ORIGINAL ARTICLE

Molecular Mechanisms of Fosfomycin Resistance in MDR *Escherichia coli* Isolates from Urinary Tract Infections

[1]RehabBahy*, [2]Wael M. Abu El-Wafa, [2]Ahmed M. Abouwarda

[1]Department of Microbiology and Immunology, Faculty of Pharmacy, Fayoum University, Fayoum, Egypt
[2]Department of Microbiology, General Division of Basic Medical Sciences, Egyptian Drug Authority (EDA), Formerly National Organization for Drug Control and Research (NODCAR)

*Corresponding Author:
Rehab Bahy

Tel: +201144738122
rhb11@fayoum.edu.eg
ORCID ID: 0000-0002-2868-928X

ABSTRACT

Background: *Escherichia coli is one of the most common pathogens in nosocomial and community-acquired infections in humans. Fosfomycin, a broad-spectrum antibiotic, prevents the production of peptidoglycan, which is necessary for developing bacterial cell walls. Urinary Tract Infections (UTIs) remain the most prevalent infectious diseases in hospital and community settings. In Egypt, E. coli is the most frequent microbe involved in UTIs. Unfortunately, Egypt has comparatively higher levels of fosfomycin resistance among Uropathogenic E. coli (UPEC) strains.* **Objective:** *we investigated the molecular mechanisms of fosfomycin resistance in seven previously reported MDR clinical isolates of E. coli.* **Methodology:** *we initially explored the tested isolates for the three plasmid-conjugated genes: fosA3, fosC2, and fosA presence. Isolates that did not show any of the three genes were then examined for mutations in the chromosomal genes: murA, glpT, and uhpT.* **Results:** *confirm that five out of the seven tested isolates were positive for the fosA3gene. No detection for fosC2 or fosA genes in any of these isolates. Mutations in murA and glpT were confirmed in the other two isolates (isolates UPEC 9 and UPEC 27). MurA and GlpT proteins showed new amino acid substitutions.* **Conclusion:** *we herein report that fosfomycin resistance in the tested UPEC isolates were due to unique amino acid changes in MurA or the loss of function of the transporters.*

INTRODUCTION

In hospital and community settings, urinary tract infections continue to be the most prevalent infectious disorders. With chronic and recurrent infections being problematic, these infections are now among the most common infectious diseases around the globe[1]. The frequency of UTI cases among pregnant women in Egypt increased from 22% to 35% in [2] to 42.3% in 2018, and 53.5%in 2019[3]. In addition, full-term neonates had a higher incidence of UTI, 70% [4]. The most prevalent bacteria causing UTI, according to several studies from Egypt, were *E. coli* (30.7-74%), *Staphylococcus aureus* (17.5-21%), and *Klebsiella* spp. (17.5-20.9%)[5]. Among the most important mechanisms of antimicrobial resistance, particularly for UPEC infections, is the production of Extended Spectrum Beta-Lactamases (ESBL). Unfortunately, added to β-lactams, these organisms frequently carry genes for quinolone and aminoglycoside resistance. Therefore, early detection of ESBL-producing bacterial isolates responsible for clinical illness is crucial for effective UTI treatment[6].

Fosfomycin, formerly known as phosphonomycin, has been used to treat uncomplicated urinary UTI since its discovery in the early 1970s. The antibiotic derived from phosphonic acid with an epoxide and a propyl group has a distinct chemical composition, and its mode of action as a broad-spectrum activity against Gram-negative and Gram-positive aerobic bacteria is unrelated to any other antibiotic family [7]. Fosfomycin works as a bactericide by preventing the first phase of peptidoglycan production. It functions as an analogue of phosphoenolpyruvate (PEP) and binds the vital enzyme MurA (UDP-N-acetyl glucosamine enolpyruvyltransferase), thereby causing bacterial cell lysis and death [8].

Older antibiotics like fosfomycin and colistin are being reassessed as potential treatment options against multidrug-resistant (MDR) bacteria, particularly ESBL and carbapenemase-producing Enterobacteriaceae. This includes using fosfomycin for primary urinary tract infections and possibly with other antibiotics[9].

Reduced drug uptake continues to be the most common resistance mechanism among clinical isolates as well as *invitro* obtained mutants among the three main fosfomycin resistance mechanisms that have been described: (a) reduced permeability to fosfomycin, (b) modification of *murA*, and (c) modification in the antibiotic. This chromosomally encoded resistance mechanism in *E. coli* is mainly due to mutations in the glycerol-3-phosphate transporter (GlpT) and glucose-6-

phosphate transporter (UhpT), the two major nutrient transport systems involved in the uptake of fosfomycin. The most frequent methods for bacteria to develop resistance towards antibiotics, generally, is through mutation in the drug target [7].However, it appears that clinical isolates rarely have *murA* alterations. It was demonstrated that over expression of gene *murA* aids in the low-fitness cost development of clinical levels of fosfomycin resistance[10]. Recently, a thorough evaluation of these chromosomal resistance pathways was published [10,8].It appears that chromosome-mediated resistance fosfomycin resistance predominates over plasmid-mediated resistance. However, two new plasmid-mediated fosfomycin-modifying enzymes, FosA3 and FosC2, were discovered in *E. coli* that produced CTX-M β-lactamases. Transferable plasmids that carry *fosA3* or *fosC2* may hasten the global spread of fosfomycin resistance[11].

Genes for fosfomycin-modifying enzymes are typically identified on plasmids, transposons, or within integrons in enterobacteriaceae. No matter where the insertion sequences are detected (chromosomal or plasmid), this indicates that the genes have been mobilized. Since fosfomycin-modifying enzymes are broadly detected in Asia, where nearly all of these genes were initially discovered, these facts allow for quick proliferation. Furthermore, the presence of other resistance genes in plasmids harboring fos-genes increases the likelihood of co-selection of resistance to fosfomycin under the selective pressure of other antibiotics[12].

In the current investigation, we intended to investigate the chromosomal and plasmid-borne fosfomycin resistance genes in the seven MDR *E. coli* isolates reported in our prior research[6].

METHODOLOGY

Bacterial strains:

Seven *E. coli* clinical isolates (UPEC2, UPEC3, UPEC7, UPEC9, UPEC12, UPEC13, and UPEC27) previously reported as fosfomycin-resistant [6] were used in this study. An isolated colony from a recently streaked selective plate was used to inoculate 5 mL of Tryptic Soy Broth (TSB) medium, and tubes were incubated for 24 hours at37°C. Bacterial cells were harvested by centrifugation at 8000 rpm for 3 minutes at room temperature. The supernatants were removed, and the pellets were resuspended in 250 µL of the resuspension solution.

Plasmid Isolation:

Plasmids were isolated using the Thermo Scientific GeneJET Plasmid Miniprep Kit (USA) according to Kit manual. The main principle was to release the plasmid DNA, pelleted bacterial cells are resuspended and subjected to SDS/alkaline lysis. To ensure that plasmid DNA can bind to the silica membrane in the spin column, the resultant lysate is neutralized by neutralizing solution. SDS and cell debris precipitate are centrifugally pelleted, and the plasmid DNA-containing supernatant is then deposited onto the spin column membrane. After being cleaned of any impurities, the adsorbed DNA is eluted using a little amount of the elution buffer (10 mMTris-HCl, pH 8.5)[13].

PCR Amplification:

The presence of the *fosA3*, *fosC2*, and *fosA* genes was detected by PCR according to a published protocol [14]. The PCR primers used to detect the *fos*-genes are listed in Table (1).

Table 1: Oligonucleotide primers used in this study[15,16]

Primer	Sequence	Product Size (bp)
FosA3-F	GCGTCAAGCCTGGCATTT	282
FosA3-R	GCCGTCAGGGTCGAGAAA	
FosC2-F	TGGAGGCTACTTGGATTTG	217
FosC2-R	AGGCTACCGCTATGGATTT	
FosA-F	ATCTGTGGGTCTGCCTGTCGT	271
FosA-R	ATGCCCGCATAGGGCTTCT	
MurA gene-F	AAACAGCAGACGGTCTATGG	1260
MurA gene-R	CCATGAGTTTATCGACAGAACG	
GlpT gene-F	GCGAGTCGCGAGTTTTCATTG	1392
GlpT gene-R	GGCAAATATCCACTGGCACC	
UhpT gene-F	TTTTTGAACGCCCAGACACC	1359
UhpT gene-R	AGTCAGGGGCTATTTGATGG	

PCR amplification performed in a PCR thermocycler (MJ Research, USA) with the following cycling conditions: initial denaturation at 94ºC for 3min followed by 35 cycles of amplification (94ºC for 30s, annealing at 57.5ºC (*fosA3*), 50.5ºC (*fosC2*), and 59.5ºC (*fosA*) for 30 s, and extension at 72ºC for 30s), and a final extension cycle of 10 min at 72ºC.

Nucleotide sequencing of the *murA*, *glpT*, and*uhpT* genes:

Genomic DNA from fosfomycin-resistant isolates that were found negative for *fosA3*, *fosC2*, and *fosA* genes were prepared using the boiling method. A mixture of 2-3 colonies of *E. coli* was incubated at 100ºC for 8 minutes after adding 250μl of 1x TE buffer. Cells were

then recovered by centrifugation twice at 8000 rpm for 10 minutes. The supernatants were then used as DNA templates in PCR [17]. PCR was used to amplify the full genes' lengths of the genes *murA*, *glpT*, and *uhpT* using their corresponding genomic DNA and the primer pairs listed in Table (1). The *murA*, *glpT*, and *uhpT* genes were amplified as follows: denaturation at 94 °C for 2 minutes, followed by 30 cycles of denaturation at 94 °C for 30 seconds, annealing at 55 °C for 30 seconds, and extension at 72°C for 2 minutes[15].

Purification of the PCR products:

The amplified PCR fragments were purified using the GeneJET Purification kit™ in accordance with the manufacturers' recommendations. Briefly, the binding buffer is added to a reaction mixture containing DNA before the mixture is put to a purification column. Proteins are denatured by a complexing substance in the binding buffer, which also facilitates DNA binding to the silica membrane in the column. The binding is an added convenience. Buffer has a colour indication that makes it simple to check the pH of the solution for optimum DNA binding. A quick wash process gets rid of impurities. The elution buffer is then used to completely remove the purified DNA from the column. The DNA has been recovered and is prepared for use in later applications[14]

Sequencing of tested genes:

The purified PCR products of the *murA*, *glpT*, and *uhpT* genes were sequenced at the GATC Company using the ABI3730xl DNA sequencer and the corresponding forward and reverse primers (Table 1).It was performed by combining the traditional Sanger technology with the new 454 technology. The sequence chromatograms were viewed using Finch TV application4.3.5 sequence analysis. The obtained sequences were analyzed using the DNASTAR V.7 (Lasergene) software, and our results were compared by BLAST program (www.ncbi.nlm.nih.gov/blast)[18].

RESULTS

The aim was to study underlying mechanisms of fosfomycin resistance in the seven clinical *E. coli* isolates; we amplified the three plasmid-coded genes *fosA3*, *fosA* and *fosC2*. Data revealed that five of the tested isolates (71.4%) were positive for *fosA3*. Neither *fosC2* nor *fosA* gene was detected among these isolates. Isolates number UPEC9 and UPEC27 were negative for the three resistance genes. Therefore, the *murA*, *glpT*, and *uhpT* nucleotide sequences of the two isolates were assessed for any possible mutations. Table (2) summarizes amino acids substitutions and sequence variations of MurA, GlpT, and UhpT proteins.

Table 2: Molecular characterization of the fosfomycin-resistant *Escherichia coli* isolates UPEC 9 and UPEC 27

E. coli Isolate	Amino acid substitutions or sequence variations		
	MurA	**GlpT**	**UhpT**
UPEC9	Ser206Asn, Gln208His, Gly236Asp	Gln58His, Pro377Ala, Thr383Ile, Ala385Gly, Gly386Arg, Thr407Pro	Phe34Ile
UPEC27	Pro99Ser, Ala102Thr, Gly118Arg	None	None

DISCUSSION

Fosfomycin was first isolated from cultures of the Streptomyces spp. (*Streptomyces fradiae*, *Streptomyces viridochromogenes*, and *Streptomyces wedomorensis*) in 1969 and has a low molecular mass (138 Da) derivative of phosphoric acid. Additionally, it was synthesized through a process that used a certain ratio of carbon and phosphorus. A phosphonic acid moiety and an epoxide group, which are both crucial for the biological activity of fosfomycin, are its two main structural components[19].

Fosfomycin is used for treating Urinary tract infections (UTIs) that are acute and simple in nature and often brought on by*E. coli* (+/- extended spectrum beta-lactamase [ESBL]), *Klebsiella* spp., *Proteus mirabilis, Staphylococcus saprophyticus*, *Enterococcus* spp., and *Streptococcus agalactiae*. Numerous European nations have also approved the use of intravenous fosfomycin to treat infections outside of the urinary system[20]. Considering this antibiotic as a monotherapy and in

addition to other antibacterial medications used to treat MDR Gram-negative infections that influence the whole body. Because of that, it is crucial to understand the underlying mechanisms of resistance towards fosfomycin, the likelihood that resistance will arise, the frequency of resistant isolates, and any potential clinical repercussions[21].

The frequency of resistance has been reported in clinical isolates of *E. coli* to be relatively low (about 1%) in several countries, despite the long-term use of fosfomycin in treatment of mild UTIs[22]. In the present study, we explored the underlying resistance mechanisms in the fosfomycin-resistant isolates of the MDR *E. coli* reported in our previous study[6].

Fosfomycin actively enters the *E. coli* cells through two nutrient transporters from the dominant facilitator superfamily: the hexose-6-phosphate transporter (UhpT), which is activated by extracellular glucose-6-phosphate (G6P), and the glycerol-3-phosphate transporter (GlpT), which is expressed in a

constitutively active state. Additionally, substantial concentrations of cyclic AMP (cAMP) and its receptor protein complex (CRP) are necessary for the complete expression of the *glpT* and *uhpT* genes. Adenylcyclase (CyaA) activity is necessary for cAMP synthesis in Enterobacteriaceae, and the phosphotransferase enzyme PstI, a part of the PEP sugar phosphor transferase transport system, also controls intracellular cAMP levels. Furthermore, the *uhpA*, *uhpB*, and *uhpC* genes locally regulate the expression of uhpT[8].

In the current study, data on the amplification of the plasmid-coded genes *fosA3*, *fosC2*, and *fosA* revealed that resistance to fosfomycin in 71.4% of isolates was mostly because of FosA3. No FosC2 or FosA-associated resistance could be detected among these isolates. This level of FosA3-linked resistance is more than that reported in previous studies, where the prevalence of *fosA3*gene was 60.5% [23] and 38.58%[24].

The plasmid-borne *fosA3* (in plasmids) has been the most often discovered *fosA* subtype over the past ten years. The first report from Japan in 2010 involved *E. coli* that produced CTX-M and effectively changed fosfomycin. Provided that there were 58 *E. coli* isolates resistant to fosfomycin carrying *fosA3* were clonally unrelated suggests that *fosA3* expanded through horizontal dissemination.

In a different survey, [25] 69 of 86 examined *E. coli* isolates were non-fosfomycin susceptible, yet 29/69 (42%) of these isolates were capable of transferring fosA3 across other *E. coli* strains.

Our analysis exhibited fosfomycin resistance in isolates UPEC9 andUPEC27 that lack the *fosA3*gene was due to either amino acid substitutions in *murA* (Ser206Asn, Gln208His, or Gly236Asp) or (Pro99Ser, Ala102Thr, Gly118Arg), respectively or as a result of the lack of an effective transporter, i.e., GlpT or UhpT.

Fosfomycin inhibits by binding the MurA enzyme to its active site nucleophile Cys-115[26]. In current study, *E. coli* isolates UPEC9 and UPEC27were found demonstrating the amino acid substitutions Ser206Asn, Gln208His, and Gly236Asp. It is not clear, however, from studying the crystal structure of *E. coli* murA complexed with fosfomycin, what role Ser206,Gln208, and Gly236play in the protein-inhibitor interaction. Further investigations are therefore required to determine how these amino acid alterations contributed to fosfomycin resistance.

Fosfomycin enters cells via two pathways: glycerol-3-phosphate transport GlpT or hexose phosphate transport UhpT. *E. coli*fosfomycin resistance has been associated with GlpT or UhpT abnormalities in several studies[27,11,15]. Only isolate number UPEC9 of tested *E. coli* was identified to have the *glpT* gene mutations in this investigation. Six amino acid substitutions were detected in GlpT: Gln58His, Pro377Ala, Thr383Ile, Ala385Gly, Gly386Arg, and Thr407Pro.Additionally, a single amino acid substitution (Phe34Ilein) in the

UhpTprotein was found. *E. coli* isolate UPEC27 didn't show any mutations either in glpT or in the uhpT gene.

Based on earlier evidence that the *fosA3* gene is likely encoded on a conjugated plasmid [28,16], this gene's mobility could promote the spread of fosfomycin resistance worldwide.

CONCLUSION

This study reported that the primary causes of fosfomycin resistance are the amino acid changes in the crucial enzyme MurA or the absence of a transporter system (GlpT or UhpT) that reduces competition with the healthy microflora *in vivo*.

This manuscript has not been previously published and is not under consideration in the same or substantially similar form in any other reviewed media. I have contributed sufficiently to the project to be included as author. To the best of my knowledge, no conflict of interest, financial or others exist. All authors have participated in the concept and design, analysis, and interpretation of data, drafting and revising of the manuscript, and that they have approved the manuscript as submitted.

Conflict of interest: Authors declare no financial and Non-financial conflict of interest

REFERENCES

1. Scribano D, Sarshar M, Prezioso C, et al. d-Mannose Treatment neither Affects Uropathogenic *Escherichia coli* Properties nor Induces Stable FimH Modifications. Molecules, 2020; 25(2).

2. Mohamed NR, Omar HHH, and Abd-Allah IM. Prevalence and risk factors of urinary tract infection among pregnant women in Ismailia City, Egypt. IOSR J Nurs Heal Sci 2017; 6, p. 62–72.

3. Mohamed Labib El-Kashif M and Eaid Elgazzar S. Maternal markers for detecting urinary tract infection among pregnant women in Port Said City, Egypt. Am J Nurs Res, 2018; 6, p. 317–326.

4. Mohamed W, Algameel A, Bassyouni R, et al. Prevalence and predictors of urinary tract infection in full-term and preterm neonates. Egypt Pediatr Assoc Gaz, 2020; 68, p. 1–7.

5. Ibrahim Shebl R and Mosaad YO. Frequency and antimicrobial resistance pattern among bacterial clinical isolates recovered from different specimens in Egypt. Cent African J Public Heal, 2019; 5, p. 36-45.

6. Mohamed Abu El-Wafa W and Abouwarda AM. In vitro assessment of the antibacterial effects of the combinations of fosfomycin, colistin, trimethoprim

and nitrofurantoin against multi-drug-resistant *Escherichia coli.* Lett Appl Microbiol, 2022; 74(3), p. 334-343.

7. Silver LL. Fosfomycin: Mechanism and Resistance. Cold Spring Harb Perspect Med, 2017; 7(2).

8. Castaneda-Garcia A, Blazquez J, and Rodriguez-Rojas A. Molecular Mechanisms and Clinical Impact of Acquired and Intrinsic Fosfomycin Resistance. Antibiotics (Basel), 2013; 2(2), p. 217-36.

9. Samonis G, Maraki S, Karageorgopoulos DE, et al. Synergy of fosfomycin with carbapenems, colistin, netilmicin, and tigecycline against multidrug-resistant *Klebsiella pneumoniae, Escherichia coli, and Pseudomonas aeruginosa* clinical isolates. Eur J Clin Microbiol Infect Dis, 2012; 31(5), p. 695-701.

10. Couce A, Briales A, Rodriguez-Rojas A, et al. Genomewide overexpression screen for fosfomycin resistance in *Escherichia coli*: MurA confers clinical resistance at low fitness cost. Antimicrob Agents Chemother, 2012; 56(5), p. 2767-9.

11. Wachino J, Yamane K, Suzuki S, et al. 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, 2010; 54(7), p. 3061-4.

12. Poirel L, Madec JY, Lupo A, et al. Antimicrobial Resistance in *Escherichia coli.* Microbiol Spectr, 2018; 6(4).

13. Scientific T, *Thermo Scientific GeneJET Plasmid Miniprep Kit #K0502, #K0503* T. Scientific, Editor 2014.

14. Scientific T, *Thermo Scientific GeneJET PCR Purification Kit #K0701, #K0702*, T. Scientific, Editor 2015.

15. Takahata S, Ida T, Hiraishi T, et al. Molecular mechanisms of fosfomycin resistance in clinical isolates of *Escherichia coli.* Int J Antimicrob Agents, 2010; 35(4), p. 333-7.

16. Hou J, Huang X, Deng Y, et al. Dissemination of the fosfomycin resistance gene fosA3 with CTX-M beta-lactamase genes and rmtB carried on IncFII plasmids among *Escherichia coli* isolates from pets in China. Antimicrob Agents Chemother, 2012; 56(4), p. 2135-8.

17. Farhadian A, Nejad QP, peerayeh SN, et al. Determination of Vancomycin and Methicillin Resistance in Clinical Isolates of *Staphylococcus aureus* in Iranian Hospitals. British Microbiology Research Journal, 2014; 4(4), p. 454-461.

18. Neumann RS, Kumar S, Haverkamp TH, et al. BLASTGrabber: a bioinformatic tool for visualization, analysis and sequence selection of massive BLAST data. BMC Bioinformatics, 2014; 15, p. 128.

19. Lu PL, Hsieh YJ, Lin JE, et al. Characterisation of fosfomycin resistance mechanisms and molecular epidemiology in extended-spectrum beta-lactamase-producing *Klebsiella pneumoniae* isolates. Int J Antimicrob Agents, 2016; 48(5), p. 564-568.

20. Bi W, Li B, Song J, et al. Antimicrobial susceptibility and mechanisms of fosfomycin resistance in extended-spectrum beta-lactamase-producing *Escherichia coli* strains from urinary tract infections in Wenzhou, China. Int J Antimicrob Agents, 2017; 50(1), p. 29-34.

21. Matthews PC, Barrett LK, Warren S, et al. Oral fosfomycin for treatment of urinary tract infection: a retrospective cohort study. BMC Infect Dis, 2016; 16(1), p. 556.

22. Kahlmeter G. An international survey of the antimicrobial susceptibility of pathogens from uncomplicated urinary tract infections: the ECO.SENS Project. J Antimicrob Chemother, 2003; 51(1), p. 69-76.

23. Galindo-Mendez M, Navarrete-Salazar H, Baltazar-Jimenez F, et al. Emergence of Fosfomycin Resistance by Plasmid-Mediated fos Genes in Uropathogenic ESBL-Producing *E. coli* Isolates in Mexico. Antibiotics (Basel), 2022; 11(10).

24. Hameed MF, Chen Y, Bilal H, et al. The Co-occurrence of mcr-3 and fosA3 in IncP plasmid in ST131 *Escherichia coli*: A novel case. J Infect Dev Ctries, 2022; 16(4), p. 622-629.

25. Li Y, Zheng B, Zhu S, et al. Antimicrobial Susceptibility and Molecular Mechanisms of Fosfomycin Resistance in Clinical *Escherichia coli* Isolates in Mainland China. PLoS One, 2015; 10(8), p. e0135269.

26. Zhu JY, Yang Y, Han H, et al. Functional consequence of covalent reaction of phosphoenolpyruvate with UDP-N-acetylglucosamine 1-carboxyvinyltransferase (MurA). J Biol Chem, 2012; 287(16), p. 12657-67.

27. Nilsson AI, Berg OG, Aspevall O, et al. Biological costs and mechanisms of fosfomycin resistance in *Escherichia coli.* Antimicrob Agents Chemother, 2003; 47(9), p. 2850-8.

28. Hou J, Yang X, Zeng Z, et al. Detection of the plasmid-encoded fosfomycin resistance gene fosA3 in *Escherichia coli* of food-animal origin. J Antimicrob Chemother, 2013; 68(4), p. 766-70.