ORIGINAL ARTICLE

DIAGNOSTIC ACCURACY OF RAPID FOSFOMYCIN NP TEST FOR DETECTION OF FOSFOMYCIN RESISTANCE IN *ESCHERICHIA COLI* IN A TERTIARY CARE HOSPITAL IN PAKISTAN

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Background: Emergence of resistance among *Escherichia coli (E.coli*) isolates against therapeutic options for UTIs (Urinary tract infections) has led to renewed interest in older antibiotics like Fosfomycin. In this study we evaluated diagnostic accuracy of Rapid Fosfomycin NP test based on glucose metabolism for rapid Fosfomycin susceptibility testing among urinary *E.coli* isolates. **Methods:** In a cross-sectional validation study conducted in the Microbiology Department, Armed Forces Institute of Pathology, Rawalpindi, Pakistan from 15th March to 15th September 2020, 149 consecutive urine specimens were included as per selection criteria. Rapid Fosfomycin NP test was performed as per protocol of Nordmann P *et al* on urinary *E.coli* isolates for detection of Fosfomycin resistance and results were compared with reference modified Kirby-Bauer disk diffusion method. **Results:** Out of total 149 *E.coli* isolates from 149 urine specimens, 80 were classified as Fosfomycin susceptible and 69 as Fosfomycin resistant by reference disk diffusion method. Sensitivity, specificity, positive predictive value, negative predictive value and diagnostic accuracy of rapid Fosfomycin NP test was found to be 94.2%, 98.75%, 98.48%, 95.2% and 96.64%, respectively. In our study reliable results were achieved after 2.5 hours of incubation. **Conclusion:** The rapid Fosfomycin NP test is valid and user-friendly technique which can be performed with minimal technical expertise. It is less time consuming than disk diffusion and E-test strips and easy to perform as compared to agar dilution method. It can be useful as alternative to agar dilution in urinary *E.coli* isolates which would help in selecting appropriate therapeutic option for UTIs.

Keywords: Urinary tract infections (UTIs); Rapid Fosfomycin Nordmann Poirel (NP) test; *Escherichia coli (E.coli*)

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INTRODUCTION

Urinary tract infections (UTIs) represent most important microbial infections which contribute to considerable morbidity in both out-patient and in-patient settings influencing around 150 million people globally every year. UTIs occur in both genders but are more frequent in females.[1,2] Consequently, due to its high prevalence from community-based origins along with hospital acquired infections, this group signifies a substantial burden to the health care systems internationally causing 100,000 hospitalization annually.[3–5]

Escherichia coli (E.coli) is the bacterial pathogen predominantly responsible for UTI in both indoor and outdoor patients, causal agent of more than 80 percent of all community acquired infections.[3,6] It displays an augmented resistance rate to broad-spectrum beta-lactam antibiotics by virtue of extended-spectrum β-lactamases (ESBLs). These ESBL producing *E-coli* frequently develop resistance against other antimicrobials which are considered as significant therapeutic options in UTIs including fluoroquinolones, aminoglycosides and co-trimoxazole. An incoherent use of antibiotics in our setup has enormously endowed to the antimicrobial resistance and development of multidrug-resistant urinary isolates.[7]

It led to renewed interest in older antibiotics like Fosfomycin, a phosphonic acid derivative, first discovered in 1969 which is often found susceptible even in ESBL producing isolates.[7–10] Fosfomycin owing to its bactericidal nature, broad-spectrum activity and less toxicity is being used in several countries for more than 20 years and it is generally preferred as a first-line antibiotic for treating uncomplicated UTIs by various treatment guidelines.[8–11]

The reference technique for susceptibility testing of Fosfomycin is agar dilution method that is a laborious method requiring 16-20 hours to be executed. Other methods like disk diffusion and E-test strips can be performed, but result interpretation requires at least 16–18 hours. E-test is not considered

as reference method and disk zones are different in European Committee on Antimicrobial Susceptibility Testing (EUCAST) and Clinical Laboratory Standard Institute (CLSI). Molecular methods have problems such as cost and availability, moreover they cannot detect all resistant mechanisms precisely.[9,11–15]There is a need to establish a rapid test for Fosfomycin susceptibility testing.

The first NP test was developed by Nordmann *et al* in September 2012, to rapidly identify carbapenemase producers in *Enterobacterales* and was named "The Carba NP test".[16] Later in June 2016, "Rapid Polymyxin NP test" was developed for identification of polymyxin resistance in *Enterobacterales*.[17] A practical and rapid test "Rapid Fosfomycin NP" based on glucose metabolism has been developed by Nordmann *et al* in January 2019 for fosfomycin susceptibility testing among *E.coli* isolates. The sensitivity and specificity of the method were found to be 100% and 98.7% respectively according to this study and the results were interpreted within 1 hour and 30 minutes of incubation.[9]The result for this test is available at least 16 hours earlier as compared with the reference agar dilution method.

After literature search it was found that no such study has been performed to date in Pakistan. In this study we evaluated diagnostic accuracy of rapid Fosfomycin NP test for Fosfomycin susceptibility testing in our setup among clinical isolates of *E.coli* from urinary specimens keeping modified Kirby-Bauer disk diffusion method as reference standard. This can help timely and reliable treatment of urinary tract infections.

MATERIAL AND METHODS

This Cross-sectional Validation study was conducted in the Microbiology Department, Armed Forces Institute of Pathology, Rawalpindi, Pakistan from 15th March to 15th September 2020. The study was approved by Institutional Review Board and Ethical Committee. *E-coli* isolates from urinary specimens were included in the study by non-probability consecutive sampling from both indoor and outdoor patients. Clinical presentation and urine routine examination findings of all patients was collected. Repeat specimens from same patient were excluded from study. Specimens with less than 5 WBCs/ High power field and specimens from asymptomatic patients were also excluded from study. All urinary specimens included in the study were inoculated as per standard microbiological methods. Isolates were confirmed by colony morphology, traditional biochemical tests, API 20E (Biomerieux, France) and VITEK-2 system (Biomerieux, France).Antimicrobial susceptibility of all recommended antimicrobials other than fosfomycin was performed as per CLSI guidelines to determine the frequency of multidrug resistant (MDR) and extensively drug resistant (XDR) isolates.[14,15] On the basis of susceptibility the isolates were classified as MDR (resistant to at least one drug in 3 or more classes of antibiotics), XDR (resistant to at least one drug in all but 2 or less antimicrobial classes).

Fosfomycin susceptibility was performed by modified Kirby-Bauer disk diffusion method on Mueller Hinton agar using Fosfomycin trometamol disk 200µg containing 50 µg glucose-6-phosphate. It was considered as reference method in our study. Since there is discrepancy in breakpoint values of *E-coli* isolates for Fosfomycin 200µg disk between CLSI[14] (susceptible: ≥16 mm, intermediate: 13-15mm, resistant: ≤12 mm) and EUCAST[15], results were interpreted using EUCAST 2020 disk diffusion cut-offs for *E-coli* in urinary tract isolates, that is, ≥24mm as susceptible and < 24 mm as resistant. *E. coli* ATCC 25922 strain was used for quality control.

The rapid Fosfomycin NP test needs two solutions, that is; rapid Fosfomycin NP solution (Solution 1) and the stock solution of Fosfomycin (Solution 2). Preparation of these solutions was performed as per Nordmann P *et al* protocol.[9]

For stock solution, a concentration of 50 mg/ml was attained by mixing Fosfomycin powder into distilled water. Rapid Fosfomycin NP solution (Solution 1) was prepared in quantity of 125ml described as follows: 112.5 ml of distilled water was taken in a container in which 3.125 g CAMHB and 6.25 mg of phenol red were mixed. Final PH of reagent was adjusted to 6.7. The solution was sterilized by autoclave for 15 min at 121°C. After lowering down the temperature of solution to room temperature, 12.5 mL of 10% anhydrous glucose was added. For storage of both rapid Fosfomycin NP solution and Fosfomycin stock solution, they were kept in aliquots at –20°C.

Before carrying out the final test, both these solutions were warmed at 37°C. Then solution 1 and solution 2 were mixed in a sterile tube attaining final concentration of Fosfomycin of 40 µg/ml (Solution 3).

McFarland was prepared by using overnight grown 10 µl loop full bacterial colonies and resuspending in 10 ml of sterile normal saline. Final McFarland optical density of around 3.0 (10^9 CFU/ mL) was achieved.

To perform final test, 96 well polystyrene microtiter plate (sterile, round bottom, Thermo Fisher Scientific, USA) was used.

Solution 1 (solution without Fosfomycin) 150ul was added in first four wells in row A, i.e., A1 to A4 while 150ul of Solution 3 (rapid Fosfomycin NP solution with 40µg/ml Fosfomycin and 25µg/ml glucose-6-phosphate) was added in first four wells in row B, i.e., B1 to B4 (Figure-1).

50ul of normal saline was mixed to wells A1 and B1. Negative control suspension which was known Fosfomycin susceptible (ATCC *E. coli* 25922) was added to wells A2 and B2 in 50ul quantity. To wells A3 and B3, 50ul of positive control, known Fosfomycin-resistant isolate (Institutional *E. coli* control) suspension was transferred. To wells A4 and B4, 50ul suspension of test isolate was transferred. In each well, the final concentration of isolates was 10^8 CFU/ ml.

After inoculation, microtiter plate was kept in incubator at 35±2 °C. The microtiter plate was not sealed to allow free flow of oxygen for metabolism of carbohydrates. Microtiter plate was inspected after 10 minutes to see if there was any spontaneous colour change. Plate was subsequently reviewed after 1 hour (h) then after every half an hour till 4 h of incubation. Result of the test was declared positive (Isolate was labelled as Fosfomycin resistant) if the isolate grew in solution 3 containing wells leading to orange to yellow colour change in the wells. On the contrary, a test result was regarded as negative (Isolate was labelled as Fosfomycin susceptible) if the isolate did not grow in solution 3 containing wells, means no colour change in the wells (stayed orange).

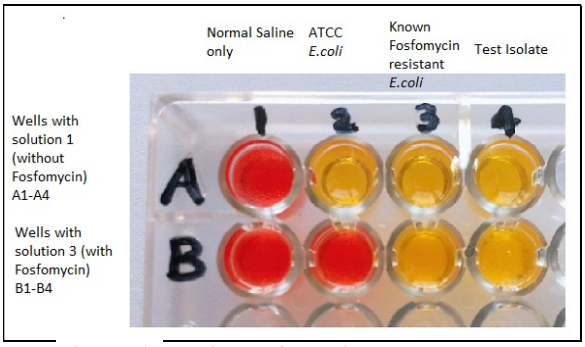

Figure-1: **Rapid Fosfomycin NP test results (representative results) after 2.5 hours of incubation on microtiter plate.**

Wells A1-A4 contained solution without Fosfomycin and wells B1-B4 had solution with Fosfomycin. Yellow colour indicates positive reaction while orange colour shows negative reaction.

Orange colour (No colour change) in wells A1 and B1 (as there was no isolate in these wells) and yellow colour in wells A2 to A4 (as there was no Fosfomycin in these wells to prevent growth of isolates) confirmed the validity of the test.

The data obtained was analysed in SPSS software (version 25) for statistical evaluation. Descriptive statistics were calculated for both qualitative and quantitative variables. Quantitative variables like age were measured as mean and standard deviation (SD). Qualitative variables like gender, rapid Fosfomycin NP test positive and negative were measured as frequency and percentage. Results were compared with disk diffusion as the reference method. Sensitivity, specificity, positive predictive value, negative predictive value and diagnostic accuracy of the Rapid Fosfomycin NP test were calculated using 2×2 table.

RESULTS

A total of 149 urine specimens yielding growth of *E.coli* from 149 different patients were included in the study. Characteristics of patients, i.e., age distribution, gender and hospitalization status have been shown in Table 1. The average age of the patients was 43.11±15.77 years. Most patients were in age group of 51–60 years. Maximum urinary specimens were midstream urine specimens followed by specimens collected by indwelling catheters. Based on antimicrobial susceptibility results of antibiotics panel for *Enterobacterales* recommended by CLSI guidelines, maximum *E.coli* isolates included in the study were classified as XDR. Disk zones of Fosfomycin susceptible isolates were mostly 26-28 mm while Fosfomycin resistant isolates were 13–15 mm (Table 2). Out of total 149 *E.coli* isolates, 80 were categorized as Fosfomycin susceptible and 69 as Fosfomycin resistant by reference disk diffusion method. Out of 80 susceptible isolates, 79 were categorized as negative by Rapid Fosfomycin NP test as there was no colour change (stayed orange) even after 4 hr of incubation but one of the isolates was found positive by Rapid Fosfomycin NP test after 1.5 hr of incubation. This isolate (false positive) had disk zone of 24 mm on disk diffusion test. Disk diffusion test and Rapid Fosfomycin NP test were repeated for this isolate and showed same results on repetition. MIC of the isolate was performed by E-test strips which showed Fosfomycin MIC of 32 µg /ml. Out of 69 isolates which were classified as resistant by Disk diffusion method, 10 showed yellow colour (positive reaction) within 1 hr of incubation, 36 isolates gave positive reaction on Rapid Fosfomycin NP test (turned yellow) within 1.5 hr, 12 isolates within 2 hr and 7 isolates were found positive at 2.5 hr of incubation.

There were 4 isolates which were found false negative, i.e., they did not give positive reaction even after 4 hr of incubation. Two of these isolates had disk zone of 22mm and two had disk zone of 23mm on disk diffusion test. All false negative isolates showed same results on repetition by both Disk diffusion and Rapid Fosfomycin NP test. E-test strips were used to determine Fosfomycin MICs for these isolates which were found to be 64 µg /ml for all 4 isolates. Sensitivity, specificity, positive predictive value, negative predictive value and diagnostic accuracy of rapid Fosfomycin NP test was found to be 94.2%, 98.75%, 98.48%, 95.2% and 96.64%, respectively (Table 3)

Table-1: Characteristics of patients and specimens

Characteristics	Total No (%)
Age groups (years)	
≤ 20	13 (8.7)
21–30	27 (18.1)
31–40	25 (16.8)
41–50	26 (17.5)
51–60	33 (22.2)
> 60	25 (16.8)
Gender	
Male	68(45.6)
Female	81 (54.4)
Hospitalization status	
Outdoor	52 (34.9)
Indoor	97 (65.1)
Type of urinary specimens	
Midstream urine specimens	74 (49.7)
Indwelling catheters	52 (34.9)
Straight catheters	20 (13.4)
Cystoscopy	03 (2)

Table 2: Characteristics of *E-coli* isolates (n=149)

Characteristics	Total No (%)
MDR isolates	57 (38.2)
XDR isolates	72 (48.3)
Fosfomycin Susceptible (Disk diffusion)	**80 (53.7)**
Disk zone 24–25mm	23 (15.4)
Disk zone 26–28mm	43 (28.9)
Disk zone ≥ 29mm	14 (9.4)
Fosfomycin Resistant (Disk diffusion)	**69 (46.3)**
Disk zone ≤ 12mm	06 (4)
Disk zone 13–15mm	23 (15.4)
Disk zone 16–18mm	17 (11.4)
Disk zone 19–21mm	06 (4)
Disk zone 22–23mm	17 (11.4)

Table-3: Comparison of Rapid Fosfomycin NP test results with results of Disk diffusion among urinary isolates of *E.coli* (n=149)

Antibiotic	Rapid Fosfomycin NP test	Disk diffusion test		Diagnostic accuracy of Rapid Fosfomycin NP test				
		R	S	Sensitivity	Specificity	PPV	NPV	DA
Fosfomycin	Positive	65	01	94.2 %	98.75%	98.5 %	95.2%	96.64%
	Negative	04	79					

PPV= positive predictive value; NPV= negative predictive value, DA= diagnostic accuracy

DISCUSSION

This is the first study on Rapid Fosfomycin NP test in Pakistan to the best of our knowledge. Urinary tract infections are most commonly caused by *E. coli* in our setup. Global rise of multidrug resistant *E. coli* isolates which acquire resistance to various oral and injectable antibiotics through the production of extended-spectrum β-lactamases (ESBLs) and other mechanisms leave very few therapeutic options.[3,6,7] Fosfomycin is not only a preferred treatment option for community and hospital acquired UTIs but there is also renewed interest in its injectable use in other severe infections caused by ESBLs and carbapenamases producing gram negative rods.[9,12,13]

Widespread use and emergence of resistance against Fosfomycin among *E.coli* isolates warrants development of a cost effective and reliable method of detection of Fosfomycin resistance.[13] The rapid Fosfomycin NP test is simple and easy to perform technique for rapid detection of Fosfomycin resistance with excellent diagnostic accuracy.[9] Previous studies performed on "The Carba NP test" by Nordmann *et al*[16] and "Rapid Polymyxin NP test" by Nordmann *et al*[17], Dalmolin TV *et al*[19] and Malli E *et al*[20] showed excellent sensitivity and specificity of the methods. The study on "Rapid Polymyxin NP test" was also conducted at our institute previously which showed reliable results and excellent diagnostic accuracy.[21]

In our study reliable results were achieved after 2.5 hours of incubation although majority of Fosfomycin resistant isolates showed positive reaction within 2 hours. These results were contrary to findings of study done by Nordmann *et al* which detected Fosfomycin resistance within 1.5 hours.[9] Even with 2.5 hours for Rapid Fosfomycin NP test, results were available 15–16 hours earlier than standard disk diffusion test and agar dilution method.

Specificity of Rapid Fosfomycin NP test in our study was 98.75% which was very much comparable to the study done by Nordmann *et al*.[9] Sensitivity of Rapid Fosfomycin NP test in our study was 94.2% which was lesser than the original study conducted in Switzerland .[9] Positive predictive value and negative predictive value of the test was 98.5% and 95.2% respectively which were comparable to previous studies done on "Rapid Polymyxin NP test".[20,21] Overall diagnostic accuracy of the test was 96.64 % which was considerably high to make it a reliable test for clinical utility.

Four out of 69 Fosfomycin resistant isolates gave false negative reaction on Rapid Fosfomycin NP test. The finding was contrary to the international study in which none of the isolates gave false negative reaction.[9] This could be due to low MICs (64 µg /ml) of those *E.coli* isolates. Their disk zones were larger as well, i.e., of 22mm and 23mm.There were 13 other isolates with disk zones 22–23 mm but they gave positive reactions on Rapid Fosfomycin NP test.

One out of 80 Fosfomycin susceptible isolates gave false positive reaction which is comparable to study done by Nordmann *et al* in which there was one susceptible isolate which gave positive reaction on Rapid Fosfomycin NP test. Contrary to that study MIC of our false positive isolate was quite high (32 µg /ml) and smaller disk zone 24mm among susceptibility category.[9] This could be the reason for false positivity in

our study. There were some limitations of our study. Firstly, we did not perform reference agar dilution method in our study rather we compared Rapid Fosfomycin NP test with Disk diffusion test. For false positive and false negative isolates, we also performed E-test strips rather than agar dilution method. Another limitation was that our study could not efficiently ascertain Fosfomycin resistance amongst isolates with low Fosfomycin MICs. Thirdly our study was focused on *E.coli* isolates and urine specimens only and did not detect Fosfomycin susceptibility in other gram negative rods.

CONCLUSION

The rapid Fosfomycin NP test is convenient, feasible and reliable technique which can be executed with minimum technical skills and has shown excellent accuracy. It is rapid as compared to disk diffusion and E-test strips and easy to perform as compared to agar dilution method. It can be utilized as an alternative to ascertain Fosfomycin resistance in urinary *E.coli* isolates which would be helpful in determining appropriate treatment option for UTIs. Further studies can be done on MDR and XDR gram negative rods and specimens other than urine so that future clinical application of the test can be enhanced in critical life-threatening infections.

Ethical approval:

Ethical approval and consent to participate approval to conduct this study was obtained from Institutional Review Board and Ethical Committee, Armed Forces Institute of Pathology.

Conflict of interest:

The authors have no financial or other interests with regard to the submitted manuscript that might be construed as a conflict of interest.

AUTHORS' CONTRIBUTION

All authors contributed equally in literature search, study design, data collection, data analysis, data interpretation, write up and proof reading.

REFERENCES

1. Anjum MU, Khan MS, Shahid AR, Shah SH. Urinary tract infections; etiological profile and antimicrobial susceptibility patterns of uropathogens. Professional Med J 2016;23(1):10–14.
2. Flores-Mireles A, Hreha TN, Hunstad DA. Pathophysiology, Treatment, and Prevention of Catheter-Associated Urinary Tract Infection. Top Spinal Cord Inj Rehabil 2019;25(3):228–40.
3. Steiger SN, Comito RR, Nicolau DP. Clinical and economic implications of urinary tract infections. Expert Rev Pharmacoecon Outcomes Res 2017;17(4):377–83.
4. McLellan LK, Hunstad DA. Urinary tract infection: pathogenesis and outlook. Trend Mol Med 2016;22(11):946–57.
5. Cortes-Penfield NW, Trautner BW, Jump RL. Urinary tract infection and asymptomatic bacteriuria in older adults. Infect Dis Clin North Am 2017;31(4):673–88.
6. Vásquez V, Ampuero D, Padilla B. Urinary tract infections in inpatients: that challenge. Rev Esp Quimioter 2017;30(Suppl 1):39–41.
7. Khan IU, Mirza IA, Ikram A, Ali S, Hussain A, Ghafoor T. In vitro activity of fosfomycin tromethamine against extended spectrum beta-lactamase producing urinary tract bacteria. J Coll Physicians Surg Pak 2014;24(12):914–7.
8. Silver LL. Fosfomycin: mechanism and resistance. Cold Spring Harb Perspect Med 2017;7(2):a025262.
9. Nordmann P, Poirel L, Mueller L. Rapid Detection of Fosfomycin Resistance in Escherichia coli. J Clin Microbiol 2019;57(1):e01531.
10. Zhanel GG, Walkty AJ, Karlowsky JA. Fosfomycin: A First-Line Oral Therapy for Acute Uncomplicated Cystitis. Can J Infect Dis Med Microbiol 2016;2016:20826937.
11. Sastry S, Doi Y. Fosfomycin: resurgence of an old companion. J Infect Chemother 2016;22(5):273–80.
12. Díez-Aguilar M, Cantón R. New microbiological aspects of fosfomycin. Rev Esp Quimioter 2019;32(Suppl 1):8.
13. Mojica MF, De La Cadena E, Hernández-Gómez C, Correa A, Appel TM, Pallares CJ, *et al*. Performance of disk diffusion and broth microdilution for fosfomycin susceptibility testing of multi-drug resistant clinical isolates of Enterobacterales and Pseudomonas aeruginosa. J Glob Antimicrob Resist 2020;21:391–5.
14. Weinstein MP, Patel JB, Bobenchik AM, Campeau S, Cullen SK, Galas MF, *et al*. M100 Performance Standards for Antimicrobial Susceptibility Testing A CLSI supplement for global application. 2020.
15. European Committee on Antimicrobial Susceptibility Testing. European Committee on Antimicrobial Susceptibility Testing Breakpoint tables for interpretation of MICs and zone diameters, Version 10.0. 2020.
16. Nordmann P, Poirel L, Dortet L. Rapid detection of carbapenemase-producing Enterobacteriaceae. Emerg Infect Dis 2012;18(9):1503.
17. Nordmann P, Jayol A, Poirel L. Rapid detection of polymyxin resistance in Enterobacteriaceae. Emerg Infect Dis 2016;22(6):1038–43.
18. Clinical and Laboratory Standards Institute. Method for dilution antimicrobial susceptibility tests for bacteria that grow aerobically 11th ed. Document M07.CLSI, Wayne, PA. 2018.
19. Dalmolin TV, Dias GÁ, de Castro LP, Ávila H, Magagnin CM, Zavascki AP, *et al*. Detection of Enterobacterales resistant to polymyxins using Rapid Polymyxins NP test. Braz J Microbiol 2019;50(2):425–8.
20. Malli E, Florou Z, Tsilipounidaki K, Voulgaridi I, Stefos A, Xitsas S, *et al*. Evaluation of rapid polymyxin NP test to detect colistin-resistant Klebsiella pneumoniae isolated in a tertiary Greek hospital. J Microbiol Methods 2018;153:35–9.
21. Shoaib M, Hussain A, Satti L, Hussain W, Zaman G, Hanif F. Evaluation of rapid polymyxin Nordmann Poirel test for detection of polymyxin resistance in clinical isolates of Enterobacteriaceae. Eur J Clin Microbiol Infect Dis 2020;39(11):2195–8.

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