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Detection of Biofilm Producing Uropathogenic Bacteria and Their Antibiotic Sensitivity Pattern

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Abstract **Original Research Article**

Background: Urinary tract infection is one the most common infection in clinical practice. Uropathogens have the ability to form biofilm in urinary tract, frequently within the indwelling catheter. Microorganism growing in a biofilm is associated with chronic and recurrent UTI and less sensitive to antimicrobial agent. So, the aim of the present study was to detect biofilm-producing uropathogenic bacteria by microtiter plate assay and antibiotic sensitivity pattern of biofilm- producing and biofilm non-producing organisms. ***Methods:*** This cross- sectional observational study was carried out in Microbiology Department, ChattogramMedical College, Bangladesh. Urine samples were collected from outpatient's department and inpatients of different wards. Standard microbiological procedures and biochemical tests were carried out. Antibiotic susceptibility test was performed by using the Kirby-Bauer disk diffusion technique. Biofilm production was detected by Microtiter plate method (MPM). ***Results:*** Out of 252 tested samples, 73(55.3%) organisms were isolated from non-catheterized urine and 74(61.66%) from catheterized urine samples. The most frequently isolated organism was *Escherichia coli* (60.27%, 50%) in both non- catheterized and catheterized patients followed by *Klebsiella spp.* (21.91%, 27.02%); *Pseudomonas* spp. (9.58%, 12.21%); *Acinetobacter* spp. (1.36%, 4.05%); *Staphylococcus aureus* (4.1%, 2.7%) respectively and 2.7% CoNS from non-catheterized patients. In the non-catheterized patients, 19 (26.02%) out of 73 bacterial isolates were biofilm-forming and in the catheterized patients, 33 (44.59%) out of 74 bacterial isolates were biofilm forming. The maximum biofilm-producing organism was *Escherichia coli* in both isolates. Biofilm- producing organism found relatively high resistance against tested antibiotics. Imipenem, Amikacin, Nitrofurantoin, and Piperacillin-tazobactam are the few microbial agents that are effective against biofilm-producing gram-negative organisms while Vancomycin and Linezolid is effective against the gram-positive organism. ***Conclusions:*** The capability of biofilm production by uropathogenic bacteria was higher in catheterized urine. Urinary catheters remain a major risk factor for biofilm formation. Biofilm producing organism showed higher antimicrobial resistance as compared to non- biofilm producing.

Keywords: Biofilm, Uropathogenic bacteria, Antibiotic Sensitivity Pattern.

INTRODUCTION

Urinary tract infection (UTI) remains one of the most common infections, both in the community and in the hospital. It is an estimated that about 150 million cases of UTI occur each year in the world [1]. Indwelling urinary catheters are standard medical devices utilized in both hospital and nursing homes setting. The risk of developing UTI increases significantly due to frequent and sometimes unnecessary use of indwelling catheters during hospitalization [2]. UTIs caused by both Gram-negative and Gram-positive bacteria, as well as by certain fungi. The most common causative agent for both uncomplicated and complicated UTIs is uropathogenic *Escherichia coli* (UPEC) [3], causing 70% - 95% of urinary tract infection [4].

Bacterial cells have found often in the form of multicellular aggregates commonly referred to as biofilm. Biofilm is defined as a microbiologically

derived sessile community characterized by cells that are irreversibly attached to a substratum or each other and embedded in a matrix of extracellular polymeric substances (EPS) that they have produce [5]. This matrix of EPS accounts for about 90% of total biofilm [6]. In medicine, biofilm associated infection have a major impact on permanent and temporary artificial implants placed in the human body (e.g. urethral catheter, ureteric and prostatic stents, artificial urinary sphincter), often with devastating consequence [7]. Biofilm associated with urinary catheters are particularly important because they can cause infection in 10% - 50% of patients who undergo catheterization. It is estimated that 65% of all hospital infections are of biofilm origin [8]. Biofilm can be found in the urothelium, prostate stones and implanted foreign bodies. Bacteria adhered to the uroepithelium and forming biofilm can invade the renal tissue causing pyelonephritis and even be responsible for chronic bacterial prostatitis [5, 9].

There are several advanced methods available for detection and studying biofilms in research laboratories, for example; flow-based cell counting, light and fluorescence microscopy, compound light and fluorescence microscopes, confocal scanning laser microscopy (CLSM), fluorescent dyes and proteins, spectrometric, piezoelectric sensors, bioluminescent assays etc. [10-12]. However, these methods are complicated and require sophisticated instrumentation and highly specialized personnel, so such methods are not applicable without reference laboratories. Therefore, simple qualitative methods, such as the Congo red agar method described by Freeman *et al.*, [13] and the tube adherence method and quantitative methods such as the microtiter plate method described by Christensen *et al.*, [14] are used in routine laboratories.

The microtiter plate method is most widely used and described as standard test for detection of biofilm production. This method allows an easy and quantitative classification of the bacterial isolates, hence can be used as a screening method for biofilm production.

The microorganisms within the biofilm are difficult to treat with antimicrobials. Therefore, detection of biofilm production by urinary pathogens can assist the physicians to initiate the proper antimicrobial treatment for UTI cases. So, this study was designed to find out the biofilm producing capability of the uropathogen by microtiter plate assay, antibiotic susceptibility pattern of biofilm producer and biofilm non-producer uropathogen from non-catheterized and catheterized urine.

AIMS AND OBJECTIVE

General Objective

Detection of biofilm producing capability of the uropathogens by microtiter plate method and to study the antimicrobial sensitivity pattern of biofilm producing and biofilm non-producing uropathogens from non- catheterized and catheterized urine.

Specific Objectives

(a) To isolate and identify of uropathogens from non-catheterized and catheterized urine by bacterial culture and biochemical test.
(b) To detection of the biofilm producing capability by microtiter plate method.
(c) To observe antimicrobial sensitivity pattern of isolated organisms.
(d) To compare the antimicrobial sensitivity pattern among biofilm producing and non-producing uropathogens isolates from catheterized and non-catheterized urine.

MATERIALS AND METHODS

It was a cross sectional observational study, done in the department of Microbiology, Chattogram Medical College, Bangladesh from January, 2018 to December, 2018. In this study sample size was 252, among them 132 were non- catheterized and 120 was catheterized urine sample. Patients with suspected urinary tract infection attending the outpatient department (OPD) and inpatients department of different wards were the study population. Informed written consent was duly taken.

For non-catheterized sample clean catch midstream urine from patients who did not have an indwelling urinary catheter in place at the time of specimen collection nor within 48 hours prior to specimen collection and having symptoms of UTI. For Catheterized sample patients who had an indwelling catheter in place for >2 days or the catheter removed within 48 hours prior to specimen collection and who were suffering from symptoms UTI. Each sample in the container was properly labelled with the patient's name, ID number etc. Then the specimens were transferred to the laboratory as quickly as possible within 2 hours after collection. Data were collected and recorded in a predesigned data sheet. The results of the experiments were recorded systemically and a computer using SPSS 20.0 analyzed the data.

Isolation and Identification of the Organism
Microscopic Examinations

5 ml of urine samples were poured into centrifuged tubes and centrifuged at 3000 RPM for 5 minutes. The supernatant fluid was discarded and one drop of sediment was transferred to a clean glass slide, covered with a cover slip and then was examined under light microscope using 10x and 40x magnifications. Media used in this study for primary isolation of

organism were Blood agar, MacConkey agar and Cystine-lactose- electrolyte-deficient agar media (CLED).

Media for biochemical tests were Kliger iron agar media (KIA), Simmon's citrate agar media and Motility indole urea agar media (MIU). Media for detection of biofilm formation is TSB- Glu 1% (Tryptone soya broth with 1% glucose)

Culture

Urine samples were inoculated in blood agar, MacConkey agar and CLED agar by calibrated wire loop (0.001ml) and were aerobically incubated at 37^{O} C for 24 hours.

Antibiotic sensitivity testing: All bacterial isolates were tested for antimicrobial sensitivity by modified Kirby-Baurer disc diffusion technique against commercially available antimicrobial agents.

Quality Control

A representative disc from each batch was standardized by testing against reference strains of *E. coli.* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *S. aureus* ATCC 25923; zone of inhibition was tested with standard value. Control strains were collected from Microbiology department, BSMMU, Dhaka.

Biofilm Detection

All isolated organisms tested for biofilm detection by microtiter plate method. Control strains were collected from Microbiology department, BSMMU, Dhaka. *Staphylococcus aureus* ATCC 25923 for positive control and *Escherichia coli* ATCC 25922 for negative control.

RESULTS

Table I: Results of urine culture among the study population (n=252)

Samples	Number of samples studied	Number of culture positive (%)	Number of culture negative (%)
Non – catheterized	132	73(55.3)	59(44.7)
Catheterized	120	74(61.66)	46(38.34)
Total	**252**	**147(58.33)**	**105(41.67)**

Table-II: Duration of catheterization in relation to culture positive among catheterized patients (n=120)

Length of catheterization	No of sample	Culture positive (%)
1 - 7 days	30	9(30.00)
8 - 14 days	44	26(59.09)
15 – 21 days	30	24(80.00)
>21 days	16	15(93.75)
Total	**120**	**74(61.66)**

χ^2 =24.082, P = <0.05; (p=.0001); highly significant.

Duration of catheterization and incidence of CAUTI is statistically significant (test done by Chi square test).

Table -III: Distribution of isolated organism between non-catheterized and catheterized patients (n=147)

Isolated Organism	Non- Catheterized (n=73)	Catheterized (n=74)	Total (n=147)	Significance (p Value)
Escherichia coli	44(60.27)	37(50.00)	81(55.10)	NS($p \geq .05$)
Klebsiella spp.	16(21.91)	20(27.02)	36(24.48)	
Pseudomonas spp.	07(09.58)	12(16.21)	19(19.92)	
Acinetobacter spp.	01(1.36)	03(4.05)	04(2.72)	
Staphylococcus aureus	03(4.10)	02(2.7)	05(3.40)	
CoNS	02(2.7)	00(00)	02(1.36)	
Total	**73(49.66)**	**74(50.34)**	**147(100)**	

NS= Non-significant., (Figure within parentheses represents percentage).

Table-IV: Result of biofilm production detected by microtiter plate method among isolated organism (n=147)

Sample	Biofilm producing organism (%)	Non–biofilm producing organism (%)
Non – catheterized (n=73)	19(26.02)	54(73.98)
Catheterized (n=74)	33(44.59)	41(55.41)
Total 147	**52(35.38)**	**95(64.62)**

χ^2 = 5.542, P= <0.05 (p=0.018); Significant.

Rate of biofilm production between non-catheterized and catheterized isolates was statistically significant (test done by Chi square test).

Table-V: Distribution of biofilm producing organism between catheterized and non- catheterized isolated organism

Organism	Non –catheterized	Catheterized	Significance (*p*-value)
Escherichia coli	11(57.89)	20(60.60)	NS($p \geq .05$)
Klebsiella spp.	04(21.05)	06(18.18)	
Pseudomonas spp.	03(15.78)	06(18.18)	
Staphylococcus Aureus	01(05.26)	01(03.03)	
Acinetobacter	0(00)	0(00)	
Total	**19(100)**	**33(100)**	

Table-VI: Distribution of biofilm producing intensity between non-catheterized and catheterized biofilm producer (n=52)

lm producing intensity	Non –catheterized (n=19)	atheterized (n=33)	Total
Weak biofilm	16 (84.21)	22 (66.66)	38 (73.07)
Moderate biofilm	02 (10.52)	03 (9.09)	05 (9.61)
Strong biofilm	01 (5.26)	08(24.24)	09 (17.30)

χ^2=3.043, P=>.05 (*p*=0.218); Non-significant.

Table–VII: Antibiotic sensitivity pattern of biofilm producer and biofilm non-producer gram negative bacteria (*Escherichia coli* and *Klebsiella* spp) (n=117)

Antibiotic	Biofilm positive (n=41)		Biofilm negative (n=76)		(*p* value)
	Sensitivity	Resistance	Sensitivity	Resistance	
Ampicillin	00(00)	41(100)	2(02.6)	74(97.4)	NS(p≥.05)
Amoxiclav	00(00)	41(100)	13(17.1)	63(82.9)	S(*p*≤.05)
Ciprofloxacin	5(12.19)	36(87.81)	26(34.21)	50(65.79)	S(*p*≤.05)
Co-trimoxazole	9(21.95)	32(78.05)	30(39.17)	46(60.83)	S(*p*≤.05)
Ceftriaxone	8(14.57)	33(80.49)	32(42.1)	44(57.9)	S(*p*≤.05)
Cefixime	5(12.19)	36(87.81)	30(39.47)	46(60.53)	S(*p*≤.05)
Cefuroxime	6(14.63)	35(85.37)	38(50)	38(50)	S(*p*≤.05)
Ceftazidime	9(21.95)	32.(78.05)	39(51.31)	37(48.69)	S(*p*≤.05)
Gentamicin	23(56.09)	18(43.91)	51(67.1)	25(32.9)	NS(p≥.05)
Amikacin	27(65.85)	14(34.15)	54(71.05)	22(28.95)	NS(p≥.05)
Nitrofurantoin	20(48.78)	21(51.22)	50(65.78)	26(34.22)	NS(p≥.05)
Imipenem	32(78.04)	9(21.96)	66(86.84)	10(13.16)	NS(p≥.05)

Table-VIII: Antibiotic sensitivity pattern biofilm producer and biofilm non-producer *Pseudomonas* spp. (n=19)

Antibiotic	Biofilm positive(n=9)		Biofilm negative(n=10)		*P* value
	Sensitivity	Resistance	Sensitivity	Resistance	
Piperacillin-tazobactam	06 (66.67)	03 (33.33)	08 (80.00)	02 (20.00)	NS(*p*≥.005)
Ciprofloxacin	01 (11.11)	08 (88.89)	03 (30.00)	07 (70.00)	
Ceftazidime	04(44.44)	05 (55.56)	06 (60.00)	04 (40.00)	
Ceftriaxone	02(22.22)	07(77.78)	04(40.00)	06(60.60)	
Gentamicin	04 (44.44)	05 (55.56)	05 (50.00)	05 (50.00)	
Amikacin	05(55.56)	04 (44.44)	06 (60.60)	04 (40.40)	
Imipenem	07(77.77)	02 (22.23)	08(80.80)	02 (20.20)	

Table-IX: Antibiotic sensitivity pattern biofilm producer and biofilm non-producer *Staphylococcus aureus* (n=5)

Antibiotic	Biofilm positive (n=2)		Biofilm negative (n=3)		Significance
	Sensitivity	Resistance	Sensitivity	Resistance	*P* value
Penicillin	0 (00)	02 (100)	0 (00)	03 (100)	NS(p≥.05)
Cotrimoxazole	0 (00)	02 (100)	01 (33.33)	02 (66.67)	
Ciprofloxacin	0 (00)	02 (100)	01 (33.33)	02 (66.67)	
Gentamicin	01(50)	01 (50)	02 (66.67)	01 (33.33)	
Cefoxitin	0 (00)	02 (100)	0 2(66.67)	01 (33.33)	
Doxycycline	0 (00)	02 (100)	01 (33.33)	02 (66.67)	
Nitrofurantoin	01 (50)	01 (50)	02 (66.66)	01 (33.34)	
Vancomycin	02 (00)	00 (00)	03 (100)	00(00)	
Linezolid	02 (100)	0 (00)	03 (100)	0 (00)	

DISCUSSION

Urinary tract infection (UTI) is the most commonly acquired bacterial infection. It poses serious health problem with respect to antibiotic resistance and biofilm formation being the prime cause for the antibiotic resistance [15]. In the present study, a total of 252 samples were collected. Among them 132 were non-catheterized urine and 120 were catheterized urine. Out of 252 samples, 147 (58.33%) were showed growth. Among the culture positive isolates, 73 (55.3%) isolates were non-catheterized urine and 74 (61.66%) isolates were catheterized urine, which is comparable with other study by Shanthi and Kaythri (2012) [16]; Mohammed and Shalakany (2015) [17] whereas 56% and 53.33% respectively culture positive in non-catheterized urine. Another study by Tomar *et al.,* (2017) [18] and Piljic *et al.,* (2013) [19] found culture positive in catheterized urine were 74% and 57.89 % respectively.

This study showed that, the incidence of UTI was higher as the days of catheterization increased. The highest incidence of CAUTI was 93.75% more than 21 catheter days, followed by 80% incidence within 15–21catheter days, 26 (59.09%) within 8-14 catheter days and 30% less than 7 catheter days. This finding is similar with Projapati *et al.,* (2015) [20], they found 100% incidence of CAUTI was more than 22 catheter days and 75% within 15-21 catheter days, 5.66% within 8-14 catheter days. However, 2.63% incidence of CAUTI was less than 1 week that is not similar with our study. Another study in Ethiopia by Awoke *et al.,* (2019) [21] who found 36.2% incidence of CAUTI was less than 7 days.

In this study, *Escherichia coli* was most frequently isolated pathogen in both non-catheterized and catheterized patients (60.27% and 50%), followed by *Klebsiella* spp. (21.91% and 27.02%), *Pseudomonas* spp. (09.58% and 16.21%), which is similar to the findings by Verma *et al.,* (2016) [22] and by Amuthamni *et al.,* (2017) [23] and by Akhter *et al.,* (2018) [24]. Other organism was *Acinetobacter* spp. (1.36% and 4.05%), *Staphylococcus aureus* (4.1% and 2.7%) and CoNS 2.7%. But a study in India by Tiwari *et al.,* (2017) [25] showed *Pseudomonas aeruginosa* was the predominant organism in CAUTI.

There are several methods to detect biofilm formation. In this study, we selected microtiter plate method because this method considered as the gold standard phenotypic test because of its higher sensitivity. We applied this method to detect ability of biofilm formation in 147 clinical isolates of non-catheterized and catheterized urine. Biofilm formation showed between non-catheterized (73) and catheterized (74) isolates was 19 (26.02%) and 33 (44.59%) respectively. This finding is similar with the study in Egypt by Abdalaah *et al.,* (2011) [26], they reported 30% biofilm produced in non- catheterized isolates and 43.3% biofilm produced in catheterized isolates. There was significant different on biofilm production between non- catheterized and catheterized urine. Biofilm was more intense in catheterized urine. This may occur because not only urinary catheter invites biofilm production, the presence of the catheter itself impairs normal defense mechanisms of the bladder. As catheter surface has no inherent mechanisms, when free swimming bacteria attaches to the surface of the catheter, they readily produced biofilm [27].

In this study, maximum biofilm producing organism was *Escherichia coli* in both non- catheterized and catheterized isolated organism 57.89% and 60.60% respectively, followed by *Klebsiella* spp. (21.05% and 18.18%), Pseudomonas spp. (15.78% and 18.18%), *Staphylococcus aureus* (05.26% and 03.03%). Pramodhini *et al.,* (2012) [28] also observed a similar rate of biofilm production in catheterized urine (63%) and Verma *et al.,* (2016) [22] found 58% biofilm producing organism was *Escherichia coli* in non-catheterized urine. But another study in India done by Tiwari and Ghnawate (2017) [25] found 41.84% biofilm producing organism was *Pseudomonas aeruginosa* and Charan *et al.,* (2015) [29] found 80.65% biofilm producing organism was *Staphylococcus aureus*. The difference of biofilm forming patterns among bacterial isolates may be due to differences in organism types, number of bacterial isolates, sample size, geographical and methodological variation to assess biofilm formation. In present study, we found that strong biofilm production was 24.24% in catheterized biofilm producer while 5.26% in non-catheterized biofilm producer.

Biofilm producing bacteria are notoriously difficult to eradicate [30]. They exhibit resistance to antibiotic by various methods like restrict penetration of antibiotic into biofilm, decreased growth rate, and expression of resistance gene.

In the present study, the antibiotic resistance of biofilm producing gram negative (*Escherichia coli*. and *Klebsiella* spp.) bacteria was found significantly higher than that of non-biofilm producers. Biofilm producing gram negative bacteria 100% resistance to Ampicillin and Amoxiclav. More than ≥ 80% resistance was observed in case of Ciprofloxacin, Cefixime, Cefuroxime and Ceftriaxone, 78.05% resistant to Co-trimoxazole and Ceftazidime, 51.16% resistant to Nitrofurantoin and 43.91% resistance to Gentamicin. Biofilm forming isolates were mostly sensitive to Imipenem (78.04%) and Amikacin (65.85%). Imipenem was least resistance among biofilm forming and non-biofilm forming isolates. This finding closely related to Panda *et al.,* (2016) [31] in India except Amikacin, which was 87.8% resistance in biofilm producing isolates. Another study in India done by Abdagire *et al.,* (2014) [32] observed Imipenem was 100% sensitive in

both isolates. Higher antibiotic resistance in biofilm producer gram negative bacteria compares to the non-biofilm producer gram negative bacteria were found in current study. This may be due to that, the penetration of antimicrobial agent through the biofilm matrix is difficult, the expression of efflux pumps increases in biofilm bacteria, and the growth rate of biofilm bacteria is slow in the center of the biofilm.

In this study, we observed effective antibiotic against biofilm producing *Pseudomonas* spp. was Impenem and Piperacillin-tazobactum (77.77% and 66.66%), where most effective antibiotic for non-biofilm forming isolates were Imipenem (80%), Piperacillin-tazobactum (80%), Amikacin (60), Ceftazidime (60%). Similarly, Majumder *et al.,* (2014) [33] in Bangladesh and in India by Nasimuddin *et al.,* (2016) [34] showed most effective antibiotic against biofilm producing *Pseudomonas* spp. was Imipenem (91.66%, 98%) and Piperacillin- tazobactum (73%). Biofilm producing *Staphylococcus aureus* were 100% resistant to most of the drugs but no resistance observed to Linezolid both the groups (biofilm forming and non-forming). A study by Charan *et al.,* (2014) [29] and by Hassan *et al.,* (2011) [35] observed Linezolid was effective against biofilm forming and non-biofilm forming *Staphylococcus aureus* and Penicillin was 100% resistance in both group which is similar to our finding. The *P* value for antibiotic sensitivity pattern of biofilm and non biofilm-producing *Pseudomonas* spp and *Staphylococcus aureus* were found to be >0.05, which means the difference in antibiotic susceptibility pattern of biofilm and non biofilm-producing these two organisms are statistically not significant. This may be attributed to lower rate of isolation of these organisms in the study.

In present study, antibiotic sensitivity pattern of biofilm non-producing *Acinetobacter* spp. to different antimicrobial showed higher resistance to Ciprofloxacin (100%), Ceftriaxone (100%), Doxycycline (100%) followed by Co-trimoxazole (75%), Gentamicin (75%) and lower resistance was observed to Piperacillin-Tazobactum (25%) and Imipenem (25%). Resistance pattern of other drug was 50% to Amikacin and Ceftazidime, which is similar with the study by Nandini and Madhusudan (2016) in India [36].

In the study, we found that the capability of biofilm production is higher in catheterized urine. Biofilm producing organisms had decreased susceptibility to commonly using antimicrobial agents for UTIs. The effective antibiotic against biofilm producing gram negative isolates was Imipenem and for gram positive isolates were Linezolid.

CONCLUSION

The most predominant bacterial isolated were *Escherichia coli*, which was 60.27% in non-catheterized patients and 50% in catheterized patients. The capability of biofilm production by uropathogen was higher in catheterized urine. Urinary catheter is remaining a major risk factor for biofilm formation. Biofilm forming isolates showed higher antimicrobial resistance as compare to non-biofilm producing. Imipenem and Piperacillin-tazobactam are the few microbial agents that are effective against biofilm producing gram negative organism while Linezolid is effective against gram positive organism. Biofilm constitute an important contribution to high incidence, recurrence and complication of UTIs, thus requiring efficient prevention and control measure. A large study is required for better understand the true impact of biofilms on antibiotic susceptibility.

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