Infection and Drug Resistance

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ORIGINAL RESEARCH

In vitro effect of fosfomycin on multi-drug resistant gram-negative bacteria causing urinary tract infections

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Background: Rising rates of resistance to antimicrobial drugs among *Enterobacteriaceae* limit the choice of therapeutic agents to treat urinary tract infections. In this context we assessed the in-vitro effect of fosfomycin against extended-spectrum beta-lactamases, AmpC beta-lactamases and carbapenemase-producing strains of *Escherichia coli, Klebsiella pneumoniae, Enterobacter* spp, and *Pseudomonas aeruginosa* isolated from the patients with urinary tract infection (UTI) and also studied the effect of fosfomycin on their biofilm formation.

Materials and methods: A total of 326 multidrug-resistant (MDR) isolates comprising of *Escherichia coli, Klebsiella pneumoniae, Enterobacter* spp, and *Pseudomonas aeruginosa* from the urine samples of the patients with a diagnosis of UTI were included in the study. MIC 50 and MIC 90 were detected by agar dilution method and the capacity to form biofilm in the presence of fosfomycin by these MDR isolates was assessed by the tissue culture plate method.

Results: The MIC50 for meropenem (0.5 µgm/mL) and nitrofurantoin (32 µgm/mL) was within the susceptible range only for *E. coli*. Fosfomycin was the only antibiotic that inhibited 100% *E.coli*, 70% *Klebsiella* spp, and 50% *Pseudomonas* spp and 40% *Enterobacter* spp which included the extended-spectrum beta-lactamases producers. It showed a similar effect on carbapenemase producers and AmpC producers. Fosfomycin disrupted biofilm in 67% (n=141) *E.coli*, 74% (n=50) *Klebsiella* spp, 88% (n=27) *Pseudomonas* spp and 36% (n=23) *Enterobacter* spp at 24 hrs of incubation with a concentration of 2 fold dilution lower than that of the MIC.

Conclusion : Fosfomycin showed a good inhibitory effect on the biofilms produced by the MDR organisms studied here.

Keywords: fosfomycin, MDR, UTI, MIC, biofilm

Introduction

The most common cause of all forms of UTIs is *Escherichia coli* (uropathogenic *Escherichia coli*), followed by other members of *Enterobacteriaceae* like *Klebsiella, Proteus, Enterobacter* spp and other gram positives like *Enterococci* and *Staphylococcus* spp.[1]

Urinary tract infections caused by drug-resistant *Enterobacteriaceae* have been on the rise.[2] The emergence of the multidrug-resistant (MDR) strains with either inherited or transmissible resistance, is resistant to most of the commonly used antibiotics has become a concern for treating UTI, both in the community as well as the hospital.[2]

The most disconcerting events are the UTIs caused by Carbapenemase-producing *Enterobacteriaceae* which are difficult-to-treat and are usually characterized

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by high mortality.[3] Many MDR pathogens are also known to produce biofilms in catheterized patients which are extremely difficult to treat.[4] In this era of increasing antimicrobial resistance, there is definitely a need for a newer drug that is orally active, has low levels of existing resistance and also has an effect on biofilms. Fosfomycin is a relatively old drug and the present study was conducted to determine the effect of fosfomycin on MDR pathogens as well as its effect on biofilm formation by these isolates.

Oral single-dose fosfomycin is considerably effective for the treatment of uncomplicated urinary tract infection.[5] Other traditional empirical antibiotic regimens which are commonly used for treating uncomplicated urinary tract infections, such as fluoroquinolones and co-trimoxazole, might be not active against these pathogens that produce ESBL and can lead to suboptimum outcomes and treatment failure.[6] Apart from fosfomycin, nitrofurantoin, and co-amoxiclav could be other options for oral antimicrobial treatment of ESBL-associated but otherwise uncomplicated urinary tract infections. Furthermore, because of its unique chemical structure and mechanism of action, fosfomycin seems to be spared from the effect of various mechanisms of resistance to antimicrobial drugs. Apart from the *Enterobacteriaceae* that produce ESBL, the very good antimicrobial effect of fosfomycin has also been reported in *Enterobacteriaceae* that are resistant to fluoroquinolones. Due to its improved pharmacokinetics, fosfomycin is increasingly used for UTIs and has been approved as an oral single-dose treatment for acute uncomplicated cystitis with mean peak urinary concentration of an oral single dose of 3 g fosfomycin tromethamine, while concentrations sufficient to inhibit the majority of the urinary pathogens can be maintained for 1 to 2 days. Though this easy dosage schedule ensures compliance but the chance of clinical cure may be compromised.[7]

Materials and methods

Study design

The study was conducted in Jawaharlal Institute of Postgraduate Medical Education & Research (**JIPMER**) which is a tertiary care referral center, an Institute of National Importance under the Ministry of Health and Family Welfare, Government of India. Urinary isolates from the in-patients with clinically diagnosed UTI (dysuria, frequency, urgency, suprapubic tenderness, the presence of pus cells in urine/high power field) admitted in the departments of Medicine, Nephrology, and Urology were included.

Inclusion criteria

All first fifty consecutive, nonrepetitive MDR isolates in a month for a period of one year (2016–2017) from the urine obtained from these patients admitted in the respective departments were included in the study. Only a single isolate from the first sample submitted in the laboratory was included from the patient. The demographic details and comorbid conditions of the respective patients were collected prospectively in a prescribed proforma.

Exclusion criteria

Pediatric patients (upto 13 yrs) were not included in the study.

Microbiological methods

The specimens were processed using the standard semi-quantitative culture method and isolates were biochemically characterized by using indole production, citrate utilization, urease production, kligler iron agar, mannitol fermentation and motility test medium, lysine and ornithine decarboxylases, arginine hydrolysis tests were used as described elsewhere.[8,9] Standard American type culture collection (ATCC) control strains (*E. coli* ATCC 25922, and *P. aeruginosa* ATCC 27853) within acceptable limits were used as quality control strains for the drugs tested. Susceptibility testing for amikacin, gentamicin, nitrofurantoin, ceftriaxone, ceftazidime, meropenem, andfosfomycin were performed as per the Clinical Laboratory Standards Institute.[10] All the isolates identified as multi-drug resistant based on the criteria of the European Centre for Disease Control (non-susceptible to ≥1 agent in ≥3 antimicrobial categories) were tested with fosfomycin.[10] The MIC of all isolates to the drugs included in the study was determined by the agar dilution method. For susceptibility testing by the agar dilution method, Mueller-Hinton agar with serial two-fold dilution of the drug was prepared from the stock antibiotic solution as described by CLSI.[10] For fosfomycin susceptibility testing by the agar dilution method, Mueller-Hinton agarsupplemented with 25 μg/mL of glucose-6-phosphate was used. The MIC of each antimicrobial agent was defined as the lowest concentration that inhibited the visible growth of the organism. Control strains, including E. coli ATCC 25922, and P. aeruginosa ATCC 27853, were included in each set of tests. The MIC of the fosfomycin were noted based on both CLSI (S ≤ 64, I =128. R> 256) guidelines for *Escherichia coli*since, CLSI do not prescribe any criteria for *Pseudomonas aeruginosa* and *Enterobacteriaceae*

other than *Escherichia coli*, EUCAST interpretative criteria ($S \leq 32$, R>32) for all isolates of *Enterobacteriaceae* and *Pseudomonas aeruginosa* other than *Escherichia colim* were used.[10,11] The various beta-lactamases namely, the ESBL, AmpC, and MBL were screened using the combination discs. ESBL producers were detected by using combination discs of ceftriaxone, ceftazidime and clavulanic acid. AmpC producers were detected by cefoxitin-EDTA disk diffusion test while MBL producers were detected by Double-disk synergy test (DDST).[10]

Biofilm formation of this MDR isolates wasperformed by tissue culture plate method described by Christensen et al[12]. Briefly, the isolates from fresh agar plates were inoculated in Trypticasesoya broth (TSB) media and incubated for 18 hrs at 37 °C in a stationary condition and diluted 1 in 100 with fresh TSB medium. Individual wells of sterile, polystyrene, 96 well-flat bottom tissue culture plate were filled with 0.2 mL aliquots of the diluted cultures and only broth without culture is used as a control to check sterility and non-specific binding of media. The tissue culture plates were incubated for 16 hrs and 24 hrs at 37 °Cseperately. After incubation, the content of each well was gently removed by tapping the plates. The wells were washed four times with 0.2 mL of phosphate buffer saline (PBS pH 7.2) to remove free-floating "planktonic" bacteria. Biofilms formed by adherent "sessile" organisms in the plate were fixed with sodium acetate (2%) and stained with crystal violet (0.1% w/v). The excess stain was rinsed off by thorough washing with deionized water and plates were kept for drying. Optical density (OD) of stained adherent bacteria was determined with a micro ELISA auto reader at a wavelength of 570 nm (OD 570 nm). These OD values were considered as an index of bacteria adhering to surface and forming biofilms. The experiment was performed in triplicate and the data were averaged and the standard deviation was calculated. To compensate for background absorbance, OD readings from sterile medium, fixative, and dye were averaged and subtracted from all test values. Then the values obtained from with and without fosfomycin were compared. The mean OD value obtained from media control well was deducted from all the test OD values. This was done in the presence of fosfomycin and without fosfomycin. Fosfomycin was used at a concentration below twofold the level of MIC of different isolates.

Statistical analysis

The stastical analysis was performed using SPSS software 19.0 version. The distribution of categorical data such as gender, clinical characteristics, antibiotic resistance profile, MDR isolates, and biofilm inhibition status was expressed as frequency and percentage. The association of the isolates on biofilm inhibition status at different time periods was carried out by using a chi-square test. The change in the biofilm inhibition status over time was carried out by using McNemar's test. All statistical analyses were carried out at 5% level of significance and p-value<0.05 was considered significant.

Results

Among these MDR organisms isolated from the patients, catheterization was the most common risk factor followed by diabetes mellitus, renal calculi and Urological surgical procedures (Table 1). MIC50 for meropenem (0.5 µgm/mL) and nitrofurantoin (32 µgm/mL) was within the susceptible range only for *E. coli* (Table 2). On the other hand, fosfomycin was the only antibiotic that good inhibitory effect on *E.coli, Klebsiella*, andamoderate effect on *Pseudomonas* spp and *Enterobacter* spp which included the extended-spectrum beta-lactamases producers, carbapenemase producers and AmpC producers with (Tables 3,4). Fosfomycin disrupted biofilm better at 24 hrs of incubation in *E.coli* at a concentration of 0.5 µgm/mL and in *Klebsiella* spp, *Pseudomonas* spp and *Enterobacter* spp at a concentration of 8 µgm/mL. The difference of inhibition of biofilm formation in *Escherichia coli* and *K.pneumoniae*, and *Pseudomonas* spp at 16 hrs and at 24 hrs was statistically significant (P-value <0.0001) (Table 5).

Discussion

A sum total of 326 non-repetitive MDR isolates was collected and subjected to MIC. Out of 326 isolates, 231 (73.4%) were from patients admitted under Medicine, 73 (21%) were from Urology and 22 (6%) were from Nephrology.

In the present study, we assessed the effect of fosfomycin in gram-negative MDR urinary isolates. *E.coli* was the most common isolate among all the MDR isolates

Table 1 The underlying comorbid conditions in the study group

S no	Underlying co-morbid factors	Number of patients	Percentage
1	Renal calculi	132	43.7%
2	Diabetes mellitus	142	47.0%
3	Urological surgical procedures	62	20.5%
4	Catheterization	145	48.0%

Table 2 Antibiotic resistance profile of different isolates in the study group

Various departments included in the study	Amikacin	Gentamicin	Meropenem	Ceftazidime	Ceftriaxone	Ciprofloxacin	Nitrofurantoin	Fosfomycin
Escherichia coli (% resistance) Total N=217								
Medicine N=158	53 (33.5%)	155 (98.1%)	30 (18.9%)	154 (97.4%)	158 (100%)	158 (100%)	19 (12%)	0
Urology N=52	21 (40.3%)	49 (94.2%)	13 (25.1%)	43 (82.6%)	52 (100%)	52 (100%)	9 (16%)	0
Nephrology N=7	4 (57.1%)	3 (42.8%)	1 (14.2%)	5 (71.4%)	6 (85.7%)	7 (100%)	1 (17%)	0
K.pneumoniae (% resistance) Total N=52								
Medicine N=44	40 (90.9%)	43 (97.7%)	33 (75%)	42 (95.4%)	44 (100%)	44 (100%)	20 (45%)	0
Urology N=5	5 (100%)	4 (80%)	3 (60%)	3 (60%)	4 (80%)	4 (80%)	2 (40%)	2 (40%)
Nephrology N=3	2 (66.6%)	3 (100%)	2 (66.6%)	2 (66.6%)	3 (100%)	3 (100%)	2 (66%)	1 (30%)
Pseudomonas spp (% resistance) Total N=32								
Medicine N=15	15 (100%)	15 (100%)	12 (80%)	12 (80%)	NA	15 (100%)	NA	0
Urology N=10	10 (100%)	10 (100%)	8 (80%)	8 (80%)	NA	10 (100%)	NA	5 (50%)
Nephrology N=7	6 (85.7%)	6 (85.7%)	6 (85.7%)	6 (85.7%)	NA	6 (85.7%)	NA	4 (57%)
Enterobacter spp (% resistance) Total N=25								
Medicine N=8	8 (100%)	8 (100%)	6 (75%)	6 (75%)	8 (100%)	8 (100%)	4 (50%)	(0%)
Urology N=10	10 (100%)	10 (100%)	8 (80%)	8 (80%)	10 (100%)	10 (100%)	8 (80%)	5 (50%)
Nephrology N=7	6 (85.7%)	6 (85.7%)	6 (85.7%)	6 (85.7%)	6 (85.7%)	6 (85.7%)	6 (85.7%)	4 (57%)

Table 3 Mechanism of beta-lactam resistance detected by phenotypic methods and effect of fosfomycin on different beta-lactam resistant isolates

Mechanism of beta-lactams resistance detected phenotypically	**Isolates with visible growth at different concentration of fosfomycin and (%) resistance of isolates to fosfomycin**						
MIC of fosfomycin [μgm/mL]	16	32	64	128	256	512	1026
ESBL producers N=319							
*Escherichia coli** N=216	0	0	0	0	0	0	0
K.pneumoniae[#] N=49	14 (30)	14 (30)	14 (30)	14 (30)	10 (20)	6 (12.2)	2 (4)
Pseudomonas spp[α] N=32	16 (50)	16 (50)	16 (50)	16 (50)	16 (50)	12 (38.4)	3 (9)
Enterobacter spp[δ] N=22	15 (68)	10 (45)	10(45)	9 (40)	9 (40)	5 (22.7)	1 (4)
MBL producers N=138							
*Escherichia coli** N=44	0	0	0	0	0	0	0
K.pneumoniae[#] N=38	19 (50)	19 (50)	19 (50)	19 (50)	19 (50)	18 (47.3)	4 (10)
Pseudomonas spp[α] N=32	16 (50)	16 (50)	16 (50)	16 (50)	16 (50)	12 (37.5)	3 (9)
Enterobacter spp[δ] N=20	9 (45)	9 (45)	9 (45)	9 (45)	8 (40)	8 (40)	2 (10)
AmpC BL Producers N=158							
*Escherichia coli** N=64	0	0	0	0	0	0	0
K.pneumoniae[#] N=38	16 (42)	16 (42)	16 (42)	15 (39)	10 (26)	10 (26)	3 (8)
Pseudomonas spp[α] N=32	16 (50)	16 (50)	16 (50)	16 (50)	16 (50)	12 (37.5)	3 (9)
Enterobacter spp[δ] N=20	8 (40)	8 (40)	8 (40)	8 (40)	6 (30)	4 (20)	1 (5)

Notes: *For *Escherichia coli* the MIC of fosfomycin according to CLSI (S $\leq$ 64 μgm/mL, I=128 μgm/mL. R> 256 μgm/mL).The MIC 50 and MIC 90 of fosfomycin in *Escherichia coli* was 1 μgm/mL and 2 μgm/mL respectively. [#]For *Klebsiella pneumoniae* the MIC of fosfomycin according to EUCAST interpretative criteria (S≤32, R>32). The MIC50 and MIC 90 of fosfomycin in *Klebsiella pneumoniae* was 32 μgm/mL and 1026 μgm/mL respectively. [α]For *Pseudomonas aeruginosa* the MIC of fosfomycin according to EUCAST interpretative criteria (S≤32, R>32). The MIC50 and MIC 90 of fosfomycin in *Pseudomonas aeruginosa* was 32 μgm/mL and 1026 μgm/mL respectively. [δ]For *Enterobacter* spp the MIC of fosfomycin according to EUCAST interpretative criteria (S≤32, R>32). The MIC50 and MIC 90 of fosfomycin in *Enterobacter* spp was 32 μgm/mL and 1026 μgm/mL respectively.

identified from different wards included in the study. Most of the MDR isolates included in the study were from patients with complicated UTI in the form of catheterization or associated diabetes mellitus, renal calculi or post-urological procedures (Table 1). In this context, MDR isolates being more commonly isolated from catheterized individuals could be a reflection of colonization and some may be due to catheter-associated UTI, though this was not determined in the study.

We observed that almost all the isolates which were obtained from patients admitted in the various departments included in the study showed variable but high resistance to most of the antibiotics while most remained sensitive to fosfomycin (Table 2). Among all isolates, *Escherichia coli* (100%) seemed to be the most susceptible to fosfomycin followed by *Klebsiella* spp (70%), and *Enterobacter* spp (60%). Other UTI pathogens like *Pseudomonas* spp also showed moderate (50–60%) susceptibility to fosfomycin

Table 4 Effect of fosfomycin on different multidrug resistant isolates

MDR isolates Total N=326	MIC of Fosfomycin [µgm/mL] (%)					
	16	32	64	128	256	512
*Escherichia coli** N=217	0	0	0	0	0	0
K.pneumoniae[#] N=52	18 (35)	18 (35)	18 (35)	18 (35)	11 (20)	6 (12.2)
Pseudomonas spp[α] N=32	16 (50)	16 (50)	16 (50)	16 (50)	16 (50)	12 (38.4)
Enterobacter spp[δ] N=25	15 (59.5)	10 (40)	10 (40)	10 (40)	9 (36)	9 (36)

Notes: *For *Escherichia coli* the MIC of fosfomycin according to CLSI (S ≤ 64 µgm/mL, I=128 µgm/mL. R> 256 µgm/mL).The MIC 50 and MIC 90 of fosfomycin in *Escherichia coli* was 1 µgm/mL and 2 µgm/mL respectively. [#]For *Klebsiella pneumoniae* the MIC of fosfomycin according to EUCAST interpretative criteria (S ≤ 32, R>32). The MIC50 and MIC 90 of fosfomycin in *Klebsiella pneumoniae* was 32 µgm/mL and 1026 µgm/mL respectively. [α]For *Pseudomonas aeruginosa* the MIC of fosfomycin according to EUCAST interpretative criteria (S ≤ 32, R>32). The MIC50 and MIC 90 of fosfomycin in *Pseudomonas aeruginosa* was 32 µgm/mL and 1026 µgm/mL respectively. δFor*Enterobacter*sppthe MIC of fosfomycin according to EUCAST interpretative criteria (S ≤ 32, R>32). The MIC50 and MIC 90 of fosfomycin in *Enterobacter* spp was 32 µgm/mL and 1026 µgm/mL respectively.

Table 5 Effect of fosfomycin on biofilm produced by different isolates

S NO	Isolate	Overall inhibited (%)	Biofilm inhibition exclusively at 16 hrs (%)	Biofilm inhibition exclusively at 24 hrs (%)	*P*-value indicating the difference between biofilm inhibition at 16 hrs and at 24 hrs exposure to fosfomycin
1	*Escherichia coli* N=141/217	96/141 (67)	76 (53)	20 (14)	*P*<0.0001
2	*K.pneumoniae* N=50/52	37/50 (74)	22 (44)	15 (30)	*P*<0.0001
3	*Pseudomonas* spp N=27/32	24/27 (88)	12 (44)	12 (44)	*P*<0.0001
4	*Enterobacter* spp N=23/25	10/23 (36)	5 (18)	5 (18)	*P*=0.068
	Total	167/241 (69.2)	115/241 (47.7)	52/241 (21.5)	*P*<0.001

(Table 2). In a study done by Falagas et al and Maraki et al, in Greece have also shown very encouraging susceptibility results similar to this study. In the study by Maraki et al, reported fosfomycin was active in vitro against a majority percentage of urinary isolates, which showed high antimicrobial resistance against the most commonly used agents for the treatment of UTIs.[13] In another study by Mittal et al, all uropathogenic *Escherichia coli* strains were found to be sensitive to fosfomycin. According to Rajendran et al, fosfomycin was the only antibiotic that effectively inhibited 90% of the strains of *Escherichia coli* and *Klebsiella* spp .[14]

Of the 326 isolates, 319 (97.8%) were resistant to the third generation cephalosporins and were also extended-spectrum beta-lactamases (ESBL) producers (Table 3). All these isolates were sensitive to fosfomycin (Table 3). In an earlier study done by Gupta et al from Chandigarh, among 150 uropathogenic strains of *Escherichia coli*, 52.6% of isolates were ESBL producers, and all strains were susceptible to fosfomycin.[15] An increasing trend of ESBL producers has been observed from India, which is attributable to the irrational use and ease of availability of antibiotics over the counter and such isolates are prevalent among hospitals as well as in the community.[16] In a study done by Cueto et al, also demonstrated 428 ESBL producing *Escherichia coli* and *Klebsiella pneumoniae* strains-were exposed to fosfomycin and it showed high in vitro activity against all these strains.[17]

In comparison to the ESBLs, AmpC beta-lactamases are known to be notorious, as they hydrolyze not only the third generation cephalosporins (3GCs) but also beta-lactamase inhibitor plus beta-lactam combinations. Though carbapenems are the drug of choice against these organisms fosfomycin has been found to be effective in vitro in earlier studies.[13,18] Fosfomycin showed a similar effect on AmpC BL producing *Escherichia coli* (100%), (57%) in *Enterobacter* spp, and (38%) in *Klebsiella pneumoniae*. It also inhibited AmpCBL *Pseudomonas* spp (30%) (Table 3). Karlowsky et al too reported 99.4% fosfomycin susceptibility against urinary isolates of *Escherichia coli*, collected from 2010 to 2013 as a part of the Canadian national surveillance study.[19] Beta-lactamase-producing isolates and AmpC-producing isolates of *E. coli* showed 94.9% and 96.6% susceptibility respectively.[19]

Carbapenemase-producing *Enterobacteriaceae* (CRE) is a major threat across the globe.[20] In vitro effect of fosfomycin has been documented on some CRE isolates in an earlier study.[21] In our study, fosfomycin showed a similar inhibitory effect on carbapenemase producing *Escherichia coli* (100%), 50% each in *Klebsiella pneumoniae* and *Enterobacter* spp, while it inhibited 30% of the isolates of *Pseudomonas* spp (Table 3). In a study done by Banerjee et al in a 380 bedded tertiary care hospital in Kolkata, similar results with 89% inhibition with fosfomycin on CRE isolates were observed.[22]

Of 217 *Escherichia coli* MDR isolates included in the study, 78 (35.9%) and 207 (95.4%) were resistant to amikacin and gentamicin respectively, while fosfomycin showed 100% inhibition in vitro against these isolates (Table 4). On the other hand, fosfomycin inhibited 60% of MDR *Klebsiella pneumoniae* and *Enterobacter* spp which were (94%) resistant to both aminoglycosides while fosfomycin inhibited 50% of *Pseudomonas* spp that are resistant to aminoglycosides (Table 4). Fosfomycin showed a similar inhibitory effect on *Escherichia coli* (100%), 60% each in *Klebsiella pneumoniae* and *Enterobacter* spp, while it inhibited 50% of the isolates of *Pseudomonas* spp that were (98%) resistant to fluoroquinolones (Table 4).

In contrary to a study done elsewhere,[23] our observation showed good in vitro susceptibility to fosfomycin and nitrofurantoin against ESBL producing *Escherichia coli*, ESBL producing *Klebsiella pneumoniae*, similar to studies published earlier.[23–27]Out of the total of 326 MDR isolates which were resistant to at least three (or more) groups of antibiotics thatar-eaminoglycosides, fluoroquinolones, and third-generationcephalosporins, 264 (87.3%) were susceptible to fosfomycin which suggests that this could be the drug of choice against such resistant isolates (Table 3). In the present study, we observed fosfomycin at a concentration of 32 µgm/mL could inhibit the 2% of isolates and 20% of isolates at 12 hrs and 24 hrs respectively, while 64 µgm/mL of fosfomycin was able to inhibit 24% of isolates and 36% of isolates at 12 hrs and 24 hrs respectively. The difference between the inhibition at 12 hrs and at 24 hrs by fosfomycin at both these concentrations (32 µgm/mL and 64 µgm/mL) was found to be statistically significant with (P=0.001) and (P=0.03) respectively. This in vitro observation supports the fact that prolonged duration of exposure to fosfomycin at 32 µgm/mL in urine can enhance the bactericidal effect thereby, further aiding clinical cure.

As a result of increased incidence and chronicity of biofilm infections, newer strategies are being developed which has a capability to reduce the incidence of biofilm infections and effectively helps in treating this chronic conditions related to the establishment of these difficult-to-eradicate bacterial structures. In this regard, the effect of fosfomycin on biofilm was studied. Due to its good renal excretion, fosfomycin getsconcentrated in urine which enhances its ability to break up biofilms.[28] In this present study, out of 326 isolates, 218 (66.87%) isolates produced biofilm which was detected by tissue culture plate method. Similar to the study by Christensen et al[29] fosfomycin could disrupt biofilms at a concentration below the MIC. In the present study, we observed a similar inhibition of biofilm formation (Table 5). Fosfomycin disrupted biofilm produced by 115 (38%) strains exclusively at 16 hrs of incubation and 167 (69.2%) strains at 24 hrs of incubation. The difference of inhibition of biofilm formation in *Escherichia coli* and *K.pneumoniae*, and *Pseudomonas* spp at 16 hrs and at 24 hrs was statistically significant (P-value <0.0001), unlike that of*Enterobacter* spp. (Table 5). In a study done by Anna Marchese et al, it was found that fosfomycin alone and in combination with N-acetylcysteine showed a decrease in biofilm formation up to 60–73% *Escherichia coli*.[30] Further, a study was done by Cai et al with fosfomycinina combination of aminoglycosides showed a decrease in biofilm formation in vitro and in vivo.[28] Fosfomycin also has antimicrobial action against gram-positive bacteria and decrease biofilm in *Staphylococcus aureus*.[31] They also suggested that bacterial biofilms that are formed in vivo appear to be more easily destroyed by antibiotics than biofilms established on the surface of catheters.[31] Fosfomycin was capable of inhibiting biofilm formation in 88% *Pseudomonas* isolates, in 74% *Klebsiella pneumoniae*, in 68% *Escherichia coli* and 43% *Enterobacter* spp (Table 5). The basis of this inhibition is still not very clear and in-depth analysis at the molecular level needs to be undertaken to unravel the mechanisms involved.

In recent years, multidrug resistance has emerged rapidly among diverse bacterial types as a consequence of irrational antibiotic use in agriculture and human and veterinary medicine. Risk factors like catheterization, diabetes mellitus, renal calculi and urological procedures with structural and functional abnormalities increase the risk of acquiring urinary tract infections with multidrug resistance strains which further increases morbidity and mortality.[32] Treatment of such infections relies on the use of broad-spectrum antibiotics like carbapenems, tigecycline, beta-lactam/beta-lactamase inhibitor combinations. The use of such antibioticsis weakened by various factors such as their parenteral route of administration which is not feasible in an outdoor setting, high cost and their added effect on the selective pressure to develop resistance. Thus, there is definitely a need for a newer drug that is orally active, has low levels of existing multi drug resistance and also doesn't encourage the emergenceof antimicrobial resistance in the future. Fosfomycin is an old antibiotic with good in vitro activity against the common pathogens causing UTI, particularly toward the *Enterobacteriaceae* acts by inactivating the enzyme that is required for peptide portion of peptidoglycan, thus disrupting bacterial cell-wall synthesis. This antibiotic has the potential to be used as an agent to treat uncomplicated UTIs. There are increasing reports of its resistance in countries where it has been used extensively such as Spain and Hong Kong, hence, caution needs to be exercised over its use.[33] Resistance to fosfomycinin *Enterobacteriaceae* is more commonly chromosomally encoded than by plasmids. However, co-transmission of resistance to fosfomycin and resistance to other antimicrobials through plasmids has been shown but are very rare.[34] Nevertheless, monotherapy of fosfomycin is not recommended as the development of resistance during therapy is a serious concern.[35]

Limitations of the present study

Most of the MDR isolates were from *Enterobacteriaceae* with a limited number of *Pseudomonas aeruginosa*. We did not study the clinical outcome with the antibiotic therapy and also, the genetic mechanism of resistance of these MDR isolates was not studied since these were not a focus of this study. The exact molecular basis of this biofilm reduction contributed by fosfomycin needs to be studied in detail.

Conclusion

In this study, we observed that fosfomycin has a good in vitro effect on most of the MDR gram-negativebacteria. It showed significantly goodactivity against ESBL, AmpC BL producing *Escherichia coli*, MBL producers like *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. Fosfomycin also had good activity on biofilm producing strains studied here. Further clinical studies using fosfomycin will add data to support fosfomycin its use in the treatment of urinary tract infections due to MDR pathogens which have the capacity to form a biofilm. Also, in-depth studies to understand the mechanism of biofilm inhibition are required.

Ethics clearance

Study was approved by the scientific advisory committee and the ethicscommittee of the Institute (NO. JIP/IEC/SC/2015/23/831). Written Informed consent was obtained from the patients prior to the collection of clinical and demographic details as per the ethicscommittee of the Institute.

Disclosure

This study is not submitted to any government departments or granting bodies. The authors do not own any stocks or shares in a company from which we procured the drug. The authors did not accept any reimbursement for preparing this article. The authors report no conflicts of interest in this work.

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