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Identification of Fosfomycin Heteroresistance Among Multidrug-Resistant Enterobacteriaceae Urinary Isolates

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Abstract
Fosfomycin is an orally bioavailable bactericidal agent which concentrates significantly in urine and is being increasingly prescribed for patients with Multidrug Resistant Enterobacteriaceae (MDRE) urinary isolates. According to the Clinical and Laboratory Standards Institute (CLSI), disc diffusion (DD) and agar dilution (AD) are the standards for fosfomycin susceptibility testing for urinary Escherichia coli and Enterococcus faecalis isolates, whereas the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines suggest both AD and broth microdilution (BD), but not DD. A prospective study was done in the Department of Microbiology in a tertiary care teaching hospital in Kolkata. MDRE urinary isolates that were resistant to oral and third-generation cephalosporins, either ciprofloxacin or levofloxacin and either of cotrimoxazole or nitrofurantoin were included in the study and tested further for fosfomycin. DD was carried out on Mueller-Hinton agar (MHA) supplemented with 25 μg/ml G6P with 50 and 200 μg discs. E-test was carried out on the same medium with drug concentrations ranging from 0.064 to 1024 μg/ml. Broth dilution was carried out on G6P supplemented Mueller Hinton broth with drug concentrations ranging from 0.25 to 1024 μg/ml. A total of 723 urine samples were obtained in a 2-month period, which yielded 79 Enterobacteriaceae, out of which 30 were MDRE. About 26 (86.67%) of these isolates were susceptible to fosfomycin by CLSI criteria and 25 (83.33%) by EUCAST criteria for minimum inhibitory concentration (MIC) by E-test and broth dilution. Only 4 (13.33%) were resistant to fosfomycin by DD. However, heteroresistant colonies were found among 21 (70%) isolates by E-test. None of these were picked up by DD or BD. The MIC of fosfomycin was between 0.25 and 512 μg/ml. These heteroresistant colonies when further subjected to E-test and BD showed resistance with MIC ≥512 μg/ml, depicting prevalence of heteroresistance. E-test can be used as a simple and effective screening method for identifying the fosfomycin heteroresistance and in turn changing the myth of the so high susceptibility rates among MDRE.

Keywords: Fosfomycin; Heteroresistant; MIC; Multidrug resistant bacteria; UTI.

1. INTRODUCTION

Urinary tract infection (UTI) is one of the most common infectious diseases encountered in clinical practice, both in community as well as hospitalized population. Patients without infection have sterile bladder urine and with proper collection, voided urine usually contains less than 10^4 bacteria/ml [1]. The most common Gram-negative organisms causing UTI, i.e., *Escherichia .coli*, *Klebsiella pneumoniae*, *Proteus* spp., *Enterobacter* spp., and *Pseudomonas* spp. [2] are all notorious for harboring multiple drug-resistance mechanisms, inherited and transmissible, chromosomal and extrachromosomal, against the commonly used oral antimicrobial agents for UTI caused by Gram-negative organisms, i.e. fluoroquinolones, cotrimoxazole, nitrofurantoin, aminopenicillins, and second- and third-generation cephalosporins [1]. With rampant overuse and misuse of these drugs, the Gram-negative bugs have become overwhelmingly resistant to all or most of these agents, making outpatient oral therapy increasingly difficult.

Fosfomycin is a bactericidal antibiotic agent. It inhibits an enzyme-catalyzed reaction in the first step of the synthesis of the bacterial cell wall. Fosfomycin interferes with the first cytoplasmic step of bacterial cell wall biosynthesis, the formation of the peptidoglycan precursor UDP *N*-acetylmuramic acid (UDPMurNAc). This inhibitory action takes place at a step earlier than the action of beta-lactams or glycopeptides. Fosfomycin reduces adherence of bacteria to urinary epithelial cells [3]. Fosfomycin is considerably active against both Gram-negative and Gram-positive pathogens.

Specifically, fosfomycin is considered active against *Enterococcus* spp. (including *Enterococcus faecalis* and *Enterococcus faecium)*, *Staphylococcus aureus* and *Staphylococcus epidermidis, E. coli, Klebsiella* spp., *Enterobacter* spp., *Serratia* spp., *Citrobacter* spp., *Proteus mirabilis, Salmonella* spp., and *Shigella* spp. while organisms like *Pseudomonas* spp., *Acinetobacter* spp., *Stenotrophomonas maltophilia*, *Burkholderia cepacia*, *Staphylococcus capitis*, *Staphylococcus saprophyticus,* and *Mycobacterium tuberculosis* are intrinsically resistant to fosfomycin. *Morganella morganii* is also found to be resistant to fosfomycin [3].

A bacterial strain, which is resistant to three or more different antimicrobial classes, is referred to as a multidrug resistant (MDR) bacterium [4]. Fosfomycin is emerging as a novel oral antibiotic with good bactericidal activity against these MDR pathogens.

The oral bioavailability of fosfomycin ranges between 34 and 58%. Absorption occurs in the small intestine and co-administration of fosfomycin with food may reduce absorption [3]. It is best absorbed if given before food intake and is excreted in urine [5]. Age does not affect absorption. Following a single 3 g dose of fosfomycin, peak urine concentrations are reached within 4 h. High urine as well as bladder tissue concentrations (128 mg/l) are retained for 1-2 days, which is sufficient to eliminate the majority of common uropathogens [3]. Fosfomycin, being derived from phosphonic acid, is chemically unrelated to other antimicrobials and used to treat community acquired UTI. With the spread of multidrug resistance, fosfomycin is a potential option, although experience with intravenous fosfomycin is scarce [6].

According to the Clinical and laboratory Standards Institute (CLSI) guidelines, disc diffusion (DD) and agar dilution (AD) are the standards for fosfomycin susceptibility testing for urinary E. coli and E. faecalis isolates [7] whereas the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines suggest both AD and broth microdilution, but not DD. The minimum inhibitory concentration (MIC) breakpoints for fosfomycin vary between these two guidelines.

The objectives of this study were to observe the *in vitro* activity along with MIC of fosfomycin besides the susceptibility profile of multidrug resistant Enterobacteriaceae (MDRE) from urinary isolates.

2. METHOD(S)

A prospective observational cross-sectional study was done in the Department of Microbiology, ESIC Medical College, Joka, for 2 months (May 9th, 2016 to July 8th, 2016) after obtaining institutional ethical clearance. The uncentrifuged urine samples received in the laboratory were examined microscopically for presence of pus cells, bacteria, casts, and other sediments. Semi-quantitative urine culture was done for all samples as per standard criteria. After culture, colony count and identification of the significant urinary isolates were done by using standard microbiological techniques like Gram stain, biochemical tests like indole test, methyl red test, citrate test, oxidase test, sugar utilization, H_2S production, etc., and automated identification by VITEK 2 Compact (BioMerieux Inc., France).

Antimicrobial susceptibility testing for amoxicillin/clavulanate, piperacillin/tazobactum, cefazolin, ceftriaxone, cefepime, ciprofloxacin, levofloxacin, cotrimoxazole, nitrofurantoin, gentamicin, amikacin, meropenem, imipenem, doxycycline, colistin, and fosfomycin for the Enterobacteriaceae isolates was done by Kirby Bauer DD method on Mueller Hinton agar (MHA), and interpretation was done according to the Clinical and Laboratory Standards Institute (CLSI) version 2016 guidelines [7]. The Gram-positive and the non-Enterobacteriaceae Gram-negative isolates were excluded from the current study. The Enterobacteriaceae isolates, which fulfilled all of the following criteria, i.e., (i) resistant to both oral and third-generation cephalosporins, (ii) resistant to either of ciprofloxacin or levofloxacin, and (iii) resistant to an alternative oral urinary antibiotic, from either of cotrimoxazole or nitrofurantoin, were included in our study and tested further.

The MICs for ampicillin, amoxicillin/clavulanate, piperacillin/tazobactum, cefuroxime, ceftriaxone, cefepime, ertapenem, imipenem, meropenem, amikacin, gentamicin, nalidixic acid, ciprofloxacin, tigecycline, nitrofurantoin, colistin, and cotrimoxazole were tested by VITEK 2 Compact, for these isolates.

Screening for extended spectrum beta-lactamase production for these isolates was done by DD method using cefotaxime disc. For further confirmation, combined disc method using cefotaxime and cefotaxime with clavulanic acid was used. Interpretation was done by the CLSI guidelines where a 5-mm increase in zone size for cefotaxime with clavulanic acid than that for cefotaxime alone was taken as positive for extended spectrum beta-lactamase production.

DD for fosfomycin was carried out on MHA supplemented with 25 μg/ml G6P with 50 and 200 μg discs. MIC for fosfomycin was tested by E-strip (BioMerieux Inc., France) and microbroth dilution method. E-test was carried out on the same medium with drug concentrations ranging from 0.064 to 1024 μg/ml. Broth dilution was carried out on G6P-supplemented Mueller Hinton broth with drug concentrations ranging from 0.25 to 1024 μg/ml. All interpretations were done by CLSI 2016 and EUCAST 2016 guidelines. If heteroresistant colonies were seen, then these heteroresistant colonies were picked up and tested for MIC for fosfomycin by E-strip and broth dilution again.

3. RESULTS

A total of 723 urine samples were obtained in a 2-month period, which yielded 79 Enterobacteriaceae, out of which 30 non-repetitive consecutive Enterobacteriaceae isolates causing UTI that fulfilled our inclusion criteria of MDRE were included in the study during a period of 2 months (May 9th, 2016 to July 8th, 2016).

Among the included isolates, 4 (13.33%) were from patients below 18 years of age, 12(40%) were in the age group of 19-40 years, 7(23.33%) were between 41 and 59 years and 7(23.33%) were above 60 years of age. Among these patients, 10 (33.33%) were male and 20 (66.67%) were female. Thus, majority of the female patients were in the reproductive age group.

The urine samples were received from various departments. About 12 (40%) were from the obstetrics and gynecology department, 8 (26.67%) were from medicine department, 6 (20%) were from surgery, and 4 (13.33%) were from

Table 1: Antimicrobial susceptibility of multidrug-resistant *Escherichia coli* (*n* = 22) and *Klebsiella pneumoniae* (*n* = 8).

Antimicrobial agent	*E. coli* (*n* = 22)	*Klebsiella pneumonia* (*n* = 8)	MIC range µg/ml	MIC_{90} µg/ml	MIC_{50} µg/ml
Ampicillin	Nil	Nil	≥32	≥32	≥32
Cefazolin	Nil	Nil	NA	NA	NA
Ceftriaxone	Nil	Nil	≥64	≥64	≥64
Cefuroxime	Nil	Nil	≥64	≥64	≥64
Cefepime	1 (4.5%)	Nil	1–≥64	≥64	≥64
Amoxicillin/clavulanic acid	4 (18.18%)	2 (25%)	4–≥32	≥32	≥32
Piperacillin/tazobactam	7 (31.82%)	1 (12.5%)	4–≥128	≥128	≥128
Cefoperazone/sulbactam	7 (31.8%)	2 (25%)	NA	NA	NA
Imipenem	12 (54.55%)	2 (25%)	0.25–≥16	≥16	≥16
Meropenem	12 (54.55%)	2 (25%)	0.25–≥16	≥16	≥16
Ertapenem	11 (50%)	1 (12.5%)	0.5–≥8	≥8	≥8
Nalidixic acid	Nil	Nil	≥32	≥32	≥32
Ciprofloxacin	Nil	Nil	≥4	≥4	≥4
Levofloxacin	Nil	Nil	NA	NA	NA
Amikacin	12 (54.55%)	2 (25%)	2–≥64	≥64	≥64
Gentamicin	7 (31.82%)	3 (37.5%)	1–≥16	≥16	≥16
Nitrofurantoin	14 (63.64%)	Nil	16 – 512	256	64
Cotrimoxazole	Nil	4 (50%)	≤20–≥320	≥320	≥320
Doxycycline	8 (36.36%)	2 (25%)	NA	NA	NA
Tigecycline	21 (95.45%)	3 (37.5%)	0.5-8	8	0.5
Colistin	22 (100%)	8 (100%)	≤0.5-2	2	≤0.5
Fosfomycin	20 (90.9%)	6 (75%)	0.25-512	256	2

the pediatrics department. Of these samples, 11 (36.67%) were from the out-patient department, 18 (60%) were from the ward, and 1 (3.33%) was from the intensive care unit. Among the 30 included samples, 22 (73.33%) were *E. coli* and 8 (26.67%) were *K. pneumoniae*. Among the 30 included samples (which fulfilled our inclusion criteria, i.e., resistant to oral cephalosporins, third-generation cephalosporins, ciprofloxacin, and levofloxacin and either of nitrfurantoin or cotrimoxazole), all (100%) were resistant to ampicillin, cefuroxime, and nalidixic acid. Among the 30 samples, 14 (46.67%) were susceptible and 16 (53.33%) were resistant to nitrofurantoin and 4 (13.33%) were susceptible and 26 (86.67%) were resistant to cotrimoxazole. These 4 isolates susceptible to cotrimoxazole were, however, found resistant to nitrofurantoin, and the 14 isolates, which were susceptible to nitrofurantoin, were found to be resistant to cotrimoxazole, and hence included in the study. However, out of all, 12 isolates were found resistant to both of these oral drugs. There were 8 isolates which were gentamicin resistant but susceptible to amikacin whereas 4 isolates were gentamicin susceptible but resistant to amikacin. Among the total isolates, 26 (86.67%) were susceptible to tigecycline. All the isolates included were sensitive to colistin (Table 1).

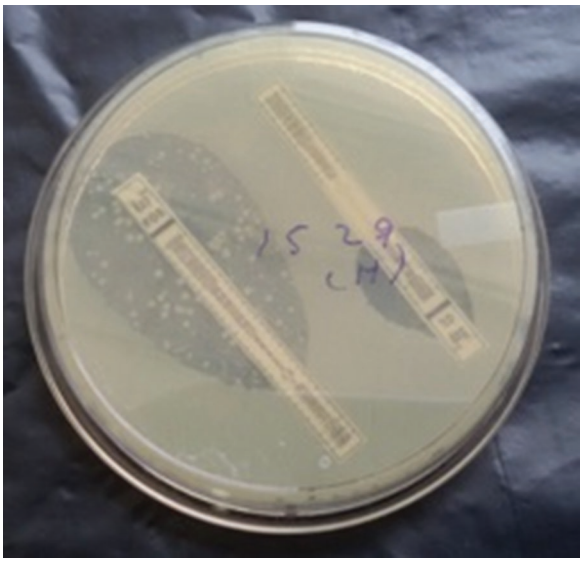
Among the 30 samples, all (100%) were positive for extended spectrum beta lactamase (ESBL) screening. On screening test with cefpodoxime, ceftriaxone, and cefotaxime, all the isolates showed resistance. They were further confirmed by double disc test using cefotaxime and cefotaxime with clavulanic acid, which showed a 5-mm increase in zone size between the zone with cefotaxime and that of cefotaxime with clavulanic acid. However, 24 of these isolates were found to be resistant to the combination of betalactams and betalactamase inhibitors.

Among the samples included, 26 (86.67%) were susceptible and 4 (13.33%) were resistant to fosfomycin. Of these 4 resistant isolates, 2 were *E. coli* and 2 were *K. pneumoniae*. About 26 (86.67%) of these isolates were susceptible to fosfomycin by the CLSI criteria and 25 (83.33%) by the EUCAST criteria for MIC by E-test and broth dilution and DD.

Among the 30 isolates included in the study, 21 (70%) had heteroresistant colonies on MHA plate when tested for MIC for fosfomycin by E-test method (Figure 1). These isolates were not detected by DD method or broth dilution methods. These included 15 *E. coli* and 6 *K. pneumoniae* isolates. The MIC of fosfomycin was between 0.25 and 512 µg/ml. These heteroresistant colonies when further subjected to E-test and BD showed resistance with an MIC ≥512 µg/ml, depicting prevalence of heteroresistance. Complete susceptibility and no heteroresistance were seen in 5 of the *E. coli* isolates.

Among the included isolates, 16 (53.33%) were resistant and 14 (46.67%) were susceptible to imipenem and meropenem. Hence, these 16 isolates were labeled as carbapenem resistant Enterobacteriaceae (CRE). Of these CRE isolates, 13 (81.25%) were susceptible to fosfomycin.

Figure 1: Heteroresistant colonies with fosfomycin in E-test method.



4. DISCUSSION

This study evaluated the *in vitro* activity of fosfomycin in MDR Enterobacteriaceae uropathogens. UTI was found to be most common in females of the reproductive age group. Of the 30 included samples, the causative agents were found to be *E. coli* in 73.33% and *K. pneumoniae* in 26.67% cases.

Among the included samples, 14 were susceptible and 16 were resistant to amikacin, while 10 were susceptible and 20 resistant to gentamicin. There were 8 isolates that were gentamicin resistant but susceptible to amikacin, whereas 4 isolates were gentamicin susceptible but resistant to amikacin. This shows that it is necessary to determine the individual *in vitro* susceptibility to each aminoglycoside, and the result obtained for one cannot be extrapolated for another. In a study by Xiao *et al.,* 86.7% of gentamicin-resistant *E. coli* were susceptible to amikacin [8].

Nitrofurantoin, which was earlier considered to be good antimicrobial for treatment of UTI [9], also showed resistance in 53.33% isolates. All the isolates were susceptible to colistin, which remains as the last resort for treatment. But, as colistin is known to be nephrotoxic [10], its use in treatment of UTI should be minimized as far as possible.

Among the included samples, all the 30 isolates were provisionally screened positive for production of ESBL, 24 of these isolates were found to be further resistant to the combination of betalactams and betalactamase inhibitors, and hence were harboring alternative methods of resistance, either independent of ESBL or coexpressed with it, most likely ampC betalactamases, efflux pumps, or other classes of beta lactamases not inhibited by the pharmacological enzyme inhibitors like sulbactam, tazobactam, or clavulanate. Further analysis of these resistant mechanisms was beyond the scope of this study.

The majority of the recently published studies evaluated the *in vitro* activity of fosfomycin against ESBL-producing *Enterobacteriaceae*, particularly *E. coli* and *K. pneumoniae.* Although studies evaluating the susceptibility of isolates recovered from blood or respiratory specimens have been published, the great majority of these studies focused on urines samples. In general, different studies show that fosfomycin was found to be more active against *E. coli* (range, 82-100%) than against *K. pneumoniae* (15-100%) [3]. We found that 25% of MDR uropathogenic *K. pneumoniae* isolates were resistant to fosfomycin, in comparison to only 9.1% of resistance among MDR uropathogenic *E. coli.* This is similar to the finding of Banerjee *et al.,* where *E. coli* was more susceptible to fosfomycin than *K. pneumonia* [11].

In the common fosfomycin- susceptible bacteria like *E. coli*, resistance develops when mutations occur in the uptake systems used as means of fosfomycin entry inside the bacteria. Mutations in the chromosomal *glpT* and *uhpT* genes, which encode fosfomycin transporters, result in blocked or decreased fosfomycin uptake. Heteroresistance to fosfomycin has been described for *S. pneumoniae* MurA1 protein [3]. In a study done by Engel H *et al.*, it was found that 10 out of 11 *S. pneumoniae* showed heteroresistance to fosfomycin [12]. In the present study, 70% of the MDR Enterobacteriaceae isolates showed heteroresistance to fosfomycin when tested by E-test method. This heteroresistance was not detected by conventional DD testing. When these heteroresistant colonies were picked up and further tested for MIC to fosfomycin, they however showed complete resistance. This may indicate that inadequate or underdosing of fosfomycin may select out or induce its own resistance. However, this finding is not correlated among non-MDR isolates and isolates obtained from treatment-naive patients with community acquired UTI. Complete susceptibility and no heteroresistance were seen in 5 of the *E. coli* isolates. There were 16 (53.33%) CRE isolates.

Of these 16 isolates, 13 (81.25%) were susceptible to fosfomycin. This shows that fosfomycin has good activity against MDR, ESBL producers, and CRE uropathogens. This is in accordance to the findings of Falagas *et al.,* who showed that 90% of the isolates were susceptible to fosfomycin in a systematic review [13] and Li *et al.,* where 92.2% of the *E. coli* isolates were susceptible to fosfomycin [14].

Hence, fosfomycin can be a good therapeutic option for treatment of UTI especially in cases of MDR, ESBL-producing, and CRE uropathogens. However, the question of heteroresistance and its impact on clinical outcome is unanswered in the present study and requires further pharmacokinetic and clinical studies for confirmation. A study has shown that initial inoculum with resistant subpopulations could partially explain the fosfomycin MIC discrepancies with respect to the AD method [15].

With the emergence of multidrug resistance, particularly in patients exposed to the hospital environment or previously used, misused, and abused antibiotic therapy, the therapeutic options for treatment of UTIs are becoming limited. As a result, physicians have been forced to use erstwhile less commonly used, older, injectable, and often more toxic antibiotics, like the polymyxins and aminoglycosides, for treatment of these infections. However, all these antimicrobials being injectable limit their use in the outpatient therapy of UTI.

5. CONCLUSION

Fosfomycin shows a high *in vitro* susceptibility of 86.67% among multidrug resistant UTI-causing isolates. However, heteroresistance to fosfomycin was seen in 70% isolates, which could not be detected by conventional DD testing and requires E-test for determination. When these heteroresistant colonies were picked up and further tested for MIC to fosfomycin, they however showed complete resistance. This may indicate that inadequate or underdosing of fosfomycin may select out or induce its own resistance.

Due to the ability of isolates to develop *in vitro* fosfomycin heteroresistance, it should be prescribed to the patients with caution in proper dosage for appropriate duration to avoid low concentration exposure of the isolates to the drug. However, this finding has to be evaluated by further *in vivo* pharmacokinetic and clinical studies to find out the actual impact of heteroresistance.

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Author Contributions
All authors contributed equally to this study.

Conflict of Interest
There is no conflict of interest.

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