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Research Article

Biofilm Formation by Uropathogens and Its Impact on Antimicrobial Susceptibility Pattern

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ABSTRACT

Background: Out of all Hospital-Associated Infections (HAIs),Urinary Tract Infection (UTI) is the second most common infection that accounts for approximately 34%, and 80% are associated with indwelling catheters and hence with biofilm formation, which invites multi-drug resistant microorganisms. The present study was designed to study in-vitro biofilm forming uropathogens and their antimicrobial susceptibility in a tertiary care hospital in north India.

Method: The present cross-sectional study consisted of 200 urine specimens collected over one year from patients with symptoms of urinary tract infection. Following their isolation and identification, all the isolates were subjected to screening for biofilm formation by Congo Red Agar (CRA) and the Tube Adherence (TA) methods. Subsequently, the Kirby Bauer-disk diffusion method performed the antimicrobial susceptibility test.

Results: Out of the total samples (n = 200), a total of 46 (23%) were positive by the CRA method, while 33 (16.5%) were positive by the TA method. Twenty-one (21%) isolates came positive by both methods. Biofilm formation was seen more commonly in females (82%). Biofilm-forming uropathogens develop a significantly higher resistance to antimicrobial drugs than non-producers.

Conclusion: The correlation was significant between biofilm production and multidrug resistance. Also, it was concluded that the CRA method could be employed to detect biofilm formation in resource-limited countries.

Keywords: Urinary tract infections; Tube adherence method; Congo red agar method; Antibacterial agents

INTRODUCTION

Talking of morbidity worldwide,Urinary Tract Infections (UTIs) is one of the leading causes of which is caused by different microorganisms. Worldwide, UTI has a prevalence of 11%(1),

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and according to an Indian study, it is 36.68% (2). These uropathogens tend to colonize the mucous membrane of the bladder and form micro bacterial communities called biofilms. The colonization by these microcolonies makes them impermeable to many antibiotics. It resultsin the evolution of multidrug-resistant strains, which is the leading causeof relapses in untreatable UTI. Biofilms consist of different layers of cells embedded in a matrix of extracellular exopolysaccharide – EEM (slime), which helps adhereto biomedical surfaces and protects them from the host immune system and antimicrobial therapy (3), and provides a survival strategy to the uropathogenic. The slimeconsists of extracellular DNA, proteins, polysaccharides, adhesin, and autolysin. It starts with the attachment of free-floating microorganisms to a surface. Initially,these areattached through weak van der Waal forces. Later, if left undisturbed, they anchor themselves more firmly via cell adhesion structures such as pili. Repulsion to water plays an essential role in determining the ability to form biofilms(4).Using his simple microscopes, Van Leeuwenhoekobserved microorganisms on tooth surfaces and can be regarded with the discovery of biofilms. Costerton*et al.,* in 1978, explained the mechanisms ofmicroorganisms' adherence to living and nonliving materials and the help provided by ecologic niche (5).

Biofilms aremainly formed in the prostate stones, urothelium, and implanted foreign bodies (6). Predisposing host factors are age, diabetes, long-term hospitalization, and catheterization (7). National Institute Health (NIH) says that among all the microbial infections, 80% are caused by biofilms (8). According to the Center for Disease Control and Prevention (CDC), USA, biofilms on indwelling medical devices consistof gram-positive or gram-negative bacteria or yeasts. The most common Gram-PositiveBacteria isolated are *Enterococcus faecalis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus viridans*, and Gram-negative bacteria*Escherichia coli*, *Klebsiella pneumonia*, *Proteus mirabilis*, and *Pseudomonas aeruginosa*. These bacteriacan originate from the skin of patients or healthcare workers or be some other source like the environment (9).Biofilms are composed of single or multiple species depending on the device and its duration of use in the patient.Biofilm on the urinary catheter is initially composed of single species, but with time, multispecies predominates(10). Biofilm-causing uropathogens have an inherent resistance to antibiotics, disinfectants, and antiseptics. Unlike planktonic populations, bacterial cells embedded in biofilmsshow intrinsic resistance to antibiotics which can be due to the inactivation of antimicrobial agents by exopolysaccharide (EPS), overexpression of stress-responsive genes, presence of oxygen gradients within the biofilm matrix, and differentiation of a subpopulation of biofilm cells into resistant dormant cells(11)(12).

In this study, our goal was to detect the biofilm-forming uropathogens and study their antimicrobial susceptibility pattern among patients suffering from UTI in a tertiary care hospital in northern India. This study will help the clinicians to decide

METHOD

This cross-sectional study was performed for one year on 200 urine specimens from out-patients (n=15) and in-patients (n=185) who were clinically diagnosed with UTI and fell under the inclusion criteria. Semi-quantitative urine culture was performed on UTI agar (HiMedia Labs) as per standardized SOPs of the department.

As described by Freeman DJ *et al*., 1989 (13), CRA was performed. CRA medium was prepared by mixing brain heart infusion broth (Oxoid, UK) 37 g/L, sucrose 50 g/L, agar No. 1 (Oxoid, UK) 10 g/L, and Congo red indicator (Oxoid, UK) 8 g/L. The Congo red stain (HiMedia Labs) was prepared separately as a concentrated aqueous solution and autoclaved (121°C for 15 min) from the rest of the other constituents. It was later added to the autoclaved brain heart infusion agar (HiMedia Labs) with sucrose at 55°C. CRA plates were then inoculated with test isolates and left for aerobic incubation at 37°C for 24 hours. For all positive isolates, CRA and TA methods detected biofilm formation. Black colonies with a dry crystalline

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consistency indicated biofilm formation, whereas no-biofilm formation was identified as red or pink crystalline colonies (Figure 1).

Figure1. Congo Red Agar method shows biofilm producers (black crystalline colonies) & non-producers (pink colonies)

Tube Adherencemethod, as described by Christensen GD *et al*. (14), 1982 is a quantitative method for biofilm detection. Test organisms were inoculated in 10 ml of trypticase soy broth with 1% glucose in test tubes and incubated at 37°C for 24 hours. After incubation, tubes were decanted, washed with phosphate-buffered saline (pH 7.3), and dried. Tubes were then stained with crystal violet (0.1%). The excess stain was washed with deionized water and dried. The scoring for the tube method was done according to the results of the control strains. Biofilm formation was considered positive when a visible film lined the wall and the bottom of the tube (Figure 2).

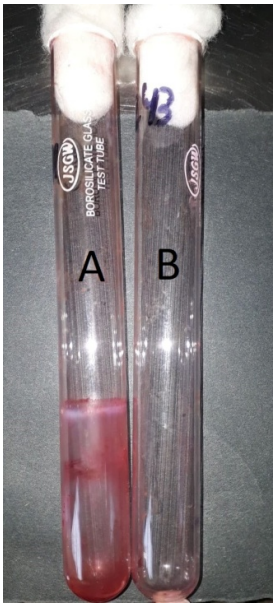

Figure2. Tube Adherence method showing biofilm producer (tube A) & non-producer (tube B)

Antimicrobial Susceptibility testing was performed using the Kirby Bauer Disk diffusion method on Mueller Hinton agar (HiMedia Labs) according to the Clinical Laboratory Standard Institute

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(CLSI 2019) guidelines (15). The antimicrobial discs used for Gram-positive isolates were Penicillin G 10 units, Gentamicin 10mcg, Ciprofloxacin 5mcg, Vancomycin 30mcg, Linezolid 30mcg, Nitrofurantoin 300mcg, and Norfloxacin 10mcg. The antimicrobial discs used for Gram-negative isolates were Piperacillin-tazobactam 100/10mcg, Cefuroxime 30mcg, Cefepime 30mcg, Amikacin 30mcg, Imipenem 10mcg, Gentamicin 10mcg, Tobramycin 10mcg, Ciprofloxacin 5mcg, Cotrimoxazole 1.25/23.5mcg, Chloramphenicol 30 mcg, Tetracycline 30 mcg, Nitrofurantoin 300mcg, Norfloxacin 10mcg, Amoxicillin/Clavulanic acid 20/10mcg and Aztreonam 30mcg.

RESULTS

A total of 185 (92.5%) were indoor patients, while 15 (7.5%) were from the outdoor department. Out of 200 urine specimens, 64 (32%) were females, and 36 (18%) were males. Most patients belonged to 51 to 70 years (36%), followed by those above 70 years (29.5%). Of all, 102 (51%) patients were catheterized. Gram-negative dominated 70% of the positive isolates, whereas Gram-positive constituted 30%. Out of 200 uropathogens, 94 (47%) were *Escherichia coli*, followed by *Enterococcus sp*. 37(18.5%) (Table 1).

Table 1. Bacterial uropathogens among UTI patients from Out Patient Department (OPD) and In-Patient Department

S. No.	Uropathogens	OPD (n=15)	IPD (n=185)	Total (n=200)
1	*Escherichia coli*	11	83	94 (47%)
2	*Enterococcus spp.*	0	37	37 (18.5%)
3	*Klebsiella pneumoniae*	0	28	28 (14%)
4	*Staphylococcus aureus*	4	18	22 (11%)
5	*Acinetobacter spp.*	0	7	7 (3.5%)
6	*Pseudomonas aeruginosa*	0	6	6 (3%)
7	*Klebsiella oxytoca*	0	4	4 (2%)
8	*Proteus mirabilis*	0	1	1 (0.5%)
9	*Morganella morganii*	0	1	1 (0.5%)

Out of 102 catheterized patients, biofilm formation was observed in 72 (36%), which was way more than in community-acquired UTI cases 18 (9%). Table 2 shows biofilm production by different methods. CRA method detected 83 isolates(46%) as biofilm producers, whereas TA method detected only 59(33%) isolates as biofilm producers.

Table 2. Biofilm production by different methods

Methods	Congo red agar method (%)	Tube adherence method (%)	Both methods (%)
Total number of isolates	83 (46%)	59 (33%)	38 (21%)

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Amoxicillin (99%) and Amoxy-clavulanic acid (100%) were resistant in most biofilm-positiveisolates. The highest degree of drug resistance was seen in biofilm-forming *Acinetobacter spp*. followed by *Klebsiella* sp. and *Pseudomonas aeruginosa*. Resistance to antibiotics like Cefuroxime, Aztreonam, Imipenem, Tobramycin, Norfloxacin, Cotrimoxazole, Chloramphenicol, Gentamicin and Tetracycline, Vancomycin was more in biofilm positive isolates (Table 3). Biofilm-forming Gram-negative bacilli (GNB) uropathogenic developed significantly higher resistance towards antimicrobial drugs.

DISCUSSION

Urinary Tract Infectionspresenta severe health threat concerning antibiotic resistance, especially with biofilm production. During the period covered by our study, 200 samples were studied, 92% of which were received from different wards, Operation Theatre (OT), Cardiac Care Unit (CCU), Intensive Care Unit (ICU), while OPD samples were only 15% of the total and maximum samples fell into the age group of 51 – 70 years. This finding was also depicted in Madigan E & Neff D (16). Our study observed that the infected patients were primarily women (82%), which can be because of anal proximity and the shorter length of the urethra. A similar finding was also reported by Kashef N *et al*. (17).The age group of 61 – 75 years predominated in catheterized patients(n=102). The maximum number of patients were on catheterization for >4 days, a similar finding by Niveditha S *et al.* (18). The detection of bacteriuria within one week of the catheterization in this study pertains to the inadequate precautions taken while handling catheters.

Escherichia coli was isolated from 94 (47%) specimens, followed by *Klebsiella pneumonia* (16%) and *Enterococcus spp.* (18.5%), *Pseudomonas aeruginosa* (3%), *Staphylococcus aureus* (11%), Acinetobacter spp. (3.5%), *Proteus mirabilis* and *Morganella morganii* (0.5% each). These findings were similar to the studies conducted by Noor AF *et al.* (19). *Escherichia coli* was responsible for the maximum number of UTI casesbecause of the ability of Uropathogenic*Escherichia coli* (UPEC) to express a variety of virulence factors like adhesins (e.g., type 1 and P fimbriae) and toxins like hemolysin. Biofilm detection by CRA and TA methods was (46%) and (33%) respectively,and this correlates with the study of Hassan A *et al.*(20). CRA is a rapid, sensitive, and reproducible method and can be recommended in resource-limited countries. A similar finding was reported by Rewatkar AR and Wadher BJ *et al.* (21). Quantification of biofilms done by the TA method showed that only 9% were strong producers, whereas 20% were moderate producers. The rest of the isolates (71%) were weak producers. This was also observed by Panda PS *et al.* (22). Biofilm formation on CAUTI was observed more than Community-acquired UTI because bacteria survive on catheters easily as CAUTI creates an ideal environment for bacterial attachment and biofilm production.

Antibiotic resistance was more among biofilm producers in comparison to non-producers. Similar results were obtained by Rewatkar AR and Wadher BJ *et al.* (21). A possible explanationis the persistence of the organism, decreased bacterial growth rate in a biofilm, and increased expression of resistance genes. Restricted penetration of antibiotics into the biofilm and the proximity of cells within a biofilm results in plasmid exchange and leads to the spread of antimicrobial resistance.

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Table 3. Antibiogram of biofilm and non-biofilm producing isolates

Antimicrobial agent	Percentage resistance		p-value
	Biofilm producers	Non-biofilm producers	
Amoxicillin	72/73 (99%)	50/53 (94%)	0.019
Amoxy Clavulanic acid	73/73 (100%)	51/53 (96%)	0.024
Piperacillin-tazobactam	40/77 (52%)	27/64 (42%)	0
Cefuroxime	71/73 (98%)	44/53 (83%)	0.004
Cefepime	77/97 (80%)	56/64 (87%)	0.004
Aztreonam	66/76 (87%)	39/56 (70%)	0
Imipenem	67/73 (92%)	35/53 (66%)	0
Amikacin	64/77 (84%)	54/64 (85%)	0.022
Tobramycin	47/77 (61%)	17/64 (27%)	0
Norfloxacin	94/103 (92%)	67/88 (77%)	0.002
Cotrimoxazole	62/74 (84%)	28/61 (46%)	0
Cefoxitin	58/100 (58%)	25/85 (30%)	0
Chloramphenicol	46/73 (63%)	26/53 (50%)	0.022
Gentamicin	98/104 (94%)	77/96 (80%)	0.022
Tetracycline	64/74 (86%)	35/61 (57%)	0
Ciprofloxacin	23/30 (77%)	31/35 (88%)	0.449
Penicillin	24/27 (89%)	27/32 (84%)	>0.5
Vancomycin	10/27 (37%)	5/32 (17%)	0
Nitrofurantoin	14/27 (52%)	6/32 (19%)	0
Linezolid	0/27 (0%)	0/32 (0%)	0.001

In the case of *Escherichia coli*, biofilm producers showed maximum resistance toamoxyclavulanic acid followed by cephalosporins, gentamicin, co-trimoxazole amikacin, and least resistance to piperacillin-tazobactam (37%). It was similar to the finding observed by Tiwari AA &Ghnawate N *et al.* (23). In the case of *Klebsiella pneumonia*, resistanceto multiple antibiotics was observed in biofilm producers, which also correlates with the study of Tiwari AA &Ghnawate N *et al.* (23). Drug tobramycin was more effective in the case of non-biofilm producers with 83% sensitivity, while it was 25% sensitive for biofilm producers. Our study concluded that *Klebsiella sp*. was maximum resistant to antibiotics, maybe because of the high prevalence of resistant strain in our region or the exhaustive use of antibiotics. *Pseudomonas aeruginosa* was highly sensitive to tobramycin in the case of biofilm producers and non-producers. So it may be considered the antibiotic of choice for *Pseudomonas aeruginosa*. Our study had only one strain of *Acinetobacter baumannii*, a biofilm producer and

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resistant to all the antibiotics. On the other side, no biofilm-producing strain was isolated in the case of *Morganella morganii* and *Proteus mirabilis*. However, the insufficient sample size makes it impossible to draw practical conclusions from this data. In the case of Gram-positive cocci, linezolid was 100% sensitive in both biofilm producers and non-producers, which shows that it can be a good reservoir. This finding correlates well with the study of Panda PS *et al.*(22). 86% of *Staphylococcus aureus*isMRSA strains, a finding similar to a study by Yousefi M *et al.* (24). Our study highlights a broad range of uropathogens and Multi-Drug Resistant (MDR) isolates among biofilm-forminguropathogens.

This study was concerned with a single tertiary setting. Thereforebroader surveillance is needed to determine the local resistance profiles of prevalent biofilm-forminguropathogens so that an optimal empirical therapy can be documented.

CONCLUSION

This study showed a considerable opportunity foruropathogens to form biofilms. We observed asignificant correlation between biofilm production and multi-drug resistance compared to non-biofilm-forming isolates. Finally, the CRA method can be employed as the routine laboratory test for in-vitro biofilm detection as it is cost-effective also.

Authors' contribution

BM and SSS contributed to the data collection and interpretation. PS, SD contributed to the data article writing and its publication.

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Conflict of interest

There is no conflict of interest in this research.

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