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RESEARCH ARTICLE

BIOFILM FORMATION AND ANTIBIOTIC SUSCEPTIBILITY OF UROPATHOGENS IN PATIENTS WITH CATHETER ASSOCIATED URINARY TRACT INFECTIONS IN IBB CITY-YEMEN

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ABSTRACT

Biofilm formation by uropathogens on the surface of indwelling medical devices can inflict obstinate or recurring infection, thought-provoking antimicrobial therapy. This study included 227 patients with indwelling urinary catheters and suffering from CAUTI. They were analyzed for biofilm formation and antibiogram susceptibility, 59.4% were males and 40.6% were females. Ensuing phenotypic identification of isolated bacteria, antibiotic sensitivity test was performed by modified Kirby–Bauer disc diffusion method following the Clinical and Laboratory Standards Institute (CLSI 2015) guidelines; Biofilm-forming uropathogens were detected by the tissue culture plate (TCA) method. The predominant uropathogen in catheter-associated UTIs (CAUTIs) was *Escherichia coli* 46.3%, followed by *K. pneumoniae* 18.5%, *P. aeruginosa 11.9%, 7%*, *S. coagulase* negative *5.7%*, *S. aureus 4.8%*, *Enterobacter* spp. 4.4%, *E. faecalis 1.3%*. The total rate of biofilm producer bacteria was 49.3% (21.1% high producers, 28.2% moderate producers). Prime biofilm producers were *E. coli 60%* with OR=8.6 (p=0.002), followed by *K. pneumoniae 57.1%* with OR=10.1 (p=0.002), and *P. aeruginosa 37%* with OR=6.6 (p=0.02). The biofilm producers bacteria were associated with >65year patients (OR=5.4, p>0.001), pre-UTI (OR=2.4, p<0.001), long duration of catheterization (OR=15.3, p<0.001), and diabetic mellitus (OR=3.5, p<0.001). Multidrug resistance associated with biofilm producers were greater than biofilm non-producers. Gram-negative biofilm producers found 100%, 100%, 88.6%, 82.9%, 81.9%, 80.9%, and 72.4%, 40%, 33% resistant to ampicillin , amoxyclave, cotrimoxazole, ceftraxone, nalidixic acid, ciprofloxacin, cefotaxime , nitrofurantoin and amikacin respectively. Gram-positive biofilm producers, however, were found 85.7%, 85.7%, 71.4%, 71.4%, 57.1% and 42.9% resistant to penicillin, erythromycin, cotrimoxazole, gentamycin, norfloxacin, and nitrofurantoin respectively. In conclusion, a high antimicrobial resistance was observed in biofilm producers than non-biofilm producers. Of recommended antimicrobial therapies for CAUTIs, ampicillin and amoxicillin-clavulanate were the least active antibiotics, whereas imipenem and amikacin were found as the most effectual for gram-negative biofilm producer. Likewise, penicillin and erythromycin were the least active antibiotics, whereas vancomycin, and rifampicin were found as the most effective antibiotic for Gram-positive biofilm producer.

Keywords: Antibiotics susceptibility, biofilm, Catheter Associated Urinary Tract Infections, uropathogens, Yemen.

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INTRODUCTION

Biofilms have been found to be involved in a wide range of microbial infections in the body, by one estimate 80% of all infections[1]. Infectious processes in which biofilms are involved include common problems such as bacterial vaginosis, urinary tract infections, catheter infections, middle ear infections, and dental plaque formation[2], gingivitis and contact lenses[3], fatal processes such as endocarditis, and inflammation in cystic fibrosis, and permanent indwelling devices like joint prostheses, heart valves, and intervertebral disc[4,5,6]. Of nearly 40 percent of all healthcare related infections, urinary tract infections (UTIs) are the

leading cause of infection. Out of this, a massive proportion, 80%, involves urinary tract infections associated with catheters (CUTI)[6,7]. Urinary catheters are used routinely in urinary tract practice; despite the progress made in the design and materials used, urinary tract infection remains the main obstacles, due to the contamination of these residential devices[5,8]. Roughly, between 12 to 16% of hospital inpatient adults have urinary tract indwelling catheter, however, it is known to be associated with high morbidity rates, high mortality rates, increased length of hospital stay, and the increase in the cost of treatment[6,7,8,9]. Furthermore, biofilm producers associated with catheters, preceding drug resistance, and thought-provoking infection control measures have been reported in previous studies, which raises concern on CAUTIs and biofilm producers in hospital environments[10,11].

A biofilm contains any syntrophic consortium of micr -organisms in which cells attach to each other and often as well to a surface[11,13]. These adherent cells become embedded within a slimy extracellular matrix that is composed of extracellular polymeric substances (EPS) [12,13]. The cells within the biofilm produce the EPS components, which are typically a polymeric conglomeration of extracellular polysaccharides, proteins, lipids and DNA[12,13,14].

The extracellular matrix facilitates communications among the cells through biochemical signals acyl-homoserine lactone in Gram-negative bacteria and oligopeptides in Gram-positive bacteria in a phenomenon called as quorum sensing[15]. Biofilms are not just bacterial slime layers but biological systems; the bacteria organize themselves into a coordinated functional community[16]. The matrix not only impedes the pathogen against the host's defence, but also attributes the antimicrobial resistance, through the subordinating antibiotic penetration, horizontal transmission of plasmid-associated drug-resistant gene, and altered microenvironment[15,17].

In this situation, early detection of biofilm producers is crucial to reduce the irrational burden of antimicrobials resulting from antimicrobial resistance in the patient; thus, it will be helpful in control of infection associated with devices in medical fields. The rationale for the current study was to clarify bacterial etiology, highlight the resistance patterns associated with biofilm producing bacteria and establish appropriate antimicrobial therapy against biofilm producers in people with CAUTIs.

MATERIALS AND METHODS

Study Design: The cross-sectional study was conducted at the Department of bacteriology, the National Center of Public Health Laboratories (NCPHL) and Al-Thorah Hospitals in Ibb city, Ministry of Health and Population, Yemen, over a period of 12 months. The study hospital is a referral centre with medical, surgical, gynecological, pediatric, geriatric, and other specialties.

Inclusion and exclusion criteria: Urine specimen was included from all catheter patients regardless of gender and age between 12 and 65 years who met the CAUTI criteria in the study. However, non-catheterized patients who were cared for in a ward or previously under antimicrobial treatment before inserting the catheter were excluded. Also more than two types of organism grown from the clinical sample, were considered as contaminated and consequently, excluded from the study. The study of biofilms is not usually done for patients with catheters, but was conducted for the purpose of research only in this group of patients.

Data collection: Data were collected through a predetermined questionnaire. Data including patient demographic data, clinical information, biofilm formation and antibiotic susceptibility; risk factors of biofilm formation, and other laboratory results.

Laboratory Methods: CAUTI is defined using a combination of signs, clinical symptoms, and laboratory standards as described by Stamm[8]. A total of 335 urine samples from catheterized patient's admitted to the hospital were treated almost quantitatively by inoculating 0.001 ml of the sample (using a titrated wire loop) on the Cystine Lactose Electrolyte Deficiency (CLED) agar to isolate and identify uropathogens[8]. Following the inoculation, the plates were incubated for 24 hours at 37°C in an aerobic atmosphere. The growth of a single organism with a count of $\geq 10^2$ colony forming units (CFU)/ml was considered to represent as CAUTIs (positive samples counted 227) and was identified using appropriate routine identification methods including colony morphology, Gram stain, and an in-house set of biochemical tests[18].

Antimicrobial Susceptibility Testing

The susceptibility of bacterial isolates against recommended antibiotics was tested by the modified Kirby–Bauer disk diffusion method on Mueller Hinton agar (Oxoid) following guidelines of Clinical and Laboratory Standards Institute (CLSI)[19]. Antibiotics that were tested in our study include amoxycillin clavulanate (amc 20/10 μg), ampicillin (amp 10 μg), amikacin (ak 30 μg), cefuroxime (cfm 30 μg), ciprofloxacin (cip 5 μg), cotrimoxazole (cot 25 μg), gentamicin (gen 10 μg), imipenem (imp 10 μg), nitrofurantoin (300 μg), nalidixic acid (NA 30 μg) penicillin (P 25 μg), erythromycin (E 15 μg), norfloxacin (Nor 10 μg), rifampicin (RA 5 μg) and vancomycin (VAN 30 μg) (Oxoid).

Biofilm production detection*:* The detection of biofilm was done by tissue culture method/microtiter plate method (TCA)[20,21]. The bacterial isolates from fresh agar plates were inoculated in 2 ml of BHI broth and incubated for 24 h at 37°C. The cultures were then diluted 1:40 with fresh medium (BHI broth supplemented with 1% glucose); 200 μl of the sample was dispensed in the individual microtitration plate and incubated further 24 h at 37°C. With a gentle tapping, the content was removed further with a subsequent washing with phosphate buffer saline (pH 7.2) three times to remove free floating sessile bacteria. The adherent bacteria, biofilm producer, were fixed with sodium acetate (2%) and stained with crystal violet (0.1% w/v) for 10–15 min. The unbound crystal violet solution was removed with a triplicate washing with PBS, and the plate, then, was kept for drying. Finally,

all wells were filled with 200 μl ethanol (95%) to release dye from the well and Optical Density (OD) was taken at the wavelength of 630 nm. OD value of each test strain and negative control were calculated, and OD cutoff values (ODc) were assessed as described previously[21].

Data Analysis

Personal, clinical and laboratory data were obtained from each subject and recorded into a pre-designed questionnaire, then the data were statistically analyzed by a software version for statistical significance (Epi Info version 6, CDC, Atlanta, USA). First rates were calculated, then from two-by-two tables, the odds ratios were calculated and *P*-value was determined using the uncorrected chi square test. Fisher's exact test was used for the small expected cell sizes with a two-tailed probability value.

Ethical approval

We obtained written consent from all cases. Assent was taken from participants before collecting the specimens. The study proposal was evaluated and approved by the Ethics Committee of Faculty of Medicine and Health Sciences, Sana'a University.

RESULTS

The study results are illustrated in 8 tables. Out of 227 culture positive cases, Gram negative organisms were predominant (88.1%). The most organisms caused UTI among catheter patients in this study were belonging to Gram negative bacteria (88.1%), while Gram positive isolates only counted 11.9%. The most frequently isolated uropathogens was *E. coli* (46.3%) followed by *K. pneumoniae* (18.5%) and *P. aeruginosa* (11.9%). Finally, the maximum biofilm production in the current study was seen in *E. coli* where 63 out of the 105 isolates (61%) showed biofilm production followed by *Klebsiella* spp. (57.1%) and *Pseudomonas* spp. (37.0%).

Table1: The age and sex distribution of patients with indwelling catheters.

Age group in years	Sex				Total	
	Male		Female			
	No.	%	No.	%	No.	%
1-15	8	61.5	5	38.5	13	5.7
16-30	25	54.3	21	45.7	46	20.3
31-45	24	46.2	28	53.8	52	22.9
46-60	28	54.9	23	45.1	51	22.5
> 60	36	55.4	29	44.6	65	28.6
Total	121	53.2	106	46.7	227	100

The present study showed that the most effective antibiotics against biofilm producing Gram negative isolates was imipenem and for Gram positive isolates was vancomycin. The biofilm strains displayed relatively high resistance against tested antibiotics than non biofilm producers. Resistance rates of biofilm strains vs. non-biofilm strains were for Ceftriaxone (82.9% vs. 36.8%), ciprofloxacin (80.9% vs. 57.9%), cefotaxime (72.4%vs.49.5%), norfloxacin (85.7% vs. 40%) and cotrimoxazol (71.4% vs. 45%). Least resistant drugs observed were nitrofurantoin (40% vs. 23.2%), gentamicin (29.5%vs.22.1%), and amikacin (33.3% vs.14.7%).

Table 2: Distribution of uropathogens from catheter urine samples

Bacteria	Number	Percentage
Escherichia coli	105	46.3
Klebsiella pneumoniae	42	18.5
Pseudomonas aeruginosa	27	11.9
Proteus mirabilis	16	7.0
Coagulase negative *Staphylococci*	13	5.7
Enterobacter spp.	10	4.4
Staphylococcus aureus	11	4.8
Enterococcus faecalis	3	1.3
Total	227	100.0

When risk factors associated with biofilm production in the catheter by bacteria were considered among patients with population catheters, there were significant risk factors for biofilm production with pre-UTI (OR=2.4, P=0.001), age group patients>60 years (OR=5.4, p <0.0001), catheter duration>7 days (OR=15.3, p<0.0001) and diabetes (OR=3.5, p <0.0001) (Table 6).

DISCUSSION

In the current study, biofilm formation was observed among 112/227 (49.3%) isolates, Out of which, 48 (21.10%) were high, 64 (28.2%) were moderate and 115 (50.7%) were non biofilm producers. The current study is in concordance with Maqbool *et al.*,[22] who observed 47.5% biofilm forming among bacterial isolates from UTI. Hassan *et al.*,[23], Abdagire *et al.*,[24] and Soto[25] performed similar studies to detected biofilm forming capacity for the uropathogens among patients with catheter associated urinary tract infections, the biofilm production was detected in about 50% of the cases, a value also closer to the one obtained in the present study (Table 4).

When the rate of biofilm formation with respect to duration of catheterization of patients was considered, there was significant increase in the rate of biofilm formation with longer duration of catheterization in which the highest rate was occurred in >7 days duration with rate equal to 92%, followed by 4-7 days duration with rate equal to 43%, while with <4 days duration the rate only was 31.9%.

Table 3: The associated Odds ratio (OR) of uropathogens in ability to produce biofilms in patients with indwelling catheters

Bacteria	Biofilm producer		Non biofilm producer		OR	CI	X^2	*P*
	No.	%	No.	%				
E. coli, n= 105	63	60	42	40	8.6	1.6-61.3	9.5	0.002
K. pneumoniae, n= 42	24	57.1	18	42.9	10.1	1.6-79	9.3	0.002
P. aeruginosa, n= 27	10	37	17	63	6.6	1.0-54	5.3	0.02
P. mirabilis, n= 16	5	31.2	11	68.8	2.7	0.3-25.9	1.17	0.28
Coagulase negative, *Staphylococci, n= 13*	4	30.8	9	69.2	1.9	0.2-23	0.4	0.52
Enterobacter spp. , *n= 10*	3	30	7	70	0.1	0-154	0.22	0.64
E. faecalis, n= 3	1	33.3	2	66.7	2	0.21-21.4	0.5	0.47
S. aureus [*], *n= 11*	2	18.2	9	81.8				

[*] *S. aureus* was used as reference strain of biofilm formation in which it show the lowest rate of biofilm producing.

Table 4: Biofilm detection by TCA method among patients with indwelling catheters.

Bacteria	Biofilm detection by TCA							
	High*		Moderate*		Non/weak*		Total	
	No.	%	No.	%	No.	%	No.	%
Escherichia coli	29	27.6	34	32.4	42	40.0	105	46.3
Klebsiella pneumoniae	11	26.2	13	30.9	18	42.9	42	18.5
Pseudomonas aeruginosa	6	22.2	4	14.8	17	63.0	27	11.9
Proteus mirabilis	0	0	5	31.2	11	68.8	16	7.0
Coagulase negative Staphylococci	1	7.7	3	23.1	9	69.2	13	5.7
Enterobacter spp.	0	0	3	30.0	7	70.0	10	4.4
Enterococcus faecalis	0	0	1	33.3	2	66.7	3	4.8
Staphylococcus aureus	1	9.1	1	9.1	9	81.8	11	1.3
Total	48	21.1	64	28.2	115	50.7	227	100

This result is similar to that previously reported in which the formation of biofilm by urinary pathogens on the surface of the catheter and drainage system occurs universally with prolonged duration of catheterization[26]. The mechanism of the biofilm forming capacity for the uropathogens among patients with catheter associated urinary tract infections can be explained by that bacteria invading urinary tract met with potent innate defenses, including neutrophil influx and epithelial exfoliation. Bacterial subversion of innate responses involves invasion into bladder superficial cell and bacteria matured into biofilm, creating pod-like bulges on the bladder surface. Pods contained bacteria covered 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 healthy host defense[27].

Table 5: The association between age groups of patients and producing biofilm in catheters.

Age group	Biofilm producer n= 112		Non biofilm producer n= 115		OR	CI	X^2	*P*
	No.	%	No.	%				
1-15 years n= 13	3	23.1	10	76.9	0.36	0.2-2.2	0.65	0.419
16-30 years n= 52	18	34.6	34	65.4	0.46	0.24- 0.87	5.85	0.016
31-45 years n= 46	18	39.1	28	60.9	0.74	0.39-1.43	0.79	0.373
46-60 years n= 51	23	45.1	28	59.9	0.53	0.28-1.01	3.84	0.059
> 60 years n= 65	50	76.9	15	23.1	5.4	2.78-10.38	27.7	<0.001

One of the aims of this study was to identify the association between biofilm production and uropathogens strains. The maximum biofilm production was seen in *E. coli* where 63 out of the 105 isolates (61%) showed biofilm production followed by *Klebsiella* spp. (57.1%) and *Pseudomonas* spp. (37.0%). This is in accordance with Niveditha *et al.,*[28] who also observed *E. coli* (42%) as the most common biofilm producers, while Deotale *et al.*[29] reported biofilm production was more frequent by *K.*

pneumoniae (76%) comparing with lower rate (50%) of *E. coli* which is different from the present study.

Considering risk factors for biofilm producing as previous incidence of UTI, catheterization, a prolonged duration of catheterization (≥7 days), diabetic mellitus and the age group > 60 years which they had approved to increase the propensity of microorganisms to form biofilms in the urinary tract[30].

Table 6: The associated risk factors of biofilm producing in catheters by bacteria among patients with indwelling catheters.

Risk factor	Biofilm producer		Non biofilm producer		OR	CI	X^2	*P* value
	No.	%	No.	%				
Male	55	45.5	66	54.5				
Female	57	53.8	49	46.2	0.72	0.42-1.21	1.6	0.211
Pre- UTI	81	57.4	60	42.6	2.4	1.3-4.33	9.8	0.001
Age group >60 years	50	76.9	15	23.1	5.4	2.7-10.9	27.7	<0.0001
Duration of catheterization >7 days	46	92.0	4	8.0	15.3	5.5-46.3	43.9	<0.0001
Diabetic mellitus	42	71.2	17	28.8	3.5	1.8-6.6	15.2	<0.0001
Renal calculi	22	56.4	17	43.6	1.4	0.67-2.99	0.94	0.33
Hypertension	20	48.9	21	51.2	0.97	0.49-1.91	0.006	0.94

In the current study, the incidence of biofilm producing increased with increase in the age of the patient, maximum incidence was from >60 years age group which had 50 biofilm producing cases out of 65 patients (76.9%) . Also when association was seen between age >60 years and age <45 years considering both males and females together, it was found to be statistically significant (*P* value <0.0001). The odd ratio was 5.4 which displayed that those aged >60 years possess the risk of developing biofilm 5.4 times more than those who aged <60 years. These results are similar to Trautner *et al.*[30] and Soto[25] who have demonstrated a positive correlation among catheterization, old patients and biofilm formation[25,30] in addition to old ages they suggested that a significant history of UTI is a major indicator for the recurrence of UTI due to biofilm formation.

Table 7: Antibiotic resistance pattern of Gram negative bacteria.

Antimicrobial agents	Biofilm producing bacteria n=105	Non-biofilm producing bacteria n=95	P value
Amikacin	33.3%	14.7%	0.002
Amoxicillin clavulanic acid	100%	93.7%	0.009
Ampicillin	100%	96.8	0.067
Cotrimoxazole	88.6%	66.3%	<0.001
Ciprofloxacin	80.9%	57.9%	<0.001
Gentamicin	29.5%	22.1%	0.233
Ceftriaxone	82.9%	36.8%	<0.001
Nalidixic acid	81.9%	86.3%	0.396
Nitrofurantoin	40%	23.2%	0.011
Cefotaxime	72.4%	49.5%	<0.001
Imipenem	8.6%	0%	0.07

In present study incidence of biofilm producing among diabetics was higher (70%). After multivariate analysis diabetes was found to have significant association with biofilm formation (p<0.0001). The odd ratio was 3.5 which displayed that those with the diabetic possess the risk of developing biofilm 3.5 times more than those who non diabetic. This is in agreement with Pramodhini *et al.*[31] who reported that the incidence of biofilm producing among diabetics was higher than that among non-diabetics patients with indwelling catheter[31]. The crucial aim of this study was identify the association between biofilm production and anti-biogram susceptibility of uropathogens strains isolated from the study patients. The antibiotic resistance was significantly higher among biofilm producers than among non biofilm producers. This finding was comparable to the studies conducted by Pramodhini *et al.*,[31]; Maqbool *et al.*,[22]; and Tayal *et al.*[32] in which antibiotic resistance was significantly higher among biofilm producers than among non biofilm producers. The degree of antibiotic resistance may be higher among biofilm producers than in non-biofilm isolates in the present study and previous studies may be due to bacterial biofilms with long term persistence of organism in various environments, decreased bacterial growth rate in a biofilm, expression of resistance genes, and restricted penetration of antibiotics into biofilm. Furthermore, proximity of cells within a biofilm can facilitate a plasmid exchange and hence enhance the spread of antimicrobial resistance as it had been described by Abdagire *et al.*,[24].

The present study showed that the most effective antibiotics against biofilm producing Gram negative isolates from UTIs were found to be imipenem and for Gram positive isolates was vancomycin. This is in agreement with Tayal *et al.*,[32] who found that the most effective antibiotics against Gram-negative bacteria were imipenem and amikacin and for Gram positive isolates was vancomycin.

Table 8: Antibiotic resistance pattern of Gram positive bacteria.

Antimicrobial agents	Biofilm producing organisms n=7	Non-biofilm producing organisms n=20	*P* value
Co-trimoxazole	71.4%	45%	0.229
Vancomycin	0%	0%	NA
Penicillin	85.7%	90%	0.756
Gentamycin	71.4%	15%	0.0048
Erythromycin	85.7%	70%	0.414
Nitrofurantoin	42.9%	25%	0.373
Norfloxacin	57.1%	40%	0.432
Rifampicin	0	0	NA

NA= Not Applicable

In the current study Imipenem, is the antimicrobial agent that is effective against both Gram positive and Gram negative organisms while, nitrofurantoin was not effective against both Gram positive and Gram negative organisms in which the resistant rate was 40% for biofilm producing bacteria (Table 7 and Table 8). These results are different from Panda *et al.*[33] study in which they noted in 2016 that nitrofurantoin was effective against both Gram positive and Gram negative biofilm producing bacteria[33].

In the present study the investigated biofilm strains displayed relatively high resistance against tested antibiotics than non biofilm producers. Resistance to five antibiotics such as Ceftriaxone (82.9% vs. 36.8%), ciprofloxacin (80.9% vs. 57.9%), cefotaxime (72.4% vs. 49.5%), norfloxacin (85.7% vs. 40%) and cotrimoxazol (71.4%, vs.45%) was comparatively higher among biofilm producers than non-biofilm producers. Least resistant drugs observed were nitrofurantoin (40% vs. 23.2%), gentamicin (29.5% vs. 22.1%), and amikacin (33.3% vs. 14.7%) (Table 7 and Table 8). Similar results were reported by Chatterjee *et al.*[34] in which the studied biofilm strains displayed relatively high resistance against previously tested antibiotics than non biofilm producers.

CONCLUSION

High antimicrobial resistance was observed in biofilm producers than non-biofilm producers. Of recommended antimicrobial therapies for CAUTIs, ampicillin and amoxicillin-clavulanate were the least active antibiotics, whereas imipenem and amikacin were found as the most effectual for gram-negative biofilm producer. Likewise, penicillin and erythromycin were the least active antibiotics, whereas vancomycin, and rifampicin were found as the most effective antibiotic for Gram-positive biofilm producers.

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CONFLICT OF INTEREST

"No conflict of interest associated with this work".

AUTHOR'S CONTRIBUTION

This research work is part of M.Sc. thesis. The candidate is the first author who conducted the works and the experiments and wrote up the thesis. The corresponding author (HAA) supervised the clinical and laboratory work, revised and edited the thesis draft and the manuscript. Other authors were co-advisor of the work and helped in revised and edited the thesis draft and the manuscript and in the laboratory works**.**

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