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Original Research Article

Deciphering fosfomycin's antimicrobial action: comparative evaluation of susceptibility testing techniques on clinical isolates

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Abstract

Background: Treating physicians consistently face a tough challenge in treating the conditions caused by Multi drug resistant (MDRO) older antimicrobial like fosfomycin is re-established as effective alternatives for managing the infections caused by these pathogens, but limited information exists on their action against clinical isolates from specimens other than urine. So, this study aimed to assess the effectiveness of fosfomycin against Enterobacterales resistant to multiple drugs isolated from various clinical specimens and to comparatively evaluate the antimicrobial testing methods for fosfomycin like disk diffusion, agar dilution and micro broth dilution by Vitek-2.

Materials and Methods: Fosfomycin susceptibility testing was carried out for 550 clinical isolates by agar dilution, disk diffusion and Vitek-2 for a period of one year. Results of disk diffusion and Vitek-2 was compared with agar dilution.

Results: The study compared fosfomycin susceptibility testing using Vitek-2 (V2) and Disk diffusion (DD) against the reference Agar dilution (AD) method. V2 showed 83% Categorical agreement (CA) but had a 5% Very major error (VME), misclassifying resistant strains as susceptible. It also had 1.09% Major Error (ME) and 11% Minor error (mE). In contrast, DD performed better with 90% CA, lower VME (1.27%), ME (7%), and mE (1%). Overall, DD was more reliable than V2, with fewer critical errors, making it the preferred method for fosfomycin susceptibility testing.

Conclusion: According to the results of our study, fosfomycin has a good antimicrobial activity against MDR Enterobacterales and adoption of the disk diffusion method for routine testing of fosfomycin susceptibility, is both practical and feasible in healthcare settings where resource availability is limited.

Keywords: Multidrug resistant, Fosfomycin, Agar dilution, Vitek-2, Disc diffusion.

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1. Introduction

The emergence and progression of variety of antibacterial resistance mechanisms within Enterobacterales as narrowed down the treatment option for managing the conditions caused by these organisms.[1] Bacterial infections caused by MDRO have persistently presented ongoing challenges for treating physicians.[2] Rapidity in spread of extended-spectrum β-lactamase (ESBLS) and carbapenemase producing Enterobacterales among the health care settings and among the community is of public health challenge that adds on to the burden on health care system, since the presence of such enzymes confers resistance to third, fourth generation cephalosporins & monobactams further restricting the choice of antimicrobials for treatment.[3] Introduction of new antimicrobials which are efficacious against such pathogens is reduced hence the usage of older antibiotic like fosfomycin and polymyxins is re-established as a promising effective alternative agent for treatment.[4] Fosfomycin is a potent bactericidal broad-spectrum antimicrobial with almost 90% susceptibility to ESBL and carbapenemase producing Enterobacterales,[5] but there is limited data that is available regarding the action of fosfomycin against clinical isolates that are commonly encountered from specimens other than urine. In vitro susceptibility testing of fosfomycin is complex and liable for error with tendency of development of resistant mutants in vitro,[6] this may hamper the wider clinical usage of fosfomycin despite being an efficient antimicrobial agent. In view of all these facts the present study is undertaken to evaluate the antimicrobial efficacy of fosfomycin against multi-drug resistant (MDR) Enterobacterales strains isolated from various clinical specimens and to comparatively evaluate the antimicrobial testing method for fosfomycin like

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disk diffusion, agar dilution and micro broth dilution by automated method (Vitek system).

2. Materials and Methods

This was a prospective study conducted in the Microbiology department of a tertiary care hospital from August 2022 to July 2023. During this period, An overall of 550 clinical samples received routinely for culture and sensitivity in Microbiology laboratory, that were processed by Vitek-2 & those that were identified as Enterobacterales group which were multi drug resistant organisms formed the study. Specimens from all age groups and both the genders were included. Criteria for exclusion were stool specimen and repeat samples that were received to laboratory from the same patient.

Vitek-2 was performed according to the standard operating procedure and results were noted same isolates was subjected for fosfomycin disk diffusion and fosfomycin agar dilution tests.

Disk diffusion was performed according CLSI described Kirby Baur disk diffusion method on Mueller–Hinton agar (MHA) using Fosfomycin disk (200 DD) (Becton Dickinson, Sparks, MD, USA).

Agar dilution method was performed on Mueller–Hinton agar (MHA) using Fosfomycin sodium disalt (Sigma Chemical Co., St Louis, MO, USA) supplemented with 25 mg/L G6P (Sigma Chemical Co.) as recommended by the CLSI. All the tests were performed with appropriate control strain (*E.coli* ATCC 25922).

Susceptibility results were interpreted according to CLSI and EUCAST 2023 guidelines.

Susceptibility breakpoints for fosfomycin according to CLSI is available only for urinary isolates of *E.coli* for the research study we have extrapolated breakpoint to all urinary isolates of Enterobacterales and the results were interpreted.

For non- urinary isolates fosfomycin (IV) susceptibility breakpoints of EUCAST 2023 guidelines was followed for result interpretation. (**Table 1**)

Results that were noted from Vitek 2 and disk diffusion were further analysed for categorical agreement, very major error, major error and minor error in comparison with agar dilution method (Reference method).

If AST result of the isolates from Disc Diffusion and Vitek-2 is similar to the reference Agar Dilution, then the test method is categorically in agreement with the standard reference method; if not, it is categorically disagreed. Categorical disagreement are further classified into very major error (VME), major error (ME), and minor error (MiE). If the test is sensitive and the reference technique is resistant, then it is (VME). If the test method is resistant while the standard reference method is sensitive then it is (ME) it is referred to as (MiE) if the reference method is sensitive or resistant while the test method is intermediate, or vice versa.

2.1. Data analysis

The collected data were entered into MS excel followed by the analyses using SPSS version 22 (licenses to the institution, JSS AHER). The demographic characteristics such as age, gender, etc. were represented using percentage. fosfomycin susceptibility results for urinary isolates. CLSI 2023 guidelines was used and for non- urinary isolates European Committee of Antimicrobial Susceptibility Testing 2023 was used.

The sensitivity, specificity for Disk Diffusion & Vitek-2 were calculated considering agar dilution as a gold standard method.

3. Results

A total of 550 clinical samples that were received for culture and sensitivity to the microbiology laboratory were included in the study. Most of the isolates were from male patients above 60 years of age.

The susceptibility of isolates to fosfomycin was evaluated across various sample types using the gold standard agar dilution method. The overall fosfomycin susceptibility rates for different sample types are summarized in **Table 2**.

Table 1: Fosfomycin minimum inhibitory concentrations and zone diameter breakpoints for Enterobacterales according to the Clinical and Laboratory Standards Institute criteria and European Committee of Antimicrobial Susceptibility Testing 2023

Standard and Organisms	**Zone diameter breakpoint (mm)**			**MIC (µg/ml)**		
	S	**I**	**R**	**S**	**I**	**R**
CLSI 2022 - Urinary Isolates of Enterobacterales						
	≥ 16	13-15	≤ 12	≤ 64	128	≥ 256
Eucast non-urinary isolates of Enterobacterales intravenous for (Systemic Isolates) Oral for uncomplicated UTI	≥ 24	NA	≤ 24	≤ 32	NA	≥ 64

Table 2: Sample-wise distribution of various isolates

Samples	No. of samples N% (n=isolate number)	Overall Fosfomycin susceptibility – based on gold standard agar dilution method N% (n=isolate number)
Urine	44 (242)	88(213)
Exudate	25(137)	77(107)
Sputum	11(61)	73(44)
Blood	8(44)	59(26)
Et	5(27)	56(15)
Bal	2(11)	36(4)
Bile	2(11)	36(4)
Other Samples	3(17)	82(14)

Table 3: Organism wise fosfomycin susceptibility testing result by agar dilution (AD), disk diffusion (DD) and Vitek-2

S. No.	Organism	Number of Isolates (N)	Susceptibilty Testing Methods	Interpretation based on CLSI 2023 Susceptibility (%)*	Interpretation based on EUCAST 2023 Susceptibility (%)**
1	*E.coli*	275	**AD**	98	93
			DD	96	90
			VITEK 2	100	99
2	*Klebsiella Species*	226	**AD**	68	55
			DD	72	46
			VITEK 2	78	59
3	*Entrobacter species*	17	**AD**	67	57
			DD	67	36
			VITEK 2	67	57
4	Other Enterobacterales	32	**AD**	93	100
			DD	75	79
			VITEK 2	100	100
	Overall Susceptibility of fosfomycin		**AD**	88	72
			DD	83	64
			VITEK 2	83	73

The asterisk (*) indicates that Fosfomycin susceptibility interpretation follows the Clinical and Laboratory Standards Institute (CLSI) 2023 guidelines, while (**) refers to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) 2023 guidelines. Differences in breakpoints between these standards may lead to variations in susceptibility classification. Agreement of Fosfomycin Susceptibility Testing Methods among Urinary Isolates

Among 550 isolates of Enterobacterales, *Escherichia coli* showed the highest susceptibility to fosfomycin, with 98% susceptibility in urinary samples and 93% in non-urinary samples. Other members of urinary isolates of Enterobacterales, including *Citrobacter, Proteus, and Serratia species*, also showed 93% susceptibility to fosfomycin. Isolates from non-urinary samples demonstrated 100% susceptibility to fosfomycin.

Klebsiella species, isolated from both urinary (68%) and non-urinary (53%) specimens, were the clinical strains most resistant to fosfomycin. This was followed by *Enterobacter species*, with urinary and non-urinary isolates showing 67% and 57% resistance, respectively.

Among a total of 242 urinary isolates of Enterobacterales, the VITEK-2 method showed a categorical agreement (CA) of 90%, very major error (VME) in 5% (11 isolates), major error (ME) in 1% (2 isolates), and a minor error (mE) in 4% (7 isolates) compared to the reference agar dilution method. In contrast, the disk diffusion (DD) method showed 91% of CA, a VME in 1% (2 isolates), an ME in 5% (12 isolates), and an mE in 3% (7 isolates) compared to the agar dilution method.

Table 4: Agreement of fosfomycin susceptibility testing methods among urinary isolates

Agreement of Urinary Isolates (%)				
Methods	**CA**	**VME**	**ME**	**mE**
VITEK-2	90%	5%	1%	4%
DD	91%	1%	5%	3%

Table 5: Agreement of fosfomycin susceptibility testing methods among non - urinary

Agreement of Non- Urinary Isolates (%)			
Methods	**CA**	**VME**	**ME**
VITEK-2	92%	6%	2%
DD	90%	2%	8%

Table 6: Fosfomycin MIC distribution of VITEK-2 compared with agar dilution for urinary isolates

	MIC range	**MIC of AD**								
	(µg/mL)	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**
MIC of Vitek	≤16	0	0	0	0	178	26	0	**4****	**5****
	32	0	0	0	0	0	6	0	0	**2****
	64	0	0	0	0	0	0	6	0	0
	128	0	0	0	0	**1***	0	0	5	0
	≥256	0	0	0	0	**1***	0	0	1	7

**VME (Very major error), *ME (Major error)

Table 7: Fosfomycin MIC distribution of Vitek-2 with reference to agar dilution (non-urinary isolates)

	MIC range	**MIC of AD**								
	(µg/mL)	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**
MIC of Vitek	≤16	0	0	0	0	181	22	**3****	**8****	**6****
	32	0	0	0	0	0	8	0	0	1
	64	0	0	0	0	0	0	20	0	1
	128	0	0	0	0	**2***	**3***	2	11	0
	≥256	0	0	0	0	**1***	**1***	5	2	31

**VME (Very major error), *ME (Major error)

Among a total of 308 non-urinary isolates, the VITEK-2 method showed 92% of CA a very major error (VME) in 6% (17 isolates) and a major error (ME) in 2% (5 isolates) compared to the gold standard agar dilution method. Similarly, the disk diffusion (DD) method showed a VME in 2% (7 isolates) and an ME in 8% (26 isolates) compared to the agar dilution method.

Out of 242 urinary isolates, the VITEK-2 method showed:

1. 4 isolates with a MIC of ≤16 µg/mL and 128 µg/mL by agar dilution.
2. 5 isolates with a MIC of ≤16 µg/mL and 256 µg/mL by agar dilution.
3. 2 isolates with a MIC of 32 µg/mL and 256 µg/mL by agar dilution.

These discrepancies indicate a total of 11 isolates showing very major errors (VMEs). Additionally, 1 isolate with a MIC of 128 µg/mL and 1 isolate with a MIC of 256 µg/mL in VITEK-2 showed a MIC of 16 µg/mL by agar dilution, indicating major errors (MEs).

Out of 308 non-urinary isolates, the VITEK-2 method showed:

1. 3 isolates with a MIC of ≤16 µg/mL and 64 µg/mL by agar dilution.
2. 8 isolates with a MIC of ≤16 µg/mL and 128 µg/mL by agar dilution.
3. 6 isolates with a MIC of ≤16 µg/mL and 256 µg/mL by agar dilution.

These discrepancies indicate a total of 17 isolates showing very major errors (VMEs). Additionally, 2 isolates with a MIC of 128 µg/mL showed 16 µg/mL by agar dilution, and 3 isolates with a MIC of 128 µg/mL showed 32 µg/mL by agar dilution. Furthermore, 1 isolate each with a MIC of ≥256 µg/mL in VITEK-2 showed 16 µg/mL and 32 µg/mL by agar dilution. Overall, 7 isolates showed major errors (MEs) by VITEK-2.

3.1. Sensitivity and specificity of Vitek-2 and disk diffusion

Sensitivity and specificity of different methods (Vitek-2 and Disk Diffusion) compared to a gold standard (Agar Dilution).

Sensitivity: ability of a test method to correctly identify the organism that is truly Susceptible to fosfomycin (true positive rate). Higher sensitivity means fewer false negative susceptibility in our study Vitek-2 method showed 99% of Sensitivity in comparison with agar dilution. In contrast to disk diffusion showed 91% sensitivity.

In terms of specificity: ability of a test method to correctly identify those organisms which are non-susceptible to fosfomycin (true negative rate). Higher specificity means fewer false susceptibility to fosfomycin according to this our study showed specificity of Vitek- 2 method of 76% whereas disk diffusion showed 93% specificity in comparison with agar dilution.

Table 8: Sensitivity and specificity of Vitek-2 and disk diffusion

Gold Std- Agar Dilution	Sensitivity (%)	Specificity (%)
Vitek-2	99	76
Disk Diffusion	91	93

4. Discussion

The emergence of multidrug-resistant organisms (MDROs) poses a significant challenge in healthcare, as these microorganisms exhibit resistance to multiple classes of antimicrobial agents. Managing infections caused by multidrug-resistant (MDR) microorganisms remains a pressing concern for healthcare providers. With the declining effectiveness of newer antimicrobials against these infections, there has been a renewed interest in the use of older antibiotics such as fosfomycin. Fosfomycin, known for its potency, broad-spectrum activity, and high susceptibility against extended-spectrum beta-lactamase (ESBL)-producing and carbapenem-resistant Enterobacterales, has regained attention as a promising alternative therapeutic agent. In light of this, our study is centred on investigating the antimicrobial efficacy of fosfomycin against Enterobacterales isolated from various clinical specimens. Additionally, we aim to assess and compare different antimicrobial testing methods for fosfomycin, including disc diffusion, agar dilution, and microbroth dilution using Vitek2. By comprehensively evaluating the antimicrobial activity of fosfomycin and comparing various testing methods, we seek to provide valuable insights into its effectiveness in combating infections caused by multidrug-resistant Enterobacterales. This research contributes to the ongoing efforts to optimize treatment strategies and preserve the effectiveness of antimicrobial agents in the face of rising antimicrobial resistance.

In the present study, 550 clinical samples that were received in department of Microbiology for culture and sensitivity were included. Specimens from all age groups and both sexes were included. Repeat samples from same patients and stool samples were excluded from the study.

The majority of samples identified as Enterobacterales were isolated from patients above 60 years of age, which is consistent with the study conducted by Avi Peretz *et al.*, where the majority of patients were also above 60 years old.[7]

In alignment with the demographic data from our study, 56% of the patients were males, while 44% were females. A corresponding study titled "Fosfomycin Susceptibility among Multidrug-Resistant and Extended-Spectrum β-Lactamase-Producing Uropathogenic *Escherichia coli* Isolates at a Tertiary Care Hospital of Western India," led by Ruchi Jain, revealed similar findings.[8]

Overall susceptibility to fosfomycin by agar dilution was 79%, with 19% resistance and 2.18% intermediate susceptibility to fosfomycin. A similar pattern of susceptibility was also noted in the study conducted by Beata Kowalska-Krochmal *et al.,* where 78% of strains were susceptible to fosfomycin by agar dilution.[9]

Out of 45 *E.coli* (CRE) isolates, 7% were resistant and 93% were susceptible. Among 110 *Klebsiella* carbapenem-resistant isolates, 59% were resistant, 35% were susceptible, and 6% were of intermediate strains. Similar findings were also noted in the study conducted by Joanna Valanie Pereira *et al.,* entitled "Comparison of in vitro fosfomycin susceptibility testing methods with agar dilution for carbapenem-resistant *Klebsiella*," where out of 56 *E.coli* (CRE) isolates, 97% were susceptible and 4% were resistant by agar dilution. Among 177 Carbapenem-resistant *K.pneumoniae*, 68.36% were resistant and 31.63% were susceptible.[10]

In comparison of disk diffusion with agar dilution (taken as the reference), we found good categorical agreement of 90%. We observed a very low rate of very major errors at 1%, along with 7% major errors, and 1% as Minor Errors. Similarly, in the study conducted by Maria Fernanda Mojica *et al.*, they also reported a categorical agreement of over 90%. However, their rates of very major errors were slightly higher at 4%, with 2% minor errors.[1]

In comparison of Vitek 2 with agar dilution among the carbapenem-resistant *E.coli* isolates, 88% showed categorical agreement, with 4% very major errors and 7% minor errors. Among 110 *K.pneumoniae* carbapenem-resistant isolates, 77.27% showed categorical agreement, with 5.45% very major errors, 3% major errors, and 15% minor errors. These findings were comparable with the study done by Ausilia Aprile *et al.*, titled "In vitro fosfomycin study on concordance of susceptibility testing methods against ESBL and carbapenem-resistant Enterobacteriaceae," in which they showed similar findings of 80% categorical

agreement by ESBL-producing *E.coli*, whereas 84% by carbapenemase-producing *K.pneumoniae*.[12]

Our findings with respect to Vitek 2 comparison with agar dilution were also comparable with another study conducted by the author Nilgun Kansak et al., showing that out of 100 *E.coli* and *K.pneumoniae* isolates, there was 100% categorical agreement and 0% very major errors and major errors with *E.coli*, but *Klebsiella* species showed 95.5% categorical agreement with 0% very major errors and 18% major errors. However, in our study, out of 170 urinary *E.coli* isolates, 87% showed categorical agreement with 2% very major errors, and 20% showed minor errors with no major errors. Out of 60 *Klebsiella* species, 73% showed categorical agreement, 7% very major errors, 2% major errors, and 18% minor errors.[13]

Overall susceptibility of fosfomycin in our study among the urinary isolates was 90%, and among non-urinary isolates was 71%. In terms of susceptibility to fosfomycin among non-urinary clinical isolates, our results were comparable with the study conducted by Ethirajulu P *et al.*, where in their study, they also reported fosfomycin susceptibility of 71% among non-urinary isolates.[5]

5. Conclusion

Our study demonstrates that fosfomycin exhibits effective antimicrobial activity against multidrug-resistant Enterobacterales, with overall susceptibility rates of 86.13% (CLSI) and 75.87% (EUCAST) across all organisms tested. Among the susceptibility testing methods, Vitek-2 showed high sensitivity (99%) but lower specificity (76%), while disk diffusion exhibited good categorical agreement (90%), sensitivity (91%), and specificity (93%). Given its feasibility and accuracy, disk diffusion is a reliable method for routine fosfomycin susceptibility testing, particularly in resource-limited settings. These findings support the potential role of fosfomycin as an alternative treatment for multidrug-resistant bacterial infections.

6. Research Quality and Ethics Statement

The authors of this manuscript declare that this scientific work complies with reporting quality, formatting and reproducibility guidelines set forth by the EQUATOR Network. The authors also attest that this study was determined to require the Institutional Ethics Committee review, and the corresponding approval number is JSSMC PG|27|2022-23|Dated 25-05-2022. The authors have not registered this study with the Clinical Trial Registry as it is not applicable.

7. Source of Funding

Self.

8. Conflict of interest

None.

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