murA, uhpT, glpT and fosA Genes of Fosfomycin Resistance in Multi-drug Resistant *E. coli* Isolated from Hematological Malignancies' Patients with Blood Stream Infection

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Authors' contributions

This work was carried out in collaboration between both authors. Author YN is the creator of the idea and designed the plan of the work up. Authors YN and GB shared in collecting samples, carrying out Lab work up and writing the manuscript. Results analysis was performed by a qualified analyst. Both authors read and approved the final manuscript.

Original Research Article

ABSTRACT

Aim: To estimate the prevalence of *murA*, *uhpT*, *glpT* and *fosA* resistance genes of fosfomycin in multidrug resistant *Escherichia coli* isolated from patients suffering from hematological malignancies with on top blood stream infection together with correlating this distribution to the rate of expression of AmpC, ESBLs and MBLs in such isolates.
Methods: 205 blood samples collected from patients with underlying hematological malignancies were cultured to isolate *E. coli strains*. Multidrug resistance was detected. PCR was done to determine fosfomycin resistance genes; *murA*, *uhpT*, *glpT* and *fosA*.
Results: A total of 83 (40.5%) *E. coli* strains were isolated from blood samples. 58 (69.9%) were found to be multidrug resistant. AmpC beta-lactamase production was deduced in 15 (25.8%) isolates. 43 (74.1%) isolates were ESBLs producers whereas 9 (15.5%) were MBL class A carbapene-mases producers. Depending on PCR results, *murA* gene was detected in one isolate

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(1.7%), *uhpT* gene in 3 (5.2%), *glpT* gene in 4 (6.9%) whereas *fosA* gene was found in 7 *E. coli strains* (12.1%).

Conclusion: Fosfomycin is a promising antibiotic with limited degree of prevalence of its resistant genes; however disseminating resistance might increase in multidrug resistant *E. coli.*

ABBREVIATIONS

Clinical Laboratory Standards Institute guidelines (CLSI); Combined disk test (CDT); Extended-spectrum beta-lactamases (ESBLs); Metallo-beta lactamases (MBLs); Polymerase chain reaction (PCR).

1. INTRODUCTION

Blood stream infection is a major life-threatening complication in cancer patients, its mortality rate can reach up to 25-32% [1]. Such patients receiving chemotherapy, suffering from neutropenia or/and having inserted medical devices are at higher risk of blood stream infection [2]. Patients with hematologic malignancies have a varying degree of immune and intestinal barrier dysfunction. Accordingly, translocation of bowel *E. coli* to blood occurs with its variant virulence factors [3].

Also multidrug resistant bacteria are an increasing emerging problem in immunosuppressed cancer patients [4]. There is an exponential increase in multidrug resistance, not only to mention AmpC producers but also the emergence and wide spreading of extended-spectrum beta-lactamases (ESBLs) and Metallo-beta lactamases (MBLs) as carbapene-mases producing strains. Difficulty of such problem is greatly growing due to the clustering of many resistance mechanisms and/or the transmission of plasmids and transposons that transport genes with additional resistance [5-6].

Alternative treatment policies are always mandatory to confer the increasing antibiotic resistance to different multidrug resistant bacteria worldwide [7]. One objective of antimicrobial stewardship programs is selection of antibiotics with no emergent multidrug resistant microorganisms trying to find alternatives to antibiotics that recorded a high degree of resistance. Cephalosporins, carbapenems and quinolones have been associated with ESBLs-producing Enterobacteria, *Clostridium difficile* and multidrug resistant *Pseudomonas* [8].

Fosfomycin is an antibiotic derived from phosphonic acid; it is characterized by an exclusive nature with specific mechanisms of action. It yields a wide-range of activity and with nearly no cross-resistance occurring with other types of antibiotics. But some resistance mechanisms are emerging; in *E. coli*, reported fosfomycin resistance included reduced drug uptake which was explained by mutations in the genes that encoded transporters called *GlpT* and *UhpT*. Also may be due to changes in the fosfomycin main target which is the enzyme responsible for catalyzing the initial step in the biosynthesis of peptidoglycan, namely *MurA* [9,10].

The FosA metallo-enzymes resistance is one of transferable mechanisms in *E. coli* that is responsible for the binding of the glutathione to the molecule of fosfomycin [11]. Four variants FosA (FosA3, FosA4, FosA5, and FosA6) out of seven are acquired determinants of resistance among *E. coli* strains [12,13]. FosA3 is the most common that lies on conjugative plasmids encoding ESBLs of the CTX-M-type [14].

On the other hand, many other Gram-negative bacteria, as *Klebsiella,* either *pneumonia* or *oxytoca* type, *Enterobacter aerogenes* or *cloacae*, *Pseudomonas aeruginosa*, *Serratia marcescens* and *Morganella morganii*, are inherently non sensitive to fosfomycin [15].

The purpose of this current work up was to estimate the prevalence of *murA*, *uhpT*, *glpT* and *fosA* fosfomycin resistance genes among multidrug resistant *E. coli* retrieved from patients suffering from hematological malignancies with on top blood stream infection, together with correlating this distribution to the rate of expression of AmpC, ESBLs and MBLs in such isolates.

2. MATERIALS AND METHODS

A total of 205 blood samples recruited from patients with underlying hematological malignancies seeking medical care at Mansoura

University Hospitals, Mansoura, Egypt were enrolled in the current study. The samples were collected over a time span of 15 months from January 2019 to April 2020. Based on previous studies showing the prevalence of fosfomycin resistance genes among multidrug resistant *E. coli* retrieved from patients suffering from hematological malignancies with on top blood stream infection, therefore, the sample size was derived with a 95% confidence interval and an estimate error of 8%.

The included patients showed evidence of blood stream infection. The undertaken study was performed in accordance with the Declaration of Helsinki besides the national and institutional standards. Its protocol was accepted by the faculty review board with a proposal code number of R. 20.04.812. Consent was recruited from each of the study members.

Five ml blood was withdrawn from each included member under complete aseptic precautions on showing fever which was defined by having a single oral temperature recorded as ≥38.3 °C or oral temperature of ≥38.0 °C that continued for over one h and/or neutropenia; defined as an absolute count of neutrophils of less than or equal 500 cells/mm^3 [16].

Blood samples were immediately inoculated into blood culture bottles (Egyptian Diagnostic Media, Cairo, Egypt) to be aerobically incubated at 37ºC, observed daily up to seven days for signs of bacterial growth as turbidity, gas production, or hemolysis. Suspected bottles were then sub-cultured on MacConkey agar (Oxoid, England).

2.1 *Escherichia coli* Isolation

Escherichia coli strains were recognized by their colonial morphology, Gram's stain, and biochemical reactions as per standard procedures [17] followed by confirmation by API 20E (bioMérieux, France). Tests were conducted following the manufacturers' guidelines and then results were explained using the suitable reference indices as hence recommended by the producer.

2.2 Detection of Multidrug Resistant *E. coli* Strains

Disk diffusion method was applied to estimate sensitivity to various antimicrobial agents following the current National Committee for the Clinical Laboratory Standards Institute guidelines

(CLSI) [18]. We tested the coming antimicrobials; pencillins: amoxicillin–clavulanic acid (30 μg), ampicillin–sulbactam (20 μg) and piperacillin–tazobactam (100 μg/10 μg), cephalosporins: cefuroxime (30 μg), cefotaxime (30 μg), ceftazidime (30 μg), ceftriaxone (30 μg), and cefepime (30 μg), besides, aztreonam (30 μg), amikacin (30 μg), gentamicin (10 μg), imipenem (10 μg), meropenem (10 μg), sulfamethoxazole/trimethoprim (1.25/23.75 μg), ciprofloxacin (5 μg) and levofloxacin (5 μg) (Oxoid, England).

Isolates were recognized as multi-drug resistant depending on what declared by the European Centre for Disease Control (if isolate was found to be non-susceptible to more than or equal one agent in more than or equal three different antimicrobial categories) [18].

AmpC beta-lactamase *E. coli* producers were confirmed by modified three dimensional test where the noticed indentation of the inhibition zone of growth around the cefoxitin disk at the junction with the slit, indicated a positive test; the release of AmpC. In short, a Lawn culture standardized to half McFarland was prepared on Muller Hinton agar plate (MHA; Bio-Rad, Marnes-La-Coquette, France) from ATCC *E. coli* 25922 strain which is non-AmpC producer then cefoxitin (30 μg) antibiotic disc was inoculated centrally in the plate [19].

Second step was creating a linear slit of 3cm length, 3 mm distant from the cefoxitin disc by a sterile surgical blade. Then a small well was created at the other end of the slit with a sterile needle. Repeated freezing and thawing of tested *E. coli* strains followed by centrifugation for 15 min at 2000 rpm was required aiming to release the AmpC enzyme if present into the fluid. Finally 20μl of the supernatant was then loaded in the well to be tilted for 5- 10 minutes allowing the liquid to diffuse into the slit and plates were then incubated at 37°C for 24h [19].

Extended spectrum beta lactamase *E. coli* producers were known by double disk synergy test where a positive result was considered when growth inhibition zones around the cephalosporin disks were deformed towards the central disk containing clavulanic acid [20].

In brief, a suspension of each *E. coli* isolate adjusted to half McFarland standard was incubated overnight to be then sub-cultured on Muller-Hinton agar plate (MHA; Bio-Rad, Marnes-La-Coquette, France), then antibiotic

discs were inoculated as following; ceftazidime & ceftazidime (30 μg) with clavulanic acid (10 μg), as well as cefotaxime & cefotaxime (30 μg) with clavulanic (10 μg) pairs with a distance of 20 mm in between. Overnight incubation at 37°C was applied. The increase of the inhibition zone in presence of clavulanic acid compared to its absence by ≥5 mm was considered an indicator of ESBLs production. *E. coli* ATCC 25922 was used as a negative control strain [20].

Metallo-β-Lactamase and class A carbapene-mases were deduced by combined disk test (CDT) using EDTA and boronic acid disc respectively. CDT was carried out on inoculating two Muller –Hinton (MHA; Bio-Rad, Marnes-La-Coquette, France) agar plates with a standard half McFarland suspension of each *E. coli* isolate, then both imipenem (10 μg) and meropenem (10 μg) antibiotic discs were placed on each plate.

On one plate, 10μL of EDTA was poured over the antibiotic discs, while the other plate was showered with 10 μL of aminophenyl boronic acid to be all incubated overnight at a temperature of 37°C. An increase of more than or equal 5 mm in the diameter of the growth inhibition zone around the carbapenem discs compared to the obtained zone on using them alone indicated the production of MBL and class A carbapene-mases [21].

2.3 Polymerase Chain Amplification of Fosfomycin Resistance Genes

Deducing of the *murA*, *uhpT*, *glpT* and *fosA* genes was performed in multidrug resistant *E. coli* strains. DNA was extracted using QIAGEN Mini Kit (Hilden, Germany) as directed by the manufacturer's instructions.

The set of primers used to amplify *murA* gene was MF that had a sequence of: 5'-AAACAGCAGACGGTCTATGG -3' and that of MR was: 5''-CCATGAGTTTATCGACAGAACG-3', for *uhpT* gene: UF sequence was: 5'-TTTTTGAACGCCCAGACACC -3' and UR was: 5'-AGTCAGGGGCTATTTGATGG   -3', the sequence used for detecting *glpT* was as follows: GF: 5'-GCGAGTCGCGAGTTTTCATTG -3' and GR: 5'-GGCAAATATCCACTGGCACC -3' and that for *fosA* was: FAF: 5'-ATCTGTGGGTCTGCCTGTCGT -3' as a forward primer and FAR: 5'-ATGCCCGCATAGGGCTTCT -3' as a reverse one. They yielded DNA fragments of 1260, 1392, 1359 and 271 bps respectively [22].

PCR amplification for the three genes (*murA, uhpT* and *glpT*) was conducted in Norwall, CT (USA) thermal cycler as mentioned: starting by 2 min at a temperature of 94∘C, then followed by 30 turns of denaturation for 30 s at 94∘C, then another 30 s at 55∘C for annealing to be followed by extension for 2 min at 72∘C then final elongation for 10 min at 72°C. Similar conditions were used aiming to amplify *fosA* gene except that the annealing temperature was 60°C [22].

2.4 Statistical Analysis

Obtained data was shown as numbers with percentages. We used SPSS (version 21) for analyzing data by the Pearson's Chi square. P value less than 0.05 stated significance.

3. RESULTS

A total of 83 (40.5%) *E. coli* strains were retrieved from blood samples collected from patients with underlying blood malignancies seeking medical care at Mansoura University Hospitals, Mansoura, Egypt and showing evidence of blood stream infection. Disk diffusion technique was carried out to assess susceptibility to various antimicrobial agents. The recorded percentages of resistance to tested antibiotics were as follows: amoxicillin–clavulanic acid (93.9%), ampicillin–sulbactam (80.7%), piperacillin–tazobactam (95.2%), cefuroxime (78.3%), cefotaxime (71.1%), ceftazidime (68.7%), ceftriaxone (71.1%), cefepime (30.1%), aztreonam (57.8%), amikacin (73.5%), gentamicin (63.9%), imipenem (21.7%), meropenem (18.1%), sulfamethoxazole/trimethoprim (69.9%), ciprofloxacin (59.0%) and, levofloxacin (54.2%) (Table1).

Out of those isolates, 58 (69.9%) were found to be multidrug resistant. On testing the retrieved isolates for AmpC beta-lactamase production, 15 (25.8%) gave positive results, similarly on applying double disk synergy maneuver to estimate ESBLs production; 43 (74.1%) isolates were ESBLs producers. On using combined disk test, 9 (15.5%) *E. coli* isolates gave positive findings indicating release of class A carbapene-mases (Table 2).

In the current study, we determined the distribution of *murA, uhpT, glpT and fosA* genes among multidrug resistant *E. coli* strains. Depending on PCR results, *murA* gene was deduced in 1 isolate (1.7%), *uhpT* gene in 3 (5.2%), *glpT* gene in 4 (6.9%) whereas *fosA*

gene was found in 7 *E. coli strains* (12.1%) (Table3).

Two *fosA* gene positive *E coli* strains were found to be AmpC producers (13.3%), whereas 3 isolates were ESBLs producers (7%). Two positive stains were proved to be MBL class A carbapenemase positive (22.2%). Regarding *murA* gene, it was only detected in a single MBL class A carbapene-mases producing *E coli* strain (11.1%). Furthermore, *uhpT gene* was only found in one AmpC *E coli* producer (6.7%) and two ESBLs *E coli* strains (2.4%). Finally, *glpT gene* was estimated in 3 (7%) strains phenotypically proved to secrete ESBLs and 1 (11.1%) class A carbapene-mases (Table 4).

4. DISCUSSION

Studying of fosfomycin resistance pattern and its genes in relation to blood stream infections in hematological malignancies is an interesting point especially in our locality due to the wide spreading burden of drug resistance. This study was conducted over 205 patients with hematological malignancies and showed the evidences of blood stream infection.

In this study, *E. coli* was our concern. It represented 40.5% of all isolated organisms from blood samples enrolled in this work up. Mandal et al study clarified that the percentage of *E. coli* isolated from blood in patients with hematological malignancies was 2.61% which was much less than our study results and the isolated ESBLs producing *E. coli* was 1.86% [23]. They surveyed the blood samples from all feverish patients with hematological malignancies but our study included only patients with clear evidences of blood stream infections, besides differences in pathogenic bacterial organisms' prevalence, types of flora & applied antibiotic policies and protocols could be reasons for noticed difference.

The reported percentages for *E. coli* isolation from cancer patients with blood stream infection were 51.23% and 52.52% as mono-microbial and poly-microbial organisms respectively as declared in Royo-Cebrecos and his colleagues study [24]. A finding that lies in consistence with our study results was the *E. coli* isolation by 38.6% from malignant hematologic disease patients with blood stream infection after cord blood transplantation [25].

The current study estimated that 69.9% of isolated *E coli* were multidrug resistant. The Amp C beta-lactamase producing *E. coli* was 25.9%.

On applying double disk synergy test, it revealed that 74.1% isolates were ESBLs producers but with combined disk test, 15.5% *E. coli* isolates showed the release of class A carbapene-mases. The ESBLs producing *E. coli* was 62.5% from blood stream infection in cases of acute leukemia [26].

Nosheen's study [27] stated that carbapene-mases producing *E. coli* from blood stream infection patients was 16% but they counted all the carbapene-mases producing *E. coli* isolated from multiple infection sites. Out of this percentage, 37.2% was from blood stream infections. Another Indian study reported 22 *E. coli* isolates but all were carbapene-mases producing ones [28]. In a study conducted in Los Angeles, five (45.4%) *E. coli* isolates out of 11 were carbapene-mases producers [29]. The small number of the isolated stains might be the cause of this high percentage. Li and his coworkers showed that fosfomycin-resistant *E. coli* were significantly more likely to be ESBLs producers [30].

By PCR, *murA* gene was present in only 1.7% of retrieved multidrug resistant *E. coli* strains, *uhpT* gene in 5.2%, *glpT* gene in 6.9% whereas *fosA* gene was found in 12.1%. In a study by Lil et al, *glpT* gene was detected in six *E. coli* strains with a truncated glp T protein resulting from substitutions or deletion of a nucleotide in encoding gene. A similar finding was also reported for *murA* gene isolates [31].

Other previous studies stated that fosfomycin resistance in *E. coli* may be attributed to defects in either GlpT or UhpT proteins [22]. *FosA3* gene is present on a conjugated plasmid thus raising the mobility of resistance between the strains and disseminating it all over the world [32]. Sometimes fosfomycin resistance may be due to reduction of the bacteria's fitness or reduction of its virulent nature [33].

Two *fosA* gene positive *E coli* strains were found to be AmpC producers (13.3%), whereas 3 isolates were ESBLs producers (7%). Mueller et al study estimated that 29% of the ESBLs producing *E. coli* were having a *fosA* gene [34]. Two positive stains were proved to be MBL class A carbapene-mases positive (22.2%). Regarding *murA* gene, it was only detected in a single MBL class A carbapene-mases producing *E coli* strain (11.1%). Furthermore *uhpT gene* was only found in one AmpC *E coli* producer (6.7%) and two ESBLs *E coli* strains (2.4%).

Table 1. Antibiotic sensitivity pattern of *E. coli* isolates by disk diffusion method

Antibiotic		Sensitive		Resistant		P value
		№ (total=83)	%	№ (total=83)	%	
Pencillins	Amoxacillin clavulinic acid	5	6.1	78	93.9	<0.001*
	Ampicillin–sulbactam	16	19.3	67	80.7	<0.001*
	Piperacillin–tazobactam	4	4.8	79	95.2	<0.001*
Cephalosporins	Cefuroxime	18	21.7	65	78.3	<0.001*
	Ceftriaxone	24	28.9	59	71.1	<0.001*
	Cefotaxime	24	28.9	59	71.1	<0.001*
	Ceftazidime	26	31.3	57	68.7	0.004*
	Cefepime	58	69.9	25	30.1	0.003*
Monobactams	Aztreonam	35	42.2	48	57.8	0.02*
Carbapenemes	Imipenem	65	78.3	18	21.7	<0.001*
	Meropenem	68	81.9	15	18.1	<0.001*
Aminoglycoside	Gentamicin	30	36.1	53	63.9	0.005*
	Amikacin	22	26.5	61	73.5	0.002*
Trimethoprim sulphamethoxazole		25	30.1	58	69.9	0.003*
Quinolones	Ciprofloxacin	34	41	49	59.0	0.06
	levofloxacin	38	45.8	45	54.2	0.07

Pearson's Chi square

Table 2. Prevalence of Amp C, ESBLs and MBLs production among studied multi-drug resistant *E. coli* isolates

Type of beta lactamase enzyme	AmpC *E. coli* producers	ESBL *E coli* producers	MBL class A carbapenemase *E coli* producers
No	15	43	9
%	25.8	74.1	15.5
Total	58 (100%)		

Table 3. Prevalence of fosfomycin resistance genes among multidrug resistant *E. coli* isolates

Studied gene	*murA* gene		*uhpT* gene		*glpT* gene		*fosA* gene		Total
	№	%	№	%	№	%	№	%	
Positive	1	1.7	3	5.2	4	6.9	7	12.1	
Negative	57	98.3	55	94.8	54	93.1	51	87.9	58(100%)

Table 4. Correlating distribution of fosfomycin resistance genes to the category of the beta lactamase enzyme produced by multidrug resistant *E. coli* isolates

	murA gene		*uhpT* gene		*glpT* gene		*fosA* gene		Total
	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	
AmpC	0(0.00%)	15(100%)	1(6.7%)	14(93.3%)	0(0.00%)	15(100%)	2(13.3%)	13(86.4%)	15(100%)
ESBLs	0(0.00%)	43(100%)	2(4.7%)	41(95.3%)	3(7%)	41(93%)	3(7%)	40(93%)	43(100%)
MBL class A carbapenemase	1(11.1%)	9(100%)	0(0.00%)	9(100%)	1(11.1%)	8(88.9%)	2(22.2%)	7(77.8%)	9(100%)

Finally, *glpT gene* was estimated in 3 (7%) strains phenotypically proved to secrete ESBLs and 1 (11.1%) class A carbapene-mases. One limiting field in the fosfomycin resistance is direct inactivation of the antibiotic by metallo-enzymes (FosA, FosB and FosX), which are transmissible. They are frequently found in ESBLs enterobacteria and carriers of carbapene-mases with specific concern to *E. coli* [14].

One Swiss study estimated the resistance in between *E. coli* strains obtained from a tertiary care center between year 2012 and year 2015 revealed a fosfomycin resistance rate of 0.9% (35). This low deduced prevalence, may predict the low risk of expansion of such fosfomycin resistance among ESBLs-producing *E. coli* [10].

Inhibition of production and action of FosA might greatly help to expand the activity of fosfomycin against Gram negative pathogens that have an inherent ability to express this enzyme. Deletion of chromosomal *fosA* in *S. marcescens* [36] or transposon-mediated disruption of *fosA* in both *P. aeruginosa* and *K. pneumoniae* was found to eliminate intrinsic fosfomycin resistance [15] which can help to support the previously mentioned hypothesis

Further studies on larger number of cases and longer periods of time should be conducted beside this current study to provide a wider knowledge about the fosfomycin resistance among the multi-drug resistant *E. coli* from blood stream infections and other types of infections in hematological malignancies patients.

5. CONCLUSION

The research concluded that although fosfomycin is a promising antibiotic with limited degree of prevalence of its resistant genes, there is a fear of disseminating resistance that might increase especially with presence of ESBLs, Amp C and MBL class A carbapene-mases. However, we hope a low dissemination rate of this resistance.

5.1 Significance and Impact of Study

Testing liability to resistance of new antimicrobial groups like fosfomycin is a must especially in communities, like our own, suffering from wide spreading of multi-drug resistant bacterial strains; so providing alternatives for treating such strains.

CONSENT

Informed consent was received from all individual participants included in the study.

ETHICAL APPROVAL

The study protocol was approved by our faculty review board (R. 20.04.812). The procedures used in this study adhere to the tenets of the Declaration of Helsinki.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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