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ORIGINAL ARTICLE

Antibacterial Effect of Fosfomycin on Uropathogenic *Escherichia Coli* and its Biofilm Forming Ability

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ABSTRACT

Background: *Fosfomycin is an old broad-spectrum bactericidal agent. It has been suggested that fosfomycin can be introduced in the treatment of urinary tract infections resulted from multi-drug resistant organisms.* ***Objective:*** *In this study, the effect of fosfomycin on uropathogenic Escherichia coli and its ability to produce biofilm was studied.* ***Methodology:*** *Sixty uropathogenic E. coli isolates were collected. Antibiotic susceptibility testing was performed using disk diffusion method. The minimum inhibitory concentration (MIC) of fosfomycin was measured using agar dilution technique. Biofilm formation was studied using tissue culture plate method. The effect of sub-inhibitory concentrations of fosfomycin on the biofilm forming ability of the isolates was assessed.* ***Results:*** *High rates of resistance were reported for most of the tested antibiotics except for fosfomycin, to which all isolates were susceptible, and imipenem, to which 58 (96.67%) isolates were susceptible. MIC testing revealed 16.67% resistance rate to fosfomycin. Twelve (20%) isolates were found to be biofilm forming. Ten of them (83.33%) lost their ability to produce biofilm under the effect of fosfomycin.* ***Conclusions:*** *This study revealed a high antibacterial efficacy of fosfomycin against uropathogenic E. coli ability to produce biofilm in vitro. Further research studies are required to assess its ability to abolish biofilm formation on urinary catheters.*

INTRODUCTION

Urinary tract infections (UTIs) represent the most commonly acquired bacterial infections in both community and hospital settings, affecting all age groups [1]. They are responsible for up to 40% of the health care-associated infections [2]. It is particularly common in females as about 60% of all women develop a UTI episode at some point through their lives. A third of these women have at least one recurrence within a year [3].

Escherichia coli has been reported as the most prevalent microbe causing UTIs [4]. During the last decade, an obvious surge in the prevalence of multi-drug resistant (MDR) *E. coli* isolates from human sources has been recorded all over the globe [5].

Biofilm formation is a mechanism exhibited by various bacterial species to survive under disadvantageous conditions. The bacterial biofilm can be described as a structured community of bacterial cells surrounded by a polymeric matrix and attached tightly to a surface. Biofilm-forming uropathogenic bacteria may account for several recurrent UTIs. Moreover, the bacteria enclosed within the biofilm show high resistance to antibiotic treatment [6]. Previous studies have shown that sub-inhibitory concentrations of some antibiotics, although unable to eradicate bacterial cells, can inhibit biofilm-formation [7].

Fosfomycin, originally named phosphonomycin, was first discovered in Spain, 1969[8]. It has been considered a first line agent for treatment of uncomplicated UTIs because of its extremely low rate of resistance in primary uropathogens, its ideal pharmacokinetic parameters, ease of administration as a single dose, good tolerability and clinical efficacy [9,10]. Fosfomycin has a relatively long half-life (mean half-life: 5.7 hours) and a low molecular weight and therefore, it readily penetrates various tissues and achieves the minimum inhibitory concentrations needed to inhibit the growth of most pathogens [11]. It has a broad spectrum activity against the vast majority of bacteria and encouraging *in vitro* activity against MDR Gram-negative pathogens [12]. According to the present susceptibility breakpoints, fosfomycin is effective against most drug resistant *Enterobacteriaceae* [13, 4].

Fosfomycin suppresses peptidoglycan synthesis through an interaction with UDP-N-acetylglucosamine enolpyruvyl transferase (MurA). This interaction leads to reduced synthesis of the peptidoglycan precursor N-acetylmuramic acid from N-acetylglucosamine and phosphoenolpyruvate [15].

Adverse effects are rarely reported with oral fosfomycin administration[16] with only 5% of patients complaining of side effects, most commonly diarrhea[17].

Therefore, fosfomycin can be considered for the treatment of serious infections including UTIs resulted from multi-drug resistant organisms. However, a

paucity of information is available regarding its pharmacokinetics and pharmacodynamics and great uncertainty considering optimal regimens for systemic infections [18].

The aim of this work was to study the antibacterial activity of fosfomycin against *E. coli* isolated from urine samples from patients with UTIs as well as to evaluate its effect on the ability of uropathogenic *E. coli* (UPEC) to produce biofilm *in vitro*.

METHODOLOGY

Subjects

This study involved 60 *E. coli* isolates from mid-stream urine samples collected from UTI patients attended the outpatient clinics of Kasr Al-Aini Hospitals. The samples were collected during the period from June to October 2016. Patients of any age group, of both sexes, complaining of symptoms suggestive of UTI such as fever, urgency, frequency, dysuria or suprapubic tenderness and whose urinary cultures showed *E. coli* with bacterial count of 10^5 CFU/ml or more were included in this study.

Methods

Collection of urine samples:

This study involved 60 UPEC isolates obtained from bacterial cultures of midstream urine samples which were collected under aseptic techniques in dry, wide mouthed sterile containers covered with a tight fitted lid and free of preservatives and detergents. The study was approved by the Research Ethics Committee of the Institutional Review Board, Faculty of Medicine, Cairo University and informed consent was obtained from all participants.

Isolation and identification of E. coli colonies:

A loopful of an uncentrifuged sample was inoculated onto MacConkey's medium using a calibrated loop to determine the number of colony forming units (CFUs) per millilitre of urine. Only *E. coli* isolates with bacterial count of $\geq 10^5$ CFU/ml were further studied. Any Gram-negative, lactose fermenting colonies were further identified using conventional biochemical reactions including indole, citrate and urease tests as well as culture on triple sugar iron.

Antimicrobial susceptibility testing:

Testing of antimicrobial susceptibility was performed using disk diffusion method according to Kirby-Bauer method. The diameter of each zone of inhibition was measured in mm and was interpreted using Clinical and Laboratory Standards Institute (CLSI) 2016 guidelines [19].

The fosfomycin MICs were measured by agar dilution method using Muller Hinton agar media supplemented with 25 µg/ml glucose-6-phosphate. Briefly, Monuril sachet (Zambon, Italy) containing 3 gram fosfomycin was dissolved in sterile distilled water. Serial fosfomycin dilutions ranging from 2560µg/ml to 5µg/ml were prepared. Each dilution was added (by a percentage 10% of the agar volume) to the agar separately and distributed in Petri dishes. Therefore, the final concentrations at 1:10 dilution in agar ranged from 256 to 0.5 µg/ml. Bacterial suspensions of each of the 60 isolates were prepared equivalent to 0.5 McFarland, and 1 µl of each suspension was inoculated on each set of fosfomycin concentrations of the prepared plates. After incubation at 37 ºC for 24 hours, the MICs were specified and interpreted according to CLSI 2016 guidelines [19].

Detection of biofilm-formation with and without sub-inhibitory concentrations of fosfomycin:

E. coli isolates were screened for biofilm-formation by tissue culture plate technique [20, 21]. Organisms isolated from fresh MacConkey agar plates were cultured in 2 ml of tryptone soy broth (Oxoid, UK) with 1% glucose. Bacterial suspensions were made equivalent to 0.5 McFarland standard (1.5×10^8 CFU/ml) followed by further dilution 1:100 with fresh medium to achieve ~10^6 CFU/ml. Three fosfomycin dilutions, 1/4, 1/8, and 1/16 of the observed fosfomycin MICs for every isolate were used.

Sterile 96-well flat-bottom polystyrene tissue culture plates were used for testing biofilm formation. Each isolate was represented in four wells. The first three wells contained 1/4, 1/8 and 1/16 of the fosfomycin MICs, whereas the last well contained the organism alone. The positive control wells contained the control organism (an organism confirmed to be a biofilm-forming). The control organism was *E. coli* isolate received from the Strain Bank of the Department of Medical Microbiology and Immunology, Faculty of Medicine, Cairo University. Sterile broth was used as negative control. The optical density (OD) of the stained adherent biofilm was obtained by using micro ELISA autoreader Stat Fax-2100 (Awareness Technology, US) at wavelength 490 nm [21]. The interpretation of biofilm-formation was done on the basis of the criteria of *Stepanovic et al.* [22] (Table 1).

Table 1: Interpretation of biofilm formation

Average OD value	Biofilm-formation
Tested OD ≤ODC	Non
Tested OD >ODC and ≤2×ODC	Weak
Tested OD >2×ODC and ≤4×ODC	Moderate
Tested OD >4×ODC	Strong

Optical density cut-off value (ODC) = average OD of negative control + 3×standard deviation (SD) of negative control *Stepanovic et al.*[22]

RESULTS

This study included 60 *E. coli* isolates collected from urine specimens of adult UTI patients. Thirty-nine specimens were collected from female patients, and 21 specimens from male patients. The age of patients ranged from 25 to 52 years (36.72 ± 7.614). All of them attended the urology outpatient clinic complaining of symptoms suggestive of UTI. All patients were neither catheterized nor received antimicrobial therapy for 48 hours prior to collecting urine samples.

Demographic Data of UTI Patients involved in the study:

Most of the patients involved in this study complained of dysuria and frequency (81.67%), 51.67% of patients had urgency, 16.67% were feverish and only 6.67% suffered from suprapubic tenderness (Table 2).

Table 2: Demographic data of patients included in the study

		No. (60)	(%)
Age	Range	25-52	-
	Mean ± SD	36.72±7.614	-
Sex	Male	21	35
	Female	39	65
Clinical symptoms	Fever	10	16.67
	Dysuria	49	81.67
	Frequency	49	81.67
	Urgency	31	51.67
	Suprapubic tenderness	4	6.67

Antimicrobial susceptibility testing:

The antibiotic susceptibility pattern of the 60 *E. coli* isolates revealed that all of the isolates (100%) were sensitive to fosfomycin, most of them were sensitive to imipenem 58 (96.67%), followed by netilmicin 51 (85%), cefoxitin 49 (81.67%) and amikacin 46 (76.67%). The antibiotic susceptibilty pattern of *E. coli* isolates is shown in Table 3.

Table 3: Antibiotic susceptibilty pattern of *E. coli* isolates to tested antibiotics using disk diffusion method

Antibiotic	Susceptible No (%)	Resistant No (%)
Fosfomycin (200µg)	60 (100)	0 (0)
Imipenem (10µg)	58 (96.67)	2 (3.33)
Netilmicin (30µg)	51 (85)	9 (15)
Cefoxitin (30µg)	49 (81.67)	11 (18.33)
Amikacin (30µg)	46 (76.67)	14 (23.33)
Ceftazidime (30µg)	28 (46.67)	32 (53.33)
Aztreonam (30µg)	26 (43.33)	34 (56.67)
Ampicillin/sulbactam (10µg/10µg)	24 (40)	36 (60)
Trimethoprim/sulfamexosazole (1.25/23.75µg)	22 (36.67)	38 (63.33)
Ceftriaxone (30 µg);	21 (35)	39 (65)
Ciprofloxacin (5 µg)	20 (33.33)	40 (66.67)
Cefuroxime (30 µg)	19 (31.67)	41 (68.33)
Cefotaxime (30 µg)	19 (31.67)	41 (68.33)
Doxycycline (30 µg)	18 (30)	42 (70)
Ampicillin (10 µg)	2 (3.33)	58 (96.67)

Agar dilution method was carried out for determination of fosfomycin MICs and revealed that 45 *E. coli* isolates (75%) were sensitive to fosfomycin, 5 (8.33%) were of intermediate resistance, and the remaining 10 isolates (16.67%) were fosfomycin resistant.

Effect of fosfomycin on biofilm-formation:

Screening of *E. coli* isolates for biofilm-formation by tissue culture plate technique (TCP) revealed that 48 (80%) of the isolates were non-biofilm-forming, whereas 12 (20%) isolates were biofilm-forming (all were weak biofilm-forming). Biofilm-forming isolates were also tested for biofilm-formation under the effect of sub-lethal concentrations of fosfomycin (1/4, 1/8, 1/16 of MIC of fosfomycin) using tissue culture plate method. MIC, 1/4 MIC, 1/8 MIC and 1/16 MIC of the biofilm-forming isolates are shown in Table 4.

Table 4: Fosfomycin MICs of the biofilm-forming isolates and the concentrations used for biofilm inhibition

Isolate SN	Fosfomycin			
	MIC µg/ml	1/4 MIC µg/ml	1/8 MIC µg/ml	1/16 MIC µg/ml
8	64	16*	8*	4*
15	16	4*	2*	1
28	256	64*	32*	16
43	64	16*	8*	4*
46	64	16*	8*	4*
48	256	64	32	16
50	256	64*	32*	16*
52	256	64*	32*	16*
53	8	2*	1*	0.5*
54	16	4	2	1
57	64	16*	8	4
60	2	0.5*	0.25*	0.125*

**MIC dilution that inhibited biofilm-forming ability*
MIC: minimal inhibitory concentration, SN: serial number

Out of the 12 biofilm-forming *E. coli* isolates, 10 isolates (83.33%) lost their biofilm-forming ability under the effect of 1/4 of the fosfomycin MIC. Only 2 isolates (16.67%) retained their biofilm-forming ability even after exposure to fosfomycin (Table 5).

Table 5: Fosfomycin effect on the 12 biofilm-forming *E. coli* isolates using tissue culture plate technique

	Effect of 1/4 MIC No (%)	Effect of 1/8 MIC No (%)	Effect of 1/16 MIC No (%)
Abolished biofilm-forming ability	10 (83.33)	9 (75)	7(58.33)
No effect on biofilm-forming ability	2 (16.67)	3 (25)	5 (41.67)

DISCUSSION

Because of growing rates of drug resistance among uropathogens once considered as first-choice agents, the role of fosfomycin has been growing [23].

In this study, using Kirby-Bauer method, *E. coli* isolates showed high resistance rates, ranging from 96.67 to 53.33%, to most of the antibiotics tested; ampicillin, doxycycline, cefuroxime, cefotaxime, ciprofloxacin, ceftriaxone, trimethoprim-sulphamethoxalzole, ampicillin/sulbactam, aztreonam and ceftazidime. Lower resistance rates were observed for amikacin (23.33%), cefoxitin (18.33%) and netlimicin (15%). However, the studied isolates showed very low resistance rates to each of imipenem (3.33%) and fosfomycin (0%). Meanwhile, using agar dilution method, 75% of the isolates were susceptible to fosfomycin. In concordance with this study, *Yilmaz and colleagues* [24] have also reported that only 4.3% of their isolates were fosfomycin resistant. Extremely low emerged resistance has been shown to fosfomycin. Many factors may have contributed to this situation including its limited use for a single indication, i.e. uncomplicated UTIs, appropriate dosing, very high concentrations reached in the urinary tract, absence of cross-resistance with other drugs, and rarity of plasmids carrying fosfomycin resistance genes [25].

In the current study, only 12/60 (20%) of *E. coli* isolates were biofilm-forming, all of which produced weak biofilm. Out of the 12 biofilm-forming *E. coli* isolates, 10 isolates (83.33%) lost their biofilm-forming ability under the effect of 1/4 of the fosfomycin MIC. Only 2 isolates (16.67%) reserved their biofilm-forming ability even after exposure to fosfomycin. Out of the 10 isolates that lost their biofilm-forming ability after exposure to fosfomycin, seven isolates were affected by the three used sublethal concentrations of the drug. *Marchese and colleagues* [26] reported that 2000 µg/ml of fosfomycin reduced preformed slimes for initial and mature biofilm. They also stated that after fosfomycin treatment at a concentration of 128 µg/ml, residual biofilm amount ranged from 30 to 45%.

One of the essential targets in the field of clinical microbiology is the development of new strategies capable of eliminating the incidence of biofilm infections and successfully treating chronic illnesses caused by the establishment of these recalcitrant bacterial structures [27]. Some antibiotics like macrolides have been recognized to possess anti-biofilm activity [6]. However, acquired resistance to macrolides has already

been described in *Enterobacteriaceae*[28]. Fosfomycin could be a promising agent in suppressing biofilm-formation especially with the emergence of resistance to macrolides and other drugs. National guidelines have been published by the UK agency, NICE (National Institute for Health and Care Excellence), they state that fosfomycin is recommended for uncomplicated UTI which is defined as infections with no fever/flank pain) caused by drug resistant *E. coli* in adults [29].

CONCLUSION

This study showed that fosfomycin exhibits a strong antibacterial activity against antibiotic resistant uropathogenic *E. coli.* Moreover, it was able to suppress *E. coli* biofilm-forming ability. Therefore, it can be suggested as an alternative to third generation cephalosporins and fluoroquinolones for eradication of uncomplicated UTI caused by uropathogenic *E. coli.* Further studies are recommended to estimate the efficacy of fosfomycin on biofilm forming organisms and the value of coating urinary catheters with the proper concentrations of fosfomycin to reduce the possibility of biofilm-formation and the incidence of catheter-associated UTIs.

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