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In-vitro Susceptibility of Fosfomycin Against XDR – *Klebsiella pneumoniae* Isolated from Urine

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Abstract

Background: Managing infections caused by extensively drug-resistant (XDR) or multidrug-resistant (MDR) *Klebsiella pneumoniae* poses a significant challenge in hospitals and medical centers.

Objectives: The present study investigated the efficacy of common and older antibiotics, including fosfomycin and colistin, among clinical isolates of *K. pneumoniae*.

Methods: In this cross-sectional study, 43 XDR *K. pneumoniae* isolates were obtained from 215 urine samples collected at Milad Hospital, Tehran, Iran, between September 2023 and November 2024. Antimicrobial susceptibility testing against fosfomycin and other antibiotics was performed using the disk agar diffusion test in accordance with CLSI recommendations. Susceptibility to colistin was determined using colistin broth disk elution and chromogenic agar. The presence of mgrB, bla VIM, bla NDM, bla KPC, bla OXA-48, and bla IMP genes was identified using polymerase chain reaction (PCR).

Results: The incidence rates of imipenem (IMP) and meropenem (MEM) resistance in *K. pneumoniae* isolates were 90.7% and 93%, respectively. The prevalence of bla IMP, bla VIM, bla KPC, and mgrB was 25.6%, 8%, 69.8%, and 93%, respectively. No bla NDM or bla OXA-48 genes were detected. The rates of sensitivity to fosfomycin and colistin were 39.5% and 7.1%, respectively. Additionally, 32.6% of *K. pneumoniae* isolates were intermediate to fosfomycin.

Conclusions: The high rate of resistance to colistin and most other antimicrobial agents among our *K. pneumoniae* isolates must be considered due to the potential for antibiotic treatment failure and increased mortality and morbidity in elderly patients in healthcare settings. The relatively low rate of susceptibility to fosfomycin suggests the need for using another appropriate antibiotic in combination with fosfomycin for effective treatment of urinary infections.

Keywords: *Klebsiella pneumoniae*, Extensively Drug-Resistant, Multidrug-Resistant, Fosfomycin, Colistin

1. Background

Klebsiella pneumoniae, a gram-negative bacillus of the Enterobacteriaceae family, is responsible for a wide range of infections, including urinary tract infections (UTIs), pneumonia, burn infections, septicemia, and meningitis. The bacterium is the second most common cause of UTIs (1, 2). Unfortunately, in recent years, with the global emergence of antibiotic resistance among pathogens, the bacterium's susceptibility to current antibiotics has decreased dramatically. It is estimated that the global drug resistance rate of *K. pneumoniae* has reached as high as 70%, and the infection-related fatality rate has also reached 40% to 70% (3). In recent years, multidrug-resistant (MDR) *K. pneumoniae* and carbapenem-resistant *Klebsiella pneumoniae* (CRKP) have emerged as major global public health problems (3, 4). Therefore, managing infections caused by antibiotic-

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resistant *K. pneumoniae* is problematic due to the bacterium's intrinsic and acquired resistance to a broad spectrum of drugs, particularly in elderly, immunosuppressed individuals, or infants with immature immunity (3, 5). The use of older antimicrobial agents such as fosfomycin and colistin has been proposed to combat MDR Enterobacteriaceae, particularly in healthcare settings (6). In adult patients attending emergency departments between 2010 and 2016, fosfomycin susceptibility among all uropathogens was 87.8%, and higher for *Escherichia coli* (97.5%) (7).

2. Objectives

The present study aimed to characterize a collection of extensively drug-resistant (XDR) *K. pneumoniae* strains isolated from urine samples in terms of antimicrobial resistance and to evaluate the in vitro efficacy of fosfomycin and colistin against carbapenem-resistant *K. pneumoniae*.

3. Methods

3.1. Sample Collection and Bacterial Identification

From a total of 215 urine specimens processed, 43 non-duplicate XDR clinical *K. pneumoniae* strains were collected from inpatients and outpatients at different wards of Milad Hospital, Tehran, Iran, between September 2023 and November 2024. The isolates were identified using bacterial culture and standard biochemical tests. In summary, the suspected isolates were streaked on MacConkey and blood agar plates (Merck, Germany) and incubated at 37°C for 24 hours. Bacterial species were identified using standard biochemical methods (8).

3.2. Antimicrobial Susceptibility Test

Antimicrobial susceptibility was performed on Mueller-Hinton agar using the Kirby-Bauer disk diffusion technique according to the Clinical and Laboratory Standards Institute's (CLSI, 2024) guidelines for the following antibiotics: Ceftriaxone (30 mg), ceftazidime (30 mg), cefotaxime (30 mg), ciprofloxacin (5 mg), trimethoprim-sulfamethoxazole (23.75/1.25 mg), gentamicin (10 mg), clindamycin, and piperacillin-tazobactam (110 mg). Results were interpreted using CLSI 2015 disc diffusion cut-offs for *E. coli* in urinary tract isolates (9). Multidrug-resistant was defined as resistance to ≥ 3 antibiotic classes; XDR as resistance to all except 1 - 2 classes. Fosfomycin susceptibility was tested by disc diffusion method using fosfomycin trometamol disc (200 µg, BD BBL, Franklin Lakes, New Jersey) containing 50 µg G6PD on Mueller-Hinton agar. *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as control strains. Results were interpreted using CLSI 2015 disc diffusion cut-offs for *E. coli* in urinary tract isolates: ≥ 16 mm as sensitive, 13 - 15 mm as intermediate, and ≤ 12 mm as resistant (9).

3.3. Detection of Colistin Resistance

Colistin susceptibility was identified using broth disk elution and chromogenic agar (CBDE) as described in CLSI ver30 (10). Briefly, in the CBDE method, four glass tubes were used, each containing 10 mL of cation-adjusted Mueller-Hinton broth (HI-media). The first tube served as a growth control (no antibiotic disc added). One disc of colistin sulfate (10 µg, BD BBL, Franklin Lakes, New Jersey) was added to the second tube, two discs to the third tube, and four discs to the fourth tube. The tubes were incubated at room temperature for 30 - 45 minutes to elute colistin from the medium. Colonies from blood agar were used to prepare a 0.5 McFarland solution in normal saline, and after proper mixing, 50 µL inoculum was added to each tube. The test tubes were mixed thoroughly and incubated at 37°C for 24 hours (10). *Pseudomonas aeruginosa* ATCC 27853 was used as a negative control. Additionally, bacterial cultivation was done on colistin Chromagar media (CHROMagar™ COL-APSE, France), and the growth of green-blue colonies was investigated after 24 hours of incubation at 37°C. The presence of mgrB was also determined using specific primers and polymerase chain reaction (PCR) as described previously (11).

3.4. Detection of Carbapenem Resistance

3.4.1. Phenotypic Method

Confirmation of carbapenem-resistant strains was performed using disk diffusion, following the Kirby-Bauer disk diffusion method according to CLSI 2024 guidelines (12). The isolated strains were also screened for carbapenem resistance using imipenem (IMP) and meropenem (MEM) discs. Isolates showing an inhibition zone diameter of ≤ 19 mm were considered screening test positive and labeled as carbapenem-resistant Enterobacteriaceae (CRE).

3.4.2. Genotyping Method

Overnight bacterial culture was used for DNA extraction using the DNA Extraction Kit (QIAGEN, Germany) in accordance with the manufacturer's instructions. Briefly, a single colony from a fresh blood agar plate was inoculated into 5 mL of Luria-Bertani (LB) broth and incubated overnight at 37°C with shaking. A 1.5 mL aliquot of the overnight culture was centrifuged at 10,000 × g for 5 minutes, and the bacterial pellet was resuspended in the appropriate lysis buffer provided in the kit. After lysis and protein digestion, DNA was purified using spin columns and eluted in 50 µL of nuclease-free water. The extracted DNA was quantified using a NanoDrop spectrophotometer and stored at -20°C until further use. The carbapenemase genes (bla VIM, bla NDM, bla KPC, bla OXA-48, and bla IMP) were detected using conventional PCR (13). Each 25 µL PCR reaction contained 12.5 µL of 2× PCR Master Mix (Amplicon, Denmark), 1 µL of each primer (10 pmol/µL), 2 µL of template DNA, and nuclease-free water up to 25 µL. The PCR cycling conditions were as follows: Initial denaturation at 94°C for 5 minutes; 35 cycles of denaturation at 94°C for 30 seconds, annealing at the primer-specific temperature for 30 seconds, and extension at 72°C for 1 minute; followed by a final extension at 72°C for 7 minutes. The PCR products were separated by electrophoresis on a 1.5% agarose gel stained with ethidium bromide and visualized under UV illumination. A 100 bp DNA ladder (Fermentas, Lithuania) was used as a molecular size marker. Positive controls included *K. pneumoniae* ATCC BAA-1705 (for bla KPC), and negative controls used nuclease-free water and *E. coli* ATCC 25922 were included in each run to ensure assay validity.

3.5. Statistical Analysis

Statistical analysis was conducted using SPSS software (version 25, Co Ltd, Tokyo, Japan). Continuous variables were presented as mean ± standard deviation, while nominal and categorical variables were expressed as frequency percentages. The Pearson chi-square test was used to compare qualitative variables between groups. Results were deemed statistically significant if P-values were ≤ 0.05.

4. Results

In this study, a total of 43 *K. pneumoniae* isolates were collected from urine samples of inpatients (n = 37, 86.04%) and outpatients (n = 6, 14.3%) who were referred to Milad Hospital, Tehran. Samples were isolated from females (n = 20, 46.5%) and males (n = 23, 53.5%), with no significant differences found regarding gender and antimicrobial resistance. The age distribution of patients was 19 - 40 years (n = 6, 13.9%), 41 - 60 years (n = 8, 18.6%), and 61 - 80 years (n = 28, 65.1%). A significant difference was observed between age groups and antimicrobial resistance (P = 0.03).

Based on our results, more than 90% of *K. pneumoniae* isolates were phenotypically confirmed as carbapenemase producers, with 93% and 90.3% resistance to MEM and IMP, respectively. Carbapenemase genes were detected in 92.7% of isolates using PCR. The most common genes identified were bla KPC (69.8%), followed by bla IMP (25.6%), and bla VIM (8%). No bla NDM or bla OXA-48 genes were detected. Moreover, the co-existence of genes was observed in combinations of bla IMP with bla VIM (4.6%), bla IMP and bla KPC (11.6%), and bla IMP, bla KPC, and bla VIM (2.3%). More than 80% of *K. pneumoniae* isolates were determined to be MDR/XDR.

As shown in Table 1, the highest level of resistance was observed against ciprofloxacin (100%), followed by ceftazidime (97.7%), cefotaxime (97.7%), cefoxitin (86%), and gentamicin (88.4%). Additionally, 92.9% (26/28) of all *K. pneumoniae* isolates were resistant to colistin. Except for one case, the results of the two phenotypic methods (chromogenic agar and CBDE) were consistent. The mgrB gene was detected in 93% (n = 40) of bacterial isolates. Among the IMP and MEM-resistant *K. pneumoniae*, 97.5% and 100% were also resistant to colistin (Table 2).

Significant differences were observed between age groups and antimicrobial resistance (P = 0.03). In Table 2, associations between fosfomycin susceptibility and MEM resistance (P = 0.04) and bla KPC presence (P = 0.05) were noted. The rate of sensitivity to fosfomycin was 39.5%. Furthermore, fosfomycin-intermediate resistance was detected among 32.6% of bacterial isolates. Additionally, 62% of carbapenemase-producing *K. pneumoniae* were also resistant to fosfomycin.

5. Discussion

One of the major challenges in healthcare settings is the effective treatment of infections caused by MDR and XDR *K. pneumoniae* (14, 15). This has dramatically

Table 1. Antimicrobial Susceptibility Results of 43 *Klebsiella pneumoniae* [a]

Antimicrobial Agents	Number of Isolates		
	Resistance	Sensitive	Intermediate
IMP	39 (90.7)	3 (7)	1 (2.3)
MEM	40 (93)	3 (7)	0
TZP	40 (93)	2 (4.7)	1 (2.3)
CAZ	42 (97.7)	1 (2.3)	0
FOX	37 (86)	4 (9.3)	2 (4.7)
CTX	42 (97.7)	1 (2.3)	0
SXT	36 (83.7)	3 (7)	4 (9.3)
GM	38 (88.4)	5 (11.6)	0
AN	18 (41.9)	15 (34.9)	10 (23.3)
CIP	43 (100)	0	0
CL	25 (58.1)	18 (41.9)	0
Colistin	42 (97.7)	1 (2.3)	0
FOS	12(27.9)	17 (39.5)	14 (32.6)

Abbreviations: IMP, imipenem; CL, clindamycin; TZP, piperacillin-tazobactam; GM, gentamycin; CAZ, ceftazidime; FOX, cefoxitin; SXT, trimethoprim-sulfamethoxazole; CTX, cefotaxime; CIP, ciprofloxacin; MEM: meropenem; AN, amikacin; FOS, fosfomycin.

[a] Values are expressed as No. (%).

Table 2. Susceptibilities of Different Antibiotics Against *Klebsiella pneumoniae* Isolates [a]

Antibiotic		Fosfomycin Statistics			P-Value
		Resistant (n = 9)	Susceptible (n = 18)	Intermediate (n = 16)	
IMP					0. 08
	Resistance	8 (88.9)	15 (83.3)	16 (100)	
	Sensitive	1 (11.1)	2 (11.1)	0 (0.0)	
	Intermediate	0 (0.0)	1 (5.5)	0 (0.0)	
MEM					0.04
	Resistance	9 (100)	15 (83.3)	16 (100)	
	Sensitive	0 (0.0)	3 (16.6)	0 (0.0)	
TZP					0.73
	Resistance	9 (100)	16 (88.9)	15 (93.75)	
	Sensitive	0 (0.0)	2 (11.1)	1 (5.5)	
CAZ					0.49
	Resistance	9 (100)	17 (94.4)	16 (100)	
	Sensitive	0 (0.0)	1 (5.5)	0 (0.0)	
FOX					0.09
	Resistance	8 (88.9)	13 (72.2)	16 (100)	
	Sensitive	0 (0.0)	4 (22.2)	0 (0.0)	
	Intermediate	1 (11.1)	1 (5.5)	0 (0.0)	
CTX					0.49
	Resistance	9 (100)	17 (94.4)	16 (100)	
	Sensitive	0 (0.0)	1 (5.5)	0 (0.0)	
SXT					0.8
	Resistance	8 (88.9)	15 (83.3)	13 (81.25)	
	Sensitive	0 (0.0)	2 (11.1)	1 (5.5)	
	Intermediate	1 (11.1)	1 (5.5)	2 (12.5)	
GEN					0.16
	Resistance	9 (100)	14 (77.7)	15 (93.75)	
	Sensitive	0 (0.0)	4 (22.2)	1 (5.5)	
CL					0.16
	Resistance	6 (66.6)	7 (38.8)	11 (68.75)	
	Sensitive	3 (33.3)	11 (61.1)	5 (31.25)	
AMS					0.57
	Resistance	9 (100)	16 (88.8)	16 (100)	
	Sensitive	0 (0.0)	1 (5.5)	0 (0.0)	
	Intermediate	0 (0.0)	1 (5.5)	0 (0.0)	
KPC					0.05
	Positive	7 (77.7)	9 (50)	14 (87.5)	
	Negative	2 (22.2)	9 (50)	2 (12.5)	
Colistin					0.32
	Resistance	9 (100)	16 (88.8)	14(87.5)	
	Sensitive	0 (0.0)	2 (11.1)	2 (12.5)	
AN					
	Resistance	6 (66.6)	7 (38.8)	5 (31.25)	
	Sensitive	1 (11.1)	6 (33.33)	8 (50)	
	Intermediate	2 (22.2)	5 (27.7)	3 (18.75)	

Abbreviations: IMP, imipenem; CL, clindamycin; TZP, piperacillin-tazobactam; GM, gentamycin; CAZ, ceftazidime; FOX, cefoxitin; SXT, trimethoprim-sulfamethoxazole; CTX, cefotaxime; CIP, ciprofloxacin; MEM: meropenem; AN, amikacin; FOS, fosfomycin; GEN, gentamycin.

[a] Values are expressed as No. (%).

increased mortality rates, particularly among elderly and immunocompromised individuals (16-18). In recent years, the use of older antibiotics such as fosfomycin and colistin has been proposed as an alternative for

treating UTI infections caused by XDR *K. pneumoniae*. In the present study, the highest percentage of *K. pneumoniae* isolates were from urine samples of patients aged 61 - 80 years (65.1%). The isolates exhibited exceptionally high resistance to carbapenems (IMP: 90.7%; MEM: 93%), consistent with prior Iranian studies reporting carbapenem resistance rates exceeding 70% (19, 20). We also found that the vast majority (83%) of *K. pneumoniae* isolates were classified as MDR/XDR. In this regard, we determined a high prevalence of resistance to various groups of antibiotics, including third-generation cephalosporins [ceftazidime (97.7%), cefoxitin (86%), and cefotaxime 97.7%)], aminoglycosides [gentamicin (88.4%)], penicillin and beta-lactamase inhibitors [piperacillin-tazobactam (93%)], trimethoprim-sulfamethoxazole (83.7%), and fluoroquinolones [ciprofloxacin (100%)]. Additionally, a very low number of *K. pneumoniae* isolates (7.1%) were sensitive to colistin, suggesting that colistin is not an effective drug for empiric treatment of *K. pneumoniae* infections. The relatively high rates of drug-resistant MDR/XDR *K. pneumoniae* observed in different studies in Iran (19-21) might be due to several factors, including the widespread use of broad-spectrum antibiotics in healthcare settings for empiric treatment of infections, prolonged antimicrobial therapy, and unnecessary antibacterial prescriptions.

We identified a high rate of resistance to colistin (93%) using the CBDE and chromogenic agar methods. The CBDE, as a simple and low-cost phenotypic method, can be used as a reference method in laboratories. Additionally, the results of molecular tests for detection of mgrB showed the role of chromosomal genes in colistin resistance. This aligns with Iranian studies attributing colistin resistance to the overuse of last-line antibiotics and clonal dissemination of resistant strains. The high concordance between phenotypic methods (CBDE and chromogenic agar) and the mgrB detection rate (93%) suggests that chromosomal mutations, rather than plasmid-mediated mechanisms, dominate colistin resistance in these isolates, a pattern observed in other Iranian studies (21).

Based on our findings, clindamycin and fosfomycin seem to be the optimal choices against KPC-KP (Table 1). In recent decades, fosfomycin-tromethamine has been introduced as a potential alternative therapy for chronic bacterial UTIs caused by MDR Enterobacteriaceae (22). Other studies have shown the efficacy of fosfomycin ranging from 39% to 100% on carbapenemase-producing strains of *K. pneumoniae* (23, 24). For example, Endimiani et al. (18) assessed the in vitro effectiveness of fosfomycin against 68 bla KPC-possessing *Klebsiella pneumoniae* (KpKPC) isolates, including 23 strains that were not susceptible to tigecycline and/or colistin. Their findings revealed that 93% of the overall KpKPC isolates were susceptible to fosfomycin (18). Although the rate of susceptibility to fosfomycin was lower in our KpKPC isolates, the relatively high prevalence of fosfomycin-intermediate KpKPC (32.6%) in the current study is concerning and should be taken into consideration. It is proposed that prolonged and intensive use of antibiotics in healthcare settings can lead to the spread of resistance to fosfomycin via mobile elements and resistance genes (25, 26). Adjusting the dosage of the medication and combination therapy could help maintain the effectiveness of fosfomycin in the treatment of urinary infections caused by KpKPC.

The dominance of bla KPC (69.8%) contrasts with studies from other parts of Iran and South Asia, where bla NDM and bla OXA-48 are more prevalent. For instance, Hashemizadeh et al. (21) reported bla NDM as the predominant carbapenemase in southwestern Iran, while bla KPC is more common in the United States and Greece. The absence of bla NDM and bla OXA-48 in this cohort may reflect localized clonal spread or hospital-specific antibiotic pressure. Notably, the co-occurrence of bla IMP with bla KPC and bla VIM (11.6% and 4.6%, respectively) suggests horizontal gene transfer, a phenomenon increasingly reported in high-resistance settings.

5.1. Limitations

Our study has several limitations. It was conducted in only one hospital (Milad Hospital, Tehran), which limits the generalizability of the findings to other hospitals and regions within Iran or globally. The relatively small sample size may not fully capture the diversity and resistance patterns of *K. pneumoniae* in the broader population. All isolates were obtained exclusively from urine samples, and the study does not include isolates from other clinically relevant sources (e.g., blood, respiratory tract, wounds), potentially overlooking differences in resistance profiles from other infection sites. Additionally, the study did not track prior antibiotic use, which may have influenced culture positivity and resistance profiles.

This study showed a high level of antibiotic resistance in *K. pneumoniae* to different classes of

antibiotics, including older antibiotics such as colistin. Additionally, the relatively high level of fosfomycin-intermediate carbapenemase-resistant *K. pneumoniae* is concerning. It is proposed that the use of fosfomycin as an alternative drug should be in combination with another appropriate antibiotic. Moreover, there is an urgent need for heightened awareness among physicians and microbiologists, active infection control committees, appropriate antimicrobial treatment, improvement of health status, and surveillance of drug-resistant isolates to better control the emergence and spread of pan-drug-resistant isolates of *K. pneumoniae* in hospitals.

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Footnotes

Authors' Contribution: P. E. conducted the experiments. M. R. served as the advisor. F. P. edited the manuscript. L. A. collected the samples. L. G. analyzed the data, wrote the manuscript, and coordinated with other members.

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