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CORRELATION BETWEEN BIOFILM FORMATION OF UROPATHOGENIC ESCHERICHIA COLI AND ITS ANTIBIOTIC RESISTANCE PATTERN.

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<u>**ABSTRACT**</u>

BACKGROUND: Microorganisms growing in multilayered cell clusters embedded in a matrix of extracellular polysaccharide (slime) which facilitates the adherence of these microorganisms to biomedical surfaces and protect them from host immune system and antimicrobial therapy. There are various methods to detect biofilm production like Tissue Culture Plate (TCP) ,Tube method (TM) ,Modified Congo Red Agar Method (MCRA),bio luminescent assay ,piezoelectric sensors and fluorescent microscopic examination.

OBJECTIVES : This study was conducted to compare three methods for the detection of biofilms and compare with antibiotic sensitivity pattern, in uropathogenic Escherichia coli.
METHOD: This study was carried out at the Department of Microbiology Dr. B. R. Ambedkar Medical College from Dec 2011 to June 2012. Total number of 107 clinical Escherichia coli isolates were randomly selected from all age groups were subjected to biofilm detection methods and their antibiotic resistance pattern was compared. Isolates were identified by standard phenotypic methods. Biofilm detection was tested by TCP, TM and MCRA methods .
Antibiotic susceptibility test of uropathogenic E coli was performed using Kirby –Bauer disc diffusion method according to CLSI guidelines.

RESULTS: From the total of 107 clinical isolate 74 (69.1 %) isolates showed biofilm formation by all the TCP, TM, CRP methods. Biofilm forming isolates from catheter associated UTI showed drug resistance to more than 6 drugs. Only 2(13.3%) isolates from Asymptomatic UTI showed biofilm by TM & MCRA methods & were sensitive all drugs. Biofilm forming isolates from symptomatic UTI showed mixed drug resistance pattern.

CONCLUSION: We conclude from our study that biofilm formation is more common in catheterized patients. TCP method is more quantitative and reliable method for the detection of biofilm forming micro-organisms as compared to TM and MCRA methods. So TCP method can be recommended for screening of biofilm as virulence marker in drug resistant E coli isolates.
KEY WORDS: Escherichia coli, Biofilm, Drug Resistance, Congo Red Agar, Tissue Culture Plate

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INTRODUCTION: E coli accounts for 70 to 95% of urinary tract infections[1,13]

Bacteria follow ascending route of infections in 90% of urinary tract infections.

These are primarily derived from fecal flora of the host, but haematogenic infections do occur.

Virulence factors like adhesins, toxins , lipo polysaccharides , iron acquisition , presence of capsule, or serum resistance , determine the uropathogenicity of E.coli strains.

Adhesion to epithelial cells of urogenital bacteria is generally accepted as being essential for Uropathogenic bacteria because otherwise the bacteria would be washed out rapidly.

In 100% E.coli isolates from pyelonephritis patients & 17% E.coli isolates from Asymptomatic bacteriuria, pap (pili associated pyelonephritis) was a significant virulence factor.

Uropathogenic E.coli (UPEC) can also express a fimbrial adhensins like AFA I , AFA III , which are encoded by gene clusters.

UPEC can invade and colonise the bladder epithelium establishing a urinary tract reservoir allowing an explanation of recurrent UTI that does not necessarily involve reinfection via the Gastrointestinal tract[1,10]

Biofilm is an aggregate of micro-organism in which the cells are irreversibly attached to substratum or to each other.

They are embedded in a matrix of extra cellular polymeric substances (EPS) in which they have produced and exhibit an altered phonotype with respect to growth rate and gene transcription[2,3]

Availability of key nutrients, chemotaxis towards surface, motility of bacteria, surface adhesins and presence of surface bacteria are some factors which influences biofilm formation[4]

The bacteria enclosed within the biofilm are extremely resistant to treatment. This may be because the drug concentration obtained may be insufficient in certain areas of the film.

The bacteria located at the base of the biofilm are metabolically inactive and are thus resistant to certain antibiotics and possess active antibiotic degradation mechanism that contribute to avoid the accumulation of an effective drug concentration.[19]

So in this study we screened 107 non Repetitive Clinical urinary Isolates of E.coli received in Department of Microbiology Dr. B.R. Ambedkar Medical college by conventional microbiological methods& were subjected to various methods of detections of biofilm production .These include Tissue Culture Plate (TCP)method,[5] Tube method(TM) [6],Modified Congo Red Agar method(MCRA) [7].

The samples received by patients were also divided into three groups namely catheter associated infection, Asymptomatic bacteriuria and Symptomatic bacteriuria.

The antibiotic susceptibility test was carried out by Kirby Bauer Disc diffusion Technique on Mueller Hinton agar.

OBJECTIVE: Evaluation of different detection methods of biofilm formation of uropathogenic *E.coli* and their drug susceptibility pattern.

MATERIALS AND METHODS:

PLACE AND DURATION OF THE STUDY: The prospective study was conducted at the Department of Microbiology, Dr. B.R. Ambedkar Medical College from Dec 2011 to June 2012 .

INCLUSION CRITERIA: Pure growth of Escherichia coli isolates were randomly selected from all age groups.

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E coli isolated from significant bacteriuria, in urine samples of catheterized patients, antenatal cases ,UTI complicated by urinary obstruction, antibiotic therapy, recurrent infections, chronic prostatitis were selected.

EXCLUSION CRITERIA; Insignificant bacteriuria, mixed growth & any other organism showing significant growth .

SELECTION OF THE ISOLATES : Total number of 107 urine E coli isolates were subjected to biofilms detection methods. The urine samples of patients were divided into three groups namely – Catheter associated infections, Symptomatic bacteriuria, and   Asymptomatic bacteriuria.

The organisms were identified by conventional microbiological methods.

The antibiotic susceptibility test was carried out by Kirby Bauer Disc diffusion method using reference strain of positive biofilm producer E.coli ATCC-25922.

Statistical method used was linear correlation.

TISSUE CULTURE PLATE METHOD[5,8]:

The organisms isolated from fresh agar plates were inoculated in Brain Heart Infusion(BHI) with 2% sucrose incubated for 24hrs at 37^0 c in stationary conditions. Broth was diluted 1:100 with fresh medium. Individual wells of sterile polystyrene 96 well flat bottom culture plate well were filled with 200ul aliquots of diluted cultures. Only medium served as control to check sterility and nonspecific binding of media.[9]

The TCP were incubated for 24hrs at 37^0 c. After incubation contents of each well was gently removed by taping the plates. The wells were washed four times with 0.2ml of phosphate buffer saline (PBS pH 7.2) to remove free floating planktonic bacteria. Biofilms formed by adherent 'sessile' organisms in plate were fixed with sodium acetate (2%) and stained with crystal violet (0.1%w/v) for one minute. Excess stain was rinsed off by thorough washing with deionized water and plates were kept for drying. Adherent E.coli cells usually formed biofilm on all side wells and were uniformly stained with crystal violet. Optical Density (OD) of stained adherent bacteria was determined with a micro ELISA auto reader (model 680, Bio rad) at wavelength of 570 nm (OD $_{570nm)}$ These OD values were considered as index of bacteria adhering to surface and forming biofilms[.8]

Experiment was performed in triplicate and repeated three times, the data was then averaged and standard deviation was calculated. To compensate for background absorbance, OD readings from sterile medium, fixation and dye were averaged and subtracted from all test values. The mean OD value obtained from media control well was deducted from all test values.

Classification of Bacteria Adherence:

We classified based on OD values obtained for individual strains of E.coli, but moderate biofilm forming isolates are taken as biofilm forming isolates**.**

Mean OD value	Adherence	Biofilm formation
< 0.120	Non	Non/weak
0.120-0.240	Moderate	Moderate
0.240	Strong	High

Table no 1; Detection of biofilm formation by mean OD values

Negative   Strong positive
OD <0.120   OD > 0.240

Fig 1 Detection of biofilm formation by mean OD values.

TUBE METHOD : A quantitative assessment of biofilm formation was determined as previously described by Christensen [et al] BHI with2% sucrose was inoculated with loopful of microorganisms from overnight culture plates incubated for 24hrs at 37 c. Tubes were decanted and washed with PBS and dried tubes were stained with crystal violet 0.1%. Excess stain was removed and tubes were washed with deionized water Tubes were then dried inverted position and observed for biofilm formation.

Biofilm formation was considered positive when visible film lined the wall and bottom of the tube ring formation at the liquid interface was not indicative of biofilm formation, and interpreted as positive (tube 1) as negative(tube2) . Experiments were performed in triplicate and repeated three times[.8] but moderate biofilm forming isolates are taken as biofilm forming isolates
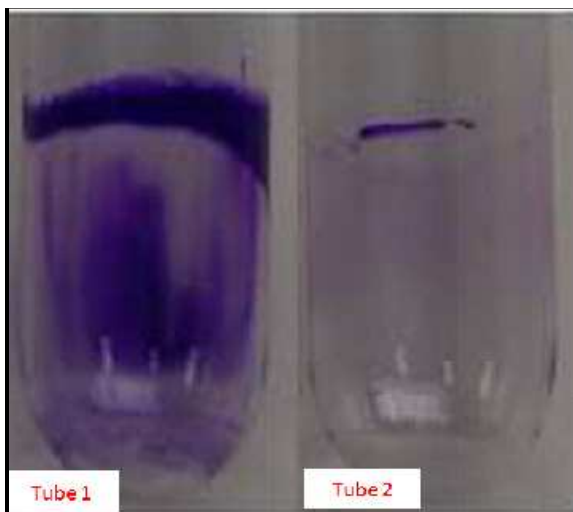
Figure 2 . Tube 1 showing strong biofilm formation. Tube 2 negative for biofilm production.

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MODIFIED CONGO RED AGAR METHOD (CRA): Freeman et al[15] had described an alternative method of screening biofilm formation by E coli isolates. In this study we use modified Congo red agar for biofilm formation. In vitro slime production ability on the published Congo red agar by Freeman et al (1989) in diffuse black pigment in the agar with growth of black pigmented colonies but pigmentation decreased with time. In the present study the modified Congo red agar (MCRA) was optimized to get strong black pigmentation at 48hrs incubation and then for 2-4 days room temperature. Black colored colonies with dry crystalline consistency interpreted- as positive biofilm producing strains. Red coloured colonies- interpreted as negative for biofilm production

Composition /Litter	Congo Red Agar	Modified Congo red agar
Congo red	0.8g	0.4g
Sucrose	36g	-
Glucose	-	10g
BHAI	52g	-
BAB-2	-	40g
Water	100ml	1000ml

Table 2; composition of Congo red agar.

fig 3 Congo red agar plate showing biofilm formation a) dry crystalline colonies-positive for biofilm, b)red colored colonies-negative for biofilm

Antibiotic susceptibility test of biofilm producing bacteria was done on Mueller Hinton agar using the following antibiotic discs[12]:
Norfloxacin, Nitrofurantoin, Ceftizoxime,ceftazidime, Amoxyclavulanic acid, Cotrimoxazole, Ampicillin ,Amikacin Imipenem,Tetracycline.
All the antibiotic disc were obtained from the Himedia. E coli ATCC 25922 were used as positive control and susceptibility testing was performed by using the Kirby Bauer Disc Diffusion Technique according CLSI guidelines.

RESULTS: Among 107 patients 65 were males and 42 were females. From the total of 107 clinical isolates 74(69.1 %) isolates showed biofilm formation by all the TCP,TM,CRP methods. Among 67 catheter associated UTI ,60 (89.5%)isolates produced biofilm by all the three methods .

Among Asymptomatic bacteriuria out of 15 isolates none produced biofilm by TCP method. 2(13.3%) produced biofilm&13(86.6%) were non biofilm producer by TM &MCRA method .

Among symptomatic UTI complicated from urinary obstructions, antibiotic therapy, recurrent infections, chronic site of infections(chronic prostatitis or stones) , 14(56%) were biofilm producers by TCPmethod,12(48%) by TM method,18(72%) by MCRA method, as shown in table3 & chart 3.

E coli isolated from	TCP		TM		MCRP	
	biofilm	nonbiofilm	Biofilm	Nonbiofilm	biofilm	Nonbiofm
Catheter associated infection (n=67)	60(89.5%)	7(10.44%)	60(89.5%)	7(10.44%)	60(89.5%)	7(10.44%)
Asymptomatic bacteriuria (n=15)	0	15	2(13.3%)	13(86.6%)	2(13.3%)	13(86.6%)
Symptomatic bacteriuria (n=25)	14(56%)	11(44%)	12(48%0	13(52%)	18(72%)	7(28%)

Table no 3 Above table shows percentage of biofilm & non biofilm forming *E.coli* isolates &its correlation with patient's condition.
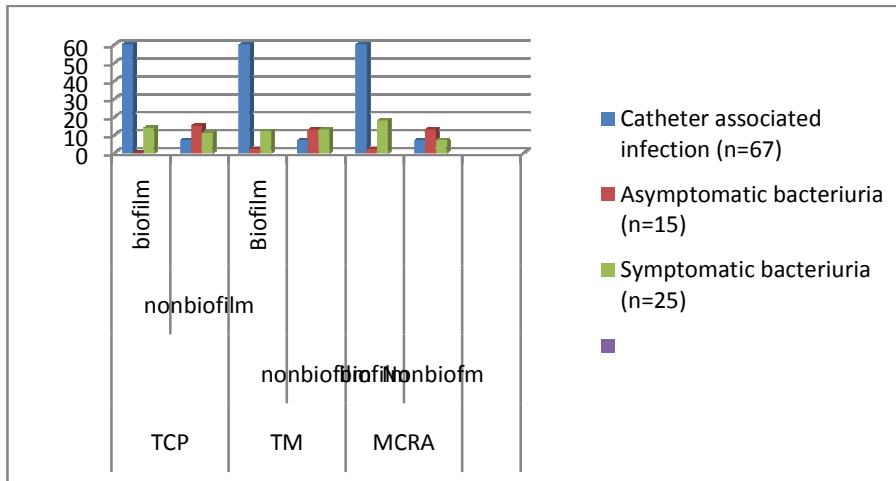

CHART NO 1 : ABOVE CHART SHOWS PERCENTAGE OF BIOFILM & NON BIOFILM FORMING *E.COLI* ISOLATES & ITS CORRELATION WITH PATIENT'S CONDITION.

Table 4 & chart 4 shows resistance percentage of each antibiotic. These biofilm producing isolates were compared with drug resistance pattern as shown table 5&chart 3. Out of 74 biofilm producing isolates ,60 were catheter associated UTI &14 were from symptomatic UTI. 70(94.5%) by TCP, 68(91.5%) by TM method,& 71(87.6%) by MCRA method showed drug resistance to more than 6 drugs. *E coli* isolates from asymptomatic UTI showed sensitive to all drugs. Isolates from symptomatic UTI showed mixed drug resistance pattern.

Antibiotic used	Number of isolates showed resistant's (%) n=107
Ampicillin(amp)	90(84%)
Cotrimoxozole	82(76.6%)
Amoxyclav	75(70%)
Amikacin	58(54.2%)
Ceftizoxime	74(69.1%)
Ceftazidme	79(73.8%)
Norflox	81(75.7%)
Nitrofurantoin	78(72.9%)
Tetracycline	74(69.8%)
Imipenem	30(28.3%)

**Table -4 Resistance percentage of each antibiotic drug to E coli isolates**
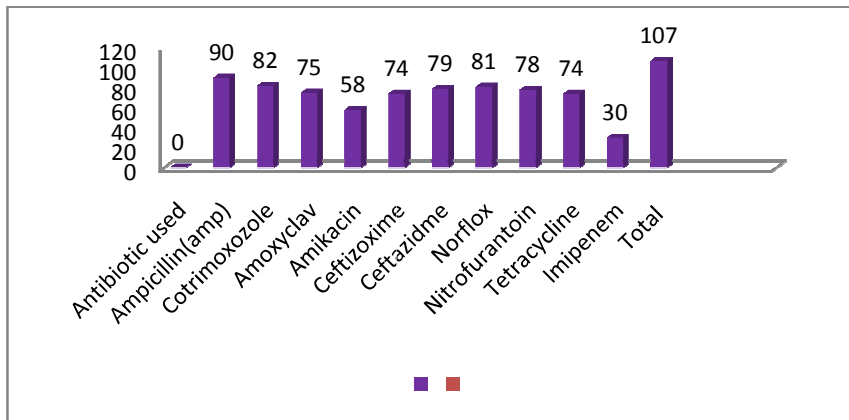
Chart 2 Resistance percentage of each antibiotic drug to E coli isolates.

	TCP	TM	CRM
Resistance to more than 6 drugs	70(94.5%)	68(91.8%)	71(87.6%)
Resistance to 5 to 6 drugs	4(5.4%)	6(8.1%)	6(7.4%)
Sensitive to all	0	0	3(4.9%)
Total	74	74	80

Table 5 The above table shows the relation between biofilm formation and antimicrobial resistance pattern.
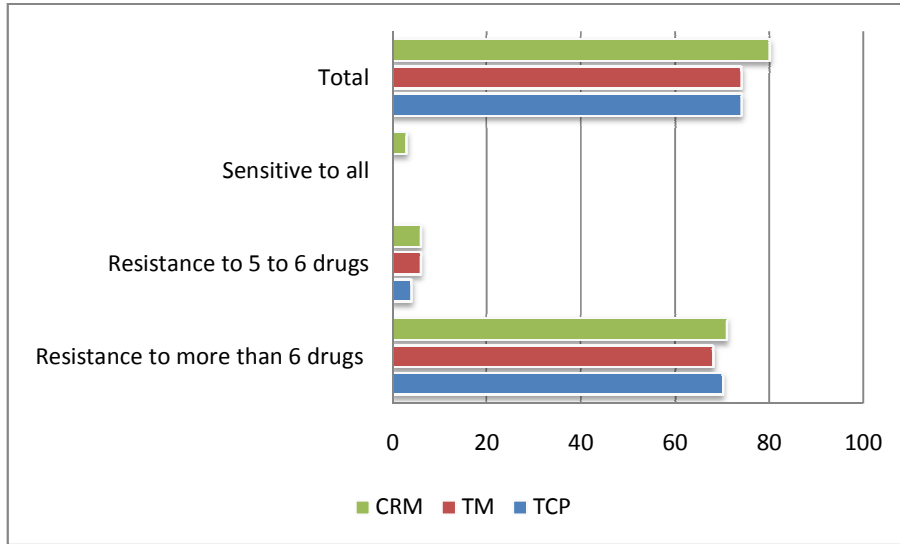
Chart 3 The relation between biofilm formation and antimicrobial resistance pattern

Antibiotics	Catheter associated infection N=60	Symptomatic bacteriuria N=14	Non biofilm Forming Isolates N =33.
Ampicillin(amp)	60	14	16
Cotrimoxozole	60	14	8
Amoxyclav	58	12	5
Amikacin	50	8	0
Ceftizoxime	60	14	0
Ceftazidme	60	14	5
Norflox	60	14	7
Nitrofurantoin	60	14	4
Tetracycline	60	14	0
Imipenem	24	6	0

Table 5 The above table shows correlation between antibiotic resistance pattern with patient's condition & biofilm formation. (N=number of biofilm positive isolates).
Chart 4 The above chart shows correlation between patient's condition ,biofilm formation & antibiotic resistance pattern. N=number of biofilm positive isolates.

DISCUSSION: Bacterial adhesion has long been considered as virulence factor contributing to infection associated with catheter and other including medical devices.[13]
There are two possible explanations for the ability of *E coli* species to colonies artificial materials . The first and the bacterial production of polysaccharide slime, the second one is the presence of adhesins for the host matrix proteins that are absorbed on to the biomedical surface.[15]
There are various methods for biofilm detection[5,6,7].

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In this study we evaluated 107 isolates from urine samples by three screening methods for their ability to form biofilms .

In our study we found that the majority of biofilm producing bacteria was form catheterised patients (89.5%) similarly , Donlan [2,3] reported in his study the association of biofilm producing bacteria with urinary catheters.

In our study we performed TCP and TM by addition of 2% sucrose to BHI broth. Sugar supplementations is essential for biofilm formation [8].This was reported by studies conducted by Mathur T et all.

In modified TCP method extended incubation for 24hrs could lead to a better discrimination between moderate and non- biofilm producing isolates[17]. This was also observed in our study.

Biofilm exhibits more resistance to broad Spectrum antibiotics[20]. This supports that biofilm adds to the virulence profile of microorganism's biofilm production. [16]

The Tube method correlates well with TCP test for strong biofilm production but it was difficult to discriminate between weak and biofilm negative isolates due to the variability is observed results by different observers so in agreement with previous report Tube test cannot be recommended as a general screening test to identify biofilm producing isolates.[18]

CONCLUSION: By this study we conclude that biofilm forming microorganisms show resistance to many drugs. TM method showed 74 isolates as biofilm formers ,TCP method also showed 74 isolates as biofilm formers but MRCA showed 80 isolates as biofilm formers .Only 74 isolates that showed biofilm formation correlated   with patients condition and antibiotic resistance pattern ,by this 6 isolates were false positive by MRCA method .TCP and TM method are more reliable for detection of biofilm formation ,when correlated with patient's clinical condition and drug resistance pattern of biofilm forming isolates TM method is more subjective ,differs with different observers, so TCP method is more reliable and accurate among all , as CRA method showed false positive. Our study concludes TCP method is better method for screening of biofilm formation as virulence marker in drug resistant E coli isolates.

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