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Detection of minimum inhibitory concentration and minimum biofilm eradication concentration among biofilm forming uropathogenic *Escherichia coli* for ciprofloxacin and nitrofurantoin

Rashmi SB[1*], Rashmi P Mahale[2]

[1] Department of Microbiology, Gulbarga Institute of Medical Sciences (GIMS), Kalaburagi, Karnataka, India

[3] Associate Professor, Department of Microbiology, Jagadguru Sri Shivarathreeswara University, Mysore, Karnataka, India

Abstract

The present study titled- *"Detection of minimum inhibitory concentration (MIC) and minimum biofilm eradication concentration (MBEC) among uropathogenic E.coli for ciprofloxacin and Nitrofurantoin"* was carried out in the Dept of microbiology, *J.S.S.* Medical College from March to June 2016 to detect MIC and MBEC among uropathogenic *E.coli*.

Total 150 uropathogenic *E.coli* isolated from patients of urinary tract infections were collected and subjected to biofilm formation among them 30 (20%) isolates showed the ability to form biofilm. Out of 150 uropathogenic isolates, 30 isolates were subjected for the detection of MIC and MBEC. Among 30 biofilm forming uropathogenic *E.coli* isolates, 12 (40%) isolates were sensitive and 18 (60%) isolates were resistant to ciprofloxacin in their planktonic state. For Nitrofurantoin antibiotic among 30 biofilm forming isolates, 23 (76.7%) isolates were sensitive and 7 (23.3%) isolates were intermediately sensitive in their planktonic form. Among 30 biofilm forming uropathogenic *E.coli*, None of the isolates showed MBEC below 16 µg/ml, 5 (16.7%) isolates showed MBEC at 16 µg/ml, 17 (56.7%) isolates showed MBEC at 32 µg/ml and rest of 8 (26.6%) isolates showed MBEC at 64 µg/ml of ciprofloxacin. For Nitrofurantoin, 23 (76.7) isolates were in the sensitive range in the planktonic state, whereas only 17 (56.7%) isolates were in the sensitive range in the biofilm state, 7 (23.3%) isolates were in the intermediate range in the planktonic state where as 8 (26.7%) isolates were in the intermediate range in the biofilm state, none were found to be in the resistant range in the planktonic state where as 5 (16.6%) isolates showed resistance to Nitrofurantoin in the biofilm state.

Keywords: *E.coli*, antibiotic resistance, uropathogenic *E.coli*, minimal inhibitory concentration (MIC) and *Minimum Biofilm Eradication Concentration* (MBEC)

1. Introduction

Uropathogenic *Escherichia coli* (UPEC) is the leading cause of urinary tract infection (UTI) and, the most common bacterial infectious diseases encountered in clinical practice [1]. The commonest bacterial agent involved in causation of UTIs is *Escherichia coli*, being the principal pathogen both in the community as well as in the hospital [2, 3]. Urinary Tract Infections (UTIs) are defined as diseases which are caused by a microbial invasion of the genitourinary tract, which extends from the renal cortex of the kidney to the urethral meatus [3]. Acute UTI caused by uropathogenic *E. coli* (UPEC) can lead to recurrent infection, which can be defined as either re-infection or relapse. *E. coli* strains causing relapse and re-infection were analysed that they produce biofilm in vitro. Yersiniabactin and aerobatic was significantly more frequent among strains causing relapse [4]. Recurrent UTIs caused by UPECs represent classical biofilm disease. Biofilms are the microbial communities of the surface-attached cells which are embedded in a self-produced extracellular polymeric matrix [3]. They can cause significant problems in many areas, both in the medical settings (e.g. persistent and recurrent infections, device-related infections) and in the non-medical (industrial) settings (e.g. biofouling in the drinking water distribution systems and in the food processing environments). Biofilm-related infections are inherently challenging to treat and difficult to fully eradicate with normal treatment regimens [5].

In general, more than 100 times the antimicrobial concentrations are needed to kill biofilm forming bacteria than to kill planktonic bacteria [5] MIC is the lowest concentration of an antimicrobial agent that require to inhibit growth of micro-organisms in a media after standard time of incubation [6]. MIC is important in laboratory to confirm resistance of micro-organism to an antimicrobial agent. The concentration of antibiotics required to eradicate cells within the biofilms is uncertain. It is therefore apparent to find out proper concentration of the drug that is capable of inhibiting the formation of biofilms or eradicating existing biofilms [7]. The minimum biofilms eradicating concentration (MBEC) assay could provide good and reliable methodology to assess the susceptibility of equalized cells growing in biofilms antibiotics [7]. The MBEC could represent the concentration of antibiotics capable of killing a biofilm. The study aimed to perform detection of MIC and MBEC among biofilm forming UPEC strains isolated from urine sample.

2. Materials and Methods

Source of data

All *E.coli* isolated from Urine samples from the clinically suspected cases of Urinary Tract Infection (UTI) from JSS hospital, Mysore.

Clinical samples

Urine samples were received to the laboratory and were subjected to routine processing as per standard operating procedures.

Phenotypic identification of *E.coli*

MacConkey agar: flat, dry, pink colonies.

Blood agar: Beta hemolytic, grey, moist, opaque colony.

Colonies were later subjected to biochemical tests such as catalase, oxidase, nitrate reduction, indole production, urease, citrate, TSI, MR-VP test as per standard guidelines

Detection of MIC by E-test method

Preparation of the medium:

Muller Hinton agar plates were prepared by dehydrated powder according to the directions specified on label.

Inoculums preparation

Inoculum was prepared from the primary culture plate. Transfer 4-5 similar colonies with a wire to 5ml Trypticase soya broth and incubate at 37 c for 2-8 hours until turbidity develops. Adjust the turbidity to standard 0.5 McFarland.

Procedure

- Prepared MHA Plates were used.
- Dip a sterile nontoxic cotton swab into the standardized inoculums and rotate the soaked swab firmly against the upper inside wall of the tube to express excess fluid.
- Streaked the entire agar surface of the plate with the swab three times, turning the plate at 60 angle between each streaks.

Detection of minimum biofilm eradication concentration (MBEC)

Assay has been performed with flat bottom, 96 well microliter plates which provided surface for the biofilm formation.

Biofilm formation

The organism isolated from fresh agar plates were inoculated in trypticase soya broth with 2/ glucose incubated for 24 hrs. at 37 c in stationary conditions. Broth was diluted 1:100 with freshly prepared medium. Individual wells of sterile polystyrene 96 well flat bottom culture plate well were filled with 200 µl aliquots of diluted cultures. Only medium served as control to check sterility and nonspecific binding of media. The TCP were incubated for 24 hrs. at 37^0c. After incubation contents of each well was gently removed by taping the plates the wells were washed four times with 0.2 ml of phosphate buffer saline (PSB pH7.2) to remove free floating planktonic bacteria. Biofilms are formed by adherent 'sessile' organisms.

Antibiotic susceptibility

We have taken ciprofloxacin and Nitrofurantoin for MIC and MBEC assay. Stock solutions were prepared as per the manufacturer guidance provided and stored at - 80°c, working solutions was prepared by diluting stock solution 1:5 by adding trypticase soya broth. Biofilms formed on the surface of standard microliter 96 well plate were inoculated with the required dilutions of antibiotics. Antibiotic plate were incubated over night at 37 c and rinsed with 2% PSB. Freshly Prepared (trypticase soya broth) medium is added to the rinsed plates and incubated at 37 c for 24 hrs. MBEC was read, by checking turbidity visually in the wells of recovery plate.

3. Results

The present study entitled "Detection of minimum inhibitory concentration (MIC) and minimum biofilm eradication concentration (MBEC) of Nitrofurantoin and Ciprofloxacin among uropathogenic *E.coli* was carried out in Department of Microbiology, J.S.S. Hospital from January 2015 to December 2016.

Biofilm Formation

In the present study, 30 biofilm forming uropathogenic *E.coli* isolated from urine were collected from clinically suspected cases of UTI. The biofilm forming uropathogenic *E.coli* isolates were subjected to detection of minimum inhibitory concentration (MIC) and minimum biofilm eradication concentration (MBEC) of ciprofloxacin and Nitrofurantoin.

Out of 30 biofilm forming uropathogenic *E.coli* 12 (40%) isolates were from male patients and 18 (60%) isolates were from female patients (Table 1 and Figure 1).

Among 30 isolates majority of the17 (57%) isolates were in the age group of above 40 years followed by 05 (17%) isolates in the age group of 0-10 years, 01(3%) isolate in the age group of 10-20 years, 06 (20%) isolates in the age group of 20-30 years and 01 (3%) isolates in the age group of 30-40 years (Table 2, Figure 2)

Maximum number of biofilm forming uropathogenic *E.coli* 8 (28%) were isolated from OPD patients, 5 among them had history of recurrent UTI followed by 7 (23%) isolates from nephrology/urology department, 2(6%) isolates from ICU, 2 (6%) isolates from emergency, 1 (3%) isolate from OBG department and 4(17%) isolates from other department includes surgery female ward and outsource (Table 3 and Figure 3).

Table 1: Sex wise distribution of the samples

Sex	Number	Percentage
Male	12	40%
Female	18	60%
Total	30	100%

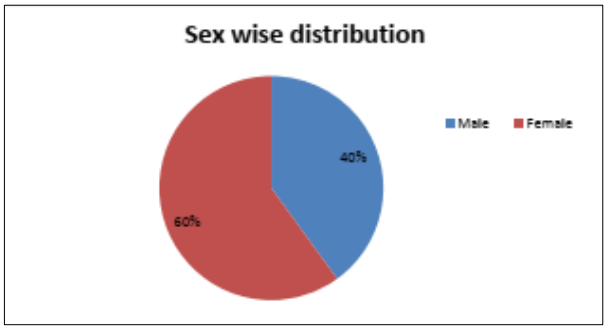

Fig 1: Sex wise distribution of the samples

Table 2: Age wise distribution

Age group	Number	Percentage
1-10 years	5	17%
10 -20years	1	3%
20 -30 years	6	20%
30- 40 years	1	3%
Above 40 years	17	57%
Total	30	100%

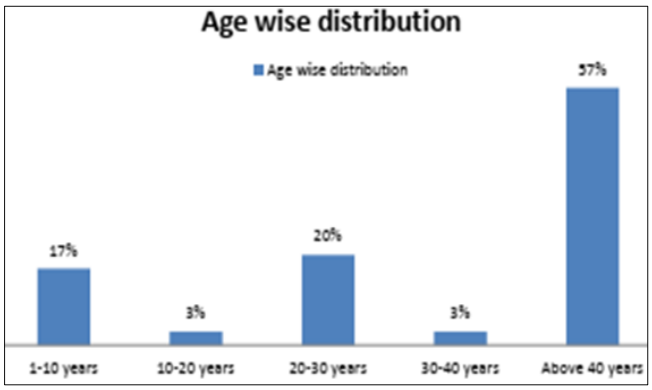

Fig 2: Age wise Distribution

Table 3: Shaming ward wise distribution

Ward wise distribution	Number	Percentage
OPD	8	26.7%
Urology/Nephrology	7	23.3%
Pediatrics	5	16.7%
ICU	2	7%
Emergency	2	7%
OBG	1	3%
Medicine	1	3%
Others	4	13.3%
Total	30	100%

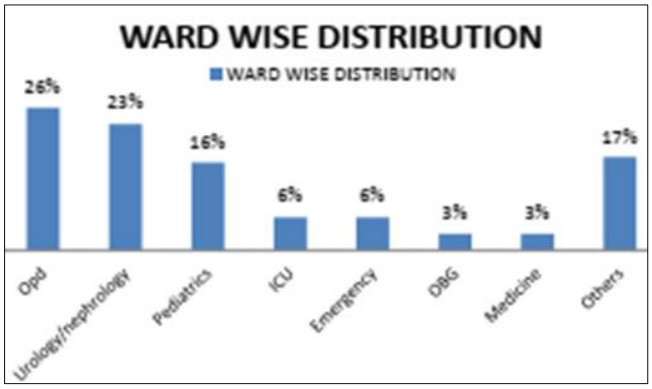

Fig 3: Showing wards wise Distribution

MIC detection by E-strip method for ciprofloxacin

The results show that there were 12 (40%) isolates sensitive and 18 (60%) isolates were resistant to ciprofloxacin in their planktonic form (Table 4, Figure 4 and 5).

Table 4: Showing results of MIC for Ciprofloxacin

S. No.	MIC µg/ml	No of isolates	Results	4%
1	0.006	4	S	40%
2	0.008	6		
3	0.125	1		
4	0.19	1		
5	4	1	R	60%
6	25	1		
	No zone	16		
Total		30		100%

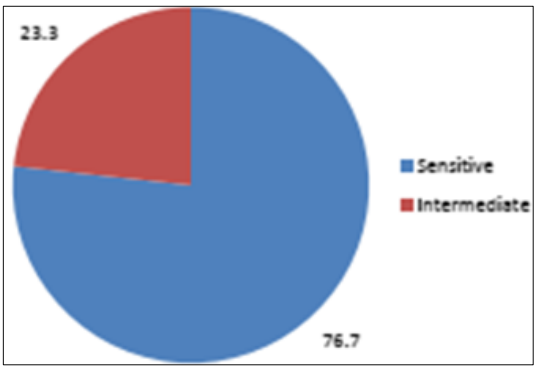

Fig 4: Showing Pattern of MIC

Fig 5: Showing sensitivity zone to Ciprofloxacin

MBEC for ciprofloxacin

None of the isolates showed MBEC below 16 µg/ml, 5 (16.7%) isolates showed MBEC at 16 µg/ml, 17 (56.7%) isolates showed MBEC at 32 µg/ml and rest of 8 (26.6%) isolates showed MBEC at 64 µg/ml of ciprofloxacin (Table 5 and Figure 6).

Table 5: showing resells of MEC of Ciprofloxacin

S. No.	MBEC values	No isolates	%
1	16µg/ml	5	16.7%
2	32µg/ml	17	56.7%
3	64µg/ml	8	26.6%
Total		30	100'.

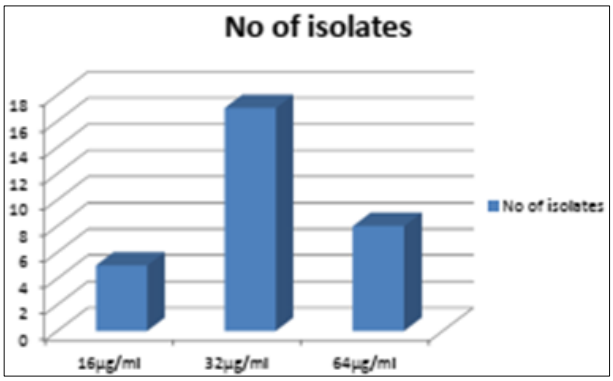

Fig 6: Showing Concentration of MBEC

MIC by E-strip method for Nitrofurantoin

The MIC results shows that 23 (76.7%) isolates were sensitive and 7 (23.3%) isolates were intermediately sensitive and none of the isolates were resistant to Nitrofurantoin in their planktonic state (Table 6 and Figure 7).

Table 6: Showing MIC results of Nitrofurantoin

S. No.	MIC µg/ml	No of isolates	Results	%
1	4	4	S	76.7%
2	6	3		
3	8	5		
4	12	7		
5	16	2		
6	21	1		
7	32	1		
S	42	7	I	23.3%
Total		30		100%

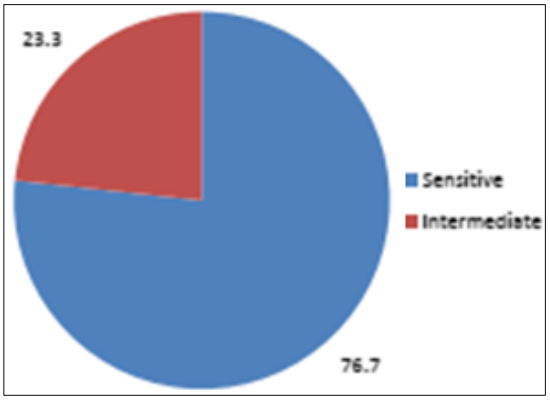

Fig 7: Showing MIC results of Nitrofurantoin

MBEC for Nitrofurantoin

None of the isolates showed MBEC below 16 µg/ml, 9 (30%) isolates showed MBEC at 16µg/ml, 8 (26.7%) isolates showed MBEC at 32 µg/ml, 8 (26.7%) at 64µg/ml, and rest of the 5 (16.6%) isolates showed MBEC at 128µg//ml to Nitrofurantoin (Table 7 and Figure 8 & 10).

Table 7: Showing results of MBEC of Nitrofurantoin

S. No.	MBMC rabies	No of isolates	%
1	16µg/m1	9	30%
2	32µg/m1	8	26.7%
3	64µg/m1	8	26.7%
4	128µg/m1	5	16.6%
Total		30	100%

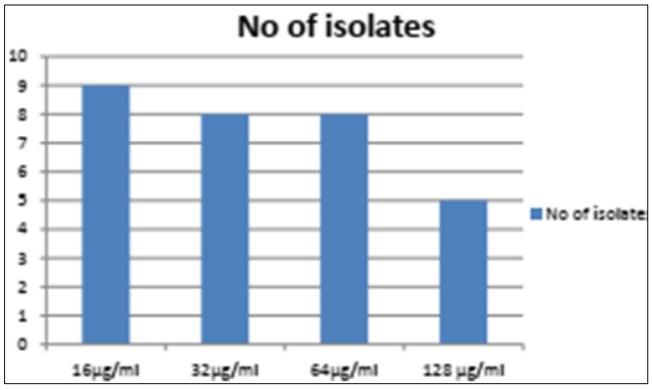

Fig 8: Showing MBEC pattern of Nitrofurantoin

Fig 9: Showing sensitivity to Nitrofurantoin in E-strip

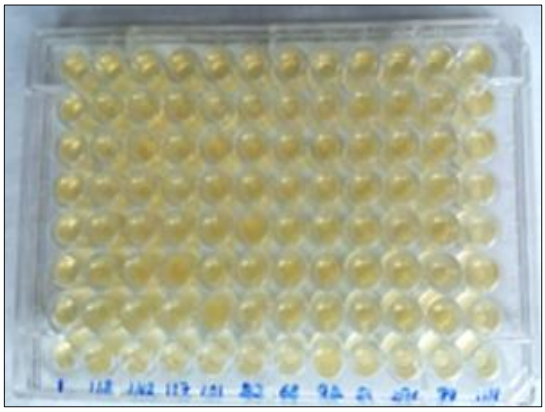

Fig 10: Showing MBEC assay in Microtiter plate

4. Discussion

Urinary tract infections (UTIs) are commonest infection encountered in clinical practice and many of the causative micro-organisms tend to respond to the urinary tract environment by biofilm formation resulting in chronic and often intractable infections.

E.coli is the most predominant pathogen causing community and nosocomial acquired UTIs [8]. Uropathogenic *E.coli* (UPEC) is the most common causative agent of UTI. UPEC present several virulent factors that allow them to colonize host mucosal epithelium [9], the most important virulent factor being ability to form biofilm. In the present study, the frequency of UTI was greater in females 60% as compared to men 40%, this in attributed to anatomic and physiologic factors. Similar results were obtained Nermeen Mohamoud Ahmed Abdallah *et al*, who have reported that frequency of UTI is greater in women 66% as compare to men 34% [10]. Similarly, in a study done by D. Nancy and D. Jagadish *et al*, they found and reported highest occurrence of UTI 61.29% female and 42.10% from males [11]. In our study, we processed 150 *E.coli* isolated from patients of urinary tract infections. Among them 30 (20%) isolates had the ability to form biofilm. Similar results was obtained by Ponnusamy Poovendra *et al*., among 324 isolates subjected to biofilm production, 66 (20.37) showed strong biofilm forming capacity [12]. UTIs are common cause of morbidity and affects person of all age group including young women, children and elderly persons.

In our study, majority of the isolates17 (57%) isolates were in the age group of above 40 years followed by, 06 (20%)

isolates in the age group of 20-30 years, 05 (17%) isolates in the age group of 0-10 years, 01(3%) isolate in the age group of 10-20 years, and 01 (3%) isolates in the age group of 30-40 years. Similar results were obtained Ponnusamy Poovendra higher number 52% of isolates were above age group of 40 years followed by 42%in below age group 40 years. In the present study, *E.coli* was isolated in higher proportion from OPD 8 (26.7%) isolates followed by, urology/nephrology 7 (23.3%) isolates, pediatrics 5 (16.7%) isolates, others include neuro surgery and female surgery ward 4 (13.3%) isolates, ICU 2 (7%) isolates, emergency 2 (7%) isolates, OBG 1(3%) isolate and medicine 1 (3%) isolate. Biofilms typically cause chronic infections, which means that the infections persist despite apparently adequate antibiotic therapy and hosts innate and adaptive defense mechanisms. Consequently, biofilm related infections are inherently challenging to treat and difficult to fully eradicate with normal treatment regimens [5]. Biofilm associated infections are generally recurrent and chronic. It's generally associated with treatment failure because of higher level of drug resistance [11]. In our study, MIC of the biofilm forming *E.coli* for ciprofloxacin and Nitrofurantoin were determined. Among 30 biofilm forming uropathogenic *E.coli* isolates, 12 (40%) isolates were sensitive and 18 (60%) isolates were resistant to ciprofloxacin in their planktonic state. For Nitrofurantoin antibiotic among 30 biofilm forming isolates, 23 (76.7%) isolates were sensitive and 7 (23.3%) isolates were intermediately sensitive in their planktonic form. Similar result were obtained N. A. Ghanwate, reported that 85.72% isolates were sensitive and 14.28% were resistant to Nitrofurantoin in their planktonic state.7 In our study, tried to detect the MBEC of biofilm forming uropathogenic *E.coli* against ciprofloxacin and Nitrofurantoin. The results shows that an increased concentration of antibiotic is required to eradicates the biofilm forming uropathogenic *E.coli* as compared to the eradication of the planktonic bacteria. Among 30 biofilm forming uropathogenic *E.coli*, None of the isolates showed MBEC below 16 µg/ml, 5 (16.7%) isolates showed MBEC at 16 µg/ml, 17 (56.7%) isolates showed MBEC at 32 µg/ml and rest of 8 (26.6%) isolates showed MBEC at 64 µg/ml of ciprofloxacin. Among 30 biofilm forming uropathogenic *E.coli* isolates, 23 (76.7) isolates were in the sensitive range in the planktonic state, whereas only 17 (56.7%) isolates were in the sensitive range in the biofilm state, 7 (23.3%) isolates were in the intermediate range in the planktonic state where as 8 (26.7%) isolates were in the intermediate range in the biofilm state, none were found to be in the resistant range in the planktonic state where as 5 (16.6%) isolates showed resistance to Nitrofurantoin in the biofilm state. Thus it is observed that biofilm forming uropathogenic *E.coli* showed 40% sensitive and 60% resistance in their MIC values for planktonic state and none of them showed sensitive in MBEC for their biofilm state for ciprofloxacin. For Nitrofurantoin, 76.7% were sensitive and 23.3% were resistant in MIC values for their planktonic form and where as 56.7% were sensitive and 43.3% were resistant in their MBEC values for Nitrofurantoin in biofilm form. Whereas, results obtained from the study done by N. Ghanwate, who has reported that MBEC of biofilm forming uropathogenic *E.coli* was increased by 950 times for ciprofloxacin and 17 fold higher for Nitrofurantoin [7]. This study concludes that *E.coli* has a high propensity to form biofilm and thus urinary tract infection (UTI) with biofilm forming uropathogenic *E.coli* (UPEC) may result in treatment failure and chances of recurrent infections as biofilm forming uropathogenic *E.coli* are commonly found to be multi drug resistant. There are several properties of biofilms that could contribute to increased resistance to antibiotics. The exopolysaccharides matrix, or "slime," that surrounds the cells may create an exclusion barrier to antimicrobials or directly complex with these agents to inactive them. Bacteria in biofilms grow more slowly and slower growth may lead to decreased uptake of the drug and other physiologic changes that could affect drug effectiveness. Evaluation of MIC and MBEC to determine changes in the pattern of antibiotic sensitivity of uropathogenic *E.coli* from planktonic to the biofilm phase of growth. It was also found that there was a difference between MBEC and MIC. A higher concentration of the antibiotics ciprofloxacin and Nitrofurantoin were required to eradicate the established biofilm, thus it is better to treat the patients with antibiotics based on biofilm susceptibility rather than planktonic susceptibilities.

5. Acknowledgments
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6. Conflict of interest statement
We declare that no conflict of interest.

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