Egyptian Journal of Medical Microbiology Volume 27 / No.4 / October 2018 87-95 Online ISSN: 2537-0979

ORIGINAL ARTICLE

Biofilm Producing Bacteria in Cases of Urinary Tract Infection at Sohag University Hospital

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Key words:
Biofilm, urinary tract infection, antibiotic resistance

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ABSTRACT

Background: *Biofilm is an important problem of great medical concern in which microorganisms are present in extracellular matrix protecting them from external environment, host immunity and antibiotic therapy. Multiple phenotypic methods are present to detect biofilm in vitro tube method, congo red and tissue culture plate methods.* ***objectives:*** *To determine the ability of bacteria that cause urinary tract infection to form biofilm , antibiotic susceptibility pattern in biofilm forming isolates and to detect some of genes responsible for biofilm formation.* ***Methodology:*** *four hundred urine samples were collected 240 samples from catheterized and160 from non catheterized patients who fulfill the inclusion criteria. Samples were cultured and colony forming unit was counted (colony forming unit > 10^5 were considered positive UTI. Identification of bacteria and their antibiotic sensitivity was done by automated system VITEK II. Multiple phynotypic biofilm detection methods were done and detection of biofilm genes was done by PCR.* ***Results****: Enterobacter spp. Were the most frequent isolated organism of Gram negative, Staph aureus was the most frequent isolated organism of Gram positive bacteria. Multiple Phenotypic methods for detection of biofilm production were done to Gram positive and Gram negative bacteria. Tube method detected 84 (68.9%) cases as positive biofilm producer in catheterised patients while 2 (5%) were positive in non catheterized patients. Congo red method detected 80 cases (65.6%) as positive in catheterized cases , 2 (5%) in non catheterized patients but tissue culture plate detected 88 cases(72%) as positive in catheterized patients. Non catheterized patients 18 (45%) were positive. PCR was done to detect biofilm genes (IcaA, IcaD in staphylococci), (BssS gene in enterobacteriaceae), IcaA, D were detected in 8 (19%) isolates of staphylococci, BssS was detected in 66/104(63.5%) of enterobacteriaceae. Sensitivity of phenotypic methods for biofilm detection in relation to genotypic revealed that tissue culture plate showed more sensitivity in Gram positive and negative bacteria.* ***Conclusion****: Multiple phenotypic methods are known for biofilm detection in vitro but tissue culture plate is the most sensitive method; so we can recommend tissue culture plate as a screening method for biofilm detection*

INTRODUCTION

Urinary tract infections are the important causes of morbidity affecting 150 million people each year and also continue to be the most common causes of infections in hospitalized patients. It is the most common bacterial infections in humans both in the community and hospitals, and in all age groups, and usually requires urgent treatment. Microorganisms associated with UTI have a property to form biofilm which could be formed by one or many bacteria with antimicrobial resistance. Host factors like age, diabetes, long term hospitalization and catheterization are the predisposing factors. Urology is the main areas of concern where biofilm can become a serious problem[1].

Bacteria in biofilm embed themselves in a self-produced extracellular matrix of exopolysaccharide , proteins and some micro molecules such as DNA. This matrix accounts for about 90% biomass. The extracellular matrix of exopolysaccharide protects the bacteria from host immunity and prevents antibiotics from gaining access to bacteria and is responsible for persistent urinary tract infections and the multidrug resistance so the present study was undertaken with the aim to: determine prevalence of biofilm formation in bacteria causing urinary tract infection. Identify the antimicrobial resistance pattern of biofilm producing uropathogens and detect some biofilm genes by molecular method (PCR).

METHODOLOGY

This study was done at Sohag University Hospital from October 2016 to March 2017 at Microbiology and Immunology Department to study biofilm producing bacteria in cases of UTI. The study was carried out after having approval from the Ethics Committee, Written consent from all the patients included in the study was taken before initiation of the study.

Isolation and Identification of Microorganisms:

Urine samples were collected from patients who fulfilled the CDC crieteria for diagnosis of UTI from patients with or without catheter in various departments (ICUs, Neurology, Tropical, Internal medicine, Urology) under complete a septic condition were sent immediately to the laboratory. Quantitative urine culture to determine colony forming unit was done as described by Forbs et al.[2] , Bacterial count $>10^5$ cfu/mL indicating UTI. Identification of bacteria was done using (Vitek II bioMérieux, France) using identification cards GN for Gram negative bacteria, AST cards for antibiotic sensitivity testing of Gram negative and positive bacteria.

Multiple Phenotypic Methods were used for Biofilm Detection in Vitro:

1. Tube method [3:]
2. Congo red agar (CRA) method [4]**:**
3. Tissue culture plate analysis [5]**:**

Table 1: Classification of bacterial adherence by microtitre plate method

Mean OD value	Biofilm formation
< 0.060	Non
0.060 - <0.124	Weak
0.124-0.240	Moderate
≥0.240	High

OD: optical density.

Molecular detection of biofilm genes: (IcaA, IcaD in staphylococci, BssS gene in enterobacteriacae.

-DNA was extracted by boiling method in both Gram positive and negative bacteria**.**

DNA extraction from Gram negative bacteria [6]:

DNA extraction from Gram positive bacteria [7]:

DNA amplification for detection of BssS gene in enterobacteriaceae, was done as mentioned by Hassan *et al.*[8]

Detection of IcaA, IcaD was done as mentioned byArciola et al [9].

After amplification, 10µl of the PCR mixture was analyzed by agarose gel electrophoresis (2% agarose in Trisborate-EDTA stained with ethidium bromide). The Gene Ruler 100 bp DNA ladder (Jena bioscience) was used as a DNA size marker visualization of bands was done using DNA documentation system.

Statistical analysis

Statistical analysis was done by spss version 22; Chi-square ($\chi 2$) test was used for comparison regarding qualitative variables, Quantitative data were expressed as means ± standard deviation. The data were tested for normality using Shapiro-Wilk test. The nonparametric Mann–Whitney test was used for data which wasn't normally distributed. A 0.05 level was chosen as a level of significance in all statistical tests used in the study.

RESULTS

This study was done at Sohag University Hospital from Octobor 2016 to March 2017 to study biofilm producing bacteria in cases of UTI. Urine samples were collected from patients with and without catheter from different departments, 400 urine samples were collected 240 from catheterized, 160 from non catheterized patients who have the symptoms of UTI, samples were cultured and colony count was determined. Samples with colony forming unit > 10^5 were considered positive UTI.

Table 2: The demographic data and sites of isolation:

Parameter	Urethral catheter (N=122)	Non-urethral catheter (N=40)	P-value
Age			
Mean± S.D.	**46.2± 17.2**	**27± 8.7**	**0.000***
Median(Range)	**50 (1.5–75)**	**31(7–33)**	
Gender			0.112
Males (%)	**50 (69.4%)**	**22 (30.6%)**	
Females (%)	**72 (80%)**	**18 (20%)**	
Department			**0.000***
Chest (%)	**6 (100%)**	**0 (0%)**	
Internal medicine (%)	**20 (50%)**	**20 (50%)**	
ICU (%)	**40 (100%)**	**0 (0%)**	
Neuro (%)	**44 (100%)**	**0 (0%)**	
Obstetrics (%)	**0 (0%)**	**2 (100%)**	
Pediatrics (%)	**0 (0%)**	**4 (100%)**	
Tropica	**12(100%)**	**0(0%)**	
Urology	**0(0%)**	**14(100%)**	

UTI was more frequent in females in catheterized patients.

Identification of bacteria to species level and their antibiotic sensitivity was done by VITEK II.

Table 3: Frequency of isolated bacteria.

Bacteria	Using catheter	Not using catheter
Burkholderia cepacia	**2 (100.0%)**	**0 (0.0%)**
Citrobacter koseri	**2 (100.0%)**	**0 (0.0%)**
E. coli	**20 (62.5%)**	**12 (37.5%)**
Enterobacter aerogenes	**12 (100.0%)**	**0 (0.0%)**
Enterobacter cloacae	**22 (61.1%)**	**14 (38.9%)**
Enterobacter cloacae complex	**4 (100.0%)**	**0 (0.0%)**
K.pneumonia	**18 (100.0%)**	**0 (0.0%)**
Pseudomonas aeuroginosa	**2 (50%)**	**2 (50%)**
Staph. Aureus	**28 (82.4%)**	**6 (17.6%)**
Staph. Galinarum	**4(100.0%)**	**0 (0.0)**
Staph. Xylosus	**2 (100.0%)**	**0 (0.0%)**
Staph.lentus	**2 (100.0%)**	**0 (0.0%)**
Stenotrophomonas maltophilia	**0 (0.0%)**	**6 (100.0%)**
Strpt. Agalactae	**4 (100.0%)**	**0 (0.0%)**

Enterobacter species were the most frequent isolated organism in Gram negative bacteria.

Fig. 1: Biofilm positive isolates by tube method.

Fig. 2: Biofilm positive bacteria by congo red method

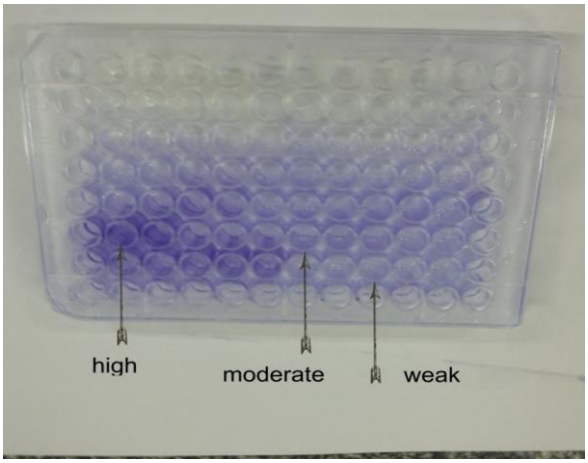

Fig. 3: Biofilm forming bacteria by tissue culture plate method.

Table 4: Frequency of biofilm positive and negative isolates using phenotypic methods (tube, congo, TCP)

Method	Using catheter (N=122)	Not using catheter (N=40)	P-value
Tube method			
Positive	**84(68.9%)**	**2 (5%)**	**0.000***
Negative	**38 (31.1%)**	**38 (95%**	
Congo red method			**0.000***
Positive	**80(65.6%)**	**2(5%)**	
Negative	**42(34.4%)**	**38(95%)**	
Tissue culture plate method			**0.013***
High	**14(11.5%)**	**2 (5%)**	
Moderate	**56(45.9%)**	**14 (35%)**	
Weak	**18(14.7%)**	**2 (5%)**	
Non	**34 (27.8%)**	**22 (55%)**	

By tube method 84(68.9%) of bacteria isolated from catheterized patients were biofilm positive by tube method, 2(5%) of non catheterized patients were positive. Congo red agar method detected 80(65.6%) of bacteria from catheterized patients and 2(5%) of bacteria in non catheterize patients but Tissue culture plate method detected 88(71.7%) biofilm positive bacteria in catheterized patients,18(45%) in non catheterized patients**.**

Table 5: Sensitivity of tube and congo red method was detected in comparison with tissue culture plate method
Tube method showed higher sensitivity and specificity than congo red agar.

Variable	Tube method	Congo red
Sensitivity	**64.2%**	**54.7%**
Specificity	**67.9%**	**57.1%**
Positive predictive value (PPV)	**79.1%**	**70.7%**
Negative predicative value (NPV)	**50 %**	**40%**

Detection of biofilm genes by (PCR) to the most frequently isolated organism both in Gram positive and Gram negative bacteria showed that in staphylococci IcaA, IcaD biofilm genes were detected in 8/42(19%) in all staphylococci as shown in table (6).

Table 6: Frequency of Ica A, D gene in different gram positive bacteria.

Bacteria	Ica A, D gene		P- value
	Positive 8 (19%)	Negative 34 (81%)	
Staphylococcus aureus	**4 (11.8%)**	**30 (88.2%)**	**0.000 ***
Staphylococcus galinarum	**4 (100%)**	**0 (0.0 %)**	
Staphylococcus xylosus	**0 (0.0 %)**	**2 (100 %)**	
Staphylococcus lentus	**0 (0.0 %)**	**2 (100 %)**	

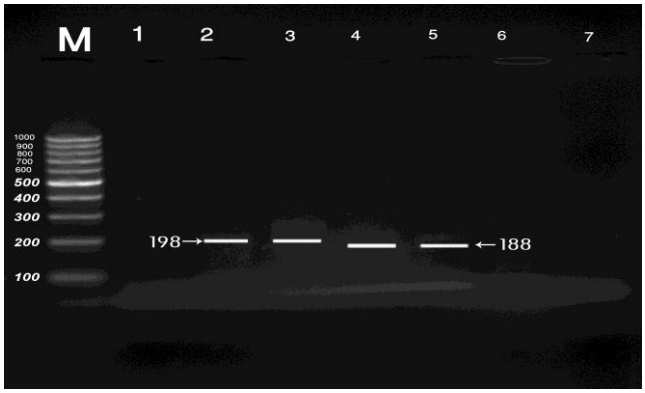

Fig. 4: Gel electrophoresis showing:
- Lane M: ladder 100-1000 bp, Lane 1: negative control, Lane 2, 3: Amplified product of Ica D gene in *Staph.* size 198 bp, Lane 4, 5: Amplified product of Ica A gene in *Staph.* size 188 bp.

BssS gene of biofilm in enterobacteriaceae was detected in 66/104(63.5%) of isolates as shown in table (7).

Table 7: Frequency of BssS gene in different Gram negative bacteria.
E.coli was the most frequent organism carrying BssS gene (93.7%).

Bacteria	BassS gene		P value
	Positive(N=66)	Negative (N=38)	
Citrobacter koseri	**2 (100%)**	**0 (0.0%)**	**0.000***
E. coli	**30 (93.7%)**	**2 (6.3%)**	
Enterobacter aerogenes	**8(66.7%)**	**4 (33.3%)**	
Enterobacter cloacae	**14 (38.9%)**	**22 (61.1%)**	
Enterobacter cloacae complex	**2 (50%)**	**2 (50%)**	
K.pneumonia	**10 (55.6%)**	**8 (44.4%)**	

By comparing methods used for detection of biofilm and BssS gene, tissue culture plate was the most sensitive method as shown in table 8.

Table 8: Sensitivity, specificity of tissue culture plate, congo red and tube methods in comparison with BssS gene(in enterobacteriaceae).

Variable	Tissue culture plate	Congo red	Tube method
Sensitivity	**63.6%**	**51.5%**	**48.5%**
Specificity	**47.6%**	**66.7%**	**66.7%**
Positive predictive value (PPV)	**65.6%**	**70.8%**	**69.6%**
Negative predicative value (NPV)	**45.5%**	**46.7%**	**45.2%**

By comparing methods used for detection of biofilm and IcaA and IcaD genes, tissue culture plate was the most sensitive method as shown in table 9.

Table 9: Sensitivity, specificity of tube, congo red and tissue culture plate methods in comparison with Ica A, D genes.

Variable	Tube method	Congo red	Tissue culture plate
Sensitivity	**100%**	**75.0%**	**100%**
Specificity	**29.4%**	**29.4%**	**23.5%**
Positive predictive value (PPV)	**25%**	**20%**	**23.5%**
Negative predicative value (NPV)	**100%**	**83.3%**	**100%**

Comparison between biofilm formation groups regarding antibiotic sensitivity among gram negative bacteria (N. =116). Biofilm forming bacteria were more resistant to Ampicillin Sulbactam, gentamycin, nitrofurantoin, trimethoprim/Sulfamethoxazole and Cefazolin than biofilm negative bacteria. The results are shown in fig.(5).

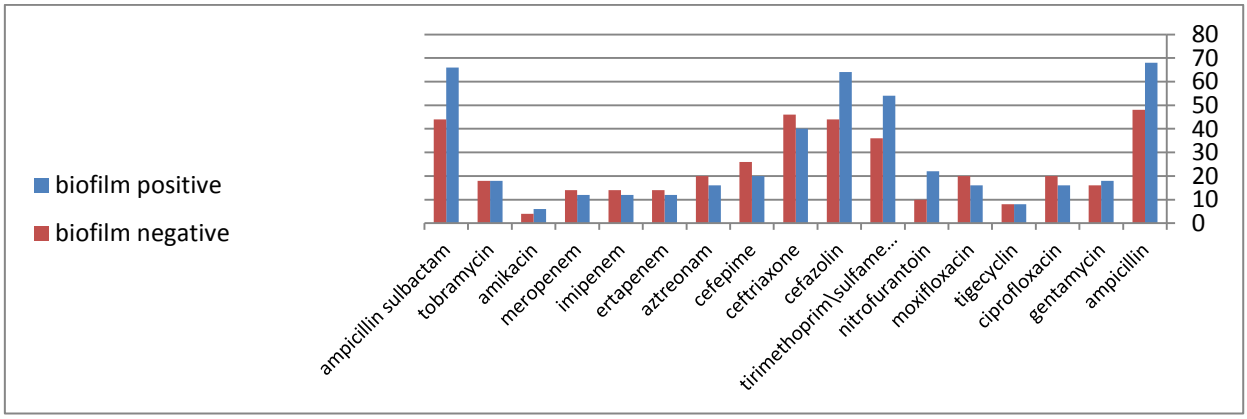

Figure 5: Antibiotic resistance pattern in biofilm positive and negative Gram negative bacteria.

Comparison between biofilm formation groups regarding antibiotic sensitivity in gram positive bacteria (N. =46). Biofilm forming bacteria were more resistant to most of antibiotics (Gentamycin, Ciprofloxacin, Tigecyclin, Nitrofurantoin, oxacillin, vancomycin, linzolie,clindamycin and erythromycin). The results are shown in fig.(6).

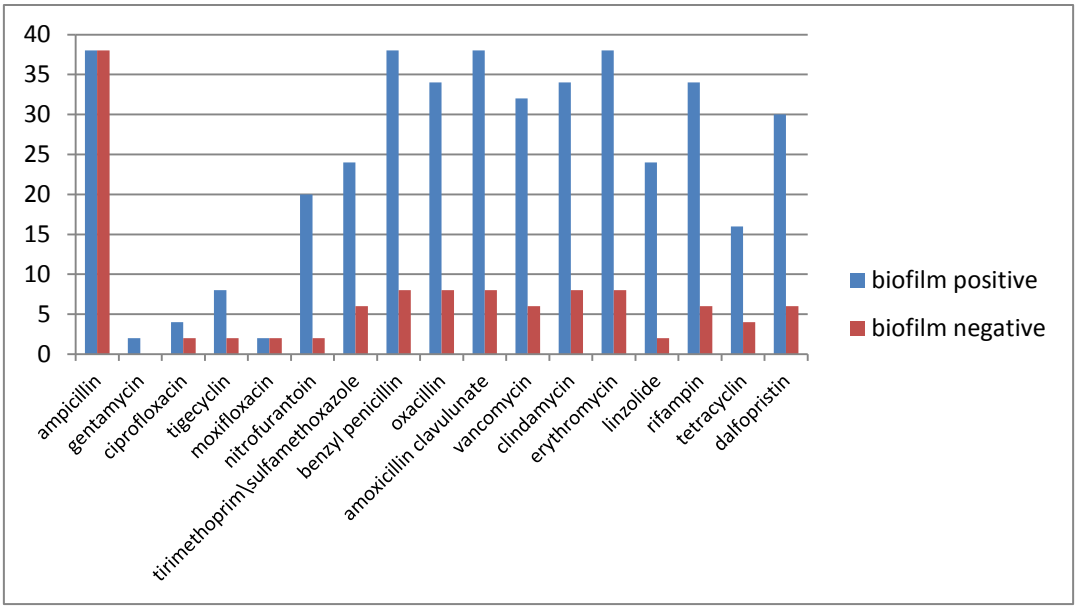

Figure 6: Antibiotic resistance pattern in biofilm positive and negative Gram positive bacteria.

Table 10: Factors associated with biofilm formation. 88(83%) of biofilm positive were catheterized patients, 22(20.8%) with renal failure, 18(17%) with hepatic problems and 2(1.9%) with prostatic enlargement as shown in table (10).

Factors	Biofilm formation		P-value
	Positive N. =106 (65.4%)	Negative N. = 56 (34.6%)	
Age			
Mean± S.D.	**41.4 ± 18.3**	**41.6 ± 16.5**	0.682
Median (Range)	**40 (1.5 – 75)**	**33 (8 – 70)**	
Gender			0.712
Male (%)	**46 (43.4%)**	**26 (46.4%)**	
Female (%)	**60 (56.6%)**	**30 (17.9%)**	
Use of Urinary catheter			
Yes (%)	**88 (83%)**	**34 (60.7%)**	**0.002***
No (%)	**18 (17%)**	**22 (39.3%)**	
Diabetes Mellitus			0.428
Yes (%)	**14 (13.2%)**	**10 (17.9%)**	
No (%)	**92 (86.8%)**	**46 (82.1%)**	
Fecal incontinence			0.849
Yes (%)	**14 (13.2%)**	**8 (14.3%)**	
No (%)	**92 (86.8%)**	**48 (85.7%)**	
Renal failure			**0.000***
Yes (%)	**22 (20.8%)**	**0 (0.0%)**	
No (%)	**84 (79.2%)**	**56 (100%)**	
Hypertension			0.798
Yes (%)	**32 (30.2%)**	**18 (32.1%)**	
No (%)	**74 (69.8%)**	**38 (67.9%)**	
Neurological problems			0.204
Yes (%)	**24 (22.6%)**	**8 (14.3%)**	
No (%)	**82 (77.4%)**	**48 (85.7%)**	
Hepatic problems			**0.001***
Yes (%)	**18 (17 %)**	**0 (0.0%)**	
No (%)	**88 (83%)**	**56 (100%)**	

DISCUSSION

Urinary tract infection, with its diverse clinical syndromes and affected host groups, remains one of the most infectious diseases encountered in clinical practice. Antimicrobial resistance is a leading problem and measures should be made to ensure an appropriate duration of therapy for symptomatic infections. The risk of developing urinary tract infection increases with the use of devices such as catheters, urethral stents or sphincters. Urinary tract infections account for an estimated 25 to 40% of nosocomial infections and represent the most common type of these infections [10]. Clinical observations have established that, the bacteria within catheter associated urinary tract infection (CAUTI) frequently develop as biofilms, directly attaching to the surface of catheters. Biofilms are microbial communities of surface-attached cells embedded in a self-produced extracellular polymeric matrix. Biofilm-based infections have higher resistance to antibiotics and disinfecting chemicals as well as resisting phagocytosis and other components of the body's immunity, when compared to planktonic cells [11].

In this study the frequency of UTI in catheterized patients (50.8%) greater than in non catheterized (49.2%) due to breaks in the sterile system or via extra luminal route, via migration along the outside of the catheter in the periurethral mucous sheath, or by the intra luminal route via movement along the internal lumen of the catheter from a contaminated collection bag or catheter-drainage tube junction[12].

The frequency of UTI was greater in women as compared to men as 55.5% of the patients were females and 44.5% were males principally owing to anatomic and physical factors. Similar results were shown by Kashef et al[13]. Kamat US et al in their study noted females are more prone to develop UTIs, due to their anatomical physiological changes like short urethra, its proximity to the anus, dilatation of the urethra and the stasis urine during pregnancy [14].

The most frequent isolated organism in catheterized and non catheterized patients is enterobacter species (31.1%) in Gram negative bacteria followed by *E.coli* (16%) , *klebsilla pneumonia (*15%) , which is consistent with H. Kumon et al.[15]. and in contrast to Niveditha *et al*.[16], Pallavi Sayal et al.[17] who reported that *E. coli* was the most prevalent organism. this may be due difference in sample size, demographic characters of the studied population.

Of Gram positive bacteria *Staph aureus* was the most prevalent organism both in catheterized (23%) and non catheterized patients (15%) followed by coagulase negative staphylococci (6.6%) this finding is coincides with that of Dardi and Maral[18].

Multiple phenotypic methods were done to detect biofilm production, Tube method detected 76/162(47%). Congo red detected 82/162(51%), maximum detection was by tissue culture plate (65%). These results were similar to those of Somya[19]

Tissue culture plate is considered the standard test to detect biofilm production so we depended on it to determine sensitivity and specificity of both tube and Congo red methods. In our study Congo red agar method showed sensitivity 54.7%, specificity 57.1%. Tube method showed sensitivity 64.2%, specificity 67.9% hence tube method is more sensitive than cogo red in phenotypic detection of biofilm this also was reported by Chanda and Annapurna [21]. This difference may be due to the inter-observer variability in the reading of results, resulting in low sensitivity and specificity in our study.

We investigated antibiotic resistance patterns of biofilm producers and non biofilm producers in Gram negative bacteria against various antibiotics, the investigated biofilm forming strains displayed relatively high resistance against tested antibiotics than non biofilm producers. Resistance to antibiotics such as nitrofurantoin (32.4%vs20.8%), Trimethoprim/ Sulfamethoxazole (79.4%vs.75%), cefazolin (94.1%vs.91.7%) and amikacin (8.8%vs.8%) were comparatively higher among biofilm producers than non-biofilm producers due to an elevated expression of the efflux pump and physiological heterogeneity which plays an important role for the development of antibiotic resistance in biofilm producing bacteria by affecting the rate of growth, metabolism, interbacterial quorum signals [23].

In our study the most effective antibiotics against isolated gram negative bacteria were found to be amikacin (91.2%), meropenem (82.4 %) and imipenem (79.5%) similar to that found by kabir et al.[24]

The most effective antibiotics against isolated gram positive bacteria were found to be gentamycin (89.4%) and ciprofloxacin (84.2 %), in contrast to the reports of Abdagire etal.[25] , Hassan et al.[8]

Molecular detection of biofilm genes was done by PCR to detect biofilm genes in both gram positive and negative bacteria. IcaA, D biofilm genes IcaA, D were detected in all biofilm forming staphylococci which is similar to results reported by

Masoud et al.[26], Gamal et al [7] who also detected IcaA, D genes in all biofilm producing isolates ; this indicates that the presence of both genes is essential for biofilm production and confirms that both genes are part of one operon, so either the entire operon is present or absent.

When comparing results of phenotypic and genotypic methods for biofilm detection we found the following results, in case of enterobacteriaceae BssS gene was detected in 66/104(63%) of cases but tube method detected 44/104 with a sensitivity of 48.5% and insignificant p value (0.121). Cogo red method detected

48/104 with a sensitivity of 51.5% and insignificant p value (0.064). Tissue culture plate detected 62/104 these 62 biofilm positive with a sensitivity of 63.6% and a significant p value (0.05) this result is less than Hassan et al.[8], who reported 100% correlation between phenotypic and genotypic methods for biofilm detection.

In case of biofilm genotypic detection in staphylococci IcaA and IcaD genes were detected in 8/42 (19%) of cases but tube method detected 32 isolates with a sensitivity of 100%, congo red detected 30 isolates with a sensitivity of 75% and tissue culture plate detected 34 isolates with a sensitivity of 100%. The result were similar to that reported by Noha et al[27] .

CONCLUSION & RECOMMENDATION

Biofilm is a serious problem of great medical concern that leads to recurrent, persistent infection and increase antimicrobial resistance. Biofilm detection and proper management is of great value for our patients so we recommend the following:

- All health care personnel should apply infection control measures when dealing with patients.
- Judicious use of antibiotics to avoid spread of multidrug resistant organisms.
- Proper catheter insertion practice
- Gentamycin, ciprofloxacin are the most effective antibiotics against isolated gram positive biofilm forming bacteria.
- Amikacin, meropenem and imipenem are the most effective antibiotics against isolated gram negative biofilm forming bacteria.
- Tissue Culture Plate method can be recommended as a general screening method for the detection of biofilm producing bacteria in laboratories.

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