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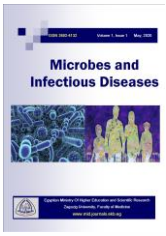

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Original article

Evaluation of *in vitro* effect of Fosfomycin on resistant Gram-negative pathogens in urinary tract infection

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ABSTRACT

Background: Urinary tract infection (UTI) is considered one of the most common infections occurring in different ages. The increasing emergence and rapid spread of multidrug-resistant (MDR) pathogens has led to reuse older antimicrobials like Fosfomycin. This study aimed to evaluate the activity of Fosfomycin on MDR pathogens beside its effect on biofilm formation. **Methods**: A total of 116 MDR Gram -negative isolates from ICU patients suffering from UTI has been included in this study. Standard microbiological tests were done to identify the isolates. Susceptibility to various antibiotics was detected by disk diffusion method. Phenotypic tests for determining various β-lactamases were done. Minimal inhibitory concentration (MIC) for Fosfomycin was detected by agar dilution method. Formation of biofilm by the isolates with and without adding Fosfomycin was assessed by microtiter plate method. **Results:** The most frequently isolated pathogen was *E. coli* (70/116); 60.3% followed by *Klebsiella spp.* (31/116); 26.7%. Fosfomycin showed a high level of inhibitory effect on most of tested isolates ; *E. coli* revealed low resistance rate of 4.2%, while *Klebsiella spp*, *Pseudomonas aeruginosa* and *Acinetobacter baumani* showed resistance rate of 16% ,36%), and 50%, respectively. A total of 72 (62.1%) isolates was ESBL producers, of which 92% isolates were Fosfomycin - sensitive , while 25(22%) isolates were MBL-positive, of which 88% were sensitive to Fosfomycin. Eighty-seven (75%) isolates were biofilm producers. Fosfomycin inhibited biofilm formation in 67(77%) isolates. **Conclusion**: ESBL and MBL producing Gram negative urinary pathogens showed high sensitivity level to Fosfomycin. Also, Fosfomycin had good inhibitory effect on their biofilm formation.

Introduction

Urinary tract infection (UTI) is considered one of the most frequent problems found in different clinical settings and accounts for about a quarter of all antimicrobial prescriptions. Every year, 150 million people worldwide are affected by this infection, including 35% of hospital infections [1]. *Escherichia coli (E.coli)* is the main pathogen responsible for uncomplicated cystitis and pyelonephritis, followed by other species of *Enterobacteriaceae*, such as *Proteus mirabilis* and mostly *Klebsiella pneumoniae*, Gram-positive pathogens, such as *Enterococcus faecalis* and *Staphylococcus saprophyticus* also may be included [2].

International guidelines for the treatment of uncomplicated UTIs include various agents, such as nitrofurantoin monohydrate, fluoroquinolones,

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trimethoprim-sulfamethoxazole, and beta lactams. Currently,a great number of uropathogens has been shown to exhibit a high level of resistance to more than one antimicrobial agent and are identified as multidrug-resistant organism (MDROs). Resistance of organisms to multiple drugs represents the most important problem arouse in antibiotic resistance era because it is difficult to manage infections by these organisms beside their exponential increase over the past few years [3]. The rapid evolution of resistance to antimicrobial agents and the decrease in introducing new drugs make it necessary to reconsider the use of older antibiotics [4].

"Fosfomycin", initially known as phosphonomycin, is an old antimicrobial agent with bactericidal and wide spectrum action, firstly identified in Spain in 1969. It belongs to the epoxide group of antibiotics which is structurally distinct from any other antimicrobial agent currently used. Fosfomycin acts by inhibiting phosphoenolpyruvate synthetase which is the first step in the synthesis of peptidoglycan so, interferes with cell wall synthesis [5]. It is available for oral use as "Fosfomycin tromethamine" and as "Fosfomycin disodium" for injection intravenously (IV). Fosfomycin is considered among the first-line therapeutic options for managing UTI [6].

Previous researches on Fosfomycin had noticed its good in vitro efficacy to ward resistant organisms like extended spectrum β lactamases (ESBL) producers, *Klebsiella pneumonia e*resistant to carbapenem and multi-resistant *P. aeruginosa*. This generated more interest in considering usage of Fosfomycin in the last few years [7].

In order to evaluate the effectiveness of Fosfomycin for the management of infections due to MDR pathogens, further studies are necessary. Hence this study on the *in-vitro* efficacy of Fosfomycin on MDR Gram negative pathogens was taken.

Materials and Methods

This cross-sectional study was carried out from (April to December 2021) at Zagazig university hospitals and Medical Microbiology and Immunology Department, Faculty of Medicine, Zagazig University.

Patients

This study included all patients admitted to ICU suffering from UTIs. Urinary tract infection is clinically manifested by the presence of pyrexia and/or any of the symptoms suggesting UTI such as increased frequency, dysuria, haematuria, suprapubic and/or flank pain. Patients who have refused to participate in the study or have received previous antibiotics within 48hrs before urine sampling and those with UTI caused by Gram positive pathogens, have been excluded from the study.

Zagazig University Institution Review Board (ZU-IRB) has approved this research (Approval code 6894). Consent was taken from all participants or their relatives.

Bacterial identification

One hundred sixteen MDR Gram-negative isolates were recovered from aseptically collected urine specimens. Cultivation of samples was done on "cysteine lactose electrolyte-deficient medium" (CLED agar,**Oxoid, UK**,) after immediate transport to the laboratory. Only isolates with a significant count have beenenrolled in our study. The isolates were identified at first by standard microbiological and biochemical tests [8,9], then verified using API 20 E, API20NE **(Bio Merieux, France)**.

Testing sensitivity of the isolates to different antimicrobial agents

On Mueller-Hinton agar (MHA) plates disc diffusion method has been performed and the results were interpreted using the guidelines of Clinical and Laboratory Standards Institute (CLSI) [10]. The antimicrobial agents used were cefotaxime (CTX:30 µg), gentamicin (CN:10 µg), trimethoprim / sulphamethoxazol (STX:1.25 / 23.75 µg), amikacin (AK:30 µg), nitrofurantoin (F:300 µg), piperacillin-tazobactam (PTZ:100 / 10 µg), imipenem (IMI, 10 µg), ciprofloxacin (CIP: 5 µg),Quality control strain "*E. coli* ATCC®25922" was used.(American Type Culture Collection [ATCC], Manassas, VA, USA).

The MDR was defined by a resistance to at least one agent in 3 or more antimicrobial groups [11]. All MDR isolates were examined for sensitivity to Fosfomycin using disc diffusion (DD) and agar diution (AD) methods. For DD, Fosfomycin disks that contain (200 µg of Fosfomycin plus 50 µg of G6P) (Oxoid, UK) have been used. Determination of MIC by AD was done by adding Fosfomycin disodium salt **(Sigma Aldrich Corporation, USA)** MHA(**Oxoid, UK**) containing 25 mg/L G6P(**Alpha Chemika,India**) to obtain serial two-fold dilutions with concentrations ranged from 0.25 to 512 mg/l.

The results of MIC of the Fosfomycin were interpreted according to CLSI guidelines for *E. coli* ($S \leq 64$, $I = 128$. $R \geq 256$), while EUCAST [12]

guidelines (S ≤ 32, R>32) were applied for all isolates of "*Enterobacteriaceae"* other than "*E. coli*" and "*Pseudomonas aeruginosa"* as CLSI do not provide any criteria for "*Pseudomonas aeruginosa"* and "*Enterobacteriaceae"*other than*"E.coli"* [13].

MIC50 and MIC90 values of Fosfomycin were calculated for tested isolates. MIC50 and MIC90 were defined as the lowest concentration of an antimicrobial at which 50% and 90% of bacterial isolates are inhibited respectively [14].

Phenotypic detection of different beta-lactamases like ESBL, and MBL (metallo β-lactamases)

- **Double disc synergy test (DDST) for ESBL detection:**

After streaking MHA plate with an inoculum of the tested organism adjusted to (0.5 McFarlandturbidity standard), cefotaxime (ctx) or ceftazidime and amoxicilin–clavulanate (AMC) discs have been placed at 30 mm (centre to centre) then incubated. The inhibition zone is enhanced towards amoxicillin-clavulanic acid (keyhole) indicating ESBL production [13].

- **DDST for MBL detection:**

On MHA plate, a disc of imipenem (10 µg) was put at a distance (20 mm center to center) from a blank disc to which 10 µL of 0.5 M EDTA (750 µg) was added. After incubation, observing potentiation of the inhibition zone in the distance between the two discs versus that on the other side of imipenem disc was considered as positive for MBL production [13].

- **Phenotypic detection of biofilm formation with and without Fosfomycin**

Assessment of biofilm formation has been carried out by microtiter plate assay, as mentioned by **Stepanovi et al.,** [15]. Fresh culture of isolates has been adjusted to a 0.5 McFarland turbidity standard. Suspensions were diluted 1:100 in 200 mL tryptic soy broth (TSB) with 1% glucose (**Oxoid, UK**) before being transferred to a sterile flat bottomed tissue culture plate. Incubation of the plates for 24 hrs at 37 °C then, the wells were rinsed three times gently with sterile phosphate buffered saline (PBS, pH 7.3) to remove planktonic bacteria. The adherent biofilms were fixed for 15 minutes in 99 percent methanol, then the solutions were withdrawn, and the plate was left to dry in air. Biofilms were stained for 5 min at room temperature by 200 µL of crystal violet 0.1% **(Sigma Chemical Co., St Louis, MO, USA**), and then washed with water and left to dry. Wash by 200 ul 95% cold ethanol in each well until complete decolorization of negative control. Using a microtiter plate reader, the optical density (OD) was measured at 570 nm (**BioTek, USA**). All experiments were done in triplicate and the average values were taken. The standard deviation was calculated.

Fosfomycin has been used with a concentration lower than MIC level of various isolates then the obtained values were compared.

Statistical analysis

All data have been analyzed using SPSS 22.0 for windows (SPSS Inc., Chicago, IL, USA). Continuous variables were displayed as mean + standard deviation (SD) and range. Quantitative data were described as percentage.

Results

In our study, a total of 116 MDR bacterial isolates were detected from urine specimens. Seventy isolates were female while (46) were male patients with their ages ranged from (29-90) years, (M±SD:55.2±14.5). The most common bacteria encountered was *E. coli* 60.3% (70/116) followed by *Klebseilla species(spp.)* 26.7% (31/116), *Pseudomonas aeruginosa* 9.5% (11/116) and *Acinetobacter baumanii*. 3.5 % (4/116). Catheterization was observed to be the main risk factor for UTI followed by urinary stones. Antibiotic susceptibility profile of isolated organisms to different antimicrobials was shown in **table (1)**. Among the differently tested antibiotics,Fosfomycin was with high level of inhibitory effect on most of tested isolates. *Esherichia coli* showed low level of resistance (4.2%), *Klebsiella spp.*(16%), *Pseudomonas aeruginosa* (36%) while *Acinetobacter baumanii*(50%). Extended spectrum β lactamases producing isolates were 72(62.06%) from which 66(91.7%) isolates were sensitive to Fosfomycin, while 25(21.5%) isolates were MBL producers and 22(88%)from these isolates showed sensitivity to Fosfomycin, **(Tables 2,3), (Figures 1 and 2).**

Regarding sensitivity of studied isolates to Fosfomycin by agar dilution method, 68(97%) of *E. coli* were sensitive according to CLSI guideline. MIC50 was 1 Ug /dl and MIC90 was 8 Ug /dl.20(64.5%) *Klebsiella spp*. ,8(72.7%) *Pseudomonas aeruginosa*, and 1(25%)

Acinteobacter baumanii were sensitive according to EUCAST breakpoints, **(Table 4)**.

Among the studied isolates, 87 (75%) were biofilm producers; (56,21,8 and 2) isolates of (*E. coli, Klebsiella, Pseudomonas aeruginosa and A. baumanii*), respectively. Fosfomycin inhibited biofilm formation at two-fold dilution lower than MIC of each isolate. Biofilm formation was inhibited in 67 isolates:(45) *E. coli* and (19) *klebsiella spp*. and (3) *Pseudomonas aeruginosa* **(Table 5)**.

Table 1. Antimicrobial resistance profile of studied isolates.

	Amikacin N (%)	**Gentamycin N (%)**	**Nitrofurantoin N (%)**	**Ceftriaxone N (%)**	**Imipeneme N (%)**	**Pipercillin/ tazobactum N (%)**	**Trimethoprim /sulfa N (%)**	**Fosfomycin N (%)**	**Ciprofloxacin N (%)**
***E.coli* (70)**	50 (71.4)	35 (50)	31 (44.2)	58(82.8)	12(17)	65(92.8)	56(80)	3(4.2)	48(68.5)
***Klebsiella spp* (31)**	18 (58)	27 (87)	19 (61.2)	25(80.6)	11(35.4)	31(100)	26(83.8)	5(16)	13(41.9)
***P. aeruginosa* (11)**	8 (72.2)	6 (54.5)	11 (100)	11(100)	5(45.4)	8(72.2)	9(81.1)	4(36)	5(45.4)
A. baumanii (4)	4 (100)	4 (100)	4 (100)	4(100)	1(25)	4(100)	4(100)	2(50)	1(25)

Table 2. Phenotypic detection of different beta-lactamases producing isolates.

Isolates (n)	**ESBL**	**MBL**
E. coli (70)	43 (61,4%)	10(14.2%)
Klebsiella spp (31)	23(74.1%)	11(35.4%)
Pseudomonas aeruginosa (11)	5(45.4%)	4(36.3%)
Acinetobacter baumanii (4)	1(25%)	0(0%)
Total (116)	72(62.06%)	25(21.5%)

Table 3. Fosfomycin effect on various beta-lactam resistant isolates.

Isolates	**R**	**S**
ESBL Producers (72)		
E.coli(43)	1 (2.3%)	42(97.7%)
Klebsiella spp. (23)	2(8.7%)	21(91.3%)
Pseudomonas aeruginosa (5)	3(60%)	2(40%)
Acinetobacter baumanii (1)	0(0%)	1(100%)
Total	6(8.3%)	66(91.7%)
MBL producers (25)	**R**	**S**
E.coli(10)	1(10%)	9(90%)
Klebsiella spp (11)	2(18%)	9(82%)
Pseudomonas aeruginosa(4)	0(0%)	4(100%)
Acinetobacter baumanii(0)	0(0%)	0(0%)
Total	3(12%)	22(88%)

Table 4. Minimum inhibitory concentration (MIC) distribution and susceptibility rates of Fosfomycin by agar dilution method against different bacterial isolates.

Isolates	0.5	1	2	4	8	16	32	64	128	256	MIC50 (ug/dl)	MIC90 (ug/dl)
***E.coli* (70)**	30	8	4	12	9	0	0	5	0	2	1	8
***Klebsiella spp.*(31)**	0	0	1	0	4	2	13	3	5	3	32	128
***Pseudomonas aeruginosa*(11)**	0	0	0	0	3	5	0	2	0	1	16	64
***Acinetobacter baumanii* (4)**	0	0	0	1	0	0	0	0	2	1	128	256

Table 5. Fosfomycin effect on biofilm produced by different isolates.

Isolates	Fosfomycin inhibit biofilm (No=67)	Fosfomycin doesn't inhibit biofilm (No=20)
E. coli	45 (67.2%)	11(55%)
Klebsiella species	19 (28.4%)	2 (10%)
Pseudomonasaeruginosa	3 (4.4%)	5 (25%)
Acinetobacter baumani	0 (0.0%)	2 (10%)

Figure 1. DDST for ESBL detection: The inhibition zone is enhanced towards amoxicillin-clavulanic acid (keyhole) .

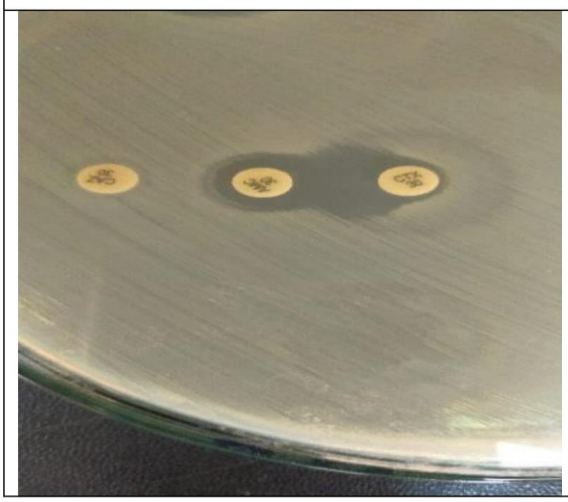

Figure 2. DDST for MBL detection:potentiation of the inhibition zone in the distance between the Imipenem &EDTA.

Discussion

Urinary tract infections are common bacterial infections caused by different pathogens and constitute a significant burden of hospitalizations and health-care cost. Currently, the rapid development of resistance to routinely used antibiotics made therapeutic options for treating UTI very limited. Hence, there is increasing interest in re-evaluation of old antimicrobial agents to overcome this problem [16]. The aim of this study was evaluation of Fosfomycin activity as analternative drug to treat UTI as well as a last line in management other infections caused by MDR Gram-negative organisms.

In this study, the most common isolated bacteria was *E. coli* 60.3%. This finding is in agreement with studies done in Egypt by **Desouky et al.** [17] and **Ali et al.** [18]. Also, other studies from different countries by **Tumturk et al.** [19].

Zare et al. [1] and **Fajfr et al**. [7] reported that *E. coli* was the most predominant isolated pathogen (66.7%,.45 %,51.3%) respectively.This documented the principal role of *E. coli* in causing UTI.

On analyzing sensitivity pattern, the highest level of sensitivity was observed to Fosfomycin followed by imipenem and ciprofloxacin.In our study, *E. coli* showed a high level of sensitivity to Fosfomycin (95.8%) followed by *Klebsiella spp* (84%), *Pseudomonas aeruginosa* (64%) while *Acinetobacter baumanii* (50%). This low level of resistance toward Fosfomycin may be attributed to non-routine usage of this antibiotic in clinical settings unlike other antibiotics. Withdrawal of an antimicrobial agent from daily regimens or excessive usage in medical field helps to great extent in removing the selective pressure on the antibiotic**.**

Concurrent observations were seen in the study done by **Kowshik and Sumana** [20], who found that Fosfomycin susceptibility was (88.88) % in *E. coli*,(80.39) % in *Klebsiella* and 60.52% in *Acinetobacter.* Also, high level of susceptibility to Fosfomycin was reported by studies conducted by **Gopichandet al. [**21] and **Fajfr et al**. [7].The promising antimicrobial activity of Fosfomycin against MDR Gram negative bacteria may be due to unique mechanism of action of this agent. In addition, Fosfomycin is found in a high concentration for a longer period of time in voided urine, its tolerability and safety are also excellent [22].

Extended spectrum β lactamases producing isolates in this study, were 72(62.06%) from which 66 (91.7%) isolates showed sensitivity to Fosfomycin. Previous studies in Egypt reported high rate of ESBL producers. **Zaiton NE et al.** [23] found that ESBL production among isolated *Enterobacteriaceae* was 49 %, while **Abdallah and colleagues** [24] at El Ahrar Hospital-Sharkia governorate detected (48.9%). Similar results were also reported by other researchers **Khater and Sherif,** [25]. An increasing rate of ESBL producers observed in Egypt may be attributableto the irrational use and availability of antibiotics over the counter.

A study conducted by **Gupta et al.** [26]. has found that 52.6% of *E. coli* were positive for ESBL, and all isolates were sensitive to Fosfomycin. In a survey conducted in Spain, there were 417 (97.4%) ESBL-producing isolates showed susceptibility to Fosfomycin[27]. Notably, **Maraki et al**. [28] have demonstrated 100% susceptibility rate to Fosfomycin among ESBL-producing *E. coli* and *K. pneumoniae* in their recent study.

Carbapenems have been one of the last choices for management of infections caused by MDR organisms but, their role has been affected by rising spread of carbapenem resistant gram-negative organisms.In our study 25(21.5%) isolates were MBL producers by phenotypic method and 22(88%)from these isolates showed sensitivity to Fosfomycin.

This frequency of MBL producing isolates, was lower than that reported in studies by **Elsheshtawy**[29], **Mariappan et al.** [30] and **Abo-Alella et al**. [31] whofound MBL-producers were(41%),(58.6%) and(79.3%) respectively. The discrepancy in the prevalence of MBL among different studies may be due to different sample size orvariances in antimicrobial regimens applied in each locality.

In this study, we performed agar dilution method for determining MIC of Fosfomycin as it the only approved method for MIC detection according to EUCAST. (97%) of *E. coli*were sensitive with (MIC50) was 1 Ug /dl and (MIC90) was 8 Ug /dl .Also, (64.5%) of *klebsiellaspp*, (72.7%) of *pseudomonasaeruginosa*and (25%) of*Acinteobacterbaumani* were sensitive. In a study conducted by **Gopichand et al.** [21] (MIC 50) and (MIC 90) of Fosfomycin in *E. coli* was 1 µgm/mL and 2 µgm/mL respectively. **Aprile et al.** [3] found the values to 1 mg/L and32 mg /L,respectively. **Anand et al**. [32] determined that Fosfomycin MIC50 &MIC90 for *E. coli* by E test stripswere 1 mg /L & 8 mg /L respectively.

Infection by biofilm -forming organisms is a serious problem leads to chronicity and treatment failure. Bacteria inside biofilm community develop resistance to antibiotics by several mechanisms suchas, hindering diffusion of antibiotics, activating efflux pumps that extrude antibiotics out,tolerance and metabolic inactivity of bacteria , horizontal gene transfer ,interaction of antibiotics with a polymeric matrix of biofilm that decreases their activity andmodifications on target cells or masking the target sites, [33]. As a result, newer strategies with ability to limit the prevalence of infections by these organisms and effectively helps in treatment of these chronic conditions should be developed.

Hence, Fosfomycin effect on biofilm has been studied., Fosfomycin has the property to be concentrated in urine that increase its capacity to

break up biofilms, this may be attributed to its good renal excretion.

Regarding this study, out of 116 isolates, **87 (75%)** isolates were biofilm producers. Similaly, **Gopichand et al.** [21] & **Christensen et** al. [34] found that Fosfomycinhas the ability to inhibit biofilms at aconcentration below the MIC level. Biofilm formation was inhibited in 67 isolates: (45) *E. coli* and (19) *Klebsiella spp*. and (3) *Pseudomonas aeruginosa*. The underling mechanisms of Fosfomycin capability to disturb biofilm are to be discussed.

Recently, there are reports of Fosfomycin resistance developed in Spain and Hong Kong due to its extensive use.**Wachino et al.** [35] from Japan identified FosA3 and FosC2, Fosfomycin-resistance genes among *E. coli* isolates. these genes are transmissible and have the ability to spread resistance to this antibiotic, hence, further studies concerning activity of Fosfomycin with other antibiotic combinations are recommended. These combinations allow usage of antibiotic at a low concentration, so decreasing the risk of adverse effect, limiting development of resistance in addition to their synergistic effect.

Limitation

Small number of studied isolates especially *Acinetobacter baumanii*. The effect of Fosfomycin with other antibiotic combinations could not be assessed due to limited financial resources.

Conclusion

In our study, ESBL and MBL producing Gram Negative urinary pathogens showed high sensitivity level to Fosfomycin. Also, Fosfomycin has good inhibitory effect on biofilm formation. Fosfomycinshould be considered as a highly effective therapeutic alternativefor UTIs caused by MDR Gram negative pathogens including ESBL and MBL producers.

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Contributors and authorship: All authors have made substantial contributions to the conception and design of the study, acquisition of data, analysis and interpretation of data, drafting the article and revising it critically for important intellectual content. Finally, they approved submissionthe version.

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