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Molecular Analysis of Fosfomycin Resistance Among *Escherichia coli* Isolates from Urinary Tract Infections in Kidney Transplant Patients During 2019 - 2020

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Abstract

Background: This study aimed to estimate the prevalence of fosfomycin resistance and the frequency of extended-spectrum beta-lactamase (ESBL) production in *Escherichia coli* isolates from three kidney transplant patients (KTPs) in Tehran.

Methods: Sixty clinical isolates of uropathogenic *E. coli* were collected from three kidney transplant centers in Tehran between April and May 2019. Antimicrobial susceptibility testing (AST), minimum inhibitory concentration (MIC) of fosfomycin, and screening for ESBL production were conducted following the protocols established by the Clinical and Laboratory Standards Institute (CLSI). The presence of the blaTEM, blaSHV, blaCTX-M, fosA3, and fosC2 genes was evaluated using polymerase chain reaction (PCR) and sequencing. Additionally, mutations in the murA, glpT, uhpT, and cya genes were assessed. The activity of the carbohydrate phosphate transporter was measured using the real-time PCR assay.

Results: According to the AST results, ampicillin showed the highest resistance rate (86%), while ertapenem and doripenem exhibited complete susceptibility (100%). According to the E-test, 1.6% of *E. coli* isolates were resistant to fosfomycin. Furthermore, 33.4% of *E. coli* isolates in KTPs were ESBL producers, with the most frequent occurrence of the blaTEM gene (55%). Additionally, mutations were identified in the murA, uhpT, and glpT genes of resistant samples. No plasmid genes for fosA3 and fosC2 were detected. The expression of the uhpT gene increased 32-fold in a susceptible isolate, as determined by qPCR.

Conclusions: The high resistance of *E. coli* isolates from urinary tract infections (UTIs) of KTPs to β-lactam antibiotics remains a significant clinical challenge. However, no correlation was found between ESBL production and resistance to fosfomycin. The resistance rate to fosfomycin was low, and the primary cause of resistance was mutations in chromosomal genes.

Keywords: ESBL, *Escherichia coli*, Kidney Transplant, Fosfomycin

1. Background

Post-kidney transplantation urinary tract infections (UTIs) are a prevalent complication, with a prevalence of 5% to 36%, increasing the risk of graft loss for patients and incurring additional costs for the healthcare system (1-4). Infection by *Enterobacteriaceae* producing extended-spectrum β-lactamases (ESBLs) is more serious in immune-deficient and transplanted patients, including kidney transplant patients (KTPs). Despite developing a broad range of antibiotics, enthusiasm for applying these drugs has decreased for several reasons. One of the main complications of conventional antibiotics is global antibiotic resistance, which is progressed to the point where most of the uropathogens, such as quinolone-resistant and β-lactamase producing bacteria, are now resistant to antibiotics, acquired through different mechanisms.

Fosfomycin, a phosphoric acid derivative, is used to combat gram-positive and gram-negative bacteria responsible for lower urinary tract infections and other systemic diseases (4-7). Like any other antibiotic, resistance to fosfomycin is a common event that could be induced either through a chromosomal- or plasmid-mediated manner (6). It should be mentioned that most mutations that lead to intrinsic chromosome-mediated resistance against this antibiotic could not easily transfer to other organisms due to the impaired uptake system. It has been reported that those mutations that target the

genes encoding fosfomycin transporters, such as *glpT* and *uhpT*, could block the entrance of the drug into the host cells, leading to the induction of acquired fosfomycin resistance. In addition to acquired mutations in transporter genes, resistance to fosfomycin can also be induced through other mechanisms, such as the plasmid's origin of fosfomycin-modifying enzymes, including FosA, FosB (8), FosC (9), and FosX (10). Additionally, the transfer of plasmids between bacteria can also result in resistance to antibiotics other than fosfomycin, such as β-lactams, aminoglycosides, and quinolones. Fosfomycin prescription in Iran during urinary tract infection is not as usual as in other countries. On the other hand, it is not the primary choice.

2. Objectives

The study aimed to estimate the prevalence of fosfomycin resistance and the frequency of ESBL production in *Escherichia coli* isolates from three KTPs in Tehran.

3. Methods

3.1. Sampling and Study Design

From April to May 2019, urine samples were collected by the mid-stream clean catch method from patients with kidney transplants referred to Labafinejad Hospital and Yekta and Gholhak Private Clinics' laboratories. *Escherichia coli* was identified based on standard bacterial tests (11). All the confirmed bacteria were kept in 10% glycerol and TSB media at -70°C.

3.2. Sample Size Calculation

The sample size was determined based on a confidence of 95% and accuracy of 0.01% using $N = Z(1 - z_{\alpha/2})^2 (p)(q)/d^2$ formula.

3.3. Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing (AST) was performed manually and interpreted according to the protocol and breakpoints recommended by the Clinical and Laboratory Standards Institute (CLSI) (12). The following antibiotics were purchased from Mast (England) and Rosco (Denmark) for AST: Ceftriaxone 30 mg, cefotaxime 30 mg, cefixime 30 mg, cefazolin 30 μg, cephalexin 30 mg, ampicillin 10 μg, ampicillin-sulbactam 20/10 μg, piperacillin/tazobactam 100/10 μg, cefpodoxime 30 μg, doripenem 10 μg, imipenem 10 μg, ertapenem 10 μg, meropenem 10 μg, gentamycin 10 μg, tobramycin 10 μg, amikacin 30 μg, ciprofloxacin 5 μg, trimetoprim 5 μg, and nitrofurantoin 200 μg.

3.4. Minimum Inhibitory Concentration

Based on the manufacturer's protocol, fosfomycin's minimum inhibitory concentration (MIC) was determined using the E-test (Liofilchem, Italy). The results were interpreted based on the CLSI guidelines approved for *E. coli* in UTIs (i.e., susceptible at MICs of $\leq$ 64 μg/mL or with zones of $\geq$ 16 mm) (12).

3.5. Phenotypic Screening of Extended-spectrum β-Lactamase Production

Cefotaxime 30 mg (CTX) and ceftazidime 30 mg (CAZ) disks, either alone or in combination with clavulanic acid 10 mg (CA), were used in the Double Disk Synergy Test (DDST) for ESBL production screening according to the CLSI 2020 guideline (12-14). Any increase in diameter of 5 mm or more around either the CTX or CAZ disk when combined with CA, compared to the diameter of these disks alone, indicated the presence of an ESBL-producing bacterial isolate. Simultaneously, two ATCC bacterial isolates, including *E. coli* ATCC 25922 and *K. pneumonia* ATCC 700603, were used as the negative and positive controls, respectively.

3.6. DNA extraction, Polymerase Chain Reaction Method, and Sequencing

The DNA was extracted as previously described (15, 16). The frequency of the bla CTX-M, TEM, and SHV genes (17, 18), as well as the fosA3, fosC2, murA, uhpT, glpT, and cyaA genes (19), which are responsible for ESBL production and fosfomycin resistance, were determined using separate polymerase chain reaction (PCR) methods in a 25 μL reaction mixture (20). The primers used in this study and the PCR program are shown in Tables 1 and 2, respectively. Electrophoresis on a 1.5% agarose gel was used for PCR product analysis. Two bacterial strains, including *K. pneumoniae* ATCC 700603 and a fosfomycin-resistant *E. coli* isolate (provided by Prof. C. Giske from Karolinska Institute, Sweden), were simultaneously used as positive controls for the PCR test.

Sequencing was done by Bioneer company, Korea, to identify the presumptive mutation(s) among encoding chromosomal fosfomycin resistance genes.

3.7. Carbohydrate Phosphate Transporter Activity

To evaluate any change in the expression rate of the transporter gene *uhpT*, real-time PCR was done on four suspicious *E. coli* isolates (one fosfomycin-resistant, one fosfomycin-intermediate, and two fosfomycin susceptible). First, a bacterial culture using Luria-Bertani (LB) broth was prepared and incubated in a 37°C shaker incubator for 24 h. Then, grown cells were harvested,

Table 1. Primers Used in Polymerase Chain Reaction and Quantitative Real-time-Polymerase Chain Reaction in This Study

Gene and Primers	Band Size	References
murA	1542	(19)
AAACAGCAGACGGTCTATGG		
CCATGAGTTTATCGACAGAAC		
uhpT	1667	(19)
TTTTTGAACGCCCAGACACC		
AGTCAGGGGCTATTTGATG		
glpT	1785	(19)
GCGAGTCGCGAGTTTTCATTG		
GGCAAATATCCACTGGCACC		
cyaA	2773	(19)
AACCAGGCGCGAAAAGTGG		
ACCTTCTGGGATTTGCTGG		
fosA3	240	(19)
CCTGGCATTTTATCAGCAGT		
CGGTTATCTTTCCATACCTCAG		
fosC2	243	(19)
TGGAGGCTACTTGGATTTG		
AGGCTACCGCTATGGATTT		
CTX-M	593bp	(19)
TTTGCGATGTGCAGTACCAGTAA		
CGATATCGTTGGTGGCATA		
TEM	800bp	(21)
TAATCAGTGAGGCACCTATCTC		
GAGTATTCAACATTTCCGTGTC		
SHV	1000bp	(19)
GCC GGG TTA TTC TTA TTT GTC GC		
ATG CCG CCA GTC A		
rpoD-qPCR	-	(22)
CAAGCCGTGGTCGGAAAA		
GGGCGCGATGCACTTCT		
uhpT-qPCR	-	(22)
AAGCCGACCCTGGACCTT		
ACGGTTTGAACCACATTTTGC		

washed twice with M9 minimum salt solution, and then inoculated into M9 minimum salt solution with or without 0.2% glucose-6-phosphate (G6P). The mixture was then incubated for 30 minutes at 37°C (1). The induction effect of G6P on the expression of the *uhpT* gene and the housekeeping gene *rpoD* was determined after RNA extraction and cDNA preparation using Bio Fact Kit (South Korea) and real-time PCR by QuantiFast SYBR Green PCR Mastermix (Qiagen). Primer sets for each gene are listed in Table 1, and the ingredients are in Table 3. Data were analyzed using the $2^{-\Delta\Delta CT}$ method normalized to the housekeeping gene *rpoD* mRNA levels.

3.8. Statistical Analysis

Statistical analysis was done using SPSS version 22, and the frequency of evaluated genes was determined as a percentage.

4. Results

Among 63 suspicious *E. coli* isolates, 60 were confirmed as *E. coli* based on standard bacterial tests. Hence, three isolates were not confirmed as *E. coli* and were excluded from this study. Also, we excluded *E. coli* isolates from UTI patients without kidney transplants. We found that 72.5% of the patients were female and 27.5% were male, aged 15 - 65.

4.1. Antimicrobial Susceptibility Testing

The susceptibility of all *E. coli* isolates was significant to doripenem and ertapenem (100%). However, the maximum resistance rate was to ampicillin, cefotaxime, and cefazolin at 85.9%, 80.1%, and 77.1%, respectively (Table 4 and Figure 1).

4.2. Minimum Inhibitory Concentration of Fosfomycin by E-Test

The fosfomycin E-test method was used for MIC detection. According to the CLSI 2020 protocol, MICs above 128 μg/mL were considered resistant. According to the results, out of 60 samples, one was resistant, and two were intermediate. Based on the results, 57/60 *E. coli* isolates were susceptible, and their MICs were between 0.125 and 48 μg/mL. Two samples were intermediate (MIC of 64 μg/mL), and one was resistant (MIC 256 μg/mL) to fosfomycin (Table 5). According to the phenotype test, resistance to fosfomycin in our samples was 1.6%.

4.3. Frequency of Extended-Spectrum Beta-Lactamase Produced by DDST

Based on the CLSI, the percentage of ESBL-producing *E. coli* in KTPs was found to be 33.5% (Figure 2).

Table 2. Polymerase Chain Reaction Programs for the Genes Evaluated in This Study

Genes	Initial Denaturation	Denaturation	Annealing Temperature (°C)	Extension	Final Extension	Cycles
blaTEM	95°C	95°C	58	72°C	72°C	35
	5 min	30 s		45 s	10 min	
blaSHV	95°C	95°C	59	72°C	72°C	30
	5 min	30 s		45 s	10 min	
blaCTX-M	94°C	94°C	57	72°C	72°C	30
	5 min	45 s		45 s	10 min	
fOSA3	5 min	45 s	58	45 s	72°C	36
	94°C	94°C		72°C	10 min	
fosC2	5 min	45 s	58	45s	72°C	36
	94°C	94°C		72°C	10 min	
murA	5 min	1 min	57	1 min	10 min	35
	94°C	94°C		72°C	72°C	
uhpT	5 min	1 min	58.5	1 min	10 min	36
	94°C	94°C		72°C	72°C	
glpT	5 min	1 min	58	1 min	10 min	36
	94°C	94°C		72°C	72°C	
cyaA	5 min	1 min	59	1 min	10 min	36
	94°C	94°C		72°C	72°C	

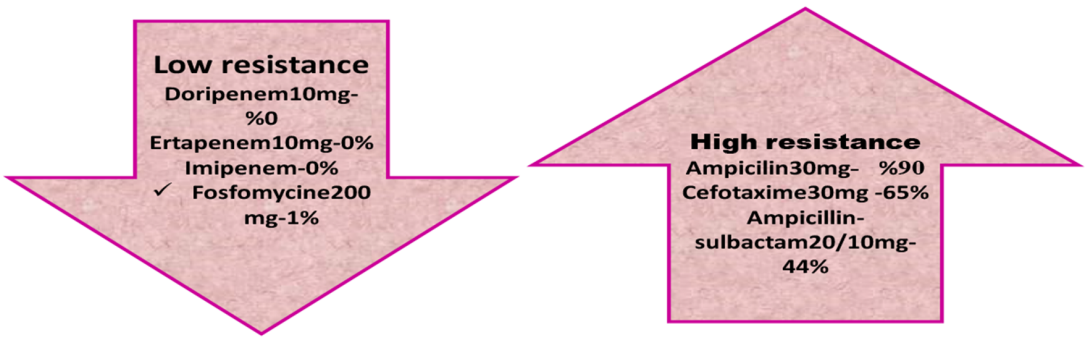

Figure 1. Frequency of the high and low antibiotic resistance rate among *Escherichia coli* isolates from kidney transplant patients (KTPs)

4.4. Frequency of Extended-Spectrum Beta-Lactamase Genes and Fosfomycin-Resistant Genes by PCR

The ESBL-responsible genes among *E. coli* isolates were bla_{TEM} (55%), bla_{CTX-M} (51.1%), and bla_{SHV} (41%). Among the fosfomycin-resistant genes, we failed to find any fos A3 and fos C2 plasmid genes despite mutations detected among the chromosomal *murA*, *glpT*, and *uhpT* genes (Table 5 and Figures 3, 4, 5 and 6).

Chromosomal mutations were identified in a resistant *E. coli* isolate and two intermediate isolates. These mutations resulted in the deletion of certain amino acid residues in the uhpT and glpT genes, respectively. In detail, after data alignment in the gene bank, two mutations in the *murA* gene, which lead to amino acid replacement (Leu 370 lle, Asp 369 Asn), were detected in two intermediate *E. coli* isolates. In the resistant *E. coli* isolate, we also detected a deletion mutation in 421 threonines encoding the *uhpT* gene. No mutation was detected in the cyaA gene among

Table 3. Ingredients of Real-time Polymerase Chain Reaction

Master Mix	Volume (μL)
Forward primer	1
Reverse primer	1
WW	6
cDNA	2
Total volume	10

Table 4. The Level of Intermediate and Sensitivity of *Escherichia coli* Isolates to Antibiotics [a]

Antibiotic (mg)	Susceptible	Intermediate	Resistance
Ampicillin 30	5 (8)	1 (2)	54 (90)
Amoxicillin-clavulanic acid 30	28 (46)	28 (46)	4 (8)
Ampicillin-sulbactam 20/10	26 (44)	8 (12)	26 (44)
Pipracilin-tazobactam 100/10	40 (67)	6 (8)	14(24)
Cefazolin 30	40 (67)	8 (12)	12 (20)
Cefepime 30	27 (45)	8 (12)	25 (43)
Cefotaxime 30	20 (34)	1 (2)	39 (65)
Doripenem 10	60 (100)	0 (0)	0 (0)
Ertapenem 10	60 (100)	0 (0)	0 (0)
Fosfomycine 200	57 (95)	2 (3)	1 (1)
Imipenem 10	57 (95)	3 (5)	0 (0)
Meropenem 10	60 (100)	0 (0)	0 (0)
Amikacin 30	40 (66)	10 (17)	10 (17)
Tobramycin 10	41 (68)	10 (17)	9 (15)
Trimethoprim 5	10 (17)	13 (22)	37 (61)
Nitrofurantoin 200	48 (83)	6 (8)	6 (8)
Ciprofloxacin 5	16 (27)	4 (6)	40 (67)
Gentamicin 10	48 (71)	6 (8)	6 (8)
Cefpodoxime 30	20 (34)	2 (2)	38 (64)

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

fosfomycin-resistant *E. coli* isolates (Table 5, Figure 7).

4.5. Evaluation of uhpT Gene Expression by Real-time PCR

According to the real-time PCR test of the *uhpT* gene on one fosfomycin-resistant, two intermediate, and two susceptible *E. coli* isolates, the expression rate of only one fosfomycin-susceptible isolate was increased by 32 times. We did not detect any increase in the expression of the *uhpT* gene in the intermediate and resistant bacterial isolates (Figures 8, 9A and B).

Figure 2. Extended-spectrum beta-lactamase detection by Double Disk Synergy Test. CAZ: Ceftazidime, CAZ+C: Ceftazidime/clavulanic acid disks. The increased diameter of the inhibition zone around the ceftazidime /clavulanic acid is shown in this figure.

5. Discussion

While some reports suggested the low incidence of drug resistance to fosfomycin, others suggested controversial results (19, 23). Moreover, in another study, the extent of resistance to other antimicrobial agents was greater in fosfomycin-resistant and intermediate *E. coli* isolates than in fosfomycin-susceptible strains. This study was conducted based on the significance of monitoring fosfomycin-resistant *E. coli* to prevent the development of cross-resistance and multidrug resistance to other antibiotics, as indicated by previous research. The current results show that resistance to fosfomycin was low (1.6%). Only one *E. coli* strain was found to be resistant to fosfomycin, and two strains were classified as intermediate out of 60 clinical isolates from UTIs of KTPs who were admitted to three main centers in Tehran in the current study. Also, we found a high resistance rate to ampicillin (86%), cefotaxime (80%), and cefazolin (77%) among *E. coli* isolates from UTIs of KTPs, which is in line with the results of previous studies conducted in Iran (24, 25). However, ESBL-producing *E. coli* infection is commonly associated with a significantly longer hospital stay and greater hospital costs (19), despite the hypothesis that there is evidence of a higher rate of fosfomycin resistance among ESBL-producing *E. coli* (20), such a relationship was not detected in this study.

Our results showed that the most frequent ESBL genes were bla_{TEM} (55%), bla_{CTX-M} (51%), and bla_{SHV} (41%). In the study of Haddadi et al. from Alborz, Iran, 61% of *E. coli* isolates harbored bla_{TEM} as the most frequent ESBL gene, similar to a recent study (18). Moreover, in

Table 5. Properties of Fosfomycin-Intermediate and Resistant *Escherichia coli* Isolates in This Study

Fosfomycin - Resistant Rate	ESBL Production	ESBL Genes			Fosfomycin MIC (μg/mL)	Fosfomycin Resistance Genes					
						Plasmid Genes		Chromosomal Genes			
R	+	*bla CTX-M* +	*bla SHV* +	*bla TEM* +	256	*fosA3* -	*fosC2* -	*murA*[a] Leu 370 Ile, Asp369 Asn	*glpT*[b] Thr 421	*uhpT*[b] Glu 429	*cyaA* NM
I	+	*bla Ctx-M* +	*bla SHV* -	*bla TEM* +	64	*fosA3* -	*fosC2* -	*murA*NM	*glpT* NM	*uhpT*NM	*cyaA*NM
I	+	*bla CtxM* +	*bla S HV* +	*blaT EM* +	64gur	*fosA3* -	*fosC2* -	*murA* NM	*glpT* NM	*uhpT* NM	*cyaA* NM

Abbreviations: ESBL, extended-spectrum beta-lactamase; MIC, minimum inhibitory concentration; I, intermediate; R, resistant; ND, not detected; NM, no mutation; +, positive for a gene; -, negative for a gene; Thr, threonine; Gl, glutamate.
[a] Type of mutation: Substitution,
[b] Type of mutation: Deletion.

Figure 3. Detection of the *murA* gene by gel electrophoresis. Wells 1 and 2 are positive controls. Wells 3 and 4 are negative controls. Wells 5 to 9 are the first to fifth samples, and well 10 is the ladder.

a recent study, only a single substitution (Leu370Ile, Asp369Asn) was identified in the *murA* gene sequence. This kind of mutation in the *murA* gene was identified in one of the intermediate *E. coli* isolates in this study (20). Furthermore, the current study is consistent with the findings of Sorlozano-Puerto et al. regarding the mutations of Asp 369 Asn and Leu 370 Ile in fosfomycin-resistant *E. coli* isolates MSC17327 and MSC17323 (20, 26). However, an inspection of the crystal structure of the *E. coli* MurA gene in complex with fosfomycin does not suggest an obvious role for Asp-369 and Leu-370 in the interaction between the protein and the inhibitor (20). In fact, fosfomycin transportation into cells is mediated by two pathways: The glycerol-3-phosphate transport system or the hexose phosphate transport system. Given this, several reports have suggested that one of the chromosomal mechanisms leading to fosfomycin resistance could be mediated through defects in the *glpT* or *uhpT* genes (20). The deletion in the coding region of the *glpT* gene leads to the formation of a truncated *glpT* gene. Other studies have also suggested that fosfomycin resistance in *E. coli* strains can be induced by any alteration in the chemical structure of fosfomycin caused by fosA3, a protein encoded by the fosA3 gene (20). However, when we evaluated the existence of *fosA3* and *fosC* genes among our *E. coli* isolates, no alteration in *fosA3* and *fosC2* genes was detected. This finding contrasts the Li et al. study from China, which declared the fosA3 gene the main cause of fosfomycin resistance among *E. coli* isolates (20). This may be related to fewer prescriptions of fosfomycin in UTI cases in Iran.

In Ghanavati et al. study (2016 - 2017) in Iran, 92.8% of isolates were fosfomycin-susceptible, and none of the ESBL-producing *Enterobacteriaceae* isolates harbored any mutated or plasmid genes (24). In the study of Bahramiyan et al. in Iran, 8% of *E. coli* isolates from different patients,

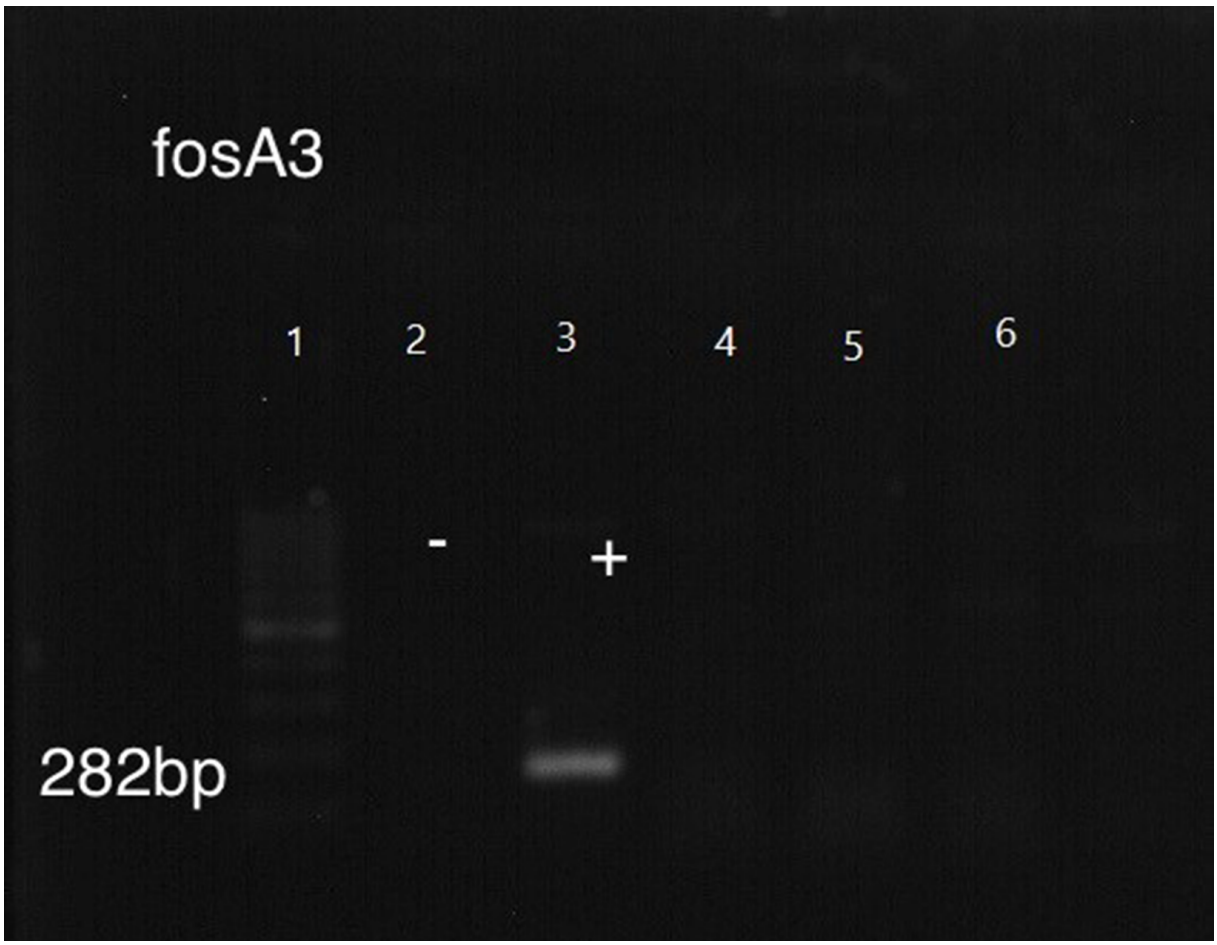

Figure 4. Detection of *fosA3* gene by gel electrophoresis. Well, 1 is the ladder, and well 2 is negative control. Well, 3 is a positive control. Wells 4, 5, and 6 are the first, second, and third samples, respectively.

including dialysis patients, were fosfomycin-resistant (25). Although the resistance rate to fosfomycin in *Enterobacteriaceae*, including *E. coli* isolates, was low, both studies reported a higher resistance rate (almost 8%) to fosfomycin than the recent study (1.6%). Also, in Bahramiyan et al. (25) study, 76% of *E. coli* isolates, and in Ghanavati et al. (24) study, 42% of *Enterobacteriaceae* isolates were ESBL producers, which was higher than 33% of *E. coli* isolates in the recent study. Such differences may be related to the variety of origins and the fact that Ghanavati et al. (24) and Bahramiyan et al. (25) studies included different members of *Enterobacteriaceae*, whereas the current study only included *E. coli* isolates from UTIs of KTPs. However, a similar resistance rate to ampicillin was detected in both studies mentioned in Iran, and the highest susceptibility was observed towards imipenem, which is consistent with the current study's findings.

Different mutations in *murA*, *glpT*, and *uhpT* chromosomal genes were detected in a recent study among *E. coli* isolates from KTPs. Ghanavati et al. (24) declared that no plasmid genes or mutation in chromosomal genes were responsible for fosfomycin resistance among *Enterobacteriaceae* isolates. Also, Bahramiyan et al. (25) showed that fosA3 and fosC2 plasmid genes were undetected among fosfomycin-resistant *E. coli* isolates. Similar to the recent study, both of these studies declared no plasmid genes responsible for fosfomycin resistance among *E. coli* isolates. Fortunately, the absence of plasmid-borne fosfomycin-resistant genes reduces the likelihood of these genes being disseminated among bacteria.

Neither Ghanavati et al. (24) nor Bahramian et al. (25) (both from Iran) reported mutations or evaluated

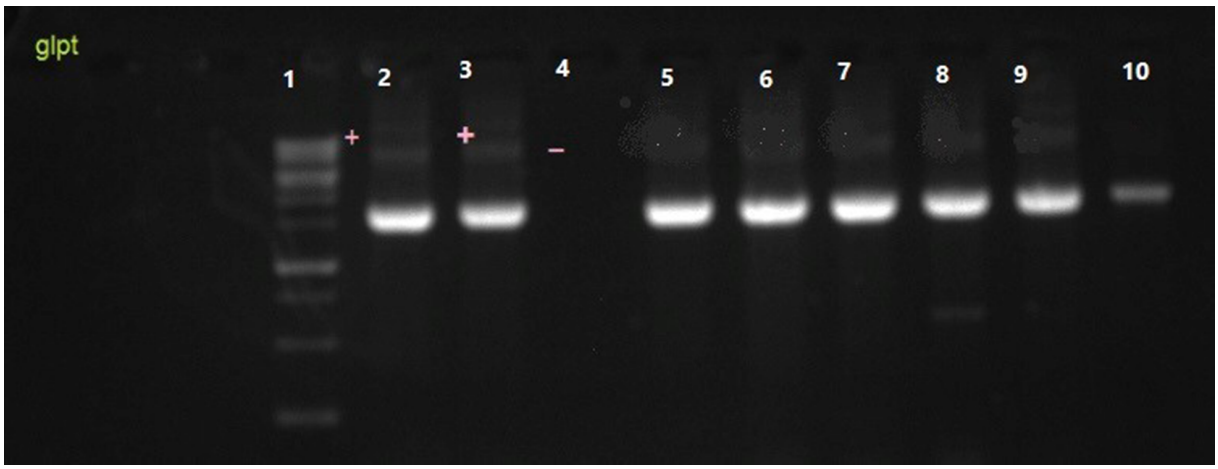

Figure 5. Detection of the *glpt* gene by gel electrophoresis. Well 1 is the ladder, well 2 is the control sample, well 3 is the positive control, and well 4 is the negative control sample. Wells 5 to 10 are the first to sixth samples, respectively.

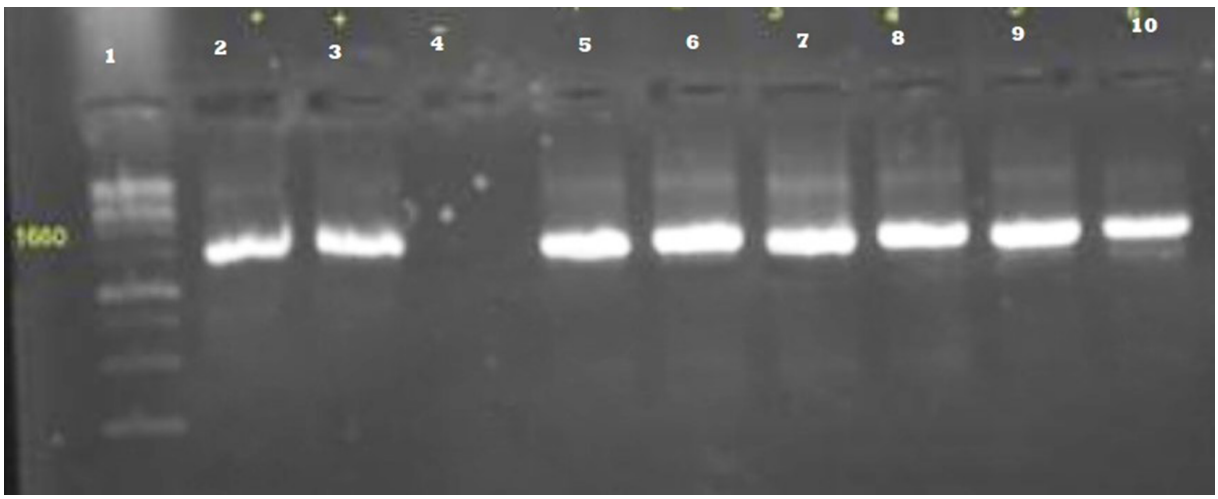

Figure 6. Detection of the *uhpT* gene by gel electrophoresis. Well 1 is the ladder, well 2 is the control sample, well 3 is a positive control, and well 4 is the negative control sample. Wells 5 to 10 are the first to sixth samples, respectively.

any fosfomycin chromosomic-resistant genes in their studies. Based on our knowledge, it is the first time that such mutations in *murA*, *glpT*, and *uhpT* genes among fosfomycin-resistant *E. coli* isolates from Tehran have been reported in the recent study. However, further studies with higher sample sizes are needed to determine the role of such chromosomal mutations.

According to Ohkoshi et al., study, higher expression of the *uhpT* gene in the presence of G6P was detected in fosfomycin-susceptible *E. coli* isolates (23). Furthermore, Kurabayashi et al. demonstrated that fosfomycin resistance in EHEC is controlled by a two-component signal transduction system called CpxAR. They demonstrated that the cpxA mutant, which lacks phosphatase activity, exhibits CpxR activity and resistance to fosfomycin (22). However, the function of the CpxAR system was not evaluated in this study. Nevertheless, it was observed that induction of susceptible isolates by G6P resulted in a 32-fold increase in uhpT expression compared to one resistant and two intermediate *E. coli* isolates.

In Seok et al. study from South Korea, the activity of fosfomycin in *E. coli* isolates from different origins was evaluated. They found that 6.7% of bacterial isolates were fosfomycin-resistant, only two isolates carried the *fosA3* gene, and diverse mutations were detected in *murA*, *uhpT*, and *glpT* genes. Although the fosfomycin resistance

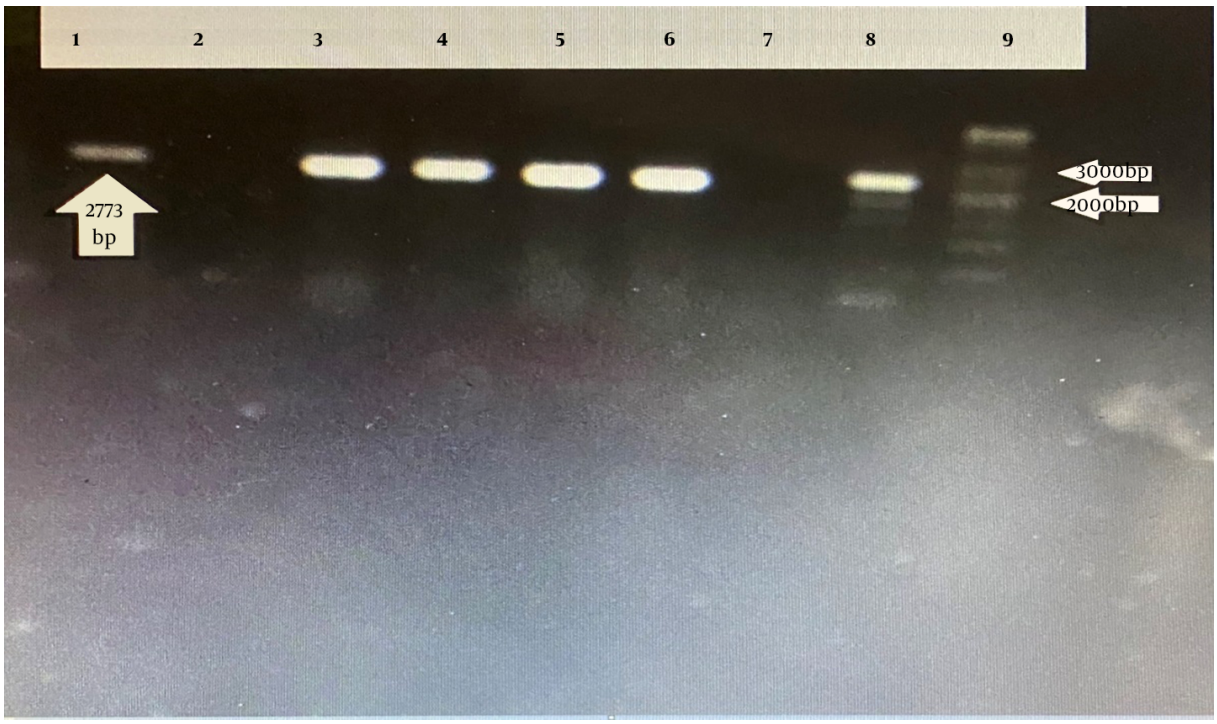

Figure 7. Detection of the *cyaA* gene by gel electrophoresis. Wells 3 to 6 are samples; wells 1, 2, 3, and 7 are the negative control, well 8 is the positive control, and well 9 is the ladder.

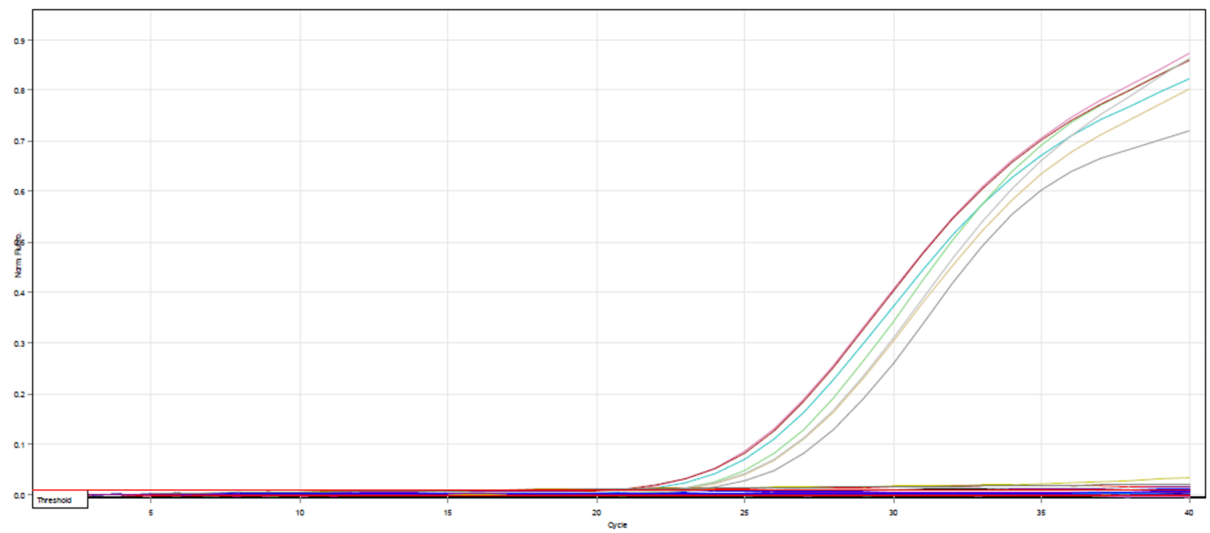

Figure 8. The graph of real-time polymerase chain reaction

rate was low in our study (1.6%) compared to 6.7% in the study by Seok et al., no fosA3 gene was detected. However, the main cause of fosfomycin resistance was mutations in three main chromosomal genes, including uhpT, glpT, and murA genes with phosphatase activity, in both studies. Similarly, amino acid substitutions or insertions in GlpT, UhpT, and MurA were found in eight, one, and two fosfomycin-resistant isolates, respectively.

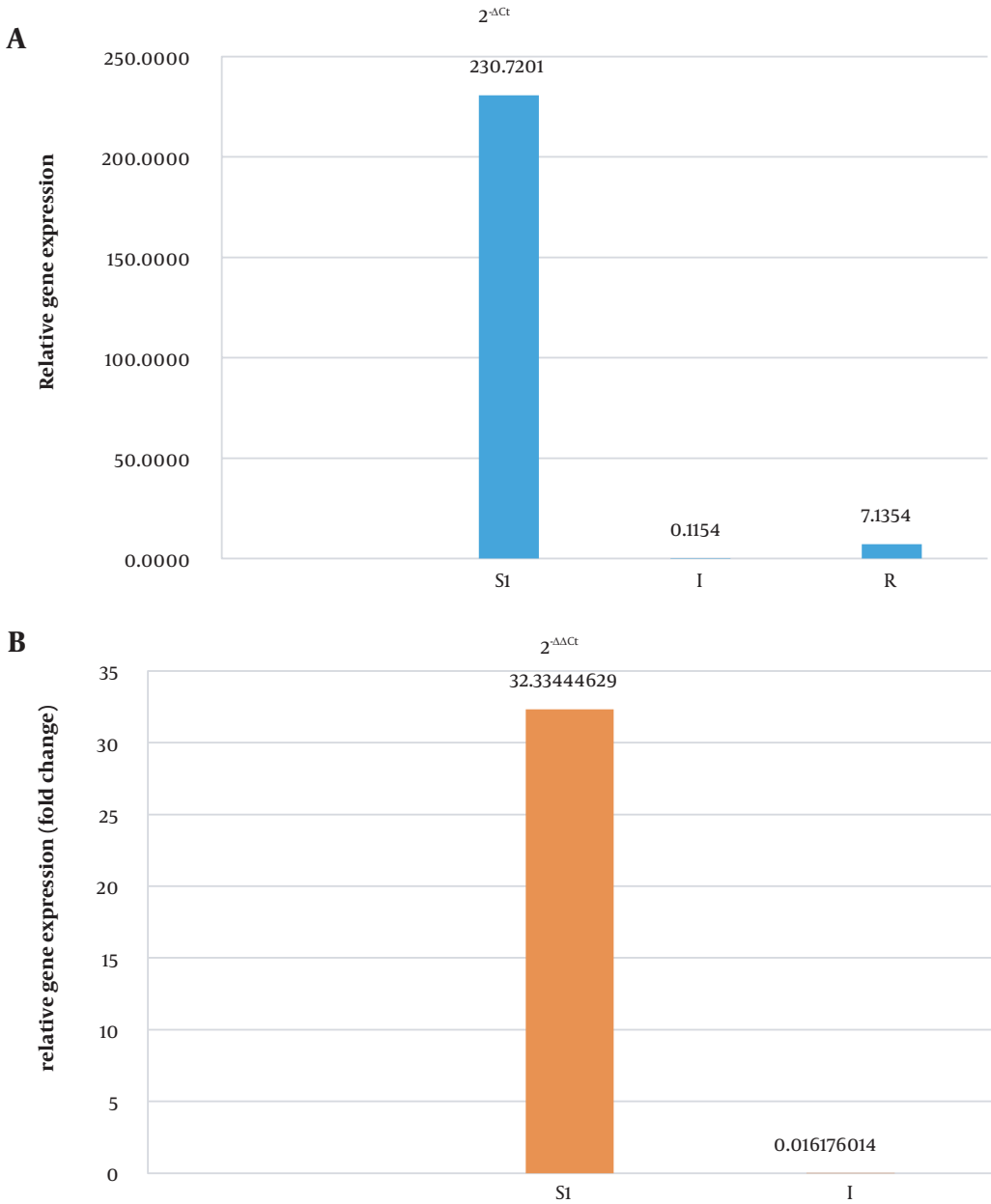

Figure 9. Relative gene expression and fold changes of S, I, and R *Escherichia coli* isolates. A, relative gene expression of susceptible (S), intermediate (I), and resistant (R) isolates; B, comparison and fold changes of *uhpT* gene expression between susceptible (S) and intermediate (I) bacterial isolates.

Only one mutation, A16T in GlpT, was identified in multiple fosfomycin-resistant *E. coli* isolates belonging to the same genotype. If the mutations in our samples differed from this mutation, it would suggest a different mechanism of resistance (27).

According to Garallah and Al-Jubori from Iraq, mutations in the glpT and uhpT genes act as efflux pumps and exclude fosfomycin from bacterial cells, leading to fosfomycin resistance in *E. coli* isolates from UTIs in their region (28). In the Bahy et al. study, similar to ours, there was no resistance to fosfomycin via plasmidic fos A and fos C2. However, over 75% of the resistance observed in this study was attributed to the presence of the *fos A3* gene (29). This result encourages us to investigate the presence

of the plasmid-borne *fosA3* gene in our upcoming study.

5.1. Conclusions

The present study showed that ESBL producers are increasing among *E. coli* isolates from UTIs of KTPs, which may lead to higher treatment costs and mortality rates. Also, this study found no association between fosfomycin-resistant and intermediate *E. coli* isolates and ESBL production or their genes. Due to the emergence of fosfomycin resistance in *E. coli* isolates in this study, we recommend continuous monitoring of antibiotic resistance mechanisms, attention to infection control guidelines, use of sensitive laboratory diagnostic methods, and close relationship between physicians and laboratories.

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Footnotes

Authors' Contribution: The subject determination, coordination with the nephrologist, and proposal designation were done by MHV. Proposal preparation, practical parts including laboratory tests such as sample collection, DNA extraction, AST, E-test, PCR, qPCR, and preparing the paper draft was done by AN under the supervision of MHV and SS. Also, supervision of sampling and introduction of kidney transplant patients with UTIs was done by SS (nephrologist). MJN participated in study design, proposal preparation, statistical analysis, and paper revision. All the authors have read the paper, accepted the results, and agreed to publish the achieved data. Also, all the authors declare that the current paper was not submitted to another journal simultaneously.

Conflict of Interests: The corresponding author (Mojdeh Hakemi-Vala) and Mohammad Javad Nasiri, the other author of this paper, are reviewers of this journal and some other Brieflands journals.

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