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ORIGINAL ARTICLE

Genomic analysis of Fosfomycin resistance in multi-drug resistant uropathogens and comparison of *in-vitro* susceptibility methods uropathogens

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ABSTRACT

Background and Objectives: Urinary tract infection is one of the most common bacterial infections causing high morbidity and mortality. The alarming rise of multidrug-resistant uropathogens worldwide forced the clinician to re-think the old drugs like Fosfomycin for its therapeutic management. Our objective was to compare agar dilution, disc diffusion and E-test method for antimicrobial susceptibility testing of Fosfomycin against different drug-resistant uropathogens.

Materials and Methods: Consecutive 181 uropathogens were tested for Fosfomycin susceptibility using agar dilution, disc diffusion and E-test. Results were interpreted using Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints. Whole genome sequencing analysis was done on the 4 XDR/PDR Fosfomycin resistant *Klebsiella pneumoniae* isolates.

Results: *Escherichia coli* was found as the most common (62.4%) uropathogen followed by *Klebsiella pneumoniae* (21%). Considering agar dilution as the gold standard, 6.1% of isolates were resistant to Fosfomycin. Following CLSI breakpoints, the susceptibility of *Escherichia coli*, *Klebsiella pneumoniae*, other *Enterobacterales* and *Pseudomonas aeruginosa* were 92.9%, 92.1%, 100%, 100%; whereas using EUCAST breakpoints the susceptibility rates were 85.7%, 86.9%, 92.9%, and 100%, respectively. The essential agreement, categorical agreement, major error, and very major error for E-test/ disc diffusion for all the organisms were 91.2%/Not Applicable, 95%/93.9%, 1.8%/4.7%, 9.1%/9.1%, respectively. Whole-genome sequencing showed mutation *UhpT* gene as well as the presence of plasmid-mediated *fosA5* or *fosA6* genes conferring Fosfomycin resistance.

Conclusion: This result supports very low resistance of *Enterobacterales* against Fosfomycin; hence should be considered a valuable option to treat multidrug-resistant uropathogens. Disc diffusion was observed to be a convenient method for Fosfomycin susceptibility testing compared to agar dilution.

Keywords: Fosfomycin; Agar dilution; Disc diffusion; E-test; *Enterobacterales*

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INTRODUCTION

Urinary tract infection (UTI) is one of the most frequent bacterial infections in both hospital and community settings, causing significant morbidity and economic burden (1-3). *Escherichia coli (E. coli)* is the most common uropathogen accounting for 70-90% followed by other *Enterobacterales* (e.g., *Klebsiella pneumoniae, Proteus mirabilis*, etc.), non-fermenters (*Pseudomonas aeruginosa*, *Acinetobacter* spp.) and Gram-positive cocci (GPC) (e.g., *Enterococcus* spp.) etc. (4, 5). The alarming rise of multidrug resistance (MDR), especially the carbapenem-resistant Gram-negative bacilli (GNB), is a pressing concern worldwide. The increasing trend of resistance to many antibiotics and the lack of newer antimicrobials in the pipeline have renewed the interest of clinicians in the usage of Fosfomycin as a viable alternative (6-8).

Fosfomycin (FP) is derived from phosphonic acid. It inactivates UDP-N- acetylglucosamine-3-enolpyruvyltransferase enzyme, that ligates phosphoenolpyruvate to the 3-hydroxyl group of UDP-N-acetylglucosamine during peptidoglycan synthesis (9). FP is recommended as the first-line agent for empirical therapy of uncomplicated UTI (5). It has bactericidal activity against many GNBs e.g., *E. coli*, *Klebsiella pneumoniae*, *Citrobacter* spp., *Enterobacter* spp., *Proteus* spp., *Serratia* spp. and GPC e.g., *Enterococcus faecalis* (9). *In-vitro* studies showed adequate FP concentrations in serum, prostate, lungs, inflamed tissues, bone, cerebrospinal fluid, and abscess fluid; hence, it could become a valuable alternative for many of the MDR *Enterobacterales* isolates other than *E. coli* (10).

CLSI approved disk diffusion (DD) test and agar dilution (AD) methods for *in-vitro* testing of FP against uropathogenic *E. coli* and *Enterococcus faecalis (E. faecalis)* (11). EUCAST recommends DD and AD methods for uropathogenic *E. coli* and AD or broth microdilution methods for other members of *Enterobacterales* (12). On the other hand, E-test always becomes an attractive method over AD or DD being easy to perform and can provide quantitative MIC results.

Hence, this study was aimed to determine FP's activity against the clinically relevant uropathogens other than *E. coli* and *E. faecalis* comparing different antimicrobial susceptibility testing (AST) methods e.g.AD, DD, and E-test.

MATERIALS AND METHODS

Bacterial isolate collection. A total of 181 consecutive urinary isolates (173 GNB and 8 GPC) from clinically suspected UTI patients attending a tertiary care hospital over one year were collected and included in this study. The study had been approved by the institutional ethical committee (IEC-305/02.06.2017, RP-41/2017). Species identification was performed using a matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF) (VITEK-MS system, BioMérieux, Marcy-l'Étoile, France). Isolates were stored at -80ºC in glycerol until further testing. Isolates identified as colonizers were excluded from the study.

Antimicrobial susceptibility testing (AST). Antibiotic susceptibility testing for all GNBs and GPCs was determined by the DD method following CLSI guidelines. *E. coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, and *Pseudomonas aeruginosa* ATCC 27853 within acceptable limits were used as quality control strains for the drugs tested. Breakpoints for *E. coli* were used to interpret FP susceptibility for all other GNB in the absence of interpretive criteria. Extended-spectrum beta-lactamase (ESBL), carbapenem resistance (CR), and high-level aminoglycoside resistance (HLAR) was interpreted according to CLSI guidelines (11).

Isolates susceptible to all the tested antibiotics were categorized as pan-sensitive (PS), non-susceptibility to at least one agent in three or more antimicrobials as multidrug-resistant (MDR), non-susceptibility to at least one agent in all but two or fewer antimicrobial categories as extremely drug-resistant (XDR), and resistance to all antimicrobials as pan drug-resistant (PDR). Isolates not fitting any of the above patterns were categorized as others (13).

FP susceptibility. Susceptibility testing of FP was performed by AD, DD, and E-test methods. Results were interpreted applying CLSI and EUCAST breakpoints/MIC. MIC_{50} and MIC_{90} for each species were determined by calculating the MIC that would inhibit 50% or 90% of the isolates, respectively. Using CLSI breakpoints (≤64 µg /mL: susceptible, 128mg/L: intermediate, ≥256 mg/L: resistant), MICs results of the E-test (Himedia, India) were interpreted. For the DD test, FP (200 µg) (Himedia, India) was used, and the result was interpreted using CLSI breakpoints (Zone

diameters ≥16mm: susceptible, 13-15mm: intermediate and ≤12mm: resistance) (11). AST results were also interpreted using EUCAST breakpoints (for AD: <32 mg/L: susceptible, ≥32mg/L: resistant; for DD: ≥24 mm: susceptible, <24 mm: resistant). In case of any disagreement, the AD method was used for reporting the test result (12). ATCC control strains *E. coli* ATCC 25922 and *E. faecalis* ATCC 29212 were used as quality control strains.

Agreement analysis. Using AD as the reference method, agreement rates were calculated for different methods. Essential agreement (EA) was defined as an E-test MIC equal to or within ±1 dilution of the AD MIC. Categorical agreement (CA) was met when E-test or DD interpretive criteria agreed (susceptible/intermediate/resistant) with AD results. A minor error (mE) was defined as E-test or DD with a susceptible or resistant result when the AD result was intermediate or when E-test or DD results were intermediate, and AD was susceptible or resistant. A major error (ME) occurred when E-test or DD results were resistant, and AD was susceptible and was calculated only for susceptible isolates. Very major errors (VME) occurred when E-test or DD results were susceptible, and AD was resistant and was calculated only for resistant isolates (14).

Whole-genome sequencing, sequence assembly, annotation, and analysis. All the XDR and PDR strains were subjected to whole-genome sequencing (WGS). DNA was extracted using Qiagen kit as per the manufacturer's instructions. The whole-genome sequencing was done with Illumina platform on the S4 flow cell of NOVASEQ 6000 using 150bp paired-end chemistry. The raw data was evaluated using FastQC v0.11.5 before and after trimming the adapters and removal of low-quality bases by Trimmomatic v0.39. SPAdes v3.15.2 was selected for the De novo genome assembly. The quality of the draft genomes was evaluated using BUSCO v5.1.2 and QUAST v5.0.2. After assessing the quality, the contigs obtained were assembled into scaffolds using SSPACE v2.0 with 500 insert size and 0.5 error size. The assembled genome was improved using Pilon v1.24 which carries out gap filling, correcting misassembles, and improving small indels and single-base differences. The quality was again evaluated using QUAST and BUSCO. The scaffolds were ordered using ABACAS v1.03 with default parameters and three reference genomes (NCBI Accession No. CP012043, FO834906, AP006725). Prokka v1.14.6 was used for the gene annotation and gene prediction of the drafted genomes. Typing was done using ResFinder, MLST, PHAST, BacMet Database, and PHASTER to predict and identify the resistant genes, alleles, and gene products.

Nucleotide sequence accession numbers. Whole-genome sequence of 4 *Klebsiella pneumoniae* have been deposited in the Sequence Read Archive under National Center for Biotechnology Information (NCBI) BioProject accession PRJNA797874. The genome sequenced files have been submitted to the NCBI SRA under accession number SRR17640812, SRR17640805, SRR17640804 and SRR17640802.

Statistical analysis. Data were analyzed using SPSS software v.20.0 (SPSS Inc., Chicago, IL) by χ^2 test. Fisher's exact test was used when data were scarce. Significance was set at $P < 0.05$ using two-sided comparisons. The correlation between E-test and DD against AD was calculated using the Pearson method.

RESULTS

Among the 181 urinary isolates, 151 belonged to *Enterobacterales*. Among the GNBs, *E. coli* was the most common bacterial isolate [62.4% (113/181)] followed by *Klebsiella pneumoniae* [21% (38/181)], *Pseudomonas aeruginosa (P. aeruginosa)* [7.7% (14/181)], *Enterobacter* spp. [2.8% (5/181)], *Citrobacter* spp. [1.1% (2/181)] and *Proteus mirabilis* [0.6% (1/181)]. Among GPCs, *Enterococcus faecium* [2.8% (5/181)] was found commonest followed by *E. faecalis* [1.7% (3/181)].

AST of FP by different methods. Considering AD as a gold standard, more than 85% of isolates were observed susceptible to FP. The results of the AD, DD, and E-test methods following CLSI and EUCAST breakpoints had been shown in the Table 1.

The percentage of susceptible, intermediate, and resistant isolates by AD method following CLSI breakpoints was 93.4%, 0.5%, and 6.1%, respectively. Regardless of any of the methods, isolates were found more susceptible to FP following CLSI breakpoints than EUCAST breakpoints (Table 1). Nitrofurantoin resistance was observed much higher than FP (27% against all uropathogens and as high as 50% among

Klebsiella pneumoniae). On the contrary, resistance to FP was found less than 10% in most of the resistant phenotypes i.e., MDR, XDR, PDR, ESBL, and Carbapenem-resistant GNB (CR-GNB) isolates (Table 2). Isolates of *Enterococcus* spp. with HLAR were observed to exhibit 25% resistance against FP. FP had MIC_{50} (8 µg/mL) and MIC_{90} (32 µg/mL) in the FP susceptible strains. The overall MIC_{50} of 8 µg/mL and MIC_{90} of 64 µg/mL were observed in AD whereas E-test showed a lower MIC_{50} value of 0.5 µg/mL and MIC_{90} of 64 µg/mL. The MIC_{50} / $_{90}$ rates of E-test in *P. aeruginosa* were similar to gold standard AD, whereas the difference between the two methods was seen in other organisms with higher MIC_{90} in E-test (Table 1).

Table 2. Characterization of urinary isolates into different resistance phenotypes and distribution of FP resistance

Resistance phenotype	Fosfomycin Sensitive (%)*
PS (30)	29 (96.7)
MDR (95)	87 (91.6)
E. coli (67)	61 (91.0)
Klebsiella pneumoniae (16)	15 (93.8)
P. aeruginosa (5)	5 (100)
Other *Enterobacterales* (2)	2 (100)
Enterococcus spp. (5)	4 (80)
XDR (23)	21 (91.3)
E. coli (7)	7 (100)
Klebsiella pneumoniae (12)	10 (83.3)
P. aeruginosa (2)	2 (100)
Other *Enterobacterales* (2)	2 (100)
PDR (2)	2 (100)
ESBL (137)	128 (93.3)
E. coli (92)	86 (93.5)
Klebsiella pneumoniae (31)	28 (90.3)
P. aeruginosa (9)	9 (100)
Other *Enterobacterale* (5)	5 (100)
Non-ESBL (36)	35 (97.2)
CRGNB (89)	80 (89.9)
Non-CRGNB (84)	83 (98.8)
HLAR (4)	3 (75)
Non-HLAR (4)	3 (100)
Others (31)	31 (100)

* Fosfomycin intermediate category is included in Fosfomycin susceptible, extended-spectrum lactamase (ESBL), carbapenem-resistant GNB (CRGNB), high-level aminoglycoside resistance (HLAR), Pan-sensitive (PS), multi-drug resistant (MDR), extremely drug-resistant (XDR) and pan-drug-resistant (PDR).

Table 1. Species wise distribution of Fosfomycin MIC $_{50}$ / $_{90}$ along with Fosfomycin and Nitrofurantoin resistance rates.

Organism	Fosfomycin MIC_{50} / $_{90}$ (mg/L) (MIC range) (mg/L)		Fosfomycin resistance (%)														NIT resistance (%)
	AD	E-test	AD						E-test						DD		
			CLSI			EUCAST			CLSI			EUCAST			CLSI			EUCAST		
			S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	
E. coli (113)	8/64(8-512)	0.25/64 (0.125-1024)	92.9	0.9	6.2	86.7	13.3	92.9	0.9	6.2	89.4	10.6	92.2	0	8.8	88.5	11.5	14.2
Klebsiella pneumoniae (38)	8/64(8-512)	8/128 (0.125-1024)	92.1	0	7.9	86.9	13.1	86.8	5.3	7.9	89.5	10.5	84.2	0	15.8	50	50	50
Other *Enterobacterales* (7)*	8/16(8-16)	0.125/64 (0.125-128)	100	0	0	100	0	87.5	12.5	0	75	25	100	0	0	87.5	12.5	28.6
Enterococcus spp. (8)	8/256(8-512)	0.125/512 (0.125-1024)	87.5	0	12.5	87.5	12.5	87.5	0	12.5	75	25	75	12.5	12.5	62.5	37.5	25*
P. aeruginosa (14)	8/32(8-64)	8/32(0.125-64)	100	0	0	92.9	7.1	100	0	0	92.9	7.1	100	0	0	92.9	7.1	NA*
Total (181)	8/64(8-512)	0.5/64(0.125-1024)	93.3	0.6	6.1	88.4	11.6	91.7	2.2	6.1	88.4	11.6	90	0.6	9.4	79.6	20.4	27.1

**Proteus mirabilis* and *Pseudomonas aeruginosa* were excluded as they are intrinsically resistant to Nitrofurantoin, DD: Disc diffusion, AD: Agar dilution, (NIT) Nitrofurantoin, S: Susceptible, I: Intermediate, R: Resistant, CLSI: Clinical and Laboratory Standards Institute, EUCAST: European Committee on Antimicrobial Susceptibility Testing, MIC: Minimum inhibitory concentration

Agreement analysis by different methods. The rates of EA, CA, mE, ME, and VME were assessed against all the organisms for DD/ E-test. Applying CLSI breakpoints, EA, CA, mE, ME, and VME for E-test/DD were found 91.2%/NA, 95%/93.9%, 2.8%/1.1%, 1.8%/4.7% and 9.1%/9.1%, respectively. Using EUCAST breakpoints, CA, ME, and VME for E-test/DD were found 88.9%/80.1%, 6.3%/16.3% and 52.4%/52.4%, respectively.

The performance of the E-test for *Klebsiella pneumoniae* was found good in comparison to *E. coli*, considering the VME result. Similar results were also observed for *Enterococcus* spp. Lastly, isolates of *P. aeruginosa* had shown no mE, ME, or VME using CLSI breakpoints of *E. coli* (Table 3).

A Scatter diagram was plotted using the FP AST result of 181 isolates by E-test and DD and compared with the AD result (Fig. 1a, 1b). One hundred out of 142 isolate with MIC ≤16mg/L by AD showed an excellent correlation with E-test. However, a poor correlation was seen between the results of the E-test and the AD result for the isolates with MIC between 64-128 mg/L (Fig.1a). Comparing the results of the DD method with AD, a good categorical agreement was observed for the isolates with MIC ≤64mg/L. Few isolates (8 out of 142) with MIC ≤ 16 mg/L by AD were classified as resistant by DD (Fig. 1b).

The correlation plot between MIC results of the E-test and AD values was drawn for the comparative analysis. Pearson R^2 value was found 0.6518, which indicates a good correlation between both methods (Fig. A1).

Whole-genome sequencing and analysis. The WGS analysis revealed that all the 4 either XDR or PDR FP resistant *Klebsiella pneumoniae* isolates have point mutation i.e., E350Q in the Hexose-6-phosphate: phosphate antiporter *UhpT* gene conferring resistance to FP. Apart from this chromosomal mutation, they also harbor plasmid-mediated *fosA5* or *fosA6* genes. Out of these 4 isolates, 2 isolates harbor the *fosA6* gene whereas 1 isolate harbor the *fosA5* gene and the rest 1 isolate haver both *fosA5* or *fosA6* genes giving rise to FP resistance.

DISCUSSION

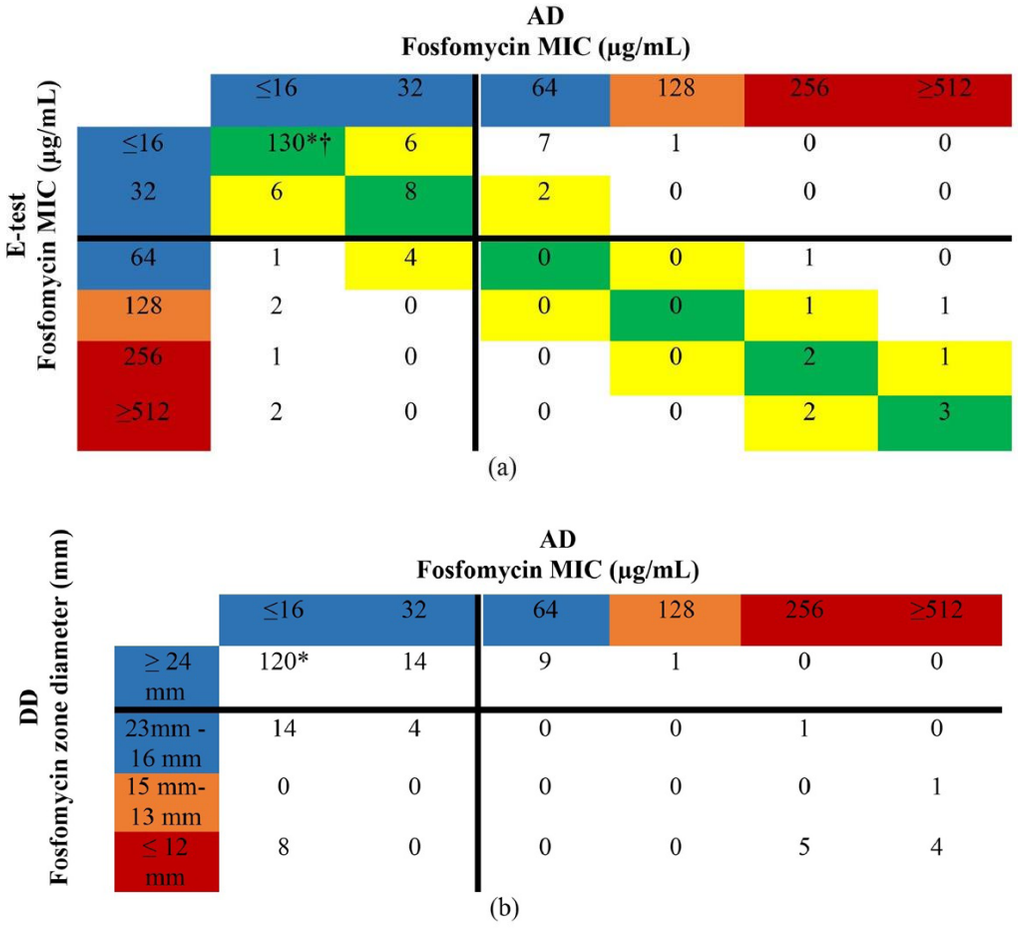
Emerging drug resistance among GNB with limited therapeutic options is a growing concern worldwide, resulting in increased morbidity, mortality, more extended hospital stay, and higher hospital cost (1, 2, 7, 9). With the spread of multidrug resistance, FP will be a potential option to this antimicrobial armamentarium (9). Currently, the therapeutic indication for FP is limited to *E. coli* and *E. faecalis* from the urinary samples, a subset of microorganisms causing UTIs (11). However, the main interest lies in assessing the *in-vitro* action of FP against infections caused by other microorganisms (16, 17). AD is recommended as the gold standard method to determine FP susceptibility, but its use is primarily confined due to the complexity to perform the test (11, 12). DD and E-test are easier to perform and take equal time to produce results.

In this study, we determined FP's activity against

Table 3. *In-vitro* antibacterial activity of FP against 181 urinary isolates using E-test and DD and categorization of errors compared with AD method

Organism	CLSI (%)									EUCAST (%)					
	DD					E-test				DD			E-test		
	CA	mE	ME	VME	EA	CA	mE	ME	VME	CA	ME	VME	CA	ME	VME
E. coli (113)	94.7	0.9	3.8	14.3	91.2	95.6	1.8	1.9	14.3	87.6	6.1	53.3	88.5	5.1	53.3
Klebsiella pneumoniae (38)	92.1	0	8.6	0	89.5	92.1	5.3	2.9	0	55.3	47.1	25	94.7	5.9	25
Other *Enterobacterales* (8)	100	0	0	0	87.5	87.5	12.5	0	0	87.5	12.5	0	87.5	12.5	0
Enterococcus spp. (8)	75	12.5	14.3	0	100	100	0	0	0	75	28.6	0	87.5	14.3	0
P. aeruginosa (14)	100	0	0	0	92.9	100	0	0	0	85.7	7.7	100	85.7	7.7	100
Total (181)	93.9	1.1	4.7	9.1	91.2	95	2.8	1.8	9.1	80.1	16.3	52.4	88.9	6.3	52.4

EA: Essential agreement, CA: Categorical agreement, mE: Minor error, ME: Major error, VME: Very major error, DD: Disc diffusion, CLSI: Clinical and Laboratory Standards Institute, EUCAST: European Committee on Antimicrobial Susceptibility Testing



DD: Disc diffusion, AD: Agar dilution, MIC: Minimum inhibitory concentration

Fig. 1. Correlation of Fosfomycin minimum inhibitory concentration and zone diameters values of E-test and disc diffusion compared to agar dilution**.** Scattergram of FP MICs obtained by (a) MICs obtained by E-test and (b) zone diameters of DD compared with AD for 181 isolates. Essential agreement highlighted in green (perfect agreement) and yellow (±1 log2 dilution). The solid horizontal and vertical lines represent the clinical breakpoint value established by EUCAST. The blue, orange and red colour represent the clinical breakpoint value established by CLSI i.e., susceptible, intermediate, and resistant respectively. Control strains *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 are represented by an * and †, respectively.

181 urinary isolates from patients causing significant bacteriuria. FP displayed consistent activity with overall susceptibility being 93.4% and 88.4% against the tested isolates following both CLSI and EUCAST criteria, respectively. MIC$_{50}$ / $_{90}$ of *E. coli* (8/64 mg/L) by AD method was found lower than MIC$_{50}$ / $_{90}$ of *Enterococcus* spp. (8/256 mg/L). So, the clinical use of FP against *Enterococcus* spp. will be helpful using susceptibility testing. Although few studies demonstrated good activity even in the presence of ESBL genes with MIC$_{90}$ ranging between 2-4 mg/L, most Asian isolates exhibited higher MIC up to 128 mg/L, and our study also showed a concordant result with them (18).

For other *Enterobacterales* including *Klebsiella pneumoniae*, the MIC$_{50}$ / $_{90}$ values were more found distributed i.e., 8/64 mg/L. The increased MIC values against FP among these isolates might be due to the patient population chosen from the hospital setting. Studies on *Klebsiella pneumoniae* isolates containing KPC carbapenemases showed 39%-100% susceptibility against FP. FP was also found to exhibit activity against strains containing *mcr-1* plasmid for colistin resistance. Hence, the study results were

worth looking at FP action against various resistant isolates of *Klebsiella pneumoniae* (9, 15, 19, 20). More *in-vitro* studies including both community and hospital isolates are required to validate the breakpoints for *Enterobacterales* other than *E. coli* before the routine usage.

Using breakpoints of *E. coli*, FP susceptibility against *P. aeruginosa* was observed considerably higher than other drugs. In the current study, the MIC_{50} / $_{90}$ value for FP ranged lower (8/32 mg/L) using both CLSI and EUCAST breakpoints in contrast to the report by Smith et al. 2020, where it was 64/256 mg/L and 124/256 mg/L, respectively (21). FP resistance in *P. aeruginosa* isolates might be due to any of the mechanisms like salvage pathway of peptidoglycan synthesis, presence of Fos metalloenzyme, which acts on *MurA* gene to block FP action, or *glpT* mutation, etc. Studies documented different results on the clinical and microbiological cure of UTI after completion of oral therapy (21). In the current study, only 22 urinary isolates of *P. aeruginosa* were analyzed. Hence, studies with a significant number of *P. aeruginosa* isolates targeting different clinical diagnoses need to be planned to establish the validity of these breakpoints and also its effect on clinical outcomes.

Our study found high resistance of nitrofurantoin among both *E. coli* and *Klebsiella pneumoniae* which is similar to the results shown in other studies (22, 23).

Susceptibility to FP ranged from 91.3%-100% among different XDR and PDR GNBs. It highlights the importance of using FP as an alternative therapeutic agent for clinically difficult management to treat infections. Similar results were observed among the isolates with ESBL, CR, and HLAR reiterating the potential use of FP in these infections (8, 24).

Our study result showed more discrepancy in the DD method in comparison to E-test comparing the various agreement rates. Categorical agreement for DD and E-test was 93.9% and 95%, respectively. Both major (4.7% and 1.8%) and very major errors (9.1% and 9.1%) were more in the DD method in comparison to E-test. Although E-test does not have CLSI recommendations, it can be considered a potential alternative to determine quantitative FP MICs in light of the absence of an approved CLSI guideline-based AD method.

It should also be noted that FP did appear to perform well against *P. aeruginosa*, *Klebsiella pneumoniae* and *Enterococcus* spp. (Table 3). Previous investigations have shown poor agreement between agar dilution and E-test for isolates of *P. aeruginosa* and *Enterobacter* spp. (7). Higher error rates were observed following EUCAST breakpoints than the CLSI. This was probably due to the stringent breakpoints, which were followed up by the EUCAST guidelines. As per our study result, E-test for FP susceptibility testing showed favorable results against Enterobacterales and *P. aeruginosa* following CLSI breakpoints.

In this study, extensive analysis of the genome of 4 XDR/PDR *Klebsiella pneumoniae* strains showed mutation E350Q in Hexose-6-phosphate:phosphate antiporter *UhpT* gene as well as the presence of plasmid-mediated *fosA5* or *fosA6* genes (25). Similar amino acid changes as well as the presence of *fosA5* or *fosA6* genes have previously been reported in the FP resistant *Klebsiella pneumoniae* isolates (26, 27). The genome sequences of 4 *Klebsiella pneumoniae* strains indicate lateral gene transfer and spontaneous mutation as major drivers of FP resistance.

This study has several limitations. The study included a relatively small number of isolates. Further, all the isolates were from a single centre. This might limit the application of our findings in other geographical areas due to differences in the mechanism of antimicrobial resistance. Our study result showed good activity of FP against multidrug-resistant microorganisms. The current study results can be used in a larger study targeting geographically diverse isolates to validate the results.

CONCLUSION

In the current study, the good activity of FP was observed against urinary *Enterobacterales* and *P. aeruginosa*. *In-vitro* studies to detect antibiotic resistance comparing various AST methods are fundamental before any therapeutic application. Therefore, it is imperative to implement a method that delivers early, easy, less expensive, and reliable results. As per our study result, alternative methods such as E-test and DD provide similar results as the gold standard agar dilution method, which may be acceptable due to low ME and VME rates. Indeed, the best performance of the E-test and DD was achieved when interpreted according to the CLSI breakpoints rather than EUCAST. Although our study has limitations, including a small number of isolates and sampling bias for

MDR isolates with more FP susceptible isolates. Our results suggest that other phenotypic methods may be useful in the interpretation of FP susceptibility in the case of *Enterobacterales* and *P. aeruginosa* using breakpoints as indicated for *E. coli* and *E. faecalis.*

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