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EVALUATION OF FOSFOMYCIN SUSCEPTIBILITY TESTING METHODS IN CLINICAL ISOLATES IN A TERTIARY CARE HOSPITAL.

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ABSTRACT Fosfomycin has received attention in recent years as an alternative for management of infection by drug resistance bugs. However, method of Invitro Susceptibility methods for Fosfomycin is cumbersome and not being standardized for all organisms. The present study aims to compare the E strip Test & Disk Diffusion Test(DDT) for Fosfomycin susceptibility taking Agar dilution Test as the reference method. Statistical analysis was performed using 95% Confidence Interval. ROC curve & Bland Altman analysis wasperformed too A total of 102 isolates obtained from urine & miscellaneous samples were tested by the three methods using standard guidelines .Most common organism was *Eschrichia coli* and overall MIC90 was 512µg/ml and MIC50 was 216µg/ml. In urinary isolates, Categorial Agreement for DDT was 43.9 % & 70.2 % for E strip. Essential Agreement for E strip was 28.4%. In non-urinary isolates,Categorial agreement for DDT was 52.8% & 75% for E strip,while Essential Agreement for E strip was 22.2%. Sensitivity & specificity were higher for E strip . Overall,E strip had lower error rates than DDT. In non-urinary isolates,there were no Major error & minor error for both the test methods. E strip had good correlation with Agar Dilution Test while DDT showed poor agreement

KEYWORDS : Fosfomycin, Agar Dilution test,Resistance, Sensitivity, E- strip

INTRODUCTION

Fosfomycin,is derived from cultures of Streptomyces spp and acts by irreversibly inhibiting MurA (UDP-N-acetylglucosamine peptidoglycan biosynthesis pathway . It enters the bacterial cytosol by two transport systems, the constitutively expressed L-α glycerophosphate (or glycerol-3-phosphate) uptake (GlpT) system and the glucose-6-phosphate(G6P) inducible hexose[1].Addition of 25µg/mL G6P in culture media optimizes induction of the UhpTsystem and hence it is added in in vitro susceptibility methods.

The Infectious Disease Society of America (IDSA) and European Society for Microbiology and Infectious Diseases (ESMID) has recommended Fosfomycin as one of the first line choice of therapy for uncomplicated cystitis and pyelonephritis[2].

However, rapidly increasing drug resistance, slowly exhausting reservoir group of antibiotic, slowness in development of new antimicrobials has led to comeback of old antibiotics like Fosfomycin because of its unique structure and no cross-resistance with other antimicrobial agents along with its broad spectrum activity. It has shown good activity against Multidrug resistant systemic infection[3].

For In vitro susceptibility testing , there is a lack of fully defined common breakpoints. As per CLSI guidelines[4] Disk Diffusion Test (DDT) and Agar Dilution Test (ADT) for urinary isolates of *Escherichia coli and Enterococcus faecalis* are approved methods. However,EUCAST recommends ADT or Broth Microdilution[5]

Studies have been conducted for determination of in vitro susceptibility testing for other organisms in urine[6,7,8,9] and in systemic infections.[10,11,12,13,14]

The present study aims to determine the susceptibility of Gram negative & Gram positive bacteria isolated from various clinical samples and evaluate the performance of DDT & E-strip test for detecting Fosfomycin susceptibility taking ADT as the reference method.

MATERIAL & METHOD:

A total of 102 consecutive non-duplicate pre-identified bacterial isolates recovered from various clinical specimens received in the laboratory were studied for Fosfomycin susceptibility testing concomitantly by the ADT, DDT & E-strip strip as per CLSI guidelines[4].

Agar dilution Test (ADT) : Mueller-Hinton Agar plates supplemented with 25 mg/liter glucose-6-phosphate and Fosfomycin in concentrations from 0.25 mg/liter to 1,024 mg/liter were prepared .An inoculum of 10^4 CFU was placed onto the agar plate and allowed to dry. The plates were incubated for 16 to 20 hour at 35°C(Celcius).

The Minimum Inhibitory Concentration (MIC) of Fosfomycin was taken as the lowest concentration that inhibited visible growth of the organism.

Disk Diffusion Test (DDT):
Fosfomycin disks (200 µg) containing 50 µg of glucose-6-phosphate(G6P) were used .The susceptibility testing of Fosfomycin for each isolate was performed twice under the same conditions on the same day. Plates were read and the mean of duplicate zone diameter of each isolate were determined after overnight incubation at 35°C in ambient air.

E strip Test
The E-strip (Biomeriuex) supplemented with G6P was used as per manufacturer's instructions.To determine the crossing point of the ellipsis with the strip, scattered colonies were taken into account if they were either in the margin of the ellipse or had a density of 5 colonies percm[2].

For quality control, ATCC *Escherichia coli* 25922, ATCC *Staphylococcus aureus* 29213, and ATCC *Pseudomonas aeruginosa* 27853 were taken

Interpretative criteria: Isolates were classified according to CLSI [4] breakpoints (susceptible, MIC of ≤64 µg ml or zone of ≥16 mm [200 µg disk]; intermediate, MIC of 128 µg /ml or zone of 13 to 15 mm; resistant, MIC of 256 ≥ µg /ml or zone of ≤12 mm *for Escherichia coli & Enterococcus* species) and EUCAST breakpoints[5](susceptible,MIC of ≤32 µg /ml; resistant, MIC of ≥32 µg /m)l for all other Enterobacterales. As zone diameters are not published for these organisms, the breakpoints proposed for DDT found by Lu et al[11] for the 200- µg Fosfomycin disk (susceptible ≥14mm;resistant, ≤14mm)

were applied

Susceptibility results of the E –strip test & DDT were compared with those obtained from the ADT . Agreement and discrepancies between the evaluated and reference method were classified as Categorial Agreement, ,Essential Agreement ,Very Major Errors(VME), Major Errors(ME), and Minor Errors(mE) as follows[15].

Essential agreement (EA) = MIC of E strip Test equal to or within ±1 dilution MIC of ADT

Categorical agreement (CA) = ADT and E strip test or DDT agree using the interpretative criteria.

Minor errors(mE) = E strip Test or DDT are Sensitive (S) or Resistant (R) and ADT is Intermediate(I), alternatively E strip Test or DDT are I and ADT is S or R .

major errors(ME) = E strip Test or DDT are R and ADT is S; the percentage of major errors is calculated only for S isolates very Major E rrors (VME)= E strip Test or DDT are S and ADT is R; the percentage of very major errors is calculated only for R isolates.

Statistical analysis:
Results were charted and % resistance to Fosfomycin by ADT was calculated taking 95% Confidence Interval (CI). Sensitivity, Specificity , Positive Predictive Value(PPV) & Negative Predictive Value (NPV) was calculated taking 95%CI. Performance characteristics of DDT and Estrip were evaluated against the. ADT using ROC curve & Bland Altman analysis.

RESULTS

Of the 102 clinical isolates ,66 isolates(64.7%) were from urinary samples and 36(35.3%) were miscellaneous (pus,respiratory,body fluid) samples The most common isolate was *Escherechia coli, Klebsiellla pneumonia, Staphylococcus aurues, Enterococcus faecalis*.
The overall MIC_{90} to Fosfomycin was 512 µg/ml and MIC_{50} was 216 µg/ml.

Applying CLSI guidelines 2017, 17 isolates (16.7%) were resistant to Fosfomycin while applying EUCAST guidelines 2018,3 isolates (37.25%) were resistant to Fosfomycin.

For urinary isolates, the resistance was 58.18% with MIC_{50} of 128 µg/ml and MIC_{90} of 512µg/ml, while resistance in non-urinary isolates was 66.67% with $MIC_{50}$of 128 µg/ml and $MIC_{90}$of 512 µg/ml.

CA was higher for E strip test as compared to DDT for in both urinary & non-urinary isolates. However, EA for E strip test was higher in urinary isolates than that in non-urinary isolates.(**Table 1)**

Table 1:Agreement for E –strip and DDT for Urinary isolates and non-urinary isolates.

Isolates(n)	Test	CA	EA	VME	ME	mE
Urinary isolates(66)	DDT	43.9	-	75%	8%	16.67
	ESTRIP	70.2	28.4	25%	3.03%	40%
Non urinary(36)	DDT	52.8	-	70.8%	0	0
	ESTRIP	75	22.2	37.5%	0	0

Table 1 shows that for Estrip Test , VME was 25% and 37.5% for urinary & non-urinary samples respectively ,while ME was 3.03% for urinary isolates and mE was 40% for urinary isolates.There was no ME and mE error in non- urinary samples**.**

The median zone diameter overall and for urinary isolates only 23 mm and 22.5 mm respectively. VME,ME and mE was 75%, 8% and 16.67% respectively .For non-urinary isolates VME was 70.8% while no ME or mE was observed for urinary isolates .

Bland Altman analysis showed small underestimation of susceptibility by E-strip as compared to ADT but with a non-significant p-value indicating thereby absence of any fixed bias. Random distribution of the errors (differences) also indicated absence of the proportional bias**(Fig 1 & 2).** Susceptibility was overestimated by DDT in Blandmann Analysis as compared to ADT and systematic differences were found only in one direction along with a significant p-value. All of these findings indicate presence of fixed bias and proportional bias. Moreover results of DDT did not agree with ADT equally throughout the range of measurements either**.(Figure 1 & 2)**

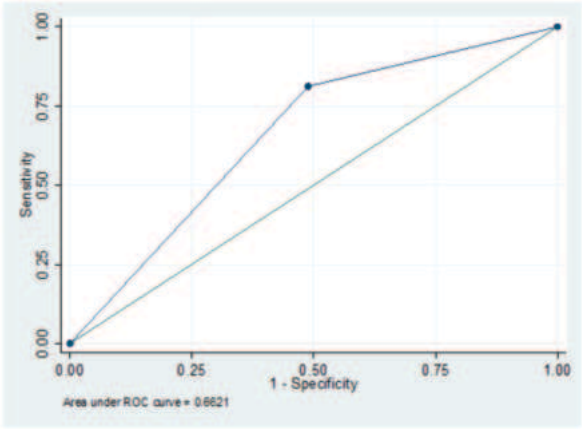

Figure 1:**ROC curve for predicting susceptibility by DDT versus ADT**

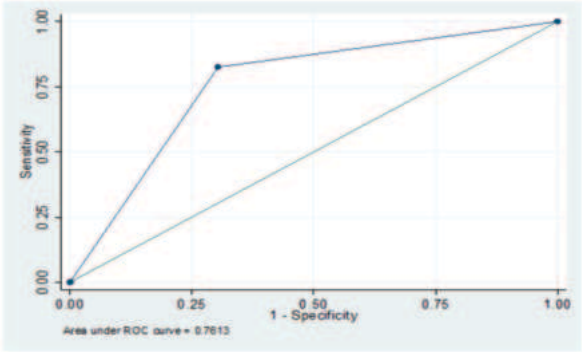

Figure 2:**ROC curve for predicting susceptibility by E strip versus ADT**

.Fairly good agreement of E strip with ADT was observed (kappa =0.51, 95%CI 0.32 -0.70, P=0.001) as compared to the poor agreement between DDT and ADT (kappa =0.16, 95% CI 0.03 -0.30, P=0.085). AUC showed better predictive value for E strip than DDT

Sensitivity of E strip (69%) was better than DDT (24%), while specificity of E-Strip & DDT was around 83% and 94% respectively. PPV, NPV & diagnostic accuracy of E-strip & DDT was 83% 70% & 75% and 81% ,51% & 56% ,respectively.

DISCUSSION

The in vitro susceptibility testing for Fosfomycin is a challenge as ADT is tedious & cumbersome while DDT & E strip has non-uniform guidelines for selected organisms.

Many studies have been performed to understand if the existing guidelines can be applied and extrapolated to other clinical isolates not only from urine but also from systemic infections**.**[12,13]

This study has tried to evaluate DDT & Estrip for Fosfomycin susceptibility testing by extrapolating the CLSI reference range to *Escherichia coli* & *Enterococcus faecalis* isolated from all types of samples & EUCAST guidelines for rest of the organisms. The results have been differentiated into urinary & non-urinary isolates for ease of understanding the pattern of resistance.

The overall resistance to Fosfomycin was 53.92%. Other studies have reported a lower resistance in urinary ESBL[8] producing strains & in MDR Enterobacterales**.**Both these studies has applied CLSI guidelines.The MIC cut off for susceptible in CLSI is >=64 µg/ml while in EUCAST it is >=32µg/ml. This wide range of MIC difference tends to mark many sensitive organisms as resistant. The difference in resistance rate in this study (16.7% using CLSI reference range versus 37.25% with EUCAST) is similar to (LV Pedago et al)[10].These studies have been performed mainly on Urinary Enterobacterales ,however present study has included even the non-Enterobacterales and Gram

positive cocci from miscellaneous samples. This could be the probable reason for lower resistance.

For E strip, an agreement of 22.2% and 28.4% in this study was very far than the 96.67% as proposed[15]. The error rates were out of acceptable rates for a test method. However in non-urinary sample absence of ME and mE shows that it can be used for screening test for detecting resistance. Of the 9 VME for non- urinary isolates, the average MIC ranges was from 0.5 to 24µg/ml. That implies even after applying strictest criteria, false sensitivity rates couldn't have been lowered. In urinary Isolates having VME ,only 1 strain had an MIC of 64 µg/ml by CLSI.

Similar to Perdago LV, et al[10], despite high VME and ME rates for E strip test both in urinary isolates & non-urinary samples, CA was frequent. Amongst 2 strains having ME in Estrip Test,MIC was very high(>1024µg/ml)which reflects false resistance. Such False resistance results can be double checked by using ADT in routine reporting, The median zone diameter in this study was comparable to Perdago LV et al[10], The error rates for DDT were are out of acceptable range in non-urinary isolates . For urinary samples there was no ME and mE but VME was again unacceptable similar to a previous report[12].

The results of Bland Altman analysis showed fairly good agreement of E-strip test with ADT was observed as compared to the poor agreement between DDT and ADT.**Figure 1 & figure 2** showed that E-strip and ADT may be used interchangeably for predicting susceptibility, though with a caution that results of the two tests might not agree equally throughout the entire range of the measurements. On the contrary, results of DDT did not agree with ADT equally throughout the range of measurements .Because of the existing bias, DDT and ADT cannot be used interchangeably for predicting susceptibility. Inadequate agreement has been reported in previous study[12].

In the present study, PPV and NPV (as indicated by AUC) of E-strip Test for susceptibility was found much better (AUC=0.76) than that of DDT (AUC=0.66).A previous study has reported an AUC 0.976 for DDT using 200µg disk with size of >=17mm for sensitive[12]. However cut off of 16mm for susceptible in *Escherichia coli* and *Enterococcus*s pecies and 14mm for all other isolates could be the reason for difference in the results.

mE are an important considerations as the 'intermediate category'[16] which represents the 'grey zone' regarding therapeutic success. It also helps to prevent serious categorization errors resulting from imprecision of inhibition zone readings. In this study, using EUCAST criteria (no defined interpretation for "intermediate") analysing results of E STRIP , out of the 8 urinary isolates with mE, 4 isolates could be converted in to VME. While in DDT, 11 Urinary isolates having mE, 1would have been fallen into ME and and 8 into VME. Thus the total VME of urinary isolates would have increased.

Limitations

1) Adequate distribution of all organisms could have better in understanding the susceptibility of Fosfomycin.
2) Applying both CLSI & EUCAST guidelines simultaneously could also have been done.

CONCLUSION

The findings of the present study shows the difference in results of predicting resistance to Fosfomycin by ADT when interpreting results by two different guidelines. Results of E test results were better than DDT both in urinary & urinary isolates .For non-urinary isolates ,extrapolating CLSI guidelines for detecting resistance to Fosfomycin can be beneficial before starting therapy .

However, a large scale of laboratory as well as clinical studies with optimum representation of isolates from different clinical samples is required to guide the use of current established standards of Fosfomycin in clinical reporting.

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