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Research Article

Comparison of Diagnostic Tests for Fosfomycin Resistance Detection in Clinically Significant Enterobacterales

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Abstract: Multidrug resistance in Enterobacterales has triggered interest in old antibiotics like fosfomycin. Various methods to determine fosfomycin resistance are time-consuming and yield unpredictable results. Hence, the study was undertaken to compare the rapid fosfomycin NP test with the recommended agar dilution test and few other tests. In this cross-sectional, time-bound, prospective study, Enterobacterales (N = 110) were tested for their susceptibility to commonly used antibiotics by Kirby Bauer disc diffusion method. Susceptibility to fosfomycin was tested by agar dilution method, Vitek 2 system, rapid fosfomycin NP test and gene detection by PCR. The results of different tests were compared with fosfomycin MIC by agar dilution test. Of the 110 Enterobacterales, sixty (54.5%) were resistant to fosfomycin by agar dilution and Vitek-2, of which twenty-three were extended spectrum beta-lactamase producers. The Vitek-2 system showed 100% sensitivity, specificity, positive and negative predictive values when compared with agar dilution method. The accuracy of rapid NP test and PCR for *fos A* gene were 81.8 and 91.1% respectively. *fos A* gene-mediated resistance was found to be common in Enterobacterales. Although the NP test is cost effective and rapid its interpretation is subjective, making it less accurate. Since antimicrobial susceptibility testing is crucial in clinical microbiology laboratories, the NP test cannot be used to detect fosfomycin resistance due to its false-positive nature. The findings of the study prompt us to use agar dilution or Vitek-2 system for detecting fosfomycin resistance in Enterobacterales.

Keywords*:* Fosfomycin, Enterobacterales, Resistance, NP Test, Minimum Inhibitory Concentration (MIC)

Introduction

The occurrence of Multi Drug Resistance (MDR) in the members of family Enterobacterales is a public health concern, which has induced renewed interest in old antibiotics like fosfomycin (Zurfluh *et al*., 2020). As a bactericidal antibiotic, fosfomycin targets an enzyme necessary for peptidoglycan synthesis, UDP N-acetylglucosamine enol pyruvyl transferase (*MurA*). Although Extended Spectrum Beta-lactamase (ESBL) producing Enterobacterales and Carbapenem Resistant Enterobacterales (CRE) are highly susceptibility to fosfomycin, increased resistance rates have been reported over the past decade (Yang *et al*., 2019). Rizvi *et al.* (2024) reports 94% susceptibility to fosfomycin among the *E. coli* isolates from ten Indian states and three union territories.

Fosfomycin resistance in Gram-negative bacilli, can be attributed to several mechanisms, including decreased permeability, mutations in peptidoglycan biosynthesis target (*MurA)* and enzymatic inactivation of fosfomycin due to *fos* genes (Castaneda-García *et al*., 2013). Metalloenzymes of *fos A* type produced by fosfomycin-resistant bacteria, can break down the conjugation of glutathione to fosfomycin, thereby deactivating the antibiotic. Among the fosfomycin inactivating enzymes,

fos A3 is the most common. Acquired resistance due to Fos A-like determinants is common in *E. coli* isolates from Southeast Asian region (Gardiner *et al*., 2019). Other Enterobacterales like *Serratia marcescens* and *Enterobacter cloacae* are known to harbour *fos A1* and *fos A2* genes, respectively (Ito *et al*., 2017).

A significant increase in the infections due to carbapenem-resistant bacteria has necessitated the use of older antibiotics like fosfomycin (Rajesh *et al*., 2021). It is commonly used in the empirical treatment of both community-acquired, and hospital-acquired urinary tract infections. In combination therapy, fosfomycin is used alongside carbapenems, aminoglycosides and tigecycline (Diez-Anguilar and Canton, 2019). Various antibiotic susceptibility testing methods used to distinguish fosfomycin resistance from susceptible strains yield different results, which is a cause of concern. Moreover, standard reference methods like agar dilution are time-consuming, taking around 20 hours, and cumbersome. Other methods, such as disc diffusion tests and E-tests require at least 18 hours to determine susceptibility results. Therefore, there is a need for a rapid test to detect fosfomycin resistance. Hence, we intend to determine susceptibility to fosfomycin using agar dilution method, disc diffusion method, Vitek 2 system, and rapid fosfomycin NP test among Enterobacterales. The results of the NP test were compared with the MIC obtained by agar dilution, Vitek 2 system, and PCR. The findings of this study will assist us in understanding the value of the rapid NP test in routine clinical laboratory screening for fosfomycin resistance.

Materials and Methods

In this cross-sectional study with a duration of six months, pure cultures of Enterobacterales (n = 110) isolated from clinical samples at the department of Microbiology were included using a convenience sampling method. In the study, clinically important Enterobacterales members that were isolated from a variety of clinical samples from both inpatients and outpatients, such as blood (29), urine (43), deep tissue (5), aspirated pus (22), bodily fluids (3), CSF (1), and ET tip (7), were included.

Identification of Bacterial Isolates & Detection of MIC

Bacterial identification was performed using VITEK-2 system. Antibiotic susceptibility to various antibiotics was performed using Kirby-Bauer disc diffusion method and Vitek-2 system, and results were interpreted as per CLSI guidelines (Clinical and Laboratory Standards Institute, 2023). The susceptibility pattern and MIC of isolates for fosfomycin were noted from Vitek reports (Aprile *et al*., 2020). All culture media, chemicals, and reagents used in this research were acquired from HiMedia Laboratories Pvt. Ltd., Mumbai, India, unless stated otherwise.

Screening for Fosfomycin Resistance

Resistance to fosfomycin in Enterobacterales was detected using the Kirby-Bauer disc diffusion method with a 200µg fosfomycin disc containing 50µg of glucose-6-phosphate for *E. coli* isolates, and results were interpreted according to CLSI guidelines (Clinical and Laboratory Standards Institute, 2023). For all the members of Enterobacterales agar dilution method was used to screen for fosfomycin resistance and results were interpreted as per EUCAST guidelines (EUCAST, 2023).

A freshly grown bacterial suspension adjusted to 0.5 McFarland turbidity was diluted 1:10 using 0.9% NaCl, and 2 µl of this suspension was spotted on the Muller Hinton agar plates containing glucose 6 phosphate (25 mg/L) and fosfomycin concentrations ranging from 0.25-256 mg/L. Inoculated plates were incubated at 37◦C for 24 hours. The lowest fosfomycin concentration that completely inhibited the growth was recorded as the Minimum Inhibitory Concentration (MIC). EUCAST breakpoints for oral fosfomycin were extrapolated to assess fosfomycin susceptibility in non-*E. coli* Enterobacterales: MIC ≤8 mg/L or diameter ≤24 mm, susceptible; and MIC >8 mg/L or diameter >24 mm, resistant (EUCAST, 2023). Fosfomycin-sensitive strain of *E. coli* ATCC 25922 was used as a control in all methods. The MIC experiment was repeated twice on each isolate and *E. coli* ATCC 25922 at a given concentrations for internal validation of the result.

Rapid Fosfomycin NP Test: (Nordmann et al., 2019)

For the rapid fosfomycin NP test we used fosfomycin stock solutions and the rapid fosfomycin NP solution.

Preparation of Fosfomycin Stock Solution

To prepare a 50 mg/ml stock solution of fosfomycin, 50 mg of fosfomycin powder (Tokyo Chemical Industry (India) Pvt. Ltd.) was dissolved in 1ml sterile distilled water. The stock solution was stored at 4°C until use (Nordmann *et al*., 2019).

Preparation of NP Solution

To prepare the rapid fosfomycin NP solution, dehydrated powder of cation-adjusted Mueller Hinton broth (2.5%/2.5 g), phenol red indicator (0.005%/0.005 g), D-glucose (1%/1g) were mixed and dissolved in 100ml of distilled water. The pH was adjusted to 7.5, and the resulting NP solution was autoclaved. This NP solution was stored at -20°C. Prior to use, the NP solution was preincubated at 37°C to prevent delayed color change and growth. Before performing the experiment, 8 µl of fosfomycin stock solution and 10 µl of glucose-6-

phosphate were added to 10 ml of NP solution and mixed well. The final fosfomycin concentration obtained in this experiment was 40 µg/ml (Nordmann *et al*., 2019).

Bacterial Inoculum Preparation

Bacterial colonies (2-3) were emulsified in 2 ml of sterile sodium chloride (0.85%) to achieve an optical density equivalent to 3.0-3.5 McFarland standard. *E. coli* ATCC 25922 was used as a fosfomycin-susceptible control. Laboratory-confirmed clinical isolates that were resistant to fosfomycin were used as fosfomycin-resistant controls (Nordmann *et al*., 2019).

Microtiter Plate Inoculation and Interpretation of the Results

A polystyrene 96-well sterile microtiter plate with a round base and lid (Labtech Medico Pvt Ltd. Kerala, India) was used. 50 µl of bacterial suspension (10^8 CFU/ml) were inoculated into two wells. The first well received 150 µl of NP solution and 25 µg/ml glucose-6-phosphate and no fosfomycin. The second well received 150 µl of NP solution, 25 µg/ml glucose-6-phosphate, and 40 µg/ml fosfomycin from the stock solution. A negative control contained 50 µl of NaCl without any bacterial suspension. Each isolate was evaluated in duplicate as described above. The microtiter plate was incubated at 37°C, and visual inspections were performed every 30 minutes for up to 2 hours to observe any color change.

A color change from red to yellow was interpreted as fosfomycin-resistant (positive), indicating that the bacterial isolate grew in the presence of fosfomycin and metabolized glucose. The result of the test was interpreted as susceptible to fosfomycin (negative) when color of the wells remained same or turned slightly orange, indicating that the bacterium failed to grow and metabolize glucose in the presence of fosfomycin (Nordmann *et al*., 2019). To minimize misinterpretation and bias from observers, the colour changes in the NP test were assessed independently by three qualified researchers who were blinded.

Detection of Fosfomycin Resistance Genes by Polymerase Chain Reaction

DNA Extraction: Four colonies of the isolate were emulsified in 100 µl of nuclease-free water, heated for 15 minutes in a dry bath (GeneI, Bangalore GeneI Pvt. Ltd, India) at 95°C, then centrifuged at 12,000 RPM for 10 minutes. One microliter of the supernatant was used as DNA template for PCR. Purity and quantity of the extracted DNA was checked using Nanodrop spectrophotometer (Epoch BioTek, USA). The primers used in the PCR for the detection *fos A, fos A3* and *murA* are shown in Table 1.

Twenty microliter PCR reaction mixture consisted of 10 µl ready-to-use master mix (Origin Diagnostics and Research, Kerala, India), 1 µl of each primer (10 pmol/µl), 1 µl of DNA template and 7 µl of Nuclease-Free Water (NFW). PCR tubes with master mix and template were loaded into a thermocycler (ProFlex^{TM} Base, Thermo Fisher Scientific, Life Technologies Holdings, Pvt. Ltd. Singapore). Master mix with nuclease free water was used as negative control. The conditions for amplifying the *fos A* gene were: Initial denaturation for 5 mins at 95°C, followed by 30 cycles at 95°C for 45 secs, 58°C for 45 secs & 72°C for 10 mins (Loras *et al*., 2021). The annealing temperature for *fos A3* gene was 52.9°C (White *et al.*, 2017). The PCR conditions for amplifying the *murA* gene were: 2 mins of denaturation at 94°C, 30 cycles at 94°C for 30 secs, 55°C for 30 secs and extension for 2 mins at 72°C (Takahata *et al*., 2010).

Agarose gel electrophoresis: Amplified products were mixed with 6X DNA loading dye (Thermo scientific, Lithuania) and separated using 1.5% agarose gel. Electrophoresis was carried out at 120 volts for 45 mins. 3 µl of 100 bp plus molecular marker (Origin Diagnostics and Research, Kerala, India) was used. The gel was stained with ethidium bromide solution for 15 minutes and observed under a UV transilluminator (Geldoc Go, Bio-Rad laboratories, India, Pvt. Ltd.) and photographed (Liu *et al*., 2020).

Data Analysis

The results obtained from Rapid NP test, PCR, and disc diffusion method were compared with agar dilution &Vitek 2 system. Results were summarized as frequency tables and percentages were worked out. Positive Predictive Agreement (PPA), Negative Predictive Agreement (NPA), Positive Predictive Value (PPV), Negative Predictive Value (NPV) and 95% confidence interval for PPA and NPA was used to analyze diagnostic accuracy of Rapid NP test and PCR in comparison with the agar dilution method (gold standard) Statistical package Jamovi version 2.3.28.0 was used for the statistical analysis of the results.

Table 1: Primers used for the detection of fosfomycin resistance genes by polymerase chain reaction

Gene	Name of the primer	Sequence 5' to 3'	Size of the product	References
fos A	Fos A-F	ATC TGTGGGTCTGCCTGTCGT	271 bp	Lu *et al*., 2016
	Fos A-R	ATG CCCGCATAGGGCTTCT		
fos A3	Fos A3-F	GCGTCAAGCCTGGCATTT	282 bp	White *et al*., 2017
	Fos A3-R	GCCGTCAGGGTCGAGAAA		
mur A	Mur A-F	AAACAGCAGACGGTCTATGG	1541 bp	Takahata *et al.*, 2010
	Mur A-R	CCATGAGTTTATCGACAGAACG		

Results

A total of 110 Enterobacterales isolated from various clinical samples, like blood (n = 29), urine (n = 43), deep tissue (n = 5), aspirated pus (n = 22), body fluids (n = 3), CSF (n = 1) and ET tip (n = 7). These isolates were tested for antibiotic susceptibility to various antibiotics using Kirby-Bauer disc diffusion method, agar dilution method and Vitek-2 system. Of the 110 Enterobacterales 70 (63.6%) were from male patients and 40 (36.4%) were from female patients. The bacterial isolates consisted of *E. coli* (38.2%), *K. pneumoniae* (56.4%), *K.oxytoca* (0.9%), *Morganella morganii* (0.9%), *Enterobacter aerogenes* (1.8%), *Citrobacter freundii* (0.9%), *Salmonella* Typhi (0.9%).

Antibiotic Susceptibility Pattern

The antibiotic susceptibility patterns of Enterobacterales isolated from different clinical samples performed by disc diffusion method are presented in Tables 2 to 4**.** As per CLSI and EUCAST guidelines agar dilution method is the reliable method for detecting fosfomycin susceptibility. Hence, agar dilution method was used as gold standard for comparing the results of Rapid NP test and PCR. The results of fosfomycin susceptibility obtained by disc diffusion, Vitek-2 system and agar dilution were concordant for *E. coli* isolates. For Enterobacterales other than *E. coli*, the results of agar dilution were found to be concordant with only Vitek-2 system. It was observed that 50 (45.45%) isolates were fosfomycin-resistant (MIC >8 mg/L) and 60 (54.55%) were susceptible (MIC <8 mg/L). The rapid NP test was performed to determine fosfomycin susceptibility for all the 110 isolates and the results were compared with agar dilution method. The results of the Rapid NP test are shown in Table 5 and Figure 1.

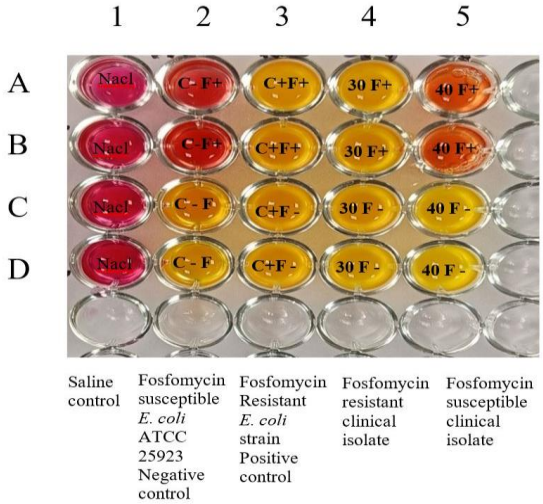

Fig. 1: Representative picture of rapid NP test that uses a microtiter plate to detect fosfomycin susceptibility. A positive NP test (Fosfomycin resistant) is shown by a yellow colour. A negative NP test (fosfomycin sensitive) is shown by a red colour

Caption for Figure 1 & Abbreviations:
Nacl: Sodium chloride; **C-:** Negative control; **C+**: Positive control; **F-**: Without fosfomycin; **F+**: With fosfomycin; **30, 40:** *E. coli* isolates from clinical samples
Column 1: Saline control without bacterial suspension - colour of the wells remains red.
Rows A2 to A5: Bacterial suspension with rapid fosfomycin NP solution supplemented with fosfomycin (F+); inoculated with negative control (C-) *E.oli* ATCC 25922, positive control (C+) laboratory confirmed fosfomycin resistant *E. coli* strain, *E. coli* isolate from clinical sample (30) resistant to fosfomycin, *E. coli* isolate from clinical sample (40) susceptible to fosfomycin.
Row B2 to B5: Replicate of A2 to A5.
Row C2 to C5: Bacterial suspension with rapid fosfomycin NP solution without fosfomycin (F-). Negative control (C-) *E.oli* ATCC 25922, Positive control (C+) laboratory confirmed fosfomycin resistant *E. coli* strain, *E. coli* isolate from clinical sample (30) resistant to fosfomycin, *E. coli* isolate from clinical sample (40) susceptible to fosfomycin.
Row D2 to D5: Replicate of C2 to C5.

Detection of Fosfomycin Resistant Genes (fos A, fos A3, murA) by PCR

All the 110 isolates were subjected to PCR for the detection of *fos A, fos A3, mur A* genes. 20 isolates were positive for *fos A* gene and negative for other genes tested. These 20 isolates were resistant to fosfomycin by agar dilution method, Vitek 2 system (>8 mg/L), and rapid NP test. The remaining 90 isolates were negative for all the genes tested. A representative agarose gel picture of the PCR for the *fos A* gene is shown in Figure 2. A comparison of the results obtained by agar dilution method, NP test and PCR is presented in Table 5 and 6. The positive and the negative predictive agreements and their 95% confidence intervals are shown in Table 7.

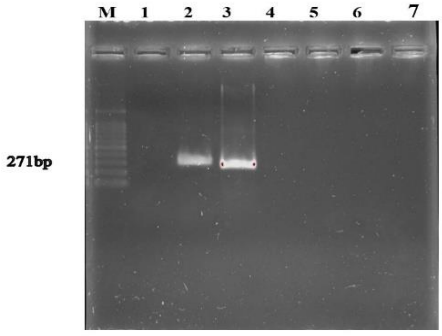

Fig. 2: A representative agarose gel picture showing the amplification product of PCR performed on Enterobacterales for the detection of *fos A* gene

Lanes:
M: Molecular marker (100bp)
1: Negative control (NC)
2: Known positive control- *E. coli* containing *fos A* gene
3: *Klebsiella* spp. positive for *fos A* gene

Table 2: The antibiotic susceptibility pattern of Enterobacterales members isolated from urine samples by disc diffusion test

Antibiotics tested	Enterobacterales from Urine (n = 43)								
	E. coli (n = 18)			*K. pneumoniae* (n = 24)			*K.oxytoca* (n = 1)		
	R	I	S	R	I	S	R	I	S
Ampicillin	13	-	5	24	-	-	1	-	-
Piperacillin+tazobactum	4	-	14	20	-	4	-	-	1
Ticarcillin	11	-	7	24	-	-	1	-	-
Cefixime	12	-	6	21	-	3	-	-	1
Cefoperazone+sulbactum	-	1	17	16	-	8	-	-	1
Ceftazidime	7	-	11	21	-	3	-	-	1
Ceftriaxome	8	-	10	21	-	3	-	-	1
Imipenem	-	-	18	13	1	10	-	-	1
Meropenem	-	-	18	13	1	10	-	-	1
Ciprofloxacin	13	-	5	22	-	2	-	-	1
Ofloxacin	11	-	7	22	-	2	-	-	1
Norfloxacin	11	-	7	21	1	2	-	-	1
Nalidixic acid	13	-	5	22	-	2	-	-	1
Amikacin	1	-	17	11	-	13	-	-	1
Gentamicin	2	-	16	12	-	12	-	-	1
Fosfomycin	-	-	18	ND			ND		
Nitrofurantoin	1	2	15	23	-	1	-	1	-
Trimethoprim/sulphamethoxazole	12	-	6	16	-	8	-	-	1

S: susceptible, I: Intermediate R: Resistant
ND: not done as disc diffusion test for Fosfomycin is recommended only for *E. coli*

Table 3: The antibiotic susceptibility pattern of Enterobacterales members isolated from Exudate samples by disc diffusion test

Antibiotics tested	Enterobacterales from exudate samples (n = 38)														
	E. coli (n = 11)			*K. pneumoniae* (n = 23)			*M. morganii* (n = 1)			*E.aerogenes* (n = 2)			*C. freundii* (n = 1)		
	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S
Amoxycillin/ clavulanic acid	7	1	3	8	-	15	-	-	1	-	-	2	-	-	1
Piperacillin+tazobactum	7	-	4	11	-	12	-	-	1	-	-	2	1	-	-
Cefepime	7	-	4	13	-	10	-	-	1	-	-	2	1	-	-
Ceftriaxone	9	-	2	15	-	8	-	-	1	1	-	1	1	-	-
Cefoperazone+sulbactum	3	1	7	9	-	14	-	-	1	-	-	2	1	-	-
Ciprofloxacin	9	1	1	13	2	8	-	-	1	-	-	2	1	-	-
Ertapenem	2	-	9	12	-	11	-	-	1	-	-	2	1	-	-
Imipenem	2	-	9	11	1	11	1	-	-	-	-	2	1	-	-
Meropenem	2	-	9	11	-	12	-	-	1	-	-	2	1	-	-
Amikacin	-	-	11	-	8	15	-	-	1	-	-	2	-	1	-
Gentamicin	1	-	10	7	1	15	-	-	1	-	-	2	1	-	
Tigecycline	11	-	-	4	-	19	-	-	1	-	-	2	-	-	1
Fosfomycin	-	-	11	ND			ND			ND			ND		
Trimethoprim/ sulphamethoxazole	7	-	4	8	-	15	-	-	1	-	-	2	-	-	1

S: susceptible, I: Intermediate R: Resistant
ND: not done as disc diffusion test for Fosfomycin is recommended only for *E. coli*

Table 4: The antibiotic susceptibility pattern of Enterobacterales members isolated from Blood samples by disc diffusion test

Antibiotics tested	Enterobacterales from blood (n = 29)								
	E. coli (n = 13)			*K. pneumoniae* (n = 15)			*S.* Typhi (n = 1)		
	R	I	S	R	I	S	R	I	S
Amoxycillin/clavulanic acid	7	1	5	9	-	6	1	-	-
Piperacillin	10	-	3	9	-	6	-	-	1
Piperacillin+tazobactum	8	-	5	9	-	6	-	-	1
Cefepime	12	-	1	9	-	6	-	-	1
Cefuroxime	13	-	-	11	-	4	1	-	-
Ceftriaxone	12	-	1	11	-	4	-	-	1
Cefoperazone+sulbactum	5	-	8	9	-	6	-	-	1
Ertapenem	5	-	8	9	-	6	-	-	1
Imipenem	5	-	8	8	-	7	-	-	1
Meropenem	5	-	8	8	-	7	-	-	1
Ciprofloxacin	10	1	2	10	2	3	-	1	-

Table 4: Continued

Amikacin	1	-	12	7	-	8	-	-	1
Gentamicin	4	-	9	7	-	8	-	-	1
Tigecycline	-	-	13	-	-	15	-	-	1
Fosfomycin	1	-	12	ND			ND		
Trimethoprim/sulphamethoxazole	8	-	5	9	-	6	-	-	1

S: Susceptible, I: Intermediate R: Resistant
ND: Not Done as disc diffusion test for Fosfomycin is recommended only for *E. coli*

Table 5: Fosfomycin susceptibility pattern of Enterobacterales obtained by agar dilution test /vitek-2 system, rapid NP test and PCR for *fos A* gene

Sample type	Bacterial Isolates (n)	Agar dilution / vitek-2		NP test		PCR	
		Resistant n (%)	Susceptible n (%)	Resistant n (%)	Susceptible n (%)	*fos A* Positive n (%)	*fos A* Negative n (%)
Urine (n = 43)	*E. coli* (18)	0	18 (100)	4 (22.2)	14 (77.7)	0	18 (100)
	K. pneumoniae (24)	24 (100)	0	21 (87.5)	3 (12.5)	5 (20.8)	19 (79.1)
	K.oxytoca (1)	0	1 (100)	0	1 (100)	0	1 (100)
Exudate (n = 38)	*E. coli* (11)	0	11 (100)	0	11 (100)	0	11 (100)
	K. pneumoniae (23)	15 (65.2)	8 (34.8)	18 (78.3)	5 (21.7)	12 (52.2)	11 (47.9)
	M. morganii (1)	0	1 (100)	1 (100)	0	0	1 (100)
	E.aerogenes (2)	2 (100)	0	2 (100)	0	1 (50)	1 (50)
	C. freundii (1)	1 (100)	0	1 (100)	0	1 (100)	0
Blood (n = 29)	*E. coli* (13)	1 (7.7)	12 (92.3)	1 (7.7)	12 (92.3)	0	13 (100)
	K. pneumoniae (15)	8 (53.3)	7 (46.7)	9 (60)	6 (40)	1 (6.7)	14 (93.3)
	*S.*Typhi (1)	0	1 (100)	0	1 (100)	0	1 (100)

Table 6: Comparison of the results of agar dilution test, rapid NP test and PCR for the detection of fosfomycin susceptibility of Enterobacterales isolated from different clinical samples

Samples tested	Members of Enterobacterales tested (n = 110)					
	Agar dilution/Vitek2 method		Fosfomycin NP test		Polymerase chain reaction (PCR)	
	Resistant n (%)	Susceptible n (%)	Colour change red to Yellow (Resistant)	No colour change remains red (Susceptible)	Fos A positive	Fos A negative
Urine (n = 43)	24 (55.9)	19 (44.2)	25 (58.1)	18 (41.9)	05 (11.6)	38 (88.4)
Exudate (n = 38)	18 (47.4)	20 (52.6)	22 (57.9)	16 (42.1)	14 (36.8)	24 (63.2)
Blood (n = 29)	09 (31)	20 (69)	10 (34.5)	19 (65.5)	01 (3.4)	28 (96.6)
Total (n = 110)	51 (46.4)	59 (53.6)	57 (51.8)	53 (48.2)	20 (18.2)	90 (81.8)

Table 7: Comparison of results obtained by rapid NP test with minimum inhibitory concentrations (MIC) of Fosfomycin obtained by Vitek 2 system, agar dilution method and detection of *fos A* gene by PCR

Tests performed	Positive predictive agreement (PPA)	Negative predictive agreement (NPA)	Positive Predictive Value (PPV)	Negative Predictive Value (NPV)	95%Confidence Interval for PPA	95%Confidence Interval for NPA
NP test	92.5%	84.5%	82%	93.8%	(72.0%, 98.0%)	(56.3%, 94.3%)
Vitek	100%	100%	100%	100%	(100%, 100%)	(100%, 100%)
Agar dilution	100%	100%	100%	100%	(100%, 100%)	(100%, 100%)
PCR for *fos A* gene	58.8%	92.3%	90.9%	63.2%	(17.9%, 44.6%)	(81.6%, 97.2%)

Discussion

Fosfomycin resistance arises from several mechanisms, including modification of the drug target *murA*, reduced penetrability to fosfomycin, and acquisition of fosfomycin resistance genes. However, the most common mechanism reported in the literature is the reduction of fosfomycin permeability (Mattioni Marchetti *et al*., 2023). Since, a single method cannot detect all types of fosfomycin resistance, we used four different methods to detect fosfomycin resistance in Enterobacterales. On the other hand, disc diffusion and Vitek-2 were employed to detect susceptibility to antibiotics other than fosfomycin.

Among the 43 urinary isolates, 24 *K. pneumoniae* (55.8%) were resistant to fosfomycin. All *E. coli* isolates from urine sample were susceptible to fosfomycin by disc diffusion, agar dilution and Vitek 2 system (Table 2). However, *E. coli* isolates from urine sample showed high (72.2%), resistance to ampicillin, nalidixic acid and ciprofloxacin followed by 66.7% resistance to trimethoprim/sulfamethoxazole and cefexime. In contrast,

K. pneumoniae from urine samples was 100% resistant to ampicillin, ticarcillin and fosfomycin followed by nitrofurantoin (95.8%). In exudate samples, 100% of *E. coli* isolates were resistant to tigecycline, followed by ciprofloxacin (81.8%). Among the *K. pneumoniae* isolates, the highest resistance rates (65.2%), were observed for fosfomycin and ceftriaxone followed by 56.5% to ciprofloxacin and cefepime as shown in Table 3. *E. coli* isolates from blood showed highest resistance (100%) to cefuroxime followed by 92.3% resistance to cefepime and ceftriaxone. *K. pneumoniae* isolates from blood exhibited 73.3% resistance to cefuroxime and ceftriaxone, followed by 66.7% to ciprofloxacin (Table 4). These susceptibility patterns of commonly used antibiotics were consistent with those reported by earlier workers (Baby *et al*., 2020; Sofia *et al*., 2019). Among the 38 exudate isolates and 29 blood isolates, 18 (47.4%) & 9 (31%) were resistant to fosfomycin, as determined by the Vitek 2 system and agar dilution method.

E. coli isolated from a urine samples in this study demonstrated 100% fosfomycin sensitivity, which is in line with findings of Tutone *et al.* (2022). A South Indian study in 2020, reported 84% of *E. coli* isolated from urine samples to be susceptible and 16% to be resistant to fosfomycin by disc diffusion method (Baby *et al*., 2020). Another Indian study reported the susceptibility rates of fosfomycin for *E. coli, K. pneumoniae, Enterobacter* spp., *Proteus* spp., and *Citrobacter* spp., as 95.5, 53.2, 71.5, 76.7, and 91.1%, respectively by using only agar dilution method (Rajesh *et al*., 2021). A study from Egypt reported 38.5% fosfomycin resistance among MDR E. coli isolated from urine sample of children (Abdelraheem et al., 2023). In our study 53.6% of Enterobacterales were susceptible to fosfomycin which included isolates from urine, blood and exudate samples (Table 5). Thus, results of our study and previous research from India and abroad indicate that the pattern of antibiotic susceptibility varies by geographic location and is dependent on the antibiotics used for empirical treatment.

Our study showed 100% agreement between the results of Vitek-2 system, agar dilution method, and disc diffusion method using fosfomycin 200µg disc containing 50 µg glucose-6-phosphate for *E. coli* isolates. Aprile *et al.,* reported concordant results for fosfomycin MICs for ESBL-producing and MDR *E. coli* using agar dilution, gradient tests, and automated Vitek -2 system. However, their study emphasizes the use of gradient tests and agar dilution test for MDR *Klebsiella,* to determine fosfomycin susceptibility (Aprile *et al.,* 2020; Clinical and Laboratory Standards Institute, 2023). We observed that the Vitek-2 system and agar dilution methods can detect fosfomycin resistance in similar manner for all the Enterobacterales. Moreover, EUCAST recommends using the agar dilution method and extrapolating breakpoints of *E. coli* to other Enterobacterales (EUCAST, 2023). In contrast, Massip *et al.* (2024) compared results of Vitek and agar dilution methods and reported higher MIC values with Vitek, indicating false resistance. Hence, there is need for more Enterobacterales to be tested by these methods form different parts of the globe.

The Rapid Fosfomycin NP test revealed that *E. coli* isolates from urine and blood samples were resistant to fosfomycin in 22.2 and 7.7% of cases, respectively. Whereas 87.5% of *K. pneumoniae* from urine samples, 78.3% from exudate and 60% from blood samples were resistant to fosfomycin by NP test (Table 5). The results and interpretation of the NP test are shown in Figure 1. Overall, the resistance exhibited by Enterobacterales was higher by NP test (51.8%) compared to the agar dilution and vitek-2 system (46.4%). Resistance to Fosfomycin was highest (55.8%) in urinary isolates other than *E. coli*, followed by exudates (47.4%) and blood isolates (31%). Notably, one *E. coli* isolate from blood was resistant to fosfomycin by agar dilution (MIC >8mg/L) and the rapid NP test but tested negative for the genes assessed by PCR (Table 5). The negative PCR result could be due to the existence of other non-*fos* gene resistance mechanisms, alternation in the expression of transporter proteins or distinct *fos* genes not targeted in this investigation (Mattioni Marchetti *et al*., 2023).

All *E. coli* isolated from urine samples were susceptible to fosfomycin by the Vitek 2 system and agar dilution methods and did not harbour the tested genes. However, four *E. coli* isolates from urine sample were falsely resistant to fosfomycin (false positive) by the NP test (Table 5). Of the 23 *Klebsiella* isolates from exudate samples, 15 were resistant to fosfomycin by agar dilution/Vitek-2 system, 18 by NP test and 12 were positive for the *fos* A gene by PCR. A comparison of the results obtained by different tests is shown in Table 6. The higher fosfomycin resistance rate observed with the NP test could be due to the reduced expression of transporters, presence of acquired fosfomycin-resistance genes or mutations in chromosomal genes similar to *fos A* in those species (Mattioni Marchetti *et al*., 2023). As reported by earlier studies, the NP test does not differentiate between chromosomally mediated and acquired types of fosfomycin resistance, which will lead to false positives (Elliott *et al*., 2019). In the present study, we too found false-positive results with the rapid NP test (Table 6).

Eleven isolates that were fosfomycin-susceptible by Vitek and agar dilution methods were resistant by NP test (false positives). Isolates showing false-positive results by NP test from urine and exudates had same MIC as that of true positives (MIC<16 & >8mg/L). However, two blood isolates showing false-positive results in NP test had an MIC of 32mg/L, which is higher than that of true positives (MIC<16 & >8mg/L). Therefore, the decrease in Positive Predictive Agreement (PPA) and negative predictive agreement (NPA) of the NP test might be due to the

difference in MIC, as reported by Yunus *et al.* (2021). Nevertheless, the NP test is a rapid, cost effective, easy, and handy technique which utilizes carbohydrate hydrolysis to detect bacterial growth in the presence of a specified fosfomycin concentration, and it detects fosfomycin resistance in *E. coli* due to various molecular mechanisms (Nordmann *et al*., 2019). Additional biochemical research can be conducted to investigate the many mechanisms underlying the variation in NP test findings, such as difference in microbial metabolism in non- *E. coli* Enterobacterales, the impact of environmental conditions or hidden mechanisms if any.

In our study 54 of the Enterobacterales [25 *E. coli* & 29 *K. pneumoniae*] were ESBL producers, of which 31 isolates [25 *E. coli* & 06 *K. pneumoniae*] were susceptible to fosfomycin. A study from six European countries reported that 97-99% of ESBL-producing *E. coli* were susceptible to fosfomycin (Sofia *et al*., 2019). In our study, 100% of ESBL-producing *E. coli* and 21% of *K. pneumoniae* were susceptible to fosfomycin. According to an Indian study from Rajasthan, uropathogenic *E. coli* that produces ESBL exhibits 50% fosfomycin resistance (Jain *et al*., 2022). These results demonstrate that antibiotic resistance rates vary geographically and depend on the antibiotics used in healthcare settings for treatment.Detection of fosfomycin-resistant genes *fos A, fos A3* and *murA* was performed using uniplex PCR. The PCR results are shown in Figure 2. Twenty fosfomycin-resistant isolates including 18 *K. pneumoniae* and one each of *E. aerogenes* and *C. freundii* were positive for the fos A gene (Table 5). One fosfomycin-resistant *E. coli* isolate from blood was negative for *fos A gene.* Moreover, all 110 isolates were negative for *fos A3* and *murA* genes. An earlier study by Castaneda-Garcia *et al.* (2013), reported that *murA*, is a very infrequent mechanism in fosfomycin-resistant clinical isolates. A study by Zurfluh *et al.* (2020), reported that *fos A* has a high prevalence in Gram negative species. However, only 20 out of the 52 Enterobacterales that were resistant to fosfomycin in our study showed positive PCR results for the *fos A* gene. Possible explanations for the fosfomycin resistance and absence of *fos A* gene seen in our isolates include altered target proteins, decreased expression of transporters including the glycerol-3-phosphate transporter (GlpT), the glucose-6-phosphate transporter (UhpT) and the adenylate cyclase (CyaA) or mutations in the *fos A* and *mur* A genes (Loras *et al*., 2021; Mattioni Marchetti *et al*., 2023). Hence, a negative PCR result for *fos A* or *mur A* gene, does not always indicate the absence of resistance. It could be due to either the existence of other non-*fos* gene resistance mechanisms, alternation in the expression of transporter proteins or distinct *fos* genes not targeted in this investigation.

Earlier researchers have also reported limitations of molecular tests in detecting fosfomycin resistance, as many known resistance mechanisms, such as loss of active transporters and mutations in *murA*, cannot be detected by PCR (Castenda- Garcia *et al*., 2013). Previous research has shown that *fos A* poses a challenge to susceptibility testing of carbapenem-resistant *Klebsiella* species using readily available methods in the clinical microbiology laboratories (Elliott *et al*., 2019; Massip *et al*., 2024). In our study, the PPA and NPA of PCR were 58.8 and 92.3%, respectively (Table 7). Further exploration of additional variants of the *fos* and *murA* genes, along with efflux pumps linked to fosfomycin resistance is necessary, which highlights one of the limitations of the current research.We found that NP test had PPA and NPA of 92.5% & 84.5%, respectively (Table 7). Earlier studies have used the NP test to detect fosfomycin resistance in *E. coli* isolated from urine samples and reported sensitivity and specificity of 92% & 98%, respectively (Elliott *et al*., 2019). In the present study agar dilution and Vitek-2 methods showed 100% concordant results. Diagnostic accuracy, positive predictive value and negative predictive value of different methods are shown in Table 7. Therefore, automated methos like Vitek-2 or agar dilution are suitable for detecting fosfomycin resistance in routine clinical microbiology laboratories. When NP test results are falsely positive, clinician may avoid fosfomycin needlessly, postpone necessary treatment, or use less effective antibiotics, which can aggravate illness or cause drug resistance. Thus, if NP test is used for screening of fosfomycin resistance the results should be confirmed by other reference methods. The rapid NP test is subjective and relies on observer interpretation bias. The positive predictive accuracy and negative predictive accuracy of the NP test observed in this study were lower than those reported in previous studies (Nordmann *et al*., 2019; Yunus *et al*., 2021). These studies have used only *E. coli* isolates in NP Test and we have used all Enterobacterales. Most of the Indian studies till date have used agar dilution, broth dilution or Kirby Bauer Disc diffusion methods for studying fosfomycin susceptibility and not the NP test (Rajesh *et al.*, 2021; Kalai *et al*., 2023). Hence, there are no Indian studies to compare our NP test results. The cost of detecting multiple genes is a constraint for routine use of PCR in diagnostic microbiology laboratories. Testing of all the variants of the *fos A* and *murA* genes, resistance mechanisms such as *murA* mutations or efflux pumps, needs to be explored in future studies, which can also be considered as limitation of this study.

Conclusion

This study aimed to determine the fosfomycin susceptibility pattern in clinical isolates of

Enterobacterales using the NP test and PCR, comparing the results with agar dilution and Vitek-2. We observed some false-positive results (n = 11) with the NP test, despite its cost-effectiveness and rapidity. Given the importance of antimicrobial susceptibility testing in clinical microbiology laboratories, the NP test alone is not suitable for detecting fosfomycin resistance. Our findings suggest the use of vitek-2 system for detecting fosfomycin resistance in Enterobacterales. In resource-limited settings phenotypic methods such as disc diffusion and agar dilution can be used, although they are time-consuming. Further studies are necessary to determine the optimal conditions for the expression and detection of different genes responsible for fosfomycin resistance. Whole-Genome Sequencing (WGS) and bioinformatics approaches may provide better insights into resistance mechanisms. Future studies will also elucidate the metabolic mechanisms behind the false-positive results of the NP test.

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Authors Contributions

Santoshi Maruti Naik: Investigation, formal analysis, writing original draft, validation, data collection, conduct of experimental work, formal analysis and interpretation.

Biranthabail Dhanashree: Conceptualization, supervision, methodology, validation, writing- reviewed and edited.

Himani Kotian: Formal analysis application of statistical techniques, Data Curation, Visualization.

All authors read and approved the final version of the manuscript.

Ethics

Institutional ethics committee (IEC KMC MLR 06/2022/270) Kasturba Medical College Mangalore has approved this study.

Competing Interest

The authors declare no competing interests.

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