

# Slime Production Among Uropathogenic Bacterial Isolates: Evaluating Different Phenotypic Detection Methods

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## ABSTRACT

**Background:** Biofilms are surface associated bacterial communities surrounded by a matrix of Exo -polymers. Biofilms contribute to the development of chronic urinary infections, refractory to antibiotic therapy.

**Methods:** This prospective study was done on 150 non-repetitive urine samples from catheterized patients. Semi-quantitative bacterial culture was performed and isolates were identified by standard biochemical tests. Biofilm formation was detected by Congo Red Agar Method (CRA), Tube Method (TM) and Tissue Culture Plate Method (TCP).

**Result:** Significant bacteriuria was observed among 113/150(74.66%) samples. Gram negative bacteria (GNB) were predominant isolates 101(89.38%) and among GNB, *Escherichia coli* was commonly isolated 30/101(29.70%). Non *E.coli* Enterobacteriaceae (NECE) isolates include *Klebsiella spp.* 25/101(24.75%), *Citrobacter spp.* 07/101(6.93%), *Proteus spp.* 17/101(16.83%) among non-fermenter bacteria *Pseudomonas aeruginosa* 21/101(20.79%). Gram positive bacteria (GPB) constitute 12/113(10.61%) among total isolates. Biofilm producers detected by TCP were 79(69.91%), TM 60(53.09%) and CRA 11(9.73%) respectively.

**Conclusion:** Detection of biofilms can be recommended for recurrent and recalcitrant infections before institution of empirical antibiotics. TCP method with good reproducibility and specificity which can be used for detection of biofilms in resource limited settings.

**Keywords:** Bacterial Biofilm, Uropathogens, TCP, Urinary Catheters

## Introduction

First described by Leeuwenhoek, as sessile bacterial communities characterized by irreversible adherence to each other or substratum embedded in matrix of extra cellular polysaccharides (EPS), called biofilm.<sup>[1]</sup> With the advancement in medicine and increased use of life saving prosthetic devices, microbial biofilm has emerged as significant clinical problem in treatment and management of infectious diseases.<sup>[2]</sup> Researchers have estimated 60-80 percent of microbial infections in body are caused by bacteria growing as a biofilm, opposed to planktonic bacteria.<sup>[3]</sup>

Over past few decades much has been learned about biofilm physiology. Biofilms display unique properties of multidrug tolerance and resistance to both opsonization and phagocytosis, enabling them to survive in hostile environmental conditions and to resist selective pressures. Because of inert nature of bacterial biofilms, host immunity is totally ineffective at clearing them. Biofilms in vivo are very difficult to diagnose essentially due to the lack of sampling methods and markers.<sup>4</sup>

Thus, this present study is designed to shed light on current understanding about biofilm phenomenon, its impact on

indwelling urinary catheter associated infection and to compare *in vitro* qualitative and quantitative detection techniques in an attempt to choose sensitive and easy to perform screening method.

## Materials and Methods

**Study Design:** This prospective study was undertaken in the Department of Microbiology in a tertiary care hospital. A total of 150 non-repetitive urine samples from catheterized patients (symptomatic and asymptomatic) admitted in various clinical wards of hospital were collected after obtaining informed consent.

**Collection And Sample Processing:** Samples collected in sterile containers with proper aseptic precautions were cultured on Mac Conkey agar and blood agar, incubated overnight aerobically at 37°C. A specimen was considered positive, if a single / two potential pathogens were cultured at a concentration of  $\geq 10^3$  Colony forming unit (CFU)/ml from catheterized urine specimens<sup>5</sup>. Organisms were identified by conventional microbiological methods<sup>5</sup> and subjected to biofilm detection by following methods.

### Biofilm Detection Method

1. **Tissue Culture Plate Method (TCP)**<sup>6</sup> Organisms isolated from fresh agar plates were inoculated in Brain

heart infusion broth (BHI) with 2% sucrose. Broth was incubated at 37°C for 18 hours. The broth culture was then diluted 1:100 with fresh medium. Individual wells of sterile 96 well flat bottom polystyrene tissue culture plates were filled with 200ul of the diluted cultures. The wells were washed with phosphate buffer saline (pH 7.2). Biofilm formed by bacteria adherent to the wells was fixed by 2% sodium acetate and stained by crystal violet (0.1%). Excess stain was removed by using deionized water and plates will be kept for drying. Optical density (OD) of stained adherent bacteria was determined with an ELISA reader at wavelength of 570nm. These OD values were considered as an index of bacterial adherence and biofilm formation.

**Table 1: Classification of bacterial adherence by TCP method.**

Mean OD	Adherence	Biofilm Formation
<0.120	Non	Non/weak
0.120-0.240	Moderate	Moderate
>0.240	Strong	High

- 2. Tube Method (TM)**<sup>7</sup> A qualitative assessment of biofilm formation was done using TM. A loopful of organisms from overnight culture plates were inoculated in 10ml of BHI with 2% sucrose in test tubes. The tubes were incubated at 37°C for 24 hours. After incubation, tubes were decanted and washed with crystal violet (0.1%). Excess stain was washed with deionized water. Tubes dried in inverted position and observed for biofilm formation. Biofilm formation was considered positive when a visible film lined the wall and bottom of the tube. Tubes were examined and the amount of biofilm formation was scored as 0-absent, 1-weak, 2-moderate and 3-strong.
- 3. Congo Red Agar Method (CRA)** -Freeman et al<sup>8</sup> had described an alternative method for screening

biofilm formation which requires the use of a specially prepared solid medium-Brain heart Infusion broth (BHI) supplemented with 5% sucrose and Congo red. The medium was composed of BHI-37gm/l, sucrose-50gm/l, agar 10gm/l and Congo red stain-0.8gm/l. Congo red was prepared as concentrated aqueous solution and autoclaved at 121°C for 15min, separately from other medium constituents and then added when the agar had cooled to 55°C. Plates were inoculated and incubated aerobically for 24 to 48 hours at 37°C. Positive results were shown as black colonies with a dry crystalline consistency.

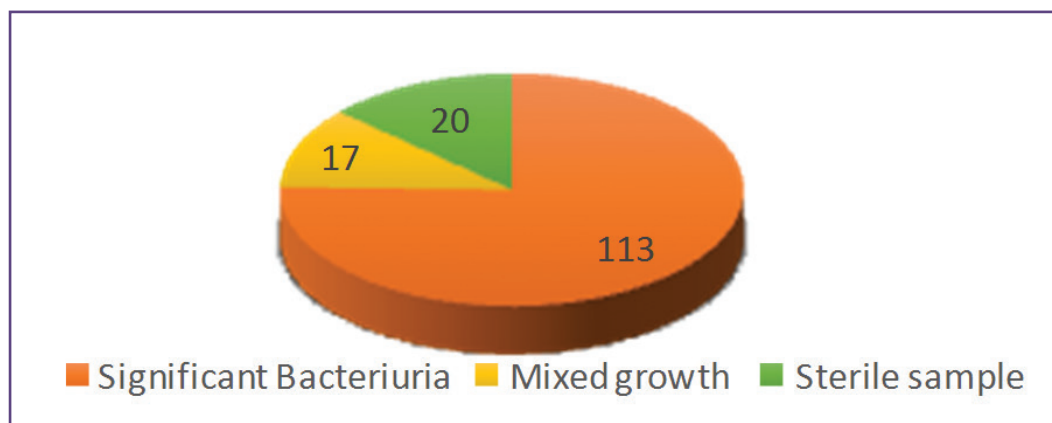
## Result

Demographic characteristics of the study subjects showed that among 150 catheterized patients (age range 16-65 years) majority were males 112 (74.66%) and 38(25.33%) were females and there was no predilection of infection to any particular gender, patients of both genders were affected, males being marginally more than females.

Significant bacteriuria was observed among 113/150 (75.33%) patients. Distribution of culture results is shown in figure 1.

Out of 113 isolates, 101(89.38%) were gram negative bacteria (GNB) and 12(10.61%) were gram positive. *Escherichia coli* was most common isolate 30/101(29.70%). Non *E. coli* *Enterobacteriaceae* (NECE) isolates include *Klebsiella spp.* 25/101(24.75%), *Citrobacter spp.* 07/101(6.93%), *Proteus spp.* 17/101(16.83%) among non-fermenter bacteria *Pseudomonas aeruginosa* 21/101(20.79%). Bacteriological profile of isolates is discussed in figure 2.

Isolates were screened for biofilm formation by TCP, TM and CRA methods. Results of phenotypic detection methods are shown in Table 2.



**Fig. 1: Distribution of culture results.**

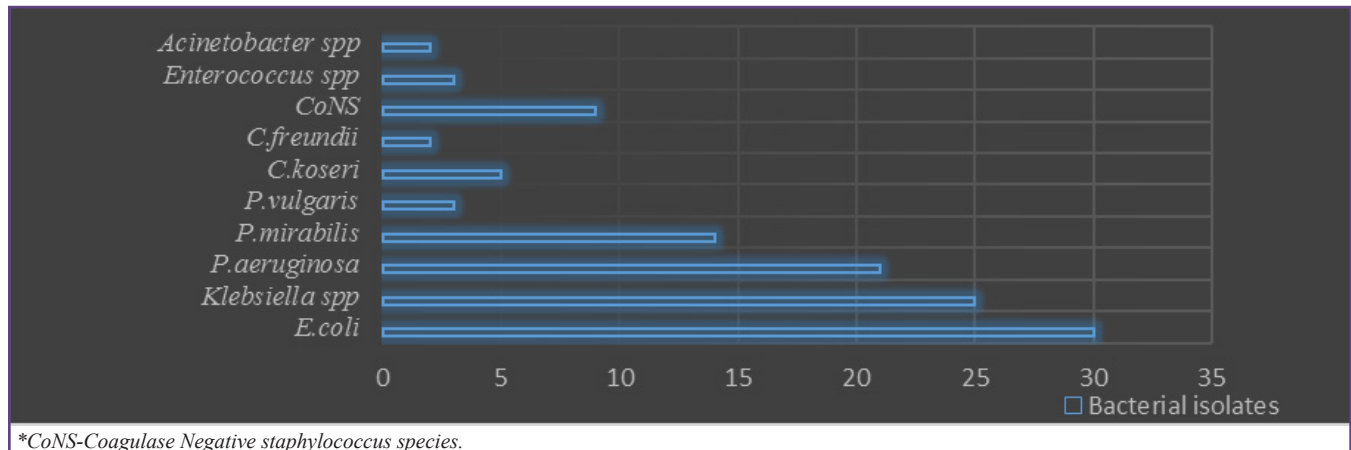


Fig. 2: Bacteriological Profile of Isolates.

Table 2: Biofilm detection by various methods.

Bacterial Isolates	Detection Methods					
	Tissue culture plate Method		Tube Method		Congo red Agar Method	
	(n)	(%)	(n)	(%)	(n)	(%)
n=113	79	69.91	60	53.09	11	09.73

Maximum biofilm producers were detected by TCP 79(69.91%) as compared to TM 60(53.09%) and CRA 11(9.73%) respectively. TM showed good correlation with the TCP assay for strong biofilm forming isolates and total 19 were picked up as strong and 41 as moderate and 53 as weak respectively. However, it was difficult to discriminate between moderate and weak biofilm producing isolates. By CRA method, most strains displayed pink to orange colonies. Only 11/113 (9.73%) isolates displayed black colonies with or without dry crystalline morphology. No correlation between colony morphology on CRA and TCP results was observed. A result of biofilm formation by various methods is shown in Table 3.

TCP method was considered the gold-standard for this study and compared with data from TM and CRA

methods. Parameters like sensitivity, specificity, negative predictive value, positive predictive value and accuracy were calculated. True positives were biofilm producers by TCP, TM and CRA method. False positive were biofilm producers by TM and CRA method and not by TCP method. False negative were the isolates which were non-biofilm producers by TM and CRA but were producing biofilm by TCP method. True negatives are those which were non biofilm producers by all the three methods. Sensitivity and specificity of TM was 73.90% and 96.85% respectively. For CRA method, sensitivity and specificity remained low and were 11.24% and 98.43% respectively (Table 4).

## Discussion

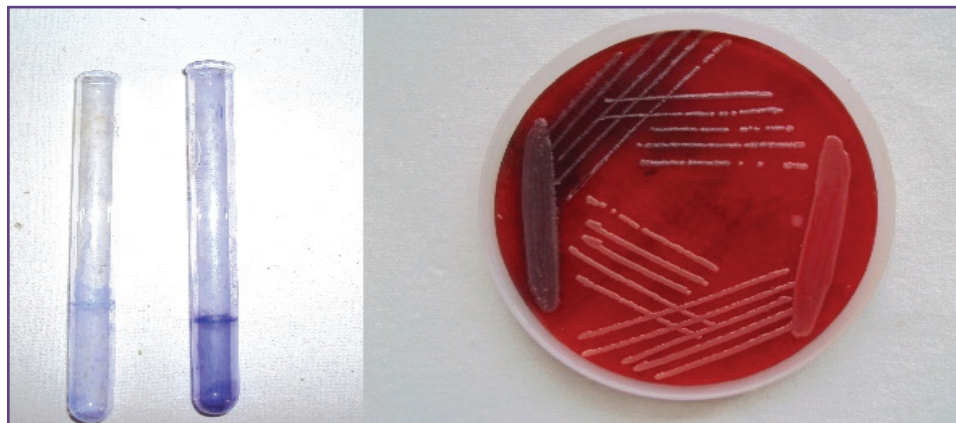
Urinary tract infection, its diverse clinical syndromes and affected host groups, remains one of the most common but widely misunderstood and challenging infectious diseases

Table 3: Detection of biofilm formation .

Biofilm Formation	Tissue Culture Plate	Tube Method	Congo Red Agar
High	25	19	11
Moderate	54	41	-
Weak/None	34	53	102

Table 4. Stastical analysis of biofilm formation by Tube method & Congo red method.

Biofilm detection Method	Sensitivity (%)	Specificity (%)	Positive Predictive Value (%)	Negative Predictive Value (%)	Accuracy (%)
TM	73.90	96.85	97.87	65.43	81.65
CRA	11.24	98.43	93.33	36.13	40.69



**Fig. 3: Biofilm detection by Tube Method and Congo Red Agar Method.**

encountered in clinical practice. Risk of developing urinary tract infection increases significantly with use of indwelling devices.<sup>[9]</sup> Catheter in urinary tract disrupts normal host defenses, resulting in over distension of the bladder and incomplete voiding that leaves residual urine for microbial growth favoring bacterial colonization. Due to introduction of foreign body, pathogens require few recognized virulence factors to colonize and establish infection than to infect fully functional urinary tract.<sup>10</sup> Biofilm mode of growth has been well documented as one of the important cause of prosthetic device related infections that are refractory of treatment.<sup>[11]</sup>

In present study significant bacteriuria was observed as 75.33% (occurrence of high incidence of single infection in our study may be explained by the heavy use of broad spectrum antibiotics). This is in concordance with Bakke et al<sup>[12]</sup> who observed 50.6% significant bacteriuria among catheterized patients. However, Teshager L and colleagues<sup>[13]</sup> observed 43.30% significant bacteriuria among catheterized. This suggested, over time urinary catheters become colonized with microorganisms living in sessile state within the biofilm, rendering them resistant to antimicrobial and host defenses and virtually impossible to eradicate without removing the catheter.<sup>[14]</sup> Not only does the urinary catheter invite biofilm formation, but presence of catheter itself impairs many normal defense mechanisms. Also, urinary catheter connects heavily colonized perineum with the normally sterile bladder, and it provides a route for bacterial entry along both its external and internal surfaces.<sup>[15]</sup>

GNB were predominant isolates 101(89.38%) and *Escherichia coli* was most common isolate 30/101(29.70%). Other isolates include *Klebsiella spp.* 25/101(24.75%), *Citrobacter spp.* 07/101(6.93%), *Proteus spp.* 17/101(16.83%) and *Pseudomonas aeruginosa* 21/101(20.79%). Gram positive (GPB) constitute

12/113(10.61%). This is in accordance with Hassan et al<sup>16</sup> and Niveditha S et al<sup>[17]</sup> who also observed *E.coli* as most common uropathogen among catheterized patients.

In current study, biofilm production was seen in 69.91% of uropathogens. Though many studies<sup>[17,18]</sup> have shown variable biofilm formation (45% -60%) among uropathogens. With respect to phenotypic biofilm detection methods we screened isolates by TCP<sup>[6]</sup>, TM<sup>[7]</sup> and CRA<sup>[8]</sup> methods because of their ease of performance in routine laboratory settings for determining biofilm formation. TCP method detected 79/113(69.91%) as biofilm producers. Out of which, 25 were high, 54 were moderate and 34 were non biofilm producers.

Tube method detected 60/113 (53.09%) as biofilm producers. Among them, 19 were strong and 41 were moderate and 53 were non biofilm producers. With CRA method most strains displayed red (pink to orange) colonies and only 11(9.73%) showed black colonies with dry crystalline morphology (Table 3). Our results are in agreement with other investigators as shown in table 5.

Considering TCP as gold standard in this study parameters like sensitivity, specificity, NPV, PPV and accuracy were calculated. Our data indicates that TM is 73.90% sensitive, 96.85% specific and 81.65% accurate method. Based on our observations we don't recommend CRA method as suitable method for biofilm screening. (Table 4). Our results are in agreement with other investigators as shown in table 6.

Other studies<sup>[23,24]</sup> comparing TM with gold standard TCP demonstrated sensitivity varies from 61% to 100% and specificity 66% to 100%.

But due to subjective variations in interpretation and lack of reproducibility among test results TM cannot be suggested as general screening test to identify biofilm



**Table 5: Biofilm formation observed by TCP, TM and CRA in other studies.**

Year	Author	Biofilm detection method		
		TCP	TM	CRA
2006	Turkyilmaz et al (19)	50.50%	55.55%	61.10%
2011	Hassan et al (20)	63.63%	54%	11%
2014	Nabajit Deka (21)	83%	57%	20%
2015	Mohamad E et al (18)	45.6%	38.11%	36.9%
2015	Tayal R et al (22)	27%	37.96%	40.88%

**Table 6: Sensitivity and specificity as observed by other authors.**

Year & Author	TM		CRA	
	Sensitivity	Specificity	Sensitivity	Specificity
2011, Hassan A et al (20)	73.00%	92.50%	11.00%	92.00%
2015, Tayal R et al (22)	94.59%	83.00%	94.59%	81.00%
Present study	73.90%	96.85%	11.24%	98.43%

producing isolates. Thus, we conclude from our study that TCP is a quantitative and reliable method to detect biofilm forming microorganisms. When compared to TM and CRA methods, TCP can be recommended as a general screening method for detection of biofilm producing bacteria in laboratories.

## Conclusion

Unique and changing properties of biofilm-positive microbes made them responsible for recalcitrant infection, which are difficult to eradicate. The presence of biofilm is probably underestimated because of lack of in vitro diagnostics. Thus, dynamic research activity is required in this field as this bacterial lifestyle may be associated with human infectious diseases.

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