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Research Article

DETECTION OF BACTERIAL BIOFILM IN PATIENTS WITH INDWELLING URINARY CATHETERS

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ABSTRACT
The microbial biofilms pose a public health problem for the persons who require indwelling medical devices, as the microorganisms in the biofilms are difficult to treat with antimicrobial agents. The present study included the isolation and the biofilm formation of the uropathogens in patients with catheter associated urinary tract infections. This prospective analysis which was carried out in the department of Microbiology, GMC, Amritsar, included 400 urine samples from catheterized patients and 100 urine samples from non catheterized patients. Following the bacterial isolation and identification, all the isolates were subjected to biofilm detection by tissue culture plate method, tube method and congo red agar method. Significant bacteriuria was observed among 79.00% of catheterized patients. *E.coli* (29.74%) was the most commonly isolated followed by *K.pneumoniae* (21.84%). In the current study 71.23% strains were positive in vitro for biofilm production. *E.coli* (28.19%) was the most common biofilm producer followed by *P.aeruginosa* (22.47%), *K.pneumoniae* (22.03%) and *P.mirabilis* (15.42%). As, Biofilm-disrupting strategies offer promise for the future but have little immediate applicability. Implementation of infection control measures to improve catheter function and remove unnecessary catheters can be done at the present time.

Keywords- *Bacterial Biofilm, Urinary Catheters, E.coli, Drug Resistance*

INTRODUCTION
Biofilm is an assemblage of the microbial cells that is irreversibly associated with a surface and usually enclosed in a matrix of polysaccharide material (Kokare *et al.*, 2009). When bacteria adhere to surfaces and become sessile, secreting a slimy glue like substance for anchorage, they form a biofilm (Costerton *et al.*, 1995) .Biofilm on indwelling medical devices may be composed of gram-positive or gram-negative bacteria or yeasts. Bacteria commonly isolated from these devices include the *Enterococcusfaecalis, Staphylococcus aureus, Staphylococcus epidermidis* and among the gram-negative bacteria *Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis* and *Pseudomonas aeruginosa*. Theseorganisms may originate endogenously or exogenously (Stickler, 1996).

30% of biofilm forming bacteria are isolated from indwelling medical devices such as endotracheal tubes followed by central venous catheters and urinary catheters being most common sites of biofilm forming bacterial colonization (Christensen *et al.*, 1985). The longer the urinary catheter remains in place, the greater is the tendency of these organisms to develop biofilms, which may result in urinary tract infections.

Biofilms have major medical significance as they decrease the susceptibility to the antimicrobial agents. Furthermore, the proximity of cells within a biofilm can facilitate a plasmid exchange and hence enhance the spread of antimicrobial resistance (Watnick and Kotler, 2000).

The present study was designed to shed light on the biofilm phenomenon formed on the indwelling urinary catheters, qualitative and quantitative detection techniques *in vitro* and the antibiotic susceptibility testing in a trail to help the clinicians make a precise decision regarding treatment of such infections.

MATERIALS AND METHODS
The present study was a prospective study, undertaken from October 2011- December 2013. The study population comprised of 400 urine samples taken from catheterized patients and 100 urine samples from

non catheterized patients taken as control group. Written & Informed consent of the patients was taken. Approval from the ethical committee was taken. Urine samples from catheterized patients were collected in sterile containers with proper aseptic precautions. Clean catch mid stream (CCMS) urine samples from non catheterized patients were taken in case of control group. Urine specimen was transported to the laboratory with minimum delay.

Processing of Specimens*:* Isolation of uropathogens was performed by a surface streak procedure on both blood and MacConkey agar using calibrated loops for semi quantitative method (Forbs *et al.*, 2007) and incubated aerobically at 37^0 C for 24 hours.

A specimen was considered positive, if a single / two potential pathogens were cultured at a concentration of $\geq 10^5$ Colony forming unit (CFU)/ml from CCMS urine or catheterized urine or $\geq 10^3$ CFU/ml of single potential pathogen from catheterized urines specimens, inoculated onto blood agar and Macconkey agar plates and incubated aerobically at 37^0C for 18-24 hours (Forbs *et al.*, 2007).

After 24 hours of incubation, the plates were observed for bacterial growth, colonies were examined, the identification of the isolated bacteria and characterization to species level was done by conventional microbiological techniques (Colle *et al.*, 2006).

Detection of the Biofilm Formation

The detection of biofilms was done by Tissue culture plate method, Tube method and Congo red agar method (Figure 1).

1. Tissue Culture Plate (TCP) (Mathur *et al.*, 2006)- Organisms isolated from fresh agar plates were inoculated in Brain heart infusion broth (BHI) with 2% sucrose. Broth was incubated at 37^0C 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 (OD_{570nm}). These OD values were considered as an index of bacteria adhering to surface and forming biofilm. Classification of bacterial adherence by TCP method

O.D value	Adherence	Biofilm formation
<0.120	Non	Non
0.120-0.240	Moderately	Moderate
>0.240	Strong	High

2. Tube Method (TM) (Mathur *et al.*, 2006)- 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 37^0C for 24 hours. After incubation, tubes were decanted and washed with crystal violet (0.1%). Excess stain was washed with deionized water. Tubes were 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) (Mathur *et al.*, 2006)- CRA medium was prepared by supplementing BHI with 5% sucrose and congo red. Medium- BHI(37g/L), Sucrose(50g/L), Agar(10g/L), Congo Red (0.8g/L).Congo Red was prepared as concentrated aqueous solution and autoclaved at 121^0C for 15min, separately from other medium constituents and then added when the agar had cooled to 55^0C.

Plates were inoculated and incubated aerobically for 24 to 48 hours at 37^0C. Positive results were shown as black colonies with a dry crystalline consistency.

The result of the different properties of bacteria was subjected to statistical analysis. The data was statistically analyzed using the Statistical Package for Social Sciences (SPSS)/16.0(Copyright@SPSS Inc.). Statistical significance was accepted at $p<0.001$.

RESULTS AND DISCUSSION

Results

The demographic characteristics of the study showed that majority of patients were males (74.50%) and (25.50%) were females and there was no predilection of infection to any particular gender, patients of both genders were affected. The age group >60 years predominated and it represented (46.25%) of the study population. Several risk factors have been cited to be associated with UTI, in this study, risk factors found to be significantly associated with acquisition of infection were diabetes mellitus, duration of catheterization (>7 days), age >60 years and prolonged hospital stay (>7 days) (Table 1). Significant bacteriuria was observed (79.00%) among catheterized as compared to controls. In our study, although *E.coli* was most commonly isolated among catheterized 94(29.74%) and noncatheterized 27(45.00%), there was significant difference in prevalence of uropathogens among both the groups. Compared to non catheterized patients, catheterized patients tended to have *P.aeruginosa*64 (20.25%), *P.mirabilis* 37(11.71%) and other rare gram negative bacteria as *Acinetobacter spp* 4(1.26%) and *Alcaligenesfaecalis* 1(0.31%) (Table 2).

We also observed difference in bacteriological profile of isolates with duration of catheterization (>7 days). *E.coli* 57(38.76%) was most commonly isolated among patients with duration of catheterization <7days whereas, *P.aeruginosa* 44(26.03%) and *P.mirabilis* 27(15.98%) were common among patients with duration of catheterization >7days (Figure 1). Other rare gram negative bacteria isolated with duration of catheterization >7days were *Acinetobacter spp* 4(2.36%), *A. faecalis* 2(1.18%). Isolates from study group were screened by TCP, TM and CRA method (Mathur *et al.*, 2006) for determining their ability to form biofilm and we also evaulated the reliability of these methods in order to determine most suitable screening method.

TCP method detected 227/316 (71.23%) biofilm producers, the TM showed good correlation with TCP assay for strongly biofilm forming isolates and total of 178/316 (56.33%) were picked up as biofilm producers. However, in CRA method most strains displayed red (pink to orange) colonies on medium and only 29(9.17%) showed black colonies with dry crystalline morphology were considered biofilm producers (Table 3). Considering TCP as gold standard, data from TM and CRA methods were compared. Parameters like sensitivity, specificity, negative predictive value (NPV) and positive predictive value (PPV) were calculated. Comparative analytical study of TM and CRA methods, with respect to TCP method which was considered gold standard in this study. Sensitivity of TM was 74.70%; specificity 96.85%; PPV 97.87%; NPV 65.42%. Sensitivity of CRA was 11.24%; specificity 98.43%; PPV 93.33%; NPV 36.13%. Thus, we observed, TCP is the better screening test for biofilm production than TM and CRA.

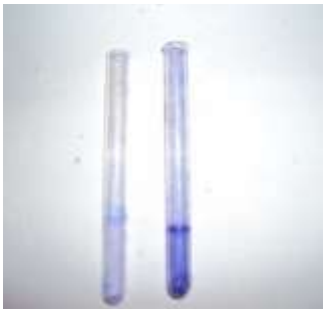
*E.coli*was the most common biofilm producer 64(28.19%) followed by *P.aeruginosa* 51(22.47%), *K.pneumoniae* 50 (22.03%) and *P.mirabilis* 35(15.42%). We also observed that biofilm production increased with duration of catheterization (Figure 2).

Discussion

The number of catheter associated urinary tract infections (CAUTIs) increases every year. The increasing number of CAUTIs bears on fact that urinary catheters became second most often used foreign body inserted into human body. Over 40% of nosocomial infections are infections of urinary tract, especially infections of catheterised patients (Gorman and Jones, 1991). Despite good aseptic management, 50% of patients have bacteriuria in first 10–14 days of catheterisation. The risk of urinary tract infections is

significantly higher in long-term inserted catheters (28 days); the percentage of infected catheters in these patients gets near to 100% (Morris and Stickler, 1998).

a. Tissue culture Plate



b. Tube Method

c. Congo Red Agar Method

Figure: Detection of Bacterial biofilm by various methods

In the present study statistically significant bacteriuria was observed among catheterized as compared to controls ($p<0.001$). This suggested that bacteriuria inevitably occurs in patients with indwelling urinary catheters over time either via breaks in the sterile system or via extraluminalroute, via migration along the outside of the catheter in the periurethral mucous sheath, or by the intraluminal route via movement along the internal lumen of the catheter from a contaminated collection bag or catheter-drainage tube junction (Saint and Chenoweth, 2003).

*E.coli*was the most frequently isolated pathogen (29.74%) followed by *K.pneumoniae* (21.84%). This was in accordance with Niveditha *et al.,* (2012) and Hassin (Hassin, 1991) and co workers who also reported *E.coli* as predominant organism.However, Pardeep *et al.,* (2013) and co workers reported *E.faecalis* followed by *E.coli* as most common biofilm producers. *E. coli*is responsible for more than 80% of all the UTIs and it causes both symptomatic UTIs and Asymptomatic Bacteriuria (ABU). The ability of the Uropathogenic *E. coli* (UPEC) to cause symptomatic UTIs is associated with the expression of a variety of virulence factors, which include adhesins (e.g., type 1 and P fimbriae) and toxins (e.g., haemolysin) (Svanborg and Godaly, 1997).

Statistical significant difference was observed in bacteriological profile of isolates with duration of catheterization more than 7 days. *E.coli* 57(38.76%) was most commonly isolated among patients with duration of catheterization <7days ($p<0.001$). Whereas, *P.aeruginosa* 44(26.03%) and *P.mirabilis* 27(15.98%) were common among patients with duration of catheterization >7days. Other rare gram negative bacteria isolated with duration of catheterization >7days were *Acinetobacter spp* 4(2.36%), *A. faecalis* 2(1.18%). Our findings were in accordance with Ko *et al.,* (2008) who observed gram negative bacteria as most common uropathogens (66.1%) and observed *E.coli* (23.4%) as the most prevalent uropathogen followed by Non E.coli Enterobactericae (NECE) (20.5%), *Pseudomonas spp* (16.4%) and other rare gram negative bacteria (5.8%) among catheterized.

The formation of biofilms by urinary pathogens on the surface of the catheter and drainage system occurs universally with prolonged duration of catheterization (Saint and Chenoweth, 2003). It appeared that catheter-associated bacteriuria results from ascending bacterial colonization within biofilm on the inside and/or outside surfaces of the catheter and drainage systems. For either short or long term catheters, the infection rate was about 5% per day (Nicolle, 2005).

Bacteria invading urinary tract met with potent innate defences, including neutrophil influx and epithelial exfoliation. Bacterial subversion of innate responses involves invasion into bladder superficial cell and bacteria matured into biofilms, creating pod-like bulges on the bladder surface. Pods contained bacteria encased in a polysaccharide-rich matrix surrounded by a protective shell of uroplakin. Thus, biofilm-like pods explains how bladder infections can persist in the face of robust host defence (Sheretz, 1997).

We observed E.coli as the most common biofilm producer (28.19%) followed by *P.aeruginosa* (22.47%), *K.pneumoniae* (22.03%) and *P.mirabilis* (15.42%). This is in accordance with Niveditha *et al.,* (2012) who also observed *E.coli* (42%) as the most common biofilm producers, where as compared to same study we observed high positivity among biofilm production in *K.pneumoniae* and *P.aeruginosa*isolates. The results of the present study were in accordance with Niveditha *et al.,* (2012) where 60% of biofilm producers were detected by TCP. Also similar results were seen by Promodhini *et al.,* (2012) who observed 83.3% isolates among catheterized patients were detected to be biofilm producers by TM. By CRA method we obtained very different results, in our study positivity rate of CRA was higher than observed by Nicolle *et al.,* (2005) who has reported 5.26% biofilm producers by CRA method, whereas our results were not in concordance with another study (Pramodhini *et al.,* 2012) which showed 23% strains were highly positive in CRA.

Thus, we observed, TCP is the better screening test for biofilm production than TM and CRA. Similar results were shown by Bose *et al.,* (2009) and by Mathur *et al.,* (2006).

The results of chi-squared test to observe statistical difference between bacterial isolates with duration of catheterization (>7 days) suggested that significant number of isolates were biofilm positive with duration of catheterization >7 days ($p<0.001$). This is in accordance with Donlan (2001) who stated that urinary catheters are tubular latex or silicone devices, which when inserted may readily acquire biofilms on the inner or outer surface. The longer the urinary catheter remains in place, the greater the tendency of the organisms to develop biofilms and result in urinary tract infections. 10%-50% of patients undergoing short term urinary catheterization(7 days) but virtually all patients undergoing long term catheterization (>28days) become infected (Stickler, 1996).

Table 1: Distribution Of Risk factors For UTI

Risk factors	Catheterized Culture positive (n/%)	Non-Catheterized Culture positive (n/%)
1.Age >60years	132 (41.77)	21 (35.00)
2.Prolonged hospital stay (>7 days)	101 (31.99)	27 (45.00)
3.Diabetes mellitus	189 (59.81)	15 (25.00)
4.Prolonged catheterization (>7 days)	169 (53.48)	-
5.Underlying chronic illness	10 (3.16)	01 (1.67)
6.Prostatic enlargement	01 (0.32)	-
7.Urological Instrumentation/ Surgery	01 (0.32)	-

Table 2: Bacteriological profile

Isolate	Catheterized(n/%)	Non Catheterized(n/%)
Escherichia coli	94/29.74	27/45.00
Klebsiella pneumoniae	69/21.84	15/25.00
Pseudomonas aeruginosa	64/20.25	-
Proteus mirabilis	37/11.71	-
Proteus vulgaris	02/00.63	-
Citrobacterkoseri	07/02.23	01/01.67
Citrobacterfreundii	10/03.16	01/01.67
Enterobacteraerogenes	-	02/03.32
Acinetobacter baumanni	02/00.62	-
Acinetobacter lwoffi	02/00.63	-
Alcaligenesfaecalis	01/00.31	-

Coagulase negative staphylococci	19/06.02	06/10.00
Staphylococcus aureus	-	04/06.67
Enterococci spp	09/02.85	04/06.67
Total	316/100	60/100

Table 3: Screening of isolates for biofilm formation by tissue culture plate, tube method and congo red agar method

Clinical Isolates	Biofilm formation	TCP(n=227) n	TCP(n=227) %	TM(n=178) n	TM(n=178) %	CRA (n=69) n	CRA (n=69) %
Catheterized	High*	95	30.06	71	22.47	29	09.17
	Moderate*	132	41.17	107	33.86	-	-
	Weak*	89	28.16	138	43.67	287	90.82
Non Catheterized	High*	09	15.00	07	11.67	01	01.67
	Moderate*	13	21.66	03	05.00	-	-
	Weak*	38	63.33	50	83.33	59	98.33

*TCP-*High-O.D(>0.240), *Moderate-O.D (0.120-0.240), *Weak/Non-O.D (<0.120), TM-*Strong-3 *Moderate-2 *Weak-1 *Non-0, CRA-*Positive-Black colonies with dry crystalline consistency, *Non-pink colonies.*

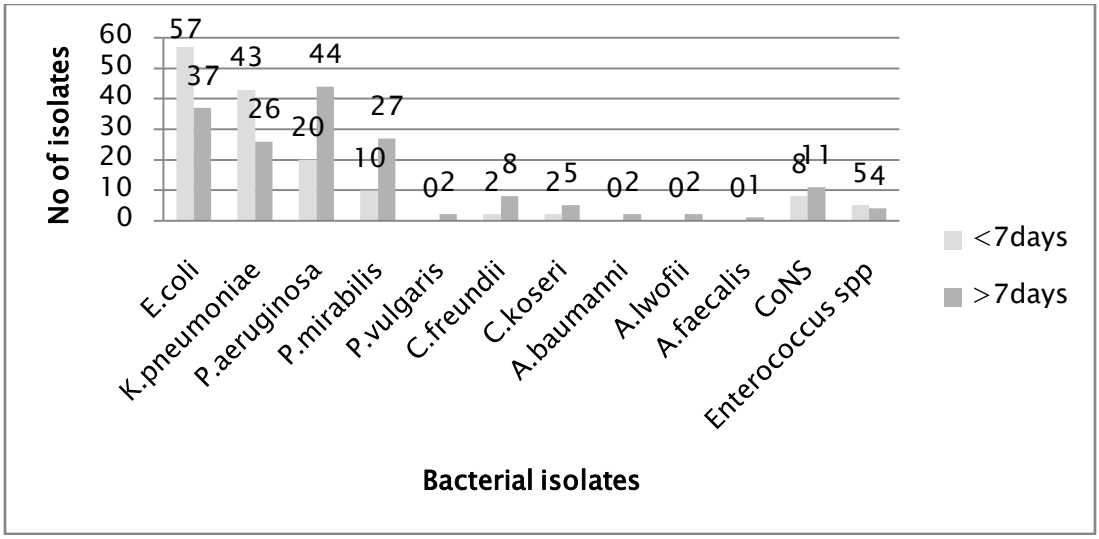


Figure 1: Distribution of Bacterial Isolates in relation to duration of catheterization

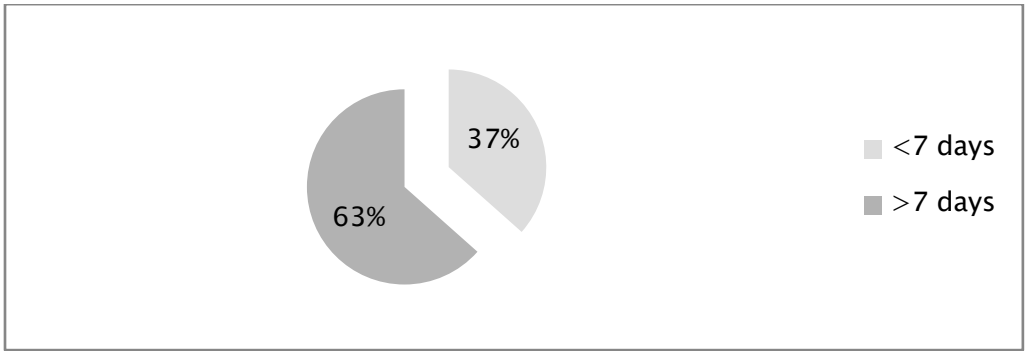


Figure 2: Distribution of biofilm forming isolates with duration of catheterization

It is seen, that the presence of biofilm on the urinary catheter leads to therapy failure (Johansen, 2004). The longer has the patient catheter, the higher diversity shows the biofilm micro flora. Progress in the area of prevention of urinary catheter–associated infections is very limited and the preventive procedures used nowadays rather only prolong the "abacterial window" than really prevent the infection. There are only few effective preventive strategies available for prevention of CAUTIs (Jacobson *et al.*, 2008).

Conclusion

To conclude, Bacteria have a basic survival strategy: to colonize surfaces and grow as biofilm communities embedded in a gel-like polysaccharide matrix. The catheterized urinary tract provides ideal conditions for the development of enormous biofilm populations. This universal resistance of biofilm cells to antibacterial agents is of course clinically important, and has to be considered when instigating antibiotic therapy for symptomatic infections. Replacing the old, biofilm-laden catheters before antibiotic treatment is a sensible option. Treatment should then be based on the susceptibility of organisms that are isolated from urine aspirated from the new catheter, as samples collected from the old catheter can contain different species and greater numbers of organisms. This study underscores the pressing need for the development of antimicrobial urinary catheters and their deployment when longer durations of catheter access are required.

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