Доклади на Българската академия на науките
Comptes rendus de l'Académie bulgare des Sciences

Tome 77, No 3, 2024

MEDICINE

Microbiology

INVESTIGATION OF FOSFOMYCIN RESISTANCE AND PLASMID-MEDIATED FOSFOMYCIN RESISTANCE GENES IN *ENTEROBACTERALES* ISOLATES

Kubra Hacieminoğlu Ulker✉, Yeliz Tanriverdi Cayci, Asuman Birinci

Received on September 12, 2023
Presented by B. Petrunov, Member of BAS, on January 30, 2024

Abstract

The study aimed to investigate the fosfomycin resistance in *Enterobacterales* isolates and the presence of plasmid-mediated fosfomycin resistance genes *fos*A3 and *fos*C2 in fosfomycin-resistant *Enterobacterales* isolates. A total of 2095 *Enterobacterales* isolates obtained from urine samples were included in the study. Identification of isolates was performed in the Vitek MS. The antimicrobial susceptibility of the isolates and detection of ESBL was determined using the Vitek 2 Compact. In 185 fosfomycin-resistant isolates, *fos*A3 and *fos*C2 genes were investigated by PCR. In addition, carbapenemase genes were investigated in carbapenem-resistant isolates. In 2095 *Enterobacterales*, 65.8% of the isolates were *E. coli*, and fosfomycin resistance was determined as 22%. ESBL production was defined as 36.5%, 58.1%, and 32.5% in all isolates, *K. pneumoniae*, and *E. coli* isolates, respectively. *K. pneumoniae* (56.2%) were the most frequently isolated bacteria among 185 fosfomycin-resistant *Enterobacterales* isolates. In these isolates, 66.95% were ESBL-producing *Enterobacterales*, and 35.7% were carbapenem-resistant isolates. According to the PCR results, two of the 185 *Enterobacterales* isolates were positive for the *fos*A3 gene, *fos*C2 gene was not detected. According to PCR for carbapenemase genes results with these isolates, $bla_{\mathrm{OXA-48}}$ positivity was 66.7%, and bla_{NDM} positivity was 3%. bla_{KPC} and bla_{VIM} positivity could not be detected. In our study, the prevalence of *fos*A3 was 1.1%. The occurrence of *fos*A3 gene

This study was prepared as a scientific research project and supported by a grant from the project management of Ondokuz Mayıs University. Project number: PYO.TIP.1904.17.008.

https://doi.org/10.7546/CRABS.2024.03.12

positivity in *Enterobacterales* isolates is important for the future treatment of UTIs.

Key words: *Enterobacterales*, fosfomycin, plasmid-mediated resistance

Introduction. Urinary tract infections (UTIs) are among the most common infections worldwide. Due to their recurring nature, these infections are a critical morbidity and mortality factor, especially for hospitalized patients [1]. *Enterobacterales* isolates are among the most common causes of uncomplicated and complicated UTIs. Increased antimicrobial resistance is a worldwide concern in these isolates, particularly associated with broad-spectrum β-lactamase (ESBL) and carbapenemase production. The agents to treat resistant *Enterobacterales* isolates are limited, so old antibiotics such as fosfomycin have gained importance [2].

In 1969, fosfomycin was discovered in Spain and isolated from *Streptomyces* species. Fosfomycin is an alternative antibiotic preferred for treating UTIs because it offers the advantage of a single dose, low side effects, and a low resistance rate to *Enterobacterales* isolates [3]. It binds to the 115th cysteine residue of the MurA enzyme, prevents the formation of uridine diphosphate–N-acetylmuramic acid, and inhibits the synthesis of a peptidoglycan layer. Fosfomycin resistance mechanisms are either chromosomal or plasmid-mediated [4]. Chromosomal-mediated fosfomycin resistance is caused by modification of the MurA or mutations in the transporter systems. On the other hand, plasmid-mediated fosfomycin resistance occurs with fosfomycin-modifying enzymes (*fos* genes). *fos* genes catalyze the opening of the fosfomycin epoxide ring, and the drug is inactivated. *fos*A subtypes and *fos*C2 are the most common causes of plasmid-mediated fosfomycin resistance in *Enterobacterales* isolates [5].

In our study, we investigated fosfomycin resistance rates and plasmid-mediated fosfomycin resistance genes *fos*A3 and *fos*C2 in the *Enterobacterales* isolates. In addition, ESBL production and presence of carbapenem resistance were evaluated in these isolates.

Materials and methods. ***Bacterial identification and susceptibility testing.*** A total of 2095 *Enterobacterales* isolates obtained from the midstream urine samples of hospitalized patients with bacterial growth greater than 10^5 CFU/ml were included in the study. Isolates were consecutively collected in Ondokuz Mayıs University Medical Faculty Hospital. Only one isolate per patient was included in the study.

Vitek MS automated system (bioMérieux, France) was used for bacterial identification. The antimicrobial susceptibility of the isolates and detection of ESBL was determined using the Vitek 2 Compact system (bioMérieux, France). Fosfomycin susceptibility was re-studied by disk diffusion method in 185 *Enterobacterales* isolates randomly selected from fosfomycin-resistant isolates.

We investigated the fosfomycin resistance genes *fos*A3 and *fos*C2 in these 185 fosfomycin-resistant isolates. In addition, the presence of carbapenem resistance

genes $bla_{\text{OXA}-48}$, bla_{NDM}, bla_{KPC}, and bla_{VIM} were investigated in these isolates. DNA extraction of isolates was performed by boiling method. The obtained DNAs were stored at $-20\,^{\circ}\text{C}$ until used as template DNA for PCR.

Detection of fosfomycin and carbapenem resistance genes. *fos*A3 and *fos*C2 genes were investigated by PCR in 185 randomly selected fosfomycin-resistant *Enterobacterales*. As described previously, fosfomycin resistance genes were amplified with the specific primers. The primers used were determined after a literature review [6, 7].

Isolates resistant to at least one of ertapenem, imipenem, and meropenem were considered carbapenem-resistant. For carbapenem-resistant isolates, $bla_{\text{OXA}-48}$, bla_{NDM}, bla_{KPC}, and bla_{VIM} were amplified with specific primers as previously described [8].

Statistical analysis. Statistical analyses were performed using IBM-SPSS-Statistics V22.0. The differences between bacterial species and fosfomycin resistance, bacterial species and ESBL positivity, and fosfomycin resistance and ESBL positivity were analyzed using the Chi-square test. $p < 0.05$ was considered statistically significant for all analyses.

Ethical aspect. Ethical approval was obtained from the Clinical Research Ethics Committee of Ondokuz Mayis University (OMU-KAEK: 2016/333, Date: 27/10/2016).

Results. Of the 2095 isolates included in the study, 65.8% were *E. coli*, and 20.5% were *K. pneumoniae*. The distribution of all isolates is shown in Table 1.

Table 1

Distribution of *Enterobacterales* isolated according to species

Species	n%	Fosfomycin		ESBL	
		Susceptible n%	Resistant n%	Negative n%	Positive n%
Escherichia coli	1380 (65.8)	1306 (94.6)	74 (5.4)	931 (67.5)	449 (32.5)
Klebsiella pneumoniae	430 (20.5)	201 (46.75)	229 (53.25)	180 (41.9)	250 (58.1)
Proteus mirabilis	78 (3.7)	51 (65.4)	27 (34.6)	57 (73.1)	21 (26.9)
Enterobacter cloacae	56 (2.7)	14 (25)	42 (75)	49 (87.5)	7 (12.5)
Morganella morganii	50 (2.4)	–	50 (100)	45 (90)	5 (10)
Klebsiella aerogenes	40 (1.9)	26 (65)	14 (35)	17 (42.5)	23 (57.5)
Klebsiella oxytoca	27 (1.3)	18 (66.7)	9 (33.3)	18 (66.7)	9 (33.3)
Providencia rettgeri	10 (0.5)	–	10 (100)	9 (90)	1 (10)
Citrobacter spp.	16 (0.8)	14 (87.5)	2 (12.5)	10 (62.5)	6 (37.5)
Serratia marcescens	5 (0.2)	2 (40)	3 (60)	3 (60)	2 (40)
Proteus vulgaris	2 (0.1)	2 (100)	–	1 (50)	1 (50)
Hafnia alvei	1 (0.05)	–	1(100)	1 (100)	–
Total	2095 (100)	1634 (78)	461 (22)	1321 (63.05)	774 (36.5)

$p < 0.05$

Table 2

The association between fosfomycin resistance and ESBL production

	Fosfomycin		Total, n%
	Susceptible, n%	Resistant, n%	
ESBL Negative	1049 (79.4)	272 (20.6)	1321 (100)
ESBL Positive	585 (75.6)	189 (24.4)	774 (100)
Total	1634 (78)	461 (22)	2095 (100)

$p < 0.05$

The rate of ESBL-producing *Enterobacterales* isolates was determined as 36.5% in our study. ESBL production was determined as 58.1% in *K. pneumoniae*, 57.5% in *Klebsiella aerogenes*, and 32.5% in *E. coli*. ESBL production was higher in *K. pneumoniae* isolates compared to *E. coli* isolates. This result was statistically significant ($p < 0.05$). Fosfomycin resistance in all isolates was 22% (461/2095). Fosfomycin resistance in *Morganella morganii*, *Providencia rettgeri*, *K. pneumoniae*, and *E. coli* isolates was 100%, 100%, 53.25%, and 5.4%, respectively (Table 1). The association between fosfomycin resistance and ESBL production is shown in Table 2. ESBL-positive isolates had higher fosfomycin resistance compared to ESBL-negative isolates. This result was statistically significant ($p < 0.05$).

Of the 461 fosfomycin-resistant isolates, 49.7% were *K. pneumoniae*, and 16.05% were *E. coli*. Fosfomycin resistance genes were investigated in 185 randomly selected isolates from these isolates. Of these 185 isolates, 66.95% were ESBL-producing *Enterobacterales*. ESBL production in *K. pneumoniae* and *E.*

Table 3

Distribution of 185 fosfomycin-resistant *Enterobacterales* isolated according to species

Species	n%	ESBL		Carbapenem Resistance* n%
		Negative n%	Positive n%	
Klebsiella pneumoniae	104 (56.2)	21 (20.2)	83 (79.8)	58 (55.7)
Escherichia coli	22 (11.9)	7 (31.8)	15 (68.2)	2 (9.1)
Enterobacter cloacae	22 (11.9)	12 (54.5)	10 (45.5)	2 (9.1)
Morganella morganii	16 (8.6)	10 (62.5)	6 (37.5)	–
Klebsiella aerogenes	6 (3.2)	3 (50)	3 (50)	–
Providencia rettgeri	6 (3.2)	2 (33.3)	4 (66.7)	3 (50)
Proteus mirabilis	5 (2.7)	5 (100)	–	–
Klebsiella oxytoca	4 (2.2)	3 (75)	1 (25)	1 (25)
Total	185 (100)	63 (34.05)	122 (66.95)	66 (35.7)

*Isolates resistant to at least one of ertapenem, imipenem, and meropenem were considered carbapenem resistant $p<0.05$.

coli was determined as 79.8% and 68.2%, respectively. According to the antibiotic susceptibility test results, 35.7% (66/185) of these isolates were carbapenem-resistant. The species distribution, ESBL production, and carbapenem resistance rates of 185 isolates are shown in Table 3.

According to the results of PCR of 185 fosfomycin-resistant isolates, two isolates (1.1%) that formed the band in the 234 bp region were suspected to be *fos*A3 gene positive, and *fos*C2 gene was not detected. Sequence analysis was performed with isolates that were suspiciously positive for *fos*A3. According to the sequence analysis, two isolates were determined as *fos*A3-positive. These isolates were *Morganella morganii* and *E. coli*. The *fos*A3-positive *M. morganii* was a non-ESBL-producing and carbapenem-susceptible isolate. The *fos*A3-positive *E. coli* was an ESBL-producing and carbapenem-susceptible isolate.

According to PCR results of carbapenem genes, 66.7% (44/66) of the isolates were $bla_{\mathrm{OXA-48}}$ positive, 3% (2/66) were bla_{NDM} positive, and all isolates were bla_{KPC}, and bla_{VIM} negative. Both of the *fos*A3 positive isolates were carbapenem susceptible. Therefore we did not investigate carbapenem resistance genes in these isolates.

Discussion. Antibiotic resistance in bacterial infections is a global public health problem. ESBL-producing isolates and carbapenem-resistant *Enterobacterales* (CRE) are among the leading causes of antimicrobial resistance. These isolates are associated with complicated infections and high morbidity and mortality. Fosfomycin is an old antibiotic for uncomplicated UTIs used by ESBL-producing *Enterobacterales*. Due to the increasing prevalence of ESBL-producing *Enterobacterales* and CRE, the frequency of fosfomycin resistance should be followed up for the treatment of urinary tract infections [9]. Our study aimed to determine the susceptibility of fosfomycin and investigate the presence of fosfomycin resistance genes, especially in ESBL-producing and non-ESBL-producing *Enterobacterales* strains.

UTIs are the most common human bacterial infections, affecting approximately 150 million people per year. In most studies with urinary system samples conducted more than 50% of the bacteria isolated were reported to be *E. coli* [10]. Similarly, 65.8% of 2095 isolates isolated from urine samples were *E. coli* in our study.

Demonstrating the presence of ESBL in Gram-negative enteric bacteria that cause UTI is critical in determining and guiding the treatment strategy. Studies indicate a high risk of developing resistance to trimethoprim/sulfamethoxazole, ciprofloxacin, aminoglycosides, and tetracycline, along with beta-lactams in urinary tract infections caused by ESBL-producing *E. coli* and *K. pneumoniae* [11]. In our study, it was noteworthy that the ESBL production rate was more than 50%, especially in *Klebsiella spp.* isolates.

Fosfomycin, an alternative treatment for many pathogens, such as MDR microorganisms, has been used for years, but the incidence of resistance has re-

mained constant for a long time. However, the increase in resistance in recent years is particularly significant in the treatment of MDR UTIs [5]. Recent studies have reported fosfomycin resistance in *Enterobacterales* isolates in 11.4–67.35% [12–14]. The fosfomycin resistance rates differ according to the characteristics of the isolates. Fosfomycin resistance is higher in East Asian studies conducted with specific isolates such as ESBL or KPC-producing *Enterobacterales*, CRE, and MDR *Enterobacterales* [9]. Our study found that fosfomycin resistance was 22% in *Enterobacterales*. We studied randomly selected fosfomycin-resistant isolates, which were not specific. Therefore, a lower rate of fosfomycin resistance may have been observed compared to East Asian studies.

Fosfomycin resistance was determined as 53.25% in *K. pneumoniae* isolates and 5.4% in *E. coli* isolates. In addition, fosfomycin resistance was 24.4% and 20.6% higher in ESBL-positive isolates than in ESBL-negative isolates, respectively. This difference between isolates was statistically significant ($p < 0.05$). According to the susceptibility results of 185 fosfomycin-resistant *Enterobacterales*, the highest ESBL positivity, and the highest carbapenem resistance was seen in *K. pneumoniae* isolates ($p < 0.05$). Considering all the results, it is seen that although *E. coli* is the most frequently isolated bacterium from urine samples, fosfomycin and carbapenem resistance is higher in *K. pneumoniae* isolates. In addition, it was determined that ESBL-producing isolates were more resistant to fosfomycin than non-ESBL-producing isolates. The reason for these results may be similar to the factors that cause antibiotic resistance and ESBL production.

In the last 15 years, studies on fosfomycin-resistant *Enterobacterales* isolates have identified the *fos*A3 gene as the most frequently isolated *fos*A subtype. In fosfomycin-resistant *Enterobacterales* isolates, *fos*A3 positivity ranged from 1.1% to 89.5% [6,7,15]. In our study, 1.1% of fosfomycin-resistant isolates were positive for *fos*A3. Comparing the results of our study with those of East Asian countries, we find that the proportion of *fos*A3 gene positivity is low. This difference could be because, in East Asian countries, livestock and domestic animals have become reservoirs for *Enterobacterales* isolates carrying the *fos*A3 gene. In East Asian countries, dietary habits and meat products sold on the street may facilitate the transmission of plasmids carrying the *fos*A3 gene between domestic animals and humans and accelerate the spread of resistance. Previous studies reported that the primary mechanism of fosfomycin resistance in KPC-producing *K. pneumoniae* and ESBL-producing *E. coli* isolates might be the *fos*A3 gene [16, 17]. However, in our study, no significant association was found with the *fos*A3 gene, neither carbapenem resistance genes nor ESBL production. The reason could be that only two *fos*A3 gene-positive isolates were determined in our study.

In *Enterobacterales* isolates, another important gene for plasmid-mediated fosfomycin resistance is the *fos*C2. Wachino et al. [18] found 14.3% *fos*C2 positivity in their study with ESBL-producing *E. coli* isolates, and Salman et al. [19] found 72.3% *fos*C2 positivity in their study with MDR gram negative

isolates. In our study, there were no positive isolates for the *fos*C2 gene as in the studies of Zhang et al. [17], and Bahy et al. [20].

In our study, fosfomycin resistance was compatible with studies conducted in our country. It was determined that fosfomycin resistance was higher in *K. pneumoniae* and ESBL-producing *Enterobacterales* isolates. This result was expected. The rate of *fos*A3 positivity was 1.1%, the rate of $bla_{\mathrm{OXA-48}}$ positivity was 66.7%, and the rate of bla_{NDM} positivity was 3%. The occurrence of *fos*A3 gene positivity in *Enterobacterales* isolates is important for the future treatment of UTIs. The analysis reported in this study was performed on samples collected between 2015 and 2016 and did not reflect current fosfomycin resistance. Therefore, further studies are needed to determine current fosfomycin resistance in *Enterobacterales* isolates in Turkey.

REFERENCES

[1] Gajdács M., M. Ábrók, A. Lázár, K. Burián (2020) Increasing relevance of Gram-positive cocci in urinary tract infections: a 10-year analysis of their prevalence and resistance trends, Sci. Rep., **10**(1), 17658.

[2] Farfour E., L. Dortet, T. Guillard et al. (2022) Antimicrobial Resistance in Enterobacterales Recovered from Urinary Tract Infections in France, Pathogens, **11**(3), 356.

[3] Falagas M. E., E. K. Vouloumanou, A. G. Togias et al. (2010) Fosfomycin versus other antibiotics for the treatment of cystitis: a meta-analysis of randomized controlled trials, J. Antimicrob. Chemother., **65**(9), 1862–1877.

[4] Falagas M. E., K. P. Giannopoulou, G. N. Kokolakis, P. I. Rafailidis (2008) Fosfomycin: use beyond urinary tract and gastrointestinal infections, Clin. Infect. Dis., **46**(7), 1069–1077.

[5] Zurfluh K., A. Treier, K. Schmitt, R. Stephan (2020) Mobile fosfomycin resistance genes in Enterobacteriaceae – An increasing threat, Microbiology Open, **9**(12), e1135.

[6] Ho P. L., J. Chan, W. U. Lo et al. (2013) Prevalence and molecular epidemiology of plasmid-mediated fosfomycin resistance genes among blood and urinary *Escherichia coli* isolates, J. Med. Microbiol., **62**(11), 1707–1713.

[7] Hou J., X. Yang, Z. Zeng et al. (2012) Detection of the plasmid-encoded fosfomycin resistance gene fosA3 in *Escherichia coli* of food-animal origin, J. Antimicrob. Chemother., **68**(4), 766–770.

[8] Doyle D., G. Peirano, C. Lascols et al. (2012) Laboratory detection of Enterobacteriaceae that produce carbapenemases, J. Clin. Microbiol., **50**(12), 3877–3880.

[9] Yang T. Y., P. L. Lu, S. P. Tseng (2019) Update on fosfomycin-modified genes in Enterobacteriaceae, J. Microbiol. Immunol. Infect., **52**(1), 9–21.

[10] Rafalskiy V., D. Pushkar, S. Yakovlev et al. (2020) Distribution and antibiotic resistance profile of key Gram-negative bacteria that cause community-onset urinary tract infections in the Russian Federation: Resource Multicentre Surveillance 2017 Study, J. Glob. Antimicrob. Resist., **21**, 188–194.

[11] Atmaca S., S. Yakut, N. Özcan et al. (2012) ESBL Positive and Negative *Escherichia coli* and *Klebsiella* spp. Resistant to at least One of the Carbapenems. Phosphomycin Susceptibility in Strains, Ankem Magazine, **32**(3), 87–93.
[12] Aprile A., G. Scalia, S. Stefani et al. (2020) In vitro fosfomycin study on concordance of susceptibility testing methods against ESBL and carbapenem-resistant Enterobacteriaceae, J. Glob. Antimicrob. Resist., **23**, 286–289.
[13] Peretz A., B. Naamneh, L. Tkhawkho et al. (2019) High Rates of Fosfomycin Resistance in Gram-Negative Urinary Isolates from Israel, Microb. Drug Resist., **25**(3), 408–412.
[14] Süzük-Yildiz S., B. Kaşkatepe, H. Şimşek et al. (2019) High rate of colistin and fosfomycin resistance among carbapenemase-producing Enterobacteriaceae in Turkey, Acta Microbiol. Immunol. Hung., **66**(1), 103–112.
[15] Cao X. L., H. Shen, Y. Y. Xu et al. (2017) High prevalence of fosfomycin resistance gene fosA3 in blaCTX-M-harbouring *Escherichia coli* from urine in a Chinese tertiary hospital during 2010–2014, Epidemiol. Infect., **145**, 818–824.
[16] Huang L., M. Cao, Y. Hu et al. (2021) Prevalence and mechanisms of fosfomycin resistance among KPC-producing *Klebsiella pneumoniae* clinical isolates in China, Int. J. Antimicrob. Agents, **57**(1), 106226.
[17] Zhang W., M. Ma, Y. Cheng et al. (2022) Spread and Molecular Characteristics of *Enterobacteriaceae* Carrying *fosA*-Like Genes from Farms in China, Microbiol. Spectr., **10**(4), e00545–22.
[18] Wachino J., K. Yamane, S. Suzuki et al. (2010) Prevalence of fosfomycin resistance among CTX-M-producing *Escherichia coli* clinical isolates in Japan and identification of novel plasmid-mediated fosfomycin-modifying enzymes, Antimicrob. Agents Chemother., **54**, 3061–3064.
[19] Salman N. A. M., M. F. Mohamed, W. A. Abu Elwafa et al. (2023) Isolation of Gram-Negative Organisms Causing Nosocomial Catheter Associated Urinary Tract Infection and Detection of Fosfomycin Effect on Multi-Drug Resistant Strains in Sohag University Hospital, Egypt. J. Med. Microbiol., **32**(3), 99–108.
[20] Bahy R., W. Abu El-Wafa, A. Abouwarda (2023) Molecular Mechanisms of Fosfomycin Resistance in MDR *Escherichia coli* Isolates from Urinary Tract Infections, Egypt. J. Med. Microbiol., **32**(2), 25–29.

Department of Medical Microbiology, Ondokuz Mayıs University,
Samsun/TURKEY, 55200
e-mails: kubra.hacieminoglu@gmail.com, yeliztanriverdi@gmail.com,
asumanbirinci@yahoo.com