mcr-1* gene in *K. pneumoniae* bloodstream infections (BSI) is relatively low in China,whereas itis more common in *Escherichia coli* (*E. coli*). The first report of the *mcr-1* gene in the United States was in 2016 in *E. coli*. In September 2016, a pandrug-resistant (PDR) *Klebsiella pneumoniae* was isolated,yet the resistance to colistin was not attributable to the *mcr-1* gene (8). Fosfomycin is an inhibitor of the MurA enzyme, which catalyzes the first step in the biosynthesis of Gram-negative bacteria peptidoglycan. The phenomenon of fosfomycin resistance in bacteria has been the subject of extensive investigation *in vitro*. In Gram-negative bacteria such as *K. pneumoniae*, resistance is exerted by three main mechanisms: (1) defective mechanisms in cytoplasmic membrane transporters (2,3), replacement of the amino acid at the active site of MurA , which mitigates the affinity to fosfomycin, and (3) the production of the inactivating enzyme encoded by the *fos* genes (9). FosA is a manganese- and potassium-dependent glutathione transferase. Glutathione-mediated transferase (FosA type) enzymes of plasmid origin include FosA3, FosA4, FosA5 and FosC2. FosA3 is the most prevalent variant of the gene, predominantly identified among clinical and environmental isolates. FosA produced by Gram-negative species, contributes to the intrinsic resistance of fosfomycin. Isolates of *E. coli* containing chromosomal fosA genes have been observed to exhibit high levels of resistance to fosfomycin. Conversely, the deletion of this gene in *Serratia marcescens* has been demonstrated to result in drug susceptibility. Nevertheless, other Gram-negative species, such as *E. coli*, have been demonstrated to exhibit reduced sensitivityto fosfomycin (10-13). The risk factors for colonization and the prevalence of multidrug-resistant (MDR) strains, such as CR-Kp strains include previous treatment or overuse of antibiotics, prolonged hospital stay, renal failure, older ages, surgical procedures and long-term residence in the intensive care unit (ICU), and mechanical ventilation (14-19). Furthermore, intestinal colonization with these strains has been linked to the progression of infection. The aim of this study was to evaluate the resistance to fosfomycin and colistin in clinical isolates of *K. pneumoniae* in Tehran, Iran.

2. Materials and Methods

2.1. Clinical Samples and Isolate Identification

A total of 73 clinical samples were collected from hospitals and laboratories in Tehran province and transferred to a research laboratory for identification. The bacterial species were isolated from clinical specimens and identified after transfer to the laboratory and stored at -70°C for further testing. To identify *K. pneumoniae* isolates, various biochemical tests were performed including fermentation of sugars in Triple Sugar Iron (TSI) agar medium, production of indole and motility in Sulfur, Indole, Motility (SIM) medium, reaction in Methyl Red & Vogues-Proskauer test (VP-MR) test, and growth in Simon citrate and urea medium. Moreover, a standard strain was employed as the control.The clinical isolates of *K. pneumoniae* were cultured in trypticase soy broth (TSB) medium (Merk, Germany) and Müller-Hinton broth (MHB) medium (iberesco) with 30% glycerol and subsequently stored at -70°C.

2.2. Bacterial DNA Extraction

The boiling method was employed for the extraction of total DNA. Accordingly, a loop of each bacterial isolate was removed from the agar medium and inoculated into a microtube containing MHB medium. The microtube was then incubated overnight in a shaker incubator at 37°C. Following this,the microtube was centrifuged and the precipitate was dissolved in sterile distilled water. The

solution was boiled for 10 minutes and then centrifuged for 5 minutes at 12,000 rpm.The supernatant containing the extracted DNA, was stored in a new microtube at -80°C.

2.3. Susceptibility to Antibiotics

The disk diffusion method was employed to assess the susceptibility of the bacterial isolates to a panel of antibiotics. The antibiotics used in this study were ceftazidime (CAZ 30 µg), cefotaxime (CTX 30 µg), gentamicin (GM 10µg), fosfomycin (FOS 200 µg), chloramphenicol (CL 10 µg).The antibiotics used were carbenicillin (CB 10 µg), cotrimoxazole (SXT, 25µg), ciprofloxacin (CP, 5 µg), piperacillin (PIP 100 µg), AMI (30 µg), and meropenem (MEN 10 µg). The method was performed in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines, version 2021. A bacterial suspension equivalent to half the McFarland standard turbidity was prepared from colonies that had been cultured for 18 to 24 hours. The quality control procedure included the use of standard strains of *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC25922.

2.4. Phenotypic Determination of ESBLs and Carbapenemase Enzymes

The test was conducted using the Müller-Hinton agar medium in accordance with the CLSI standards and a bacterial suspension equivalent to half McFarland. In this experiment, CAZ30 + clavulanic acid10 and CTX 30 + clavulanic acid 10 with ceftazidime and cefotaxime singly were utilized and incubated at 37°C for 24 h. The production of ESBLs was confirmed by an increase of the diameter of the growth inhibition zone by 5mm in comparison to ceftazidime and cefotaxime disks alone. Furthermore, the CARBA-NP and modified HODGE tests were employed to ascertain the production of carbapenemases (12,18).

2.5. Phenotypic Biofilm Formation

The microtiter tissue plate assay was employed for the determination of biofilm formation (12,18). The bacterial isolates were initially cultivated in the TSB medium supplemented with 1% glucose and incubated for 24 hours. Subsequently, a dilution of half McFarland was prepared from the bacterial suspension and 20 µL of the suspension along with 180µL of TSB medium was added to 96 -well plates. The plates were then incubated at 37°C for 24 h (with each experiment being performed in triplicate). Subsequently, the wells were washed three times with phosphate- buffered saline (PBS) and 150 µL of methanol was used to fix them. Subsequently, the wells were treated with crystal violet dye was added to wells for 15 minutes. Following a wash with distilled water, 150 µL of 95% ethanol was added and the level of biofilm formation was obtained using enzyme linked immunosorbent assay (ELISA) reader at optical density (OD) of 490nm in triplicate. The calculation of the biofilm formation level was performed in accordance with the specifications outlined in Table 1. The PCR technique was employed to amplify the *fosA3*, *mcr-1* and *mcr-2* genes using specific primers, as detailed in Table 2. The highest resistance rate was observed against ampicillin (97%), chloramphenicol (90%), and ciprofloxacin (87%), respectively, In contrast, the lowest resistance rate was noted against gentamicin (4%), amikacin (10%), and cotrimoxazole (18%). Furthermore, 44 and 23 isolates were identified as ESBL and CP-*K. Pneumonia*, respectively. Of the Forty eight isolates were identified as strong biofilm-forming *K. pneumoniae*, one isolate was determined to be a non-biofilm producer the conditions for the. PCR reaction and preparation of the master mix were as previously described (12,18).

Table 1. The calculation of biofilm formation levels

Biofilm formation ability	Calculation of cut-off level	OD calculated results
Strong	OD>ODc*4	0.33296 > OD
Moderate	ODc*4≤2*ODc<OD	0.33296≤0.16648<OD
Weak	*2ODc≤ODc<OD	0.16648 ≤0.083324<OD
No binding	0.08324 ≤OD	0.08324≤OD

ODc: control optical density

Table 2. The sequences of primers utilized in this study

primer	Sequence: 5' ⟶ 3'	Product size (bp)	Reference
fosA3	F: GGCATTTTATCAGCAGT R: AGACCATCCCCTTGTAG	350	(2)
mcr-1	F: AGTCCGTTTGTTCTTGTGGC R: AGATCCTTGGTCTCGGCTTG	320	(3)(2)(2)
mcr-2	F: CAAGTGTGTTGGTCGCAGTT R: TCTAGCCCGACAAGCATACC	715	This study

3. Results

3.1. Patients and Clinical Isolates

A total of 73 clinical isolates of Klebsiella *pneumoniae* were collected from emergency settings, and intensive care units (ICUs) and cardiac care unit (CCU_s). The majority of isolates were derivedfrom stool samples (n=53), followed by respiratory samples (n=14) and blood samples (n=6). The demographic data for these patients is presented in Table 3. The proportion of male and female patients was not significantly different, with 45% and 55%, respectively. Additionally, the age range of 21 to 30 years (38%) and 61 to 70 years (20%) exhibited the highest prevalence of *K. pneumonia* infection. It is noteworthy that no other significant risk factor was identified.

3.2. Antibiotic Susceptibility Testing

As evidenced by the data presented in Table 4, the most prevalent resistance observed was to ampicillin (97%, n=71), followed by chloramphenicol (90%, n=66) and piperacillin (87%, n=64). Conversely,the lowest resistance levels were noted for (0%), gentamicin (2%, n=2.8) and trimethoprim-sulfamethoxazole (7%, n=5) (Table 4).

3.3. Phenotypic Determination of ESBLs and Carbapenemases

A total of 44 (60%) isolates were found to be positive in the combine disk test used to determine the presence of ESBLs. Furthermore,the phenotypic test for carbapenemases determination yielded positive results for 23 (31%) of the isolates.

3.4. Phenotypic Biofilm Formation Experiments

In the biofilm formation test, 48 of the 53 MDR-*K. pneumoniae* isolatesproduced a strong biofilm, two exhibited moderate biofilm formation, two demonstrated weak biofilm formation and one isolate did not produce any biofilm (Table 5).

3.5. PCR test to determine colistin and fosfomycin resistance genes

The PCR test revealed thatfour isolates amplified the *fosA2* gene and one *mcr-2* gene, respectively.However, none of the isolates demonstrated amplification of either the *fosA3* or *mcr-1* genes amplification (Figure 1).

Table 3. The demographic data of patients

Patients demography	N (%)	Significance
Male	33 (45)	No
Female	40 (55)	No
Wards	Emergency 36 (49) ICU 21 (29) CCU 16 (22)	Yes No No
Age range	9 months-76 years	--
Pregnancy	7 (9.6)	No
Abortion	0.00	--
Antibiotic consumption	26 (36)	No
Diabetes	2 (3)	No
Cancers	0.00	--
Tissue transplantation	0.00	--
Smoking	8 (11)	No
Cardiovascular diseases	3 (4)	No
Renal impairment	1 (1.5)	No
Hepatic impairment	0.00	--
Catheter	5 (7)	No
Cortone receivement	0.00	--
Immune deficiency	0.00	--

Table 4. Results of antibiotic susceptibility test

Disk/Resistance (N=73)	Susceptibility N (%)	Intermediate N (%)	Resistance N (%)
CAZ	36 (49)	13 (18)	24 (33)
CTX	29 (40)	16 (22)	28 (38)
GM	70 (96)	1 (1)	2 (2.8)
FOS	29 (40)	24 (33)	20 (27)
CL	0 (0)	7 (10)	66 (90)
CB	48 (66)	5 (7)	20 (27)
AM	2 (3)	0 (0)	71 (97)
SXT	60 (82)	8 (11)	5 (7)
CP	34 (46)	25 (34)	14 (20)
PIP	2 (3)	7 (10)	64 (87)
AN	66 (90)	7 (10)	0 (0)
MEN	40 (55)	28 (38)	5 (7)

CAZ: ceftazidime, CTX: cefotaxime, GM: gentamicin, FOS: fosfomycin, CL: chloramphenicol, CB: carbenicillin, AM: ampicillin, SXT: cotrimoxazole, CP: ciprofloxacin, PIP: piperacillin, AN: amikacin, MEN: meropenem

Table 5. A phenotypic biofilm formation test for 53 clinical isolates of MDR-*K. pneumoniae*

Isolate	Number Average	Biofilm formation
1	0.443	Strong
2	0.134	weak
3	0.413	Strong
4	0.296	Moderate
5	0.163	weak
6	0.041	No biofilm
7	0.347	Strong
8	0.39	Strong
9	0.7	Strong
10	0.766	Strong
11	0.566	Strong
12	0.376	Strong
13	0.723	Strong
14	0.836	Strong
15	0.586	Strong
16	0.561	Strong
17	0.648	Strong
18	0.668	Strong
19	0.564	Strong
20	0.382	Strong
21	0.54	Strong
22	0.674	Strong
23	0.554	Strong
24	0.3	Strong
25	0.497	Strong
26	0.398	Strong
27	0.583	Strong
28	0.496	Strong
29	0.3	Strong
30	0.283	Moderate
31	0.472	Strong
32	0.336	Strong
33	0.778	Strong
34	0.966	Strong
35	0.76	Strong
36	0.48	Strong
37	0763	Strong
38	0.927	Strong
39	1.458	Strong
40	0.983	Strong
41	0.898	Strong
42	0.926	Strong
43	1.419	Strong
44	1.18	Strong
45	0.493	Strong
46	0.934	Strong
47	1.366	Strong
48	1.133	Strong
49	0.497	Strong
50	1.596	Strong
51	1.461	Strong
52	1	Strong
53	0.788	Strong

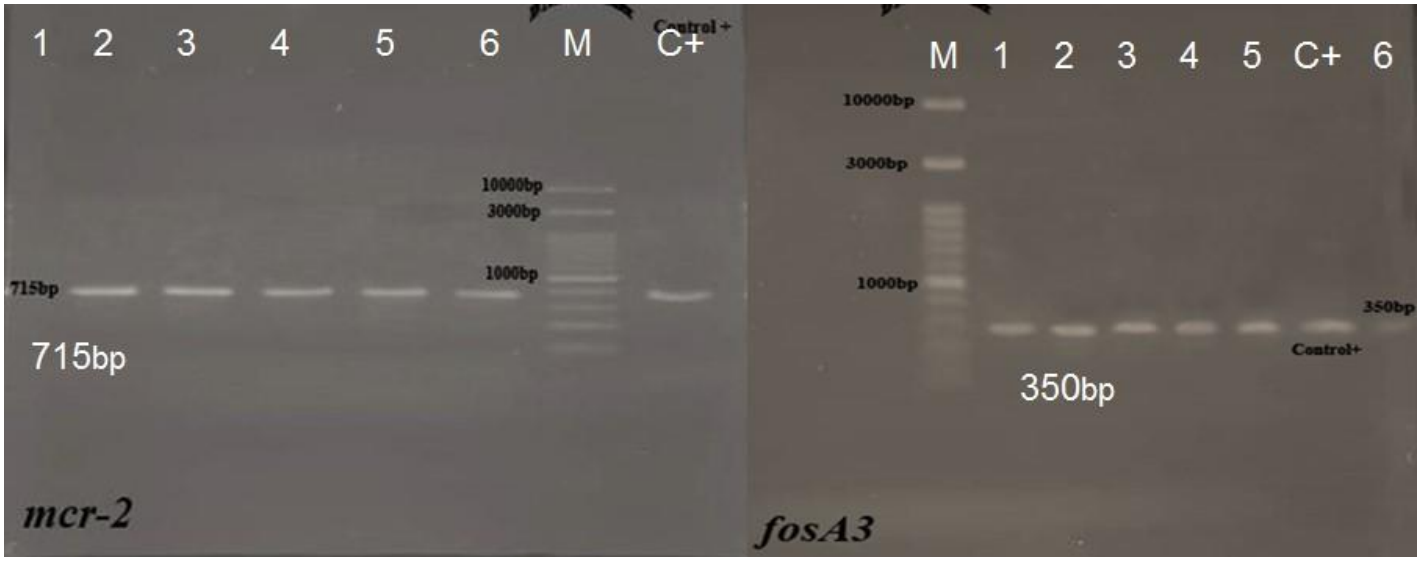

Figure. 1. PCR products of the *mcr-2* and *fosA3* genes; M: 100 bp DNA marker, C+: positive control

4. Discussion

Klebsiella. pneumoniae is regarded as an opportunistic pathogen, particularly in the context of infection in inpatients or those with immune system disorders (1-3). In fact, *Klebsiella* species are recognized as the most prevalent cause of pneumonia. Furthermore,*Klebsiella* species is also a significant contributor to ventilator-associated pneumonia (VAP) among patients in intensive care units (ICUs), accounting for 83% of nosocomial pneumonias. The mortality rate associated with Klebsiella pneumoniae is estimated to be as high as 50% (21). Type 3 fimbriae have been demonstrated to play a major factor in the formation of biofilms on biological and abiotic surfaces,as well as their attachment to endothelial and bladder epithelial cell lines (22). Extended-spectrum beta-lactamases (ESBLs) and carbapenemase-producing *Enterobacteriaceae* (CPE), including *E. coli* and. *Klebsiella pneumoniae*, represent a significant challenge to clinical management, with a high mortality rate in hospital settings. In these cases, treatment is typically administered with tigecycline or colistin. Furthermore, prolonged administration of fosfomycin has also been demonstrated to possess therapeutic potential against these bacterial species. Fosfomycin is administered orally or intravenously for uncomplicated simple urinary tract infections caused by *Enterobacteriaceae* that produce ESBL or CRE (23). In this study, 45% of the patients were male and 55% were female. The age groups 21 to 30 years (38%) and 61 to 70 years (20%) exhibited the highest prevalence of *K. pneumonia*. The results of the susceptibility test demonstrated that most of isolates were sensitive to gentamicin (96%), amikacin (90%), cotrimoxazole (82%), carbenicillin (66%), and meropenem (55%). Additionally, the highest rate of antibiotic resistance were observed against ampicillin (97%), chloramphenicol (90%), and piperacillin (87%). In their study, entitled "High levels of colistin resistance in patients with CR-Kp infection lead to higher mortality" Capone et al. (2013) observed that showing overall, 36% and 20% of the strains, respectively, exhibited overall resistance to colistin. The isolates demonstrated resistance to colistin and tigecycline. Infection was detected in 91 patients who received 73%, 59% and 28% of the appropriate antibiotic therapy, combination therapy, and removal of the infectious source, respectively. The current leading cause of CR-KP infection in central Italy is the occurrence of outbreaks of CR-Kp, predominantly of the ST258 strain.The study demonstrated a high prevalence of colistin resistance, which was independently associated with a concerning outcome (24). In this study, the combine disk was employed to determine the presence of ESBLs, with 44 (60%) of the isolates exhibiting a positive result. Furthermore, the phenotypic test for carbapenemases determination yielded positive results for23 (31%) of the isolates. In the biofilm formation test, 48 of the 53 MDR-*K. pneumoniae* isolates demonstrated strong biofilm formation, two exhibited moderate biofilm formation, two exhibited weak biofilm formation and one isolate did not produce a biofilm. In the PCR test, four isolates amplified the fosA2 gene and one mcr-2 gene, respectively, while no amplification was observed for either the *fosA3* or *mcr-1* genes. In a study conducted by Cannatelli et al. (2014), the status of the *mgrB* gene was examined in a set of 66 clinical strains of KPC-KP colistin-resistant Klebsiella pneumoniae from various hospitals in Italy and Greece. Thirty-three (35%) strains exhibited mutations in the *mgrB* gene, including the placement of different types of mobile elements (such as IS5, such as IS1F, or ISKpn14), point mutations, and partial deletions within the gene. Therefore, the, mechanism of *mgrB* mutations was found to be prevalent among KPC-KP strains (21). In a study conducted by Monaco et al. (2014), 191 clinical isolates of CRE, KPC-KP exhibited resistance to colistin in 76 (43%) of the isolates. All participating laboratories identified Colistin-resistant strains of KPC-KP. This highlights a new development in colistin resistance in pathogenic strains of KPC-KP (22). Giacobbe et al. investigated the risk factors for CP-KP bloodstream infections (BSIs) and the clinical characteristics of

these infections. The study demonstrated that previous treatment, KPC-KP colonization, previous hospitalization for more than three months, Charlson score greater than three, and neutropenia were significant risk factors for CP-KP BSI (21). The objective of this study was to investigate the effect of fosfomycin on CP-KP strains exhibiting multidrug resistance (MDR) and extensive drug resistance (XRD).Theclinical outcomes of colistin, fosfomycin and tigecycline were successful on day 14 in 54% of patients.However, inconsistency, indeterminate outcomes, and very severe infections were recorded in 33%, 6%, and 6%, respectively. The overall mortality rate at day 28 was 37%. Bacterial eradication was observed in 56% of cases. Three cases demonstrated resistance to fosfomycin. The main side effect of hypokalemia was reversible. Consequently, fosfomycin can be employed in the treatment of Gram-negative XDR and PDR infections in critically ill patients (22). In their study, Ito et al. (2017) elucidated the presence of *fosA* homologuesin the DNA of some species including *Pseudomonas aeruginosa, Klebsiella*, *Enterobacter* and *S. marcescens*In contrast,they are absent in the majority ofother species,including *E. coli, Acinetobacter baumannii,* and *Burkholderia cepacia*. FosA proteins exhibit significant divergence among bacterial pathogens, yet key amino acids are preserved in the active site. The chromosomal *fosA* gene is responsible for mediating high levels of resistance to fosfomycin. The findings indicate that the *fosA gene* is present in clinical strains and contributes to the intrinsic resistance to fosfomycin (24). Klontz et al. (2017) demonstrated that the expression of *fosA3* in *E. coli* TOP10 and *fosA* KP resulted in a notable increase in resistance to fosfomycin compared to *fosA* PA. Interestingly enough, these differences in enzymatic activity cannot be attributed to structural differences in the active sites. The results demonstrated that the dimer interface loop in *fosA* KP and *fosA3* is longer and wider than that of FosA PA, which is the reason for its more effective activity. In conclusion, these findings offer novel insights into the mechanisms of fosfomycin resistance,which may inform for the development of new strategies for FosA inhibition and enhance the efficacy of fosfomycin (25). However, the expression of neither the *fosA3* nor the *mcr-2* genes was evaluated. This study also had several other limitations, including thelack of detection of minimum inhibitory and bactericidal concentrations,the relatively low number of isolates and the vague risk factors contributing to the spread of colistin- and fosfomycin-resistant *K. pneumoniae*. In conclusion, the emergence of resistance to last-line antibiotics represents a significant challenge, particularly in the context of colistin and fosfomycin. In this study, the observed resistance phenotype was unusual and exhibited a low prevalence. However,the emergence of these strains represents a significant concern that requires their prompt identification and effective control measures.

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Authors' Contribution

S.F, A.G and S.K.S. conceptualized the study. A.B. performed the work. A.G. wrote the manuscript and edited. M.K edited and contributed during revision stage. M.R. contributed in revision stage.

Ethics

The study was approved by the Research Ethics Committee of Islamic Azad University, Tehran, Iran [ethical code: IR.IAU.PS.REC.1398.230].

Conflict of Interest

The authors declare that they have no conflict of interest.

Data Availability

The data that support the findings of this study are available on request from the corresponding author.

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