

Original Research Article

Influence of urine on Antimicrobial gradient and Disk diffusion susceptibility testing of Co-trimoxazole, Fosfomycin, Norfloxacin and Nitrofurantoin against urinary *Escherichia coli*.

ABSTRACT

Aim: The aim of present study is to examine the influence of urine on zone of inhibition and Minimum inhibitory concentration by antimicrobial gradient strips of Co-trimoxazole, Fosfomycin, Norfloxacin and Nitrofurantoin against urinary *E. coli*.

Study design: Cross-sectional study

Place and Duration of Study: Department of Medical Microbiology, School of Medical Education, Kottayam, Kerala, India. Between January 2023 and November 2023.

Methodology: A total of 75 *E. coli* isolates collected from various diagnostic microbiology laboratories were included in the study. Identification of isolates and antimicrobial susceptibility testing was done. Direct v/s standard zone of inhibition and MIC was determined. The data was statistically analysed using Cohen's kappa for interrater reliability and Intraclass correlation coefficient for consistency level.

Results: Current study evaluated the effect of urine on zone of inhibition and MIC by disc diffusion and antibiotic gradient testing respectively. The kappa value, a measure of agreement between direct v/s standard zone of inhibition and MIC for Co-trimoxazole, Fosfomycin, Norfloxacin was a perfect 1 ($P=.000$), indicating complete agreement. While, The kappa value, a measure of agreement between direct and standard MIC determination for Nitrofurantoin, was .67 ($P=.000$), indicating substantial agreement.

Conclusion: The present study suggests that direct antimicrobial susceptibility testing can be employed for Co-trimoxazole, Fosfomycin, Norfloxacin and Nitrofurantoin which are important drugs in the management of UTI, after further standardization.

Keywords: *Escherichia coli*, *Co-trimoxazole*, *Fosfomycin*, *Norfloxacin*, *Nitrofurantoin*, *Direct sensitivity testing*, *Antimicrobial susceptibility testing*, *Urinary tract infection*

INTRODUCTION

Urinary tract infections (UTIs) were first documented in Egypt in 1550 BC, and are still among the most common bacterial infections in the world [1]. It is estimated to affect 150 million people each year worldwide, with an annual incidence of 12.6% in women and 3% in men [2,3]. These common bacterial infections have been easily treated and cured with antibiotics. Unfortunately, an increase in the use of antibiotics has resulted in antibiotic resistance. When bacteria become resistant to the medicines used to treat them a number of antibiotics routinely employed for UTIs have become ineffective, leading to more severe illness, hospitalizations and mortality while driving up medical cost. Antibiotic resistance occurs naturally, but the use and misuse of antibiotics in humans and livestock have accelerated it. The most common uropathogen identified in patients with UTI is *Escherichia coli*, which account for approximately 75–95% of cases [4]. A

study conducted in 2020, found 66-77% of *E. coli* isolated from urine were Extended- spectrum beta-lactamases (ESBL) positive. In order to reduce the unnecessary use of antibiotics the World Health Organisation (WHO) has advised enhanced use of diagnostic tests [5].

When urine samples are sent for routine culture and sensitivity to the microbiological laboratory, it may take up to three days to receive the antibiogram. Delay of microbiological testing increases the risk of initiating inappropriate antibiotic treatment, in patients presenting with typical symptoms of UTI before the test result is obtained [6]so, faster antimicrobial susceptibility testing allows early detection of resistance patterns, which are necessary for adequate clinical and therapeutic management of patients and inappropriate prescribing of antibiotics. Although many studies have evaluated direct antimicrobial disk diffusion method only a few studies [7,8,9] have evaluated the effect of urine on antibiotic gradient and disk diffusion testing. The aim of the current study was to evaluate the effect of urine on MIC values and zone of inhibition with standard testing methods, so the turnaround time to receive antibiogram can be reduced.

MATERIALS AND METHODS

The present study was conducted at Department of Medical Microbiology, School of medical education, (SME),CPAS, Kottayam, Kerala,India betweenJanuary 2023 to November 2023.During the period of study 75 isolates of *E. coli* was collected, from various diagnostic microbiology laboratories in Kerala.

Identification of isolates and antimicrobial susceptibility testing

All the isolates were identified by routine biochemical testing. The samples were inoculated onto plating media such as MacConkey Agar medium (HiMedia Laboratories Pvt. Ltd.). On the next day the isolated colonies were microscopically examined by Gram's staining. The biochemical tests were performed to identify the isolates and the biochemical tests performed were Indole test, Mannitol test, Citrate utilization test, Urease test, Triple sugar iron agar test.

Antimicrobial susceptibility testing by disc diffusion as prescribed by Clinical Laboratory Standards Institute (CLSI) guidelines M02-A13[10,11] and are categorized into MDR, XDR and Non- MDR based on CDC/ECDC guidelines [12]. The following antibiotics were tested: Gentamicin (10 µg), Amikacin (10 µg), Imipenem (10 µg), Cefuroxime (30 µg), Cefoxitin (30 µg), Ciprofloxacin (5 µg), Aztreonam (30 µg), Ampicillin (10 µg), Amoxyclav (20/10 µg), Tetracycline (30 µg), Cefixime (5 µg), Ceftazidime (30 µg), Ceftazidime/Clavulanic Acid, Cefotaxime (30µg), Cefotaxime/Clavulanic Acid,Co-trimoxazole(1.25/23.751µg), Nitrofurantoin (200µg), Norfloxacin (10µg), Fosfomycin (200µg).

Direct v/s standardised zone of inhibition determination

Urinecollected from volunteers without any antibiotic history since last 2 weeks. Antibiotic free pooled urine of pH 6.4 was sterilized by passing through a 0.2 micrometre filter. *E. coli* isolates were inoculated in both, 2-fold serially diluted cation adjusted Mueller Hinton Broth and human urine and the turbidity was adjusted to 0.5 McFarland standard and was incubated at 37°Cfor 1hour. After incubation AntimicrobialSusceptibility Testing was performed andzone of inhibition of Co-trimoxazole, Fosfomycin, Norfloxacin, Nitrofurantoin was determined.

Direct v/s standardised MIC determination

E. coli isolates were inoculated in both, 2-fold serially diluted cation adjusted Mueller Hinton Broth and human urine and the turbidity was adjusted to 0.5 McFarland standard, which was incubated at 37°C for 1hour. MICs of Co-trimoxazole, Fosfomycin, Norfloxacin, Nitrofurantoin(HiMedia laboratories Pvt. Ltd), was determined by antibiotic gradients strips. *E. coli* ATCC 25922 used as control.

Statistical analysis

Data was entered into MS. Excel 2019 and analysed using SPSS 16. Descriptive statistics such as intraclass correlation for consistency level and Cohen's kappa for interrater reliability was used.

RESULTS

In this investigation, 75 isolates of *E. coli* were obtained from diverse clinical samples. The results revealed varying antibiotic susceptibility patterns, with *E. coli* displaying notable sensitivity and resistance percentages across different antibiotics. For instance, Gentamicin exhibited high sensitivity (96%) and low resistance (4%), while Amikacin showed a sensitivity of (84%), with (14.66%) intermediate and (1.33%) resistant, while Imipenem showed a sensitivity of (82.66%), with (5.33%) intermediate and (12%) resistant, Cefuroxime showed a sensitivity of (29.33%), with (18.66%) intermediate and (52%) resistant, Cefoxitin showed a sensitivity of (77.33%), with (22.66%) resistant, Ciprofloxacin showed a sensitivity of (33.33%), with (6.66%) intermediate and (60%) resistant, Aztreonam showed a sensitivity of (34.66%), with (16%) intermediate and (49.33%) resistant, Ampicillin showed a sensitivity of (17.33%), with (5.33%) intermediate and (77.33%) resistant, Amoxyclova showed a sensitivity of (44%), with (29.33%) intermediate and (26.66%) resistant, Tetracycline showed a sensitivity of (62.66%), with (2.66%) intermediate and (34.66%) resistant, Cefixime showed a sensitivity of (29.33%), with (5.33%) intermediate and (65.33%) resistant, Ceftazidime showed a sensitivity of (14.66%), with (22.66%) intermediate and (62.66%) resistant, Cefotaxime showed a sensitivity of (13.33%), with (9.33%) intermediate and (77.33%) resistant, Fosfomycin showed a sensitivity of (97.33%), with (2.66%) resistant, Cotrimoxazole showed a sensitivity of (57.33%), with (42.66%) resistant, Norfloxacin showed a sensitivity of (57.33%) sensitive and (42.66%) resistant, Nitrofurantoin showed a sensitivity of (92%), with (2.66%) intermediate and (5.33%) resistant as shown in Fig.1. These findings contribute to our understanding of the antimicrobial susceptibility profile of *E. coli* in the studied population, providing valuable insights for clinical management and future research endeavours.

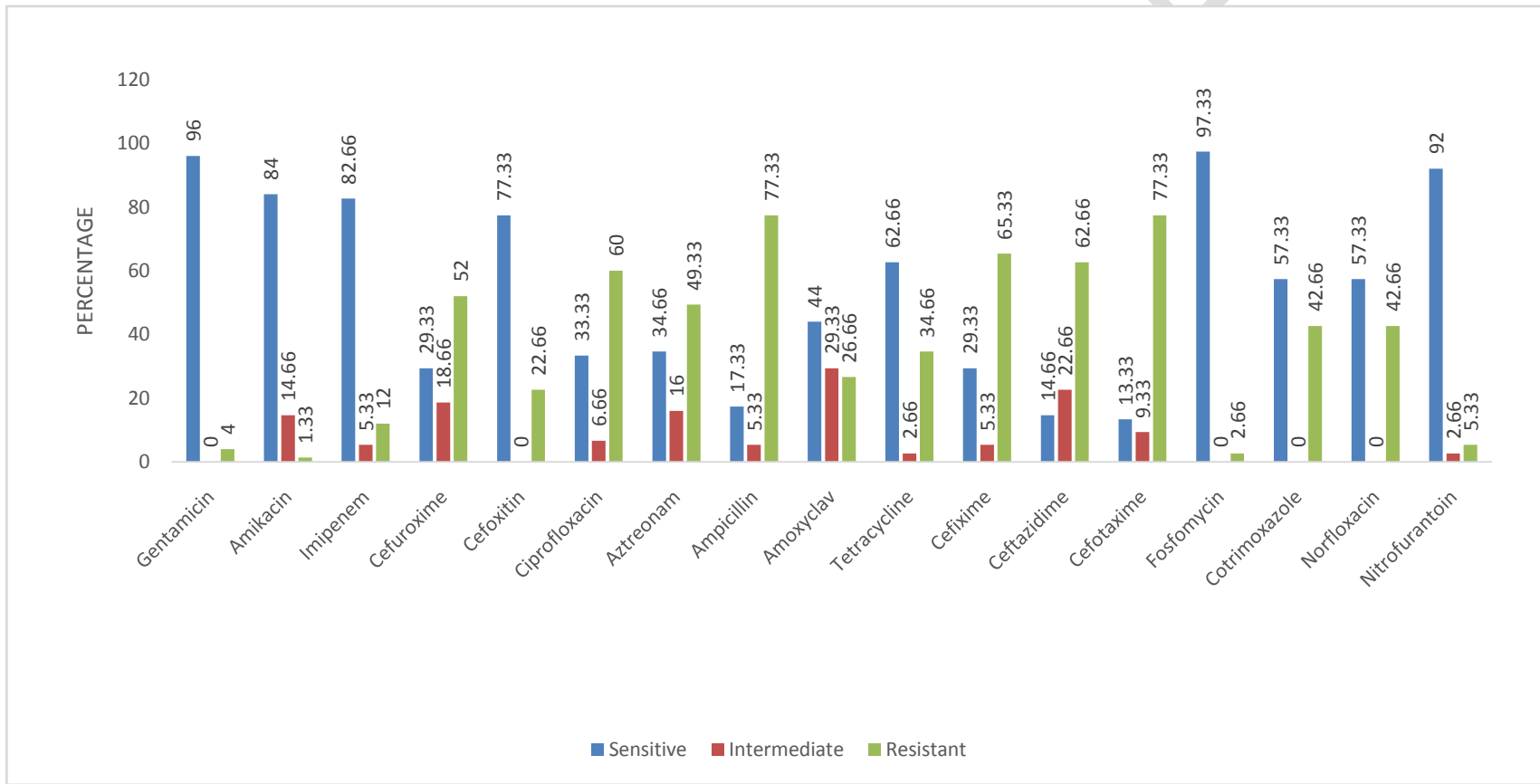


Fig. 1. Antibiotic susceptibility pattern of *E. coli*

For Norfloxacin, among 75 *E. coli* isolates, the MIC results obtained using antibiotic gradient strips without urine indicated a sensitivity of 57.33% and resistance of 42.67%, with no discernible difference when compared with urine samples. Similarly, for Fosfomycin, the MIC analysis of 75 *E. coli* isolates without urine exhibited a sensitivity of 97.33%, resistance of 2.67%, and no significant difference when contrasted with urine samples. In the case of Cotrimoxazole, the MIC results from 75 *E. coli* isolates without urine showed a sensitivity of 57.33% and resistance of 42.67%, with no observable difference compared to urine samples. For Nitrofurantoin, the MIC analysis of 75 *E. coli* isolates without urine displayed a sensitivity of 82.67%, intermediate resistance of 12%, and resistance of 5.33%. Interestingly, a minimal discrepancy of 2.67% in sensitivity and intermediate was noted, with no difference in resistance when compared to urine samples, as illustrated in Fig. 2.

In the context of normal disc diffusion, the results for Norfloxacin, Fosfomycin, Cotrimoxazole, and Nitrofurantoin, based on 75 *E. coli* isolates without urine, indicated sensitivities of 57.33%, 97.33%, 57.33%, and 92%, respectively. Correspondingly, resistance percentages of 42.67%, 2.67%, 42.67%, and 5.33% were observed. Notably, no significant differences were identified when these findings were compared with results obtained from urine samples. Interestingly, a minimal discrepancy of 2.67% in sensitivity and intermediate was noted in case of nitrofurantoin, with no difference in resistance when compared to urine samples, as illustrated in Fig. 2.

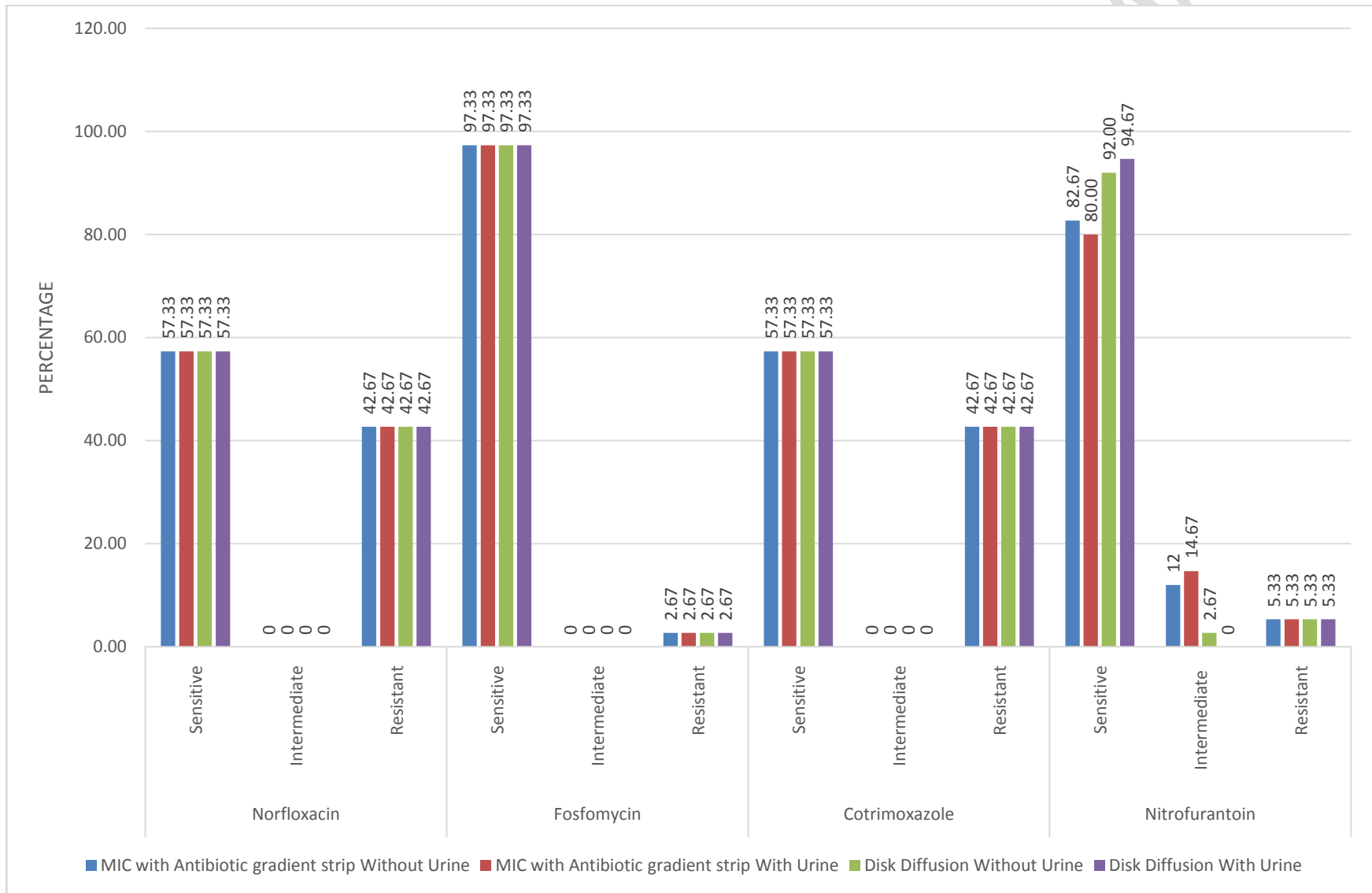


Fig. 2. Antibiotic susceptibility pattern of *E. coli* with and without urine using Antibiotic gradient strip and disk diffusion method

In this study, we aimed to assess the degree of consistency and agreement between the direct and standardized measurements of zone of inhibition and MIC determination (fig.3)

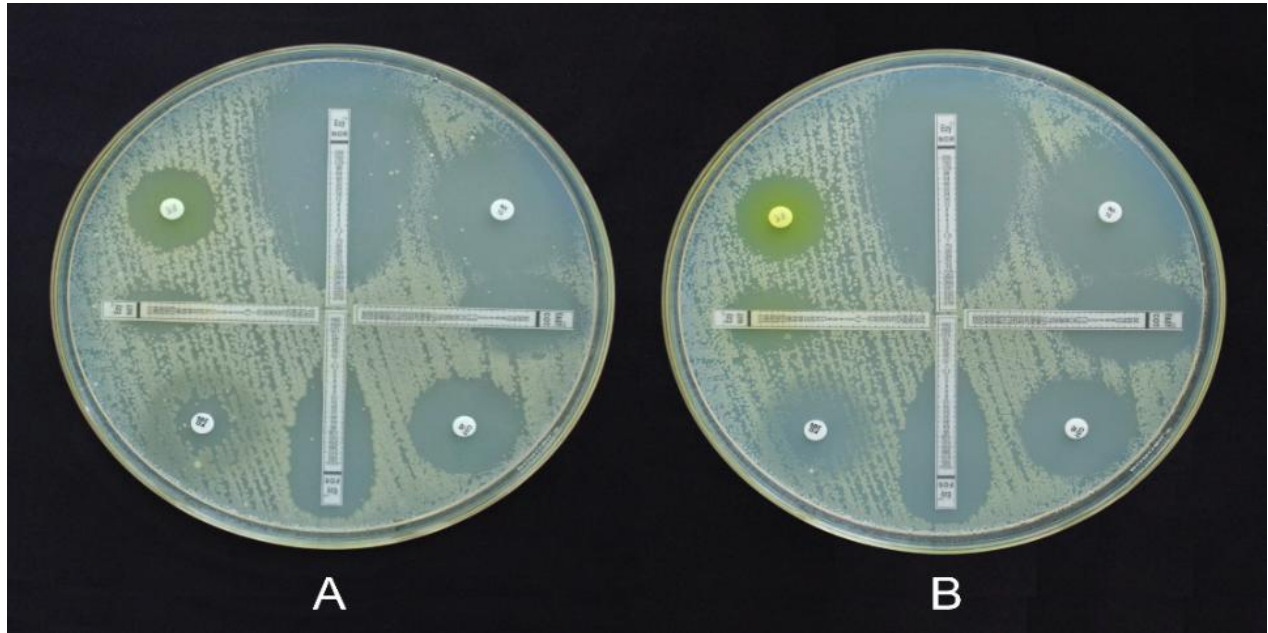


Fig. 3. Antibiotic disc and gradient diffusion method (A) with urine (B) without urine for Norfloxacin, Co-trimoxazole, Fosfomycin and Nitrofurantoin (in clockwise direction)

We employed intraclass correlation coefficient and kappa statistics to quantify these aspects, respectively.

The intraclass correlation coefficient (ICC) between direct and standardised zone of inhibition determination for Co-trimoxazole was exceptionally high at .99, indicating a robust consistency. The 95% confidence interval, along with the F test ($P = .000$), further underscored the statistical significance of this strong correlation. Both single and average measures of ICC reflected a high level of reliability in the determination of Co-trimoxazole zone diameter with and without urine.

Table 1: Intraclass correlation of Co-trimoxazole zone diameter with and without urine

		95% Confidence Interval			F Test with True Value 0		
	Intraclass Correlation	Lower Bound	Upper Bound	Value	df1	df2	Sig
Single Measures	.99	.99	.99	328.63	74	74	.000
Average Measures	.99	.99	.99	328.63	74	74	.000

The intraclass correlation coefficient (ICC) between direct and standardized zone of inhibition determination for Fosfomycin was exceptionally high at .89, indicating a robust consistency. The 95% confidence interval, along with the F test ($P= .000$), further underscored the statistical significance of this strong correlation. Both single and average measures of ICC reflected a high level of reliability in the determination of Fosfomycin zone diameter with and without urine.

Table 2: Intraclass correlation of Fosfomycin zone diameter with and without urine.

		95% Confidence Interval			F Test with True Value 0		
	Intraclass Correlation	Lower Bound	Upper Bound	Value	df1	df2	Sig
Single Measures	.89	0.83	0.93	17.85	74	74	.000
Average Measures	.94	0.91	0.97	17.85	74	74	.000

The intraclass correlation coefficient (ICC) between direct and standardised zone of inhibition determination for Norfloxacin was exceptionally high at .99, indicating a robust consistency. The 95% confidence interval, along with the F test ($P= .000$), further underscored the statistical significance of this strong correlation. Both single and average measures of ICC reflected a high level of reliability in the determination of Norfloxacin zone diameter with and without urine.

Table 3: Intraclass correlation of Norfloxacin zone diameter with and without urine

		95% Confidence Interval			F Test with True Value 0		
	Intraclass Correlation	Lower Bound	Upper Bound	Value	df1	df2	Sig
Single Measures	.99	.99	.99	1896.00	74	74	.000
Average Measures	.99	.99	1	1896.0	74	74	.000

The intraclass correlation coefficient (ICC) for Nitrofurantoin zone diameter, both for single and average measures, was high, indicating strong consistency. The 95% confidence interval further supports this, and the F test with a true value of 0 was statistically significant, emphasizing the reliability of the correlation.

Table 4: Intraclass correlation of Nitrofurantoin zone diameter with and without urine

		95% Confidence Interval			F Test with True Value 0		
	Intraclass Correlation	Lower Bound	Upper Bound	Value	df1	df2	Sig
Single Measures	.96	.94	.98	53.38	74	74	.000
Average Measures	.98	.97	.98	53.38	74	74	.000

The kappa value, a measure of agreement between direct and standardised zone of inhibition determination for Co-trimoxazole, Fosfomycin, Norfloxacin was a perfect 1, indicating complete agreement. A kappa value of 1 is interpreted as total agreement, >0.8 good agreement, 0.6-0.8 substantial agreement and 0.4-0.6 moderate agreement. This strong agreement was statistically significant, supported by a *P*-value of .000. The symmetric measures, including kappa, emphasized the robustness of the agreement, with no observed errors. The analysis was conducted on 75 valid cases, further reinforcing the reliability of the agreement assessment. In case of Nitrofurantoin one parameter was absent, so it was excluded from kappa testing.

Table 5: Kappa value of Co-trimoxazole, Fosfomycin, Norfloxacin zone diameter with and without urine

Antibiotics	Kappa Value	Asymptotic Standard Error	Approximately significant
Co-trimoxazole	1	0	.000
Fosfomycin	1	0	.000
Norfloxacin	1	0	.000

The intraclass correlation coefficient (ICC) between direct and standardised MIC determination for Co-trimoxazole was exceptionally high at .97, indicating a robust consistency. The 95% confidence interval, along with the F test (*P*= .000), further underscored the statistical significance of this strong correlation. Both single and average measures of ICC reflected a high level of reliability in the determination of Co-trimoxazole MIC with and without urine.

Table 6: Intraclass correlation of Co-trimoxazole MIC with and without urine

		95% Confidence Interval			F Test with True Value 0		
	Intraclass	Lower	Upper	Value	df1	df2	Sig

	Correlation	Bound	Bound				
Single Measures	.97	.95	.98	59.14	74	74	.000
Average Measures	.98	.97	0.98	59.14	74	74	.000

The intraclass correlation coefficient (ICC) between direct and MIC determination for Fosfomycin was exceptionally high at .84, indicating a robust consistency. The 95% confidence interval, along with the F test ($P= .000$), further underscored the statistical significance of this strong correlation. Both single and average measures of ICC reflected a high level of reliability in the determination of Fosfomycin MIC determination with and without urine.

Table 7: Intraclass correlation of Fosfomycin MIC with and without urine

		95% Confidence Interval			F Test with True Value 0		
	Intraclass Correlation	Lower Bound	Upper Bound	Value	df1	df2	Sig
Single Measures	.84	.75	.89	11.24	74	74	.000
Average Measures	.91	.86	.94	11.24	74	74	.000

The intraclass correlation coefficient (ICC) between direct and standardised MIC determination for Norfloxacin was exceptionally high at 1, indicating a robust consistency. The 95% confidence interval, along with the F test ($P= .000$), further underscored the statistical significance of this strong correlation. Both single and average measures of ICC reflected a high level of reliability in the determination of Norfloxacin MIC determination with and without urine.

Table 8: Intraclass correlation of Norfloxacin MIC with and without urine

		95% Confidence Interval			F Test with True Value 0		
	Intraclass	Lower Bound	Upper	Value	df1	df2	Sig

	Correlation		Bound				
Single Measures	1.00	1	1	62560.00	74	74	.000
Average Measures	1.00	1	1	62560.00	74	74	.000

The intraclass correlation coefficient (ICC) for Nitrofurantoin MIC, both for single and average measures was high, indicating strong consistency. The 95% confidence interval further supports this, and the F test with a true value of 0 was statistically significant, emphasizing the reliability of the correlation.

Table 9: Intraclass correlation of Nitrofurantoin MIC with and without urine

		95% Confidence Interval			F Test with True Value 0		
	Intraclass Correlation	Lower Bound	Upper Bound	Value	df1	df2	Sig
Single Measures	.85	.77	.89	11.92	74	74	.000
Average Measures	.92	.87	.95	11.92	74	74	.000

The kappa value, a measure of agreement between direct and MIC determination for Co-trimoxazole, Fosfomycin, Norfloxacin was a perfect 1, indicating complete agreement. This strong agreement was statistically significant, supported by a *P*-value of .000. While, kappa value, a measure of agreement between direct and standardised MIC determination for Nitrofurantoin, was .67, indicating substantial agreement. This substantial agreement was statistically significant, supported by a *P*-value of .000. The symmetric measures, including kappa, emphasized the robustness of the agreement, with no observed errors. The analysis was conducted on 75 valid cases, further reinforcing the reliability of the agreement assessment.

Table 10: Kappa value of Co-trimoxazole, Fosfomycin, Norfloxacin, Nitrofurantoin MIC with and without urine

Antibiotics	Kappa Value	Asymptotic Standard Error	Approximately significant
Co-trimoxazole	1	0	.000
Fosfomycin	1	0	.000
Norfloxacin	1	0	.000
Nitrofurantoin	.67	.11	.000

DISCUSSION

In clinical microbiology laboratories the standard procedure for handling specimens for diagnosis of bacteriuria has been semiquantitative culture followed by antimicrobial susceptibility testing of standardized inocula of bacteria obtained from isolated colonies with simultaneous identification of the microorganisms which requires at least 48hrs [13]. In this context of bacterial resistances, decreasing the delay for obtaining AST results is a key to avoid therapeutic failures[14]. Direct antimicrobial susceptibility testing of urine simultaneously with culture for diagnosis of bacteriuria has been advocated with good results by some investigators [15,16,17]. The issue is, meanwhile, controversial and many authorities have in the past discouraged the method [18,19,20]. With conflictive results concerning the usefulness of routine DST, the present study was to evaluate the effect of normal pooled urine on zone of inhibition and MIC value obtained by Kirbybauer disk diffusion method and antibiotic gradient testing respectively.

The present study the prevalence of XDR was 60% which is almost comparable to the study by Sah BK et. al's 63% and in their study MDR was 70.8% which is higher than the present study (10.33%) which may be due to their higher sample size. The antimicrobial susceptibility of other drugs was also comparable to Sah BK et. al's except Fosfomycin. The resistance to Fosfomycin was only 2.66%, various other studies also reported high sensitivity to Fosfomycin [21,22,23]. The prevalence of ESBL production in the present study among *E.coli* was 80% which is in agreement with Kareem et. al and Abayneh et. al which had also a smaller size 40 and 63 respectively. The higher percentage of ESBL production in these studies and be attributed to smaller size while studies with large sample size have shown a lesser percentage of ESBL production as in studies of Behera et. al reported 43.08% in 515 samples, and also by Castillo-Tokumori et al. reported 40.85% in 1158 samples.

The present study evaluated whether pooled filtered urine inoculated with known amount of *E. coli* and any effect on antimicrobial susceptibility pattern. The current study evaluated if urine had any effect on zone of inhibition and MIC by disk diffusion and antimicrobial gradient testing respectively. The zone of inhibition and MIC was compared between routine AST and direct antimicrobial susceptibility using Cohen's kappa test. For antimicrobials Cotrimoxazole, Fosfomycin and Norfloxacin there was a total agreement (kappa value 1) between the methods for zone of inhibition and MIC. These results are in agreement with studies of Mugiraneza et.al, they had categorical agreement for these drugs. In the above study nitrofurantoin exhibited 0.44 kappa value that is moderate agreement, in the present study nitrofurantoin was the only drug exhibited substantial agreement (0.66). various other studies Breteler et.al, Bronnestam et. al and Mohammad RN et. al showed the usability of direct antimicrobial susceptibility testing.

There are two major limitations in this study that could be addressed in future research. One, filter sterilized urine was used which can be replaced with urine smear-positive samples from cases of UTI. Two, only a single uropathogen was employed, other uropathogens singly or in combination can be used.

CONCLUSION

In conclusion, the present study suggest that direct antimicrobial susceptibility testing can be employed for Cotrimoxazole, Fosfomycin, Norfloxacin and Nitrofurantoin which are important drugs in the management of UTI, after further standardization. With direct susceptibility testing the turnaround time could be lowered and antimicrobial therapy can be initiated early. As clinical microbiology diagnostics continues to evolve rapid direct susceptibility testing can be standardised with innovative approaches in near future, thus decreasing the turnaround time.

ETHICAL APPROVAL

The study was approved by Institutional Ethical Committee, Ref No:IEC/30/MICRO/SME-GNR/2022

UNDER PEER REVIEW

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