

Microbiological Aspects in Biofilm Produced by some Uropathogens Isolated from Patients with Indwelling Bladder Catheters

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ABSTRACT

Seventy five catheter urine specimens obtained from sixty four patients and admitted to Urology and other departments in Ramadi Teaching Hospital were studied. qualitative and quantitative detection for biofilm production for all isolates were achieved. Antimicrobial susceptibility test for planktonic cells of study isolates was performed by broth dilution method and the MICs were detected. Further, *In Vitro* model for formation of pseudomonal biofilm on catheter segments by biofilm producer-isolates of the same bacteria was achieved. BICs and MBECs were detected for all sessile study isolates of *Pseudomonas aeruginosa* against ciprofloxacin, amikacin and cefotaxime.

Out of 75 catheter urine specimens, 61 (81.4%) were positive for culture including: *Escherichia coli* 17 (27.9%) followed by *Klebsiella* spp. 13 (21.3%), *Pseudomonas aeruginosa* 12 (19.7%), and in spectrophotometric method, the results showed that all of 12 isolates of *Pseudomonas aeruginosa*, 7 isolates of *Proteus mirabilis*, 17 isolates of *Escherichia coli* and 13 isolates of *Klebsiella*, 12 (100%), 7 (100%), 12 (70.6%) and 12 (92.3%) isolates produced biofilm in the glucose supplemented media. In biofilm antimicrobial susceptibility, the sessile study isolates of *Pseudomonas aeruginosa* were required 10-50 X MICs from ciprofloxacin and amikacin to inhibit bacterial biofilm and 50-100 X MICs to eradicate it from the surfaces of catheter in contrast to cefotaxime of which BICs were from 50-100 X MICs and MBECs from 100-1000 X MICs.

The study concluded that *Escherichia coli* was the most common uropathogen followed by *Klebsiella* spp., and *Pseudomonas aeruginosa*. Also, all study isolates of *Pseudomonas aeruginosa* and *Proteus mirabilis* produced biofilm qualitatively and quantitatively. Further, with regard to ciprofloxacin and amikacin, the study isolates were also required 10-50 X MICs to inhibit bacterial biofilm and 50-100 X MICs to remove pseudomonal adhesion from the catheters while those for cefotaxime were 50-100 X MICs and 100-1000 MICs for inhibition and eradication of bacterial biofilm respectively.

Keywords: Bacterial biofilm, Catheterized UTI, BICs, MBECs.

organisms of these biofilms produce urease, which hydrolyzes urea in the patient's urine to ammonium hydroxide, the elevated pH results in precipitation of minerals. These mineral-containing biofilms form encrustations and consecutive obstruction that may completely block the inner lumen of the catheter (Donalan, 2002). Bacteria may ascend the inner lumen into the patient's bladder in 1 to 3 days; this rate may be influenced by the presence of swarming organisms such as *Proteus* spp. (Donalan, 2001). These infections may be complicated by reaching to the kidney causing pyelonephritis (Jones *et al.*, 2005).

Bacterial biofilms are inherently resistant to antimicrobial agents in addition to host immunity (Costerton *et al.*, 1999). *In Vitro* biofilms often require from 10 to 1,000-fold the concentration of antibiotic to be eradicated as compared to planktonic bacteria. Often the MIC concentration of antibiotic, which by definition is effective against the planktonic cells, was not effective against biofilm cultures of the same organism (Olson *et al.*, 2002). Due to importance of biofilm related infections, the treatment is performed by prolonged and high-dose of antimicrobial therapy as well as elimination of infected medical device are the cornerstones of a successful therapy (Fux *et al.*, 2003). New susceptibility parameters are introduced to clinical practice like biofilm inhibitory concentration (BIC) and minimal biofilm eradication concentration (MBEC). Thus, this study has been undertaken to shed a light on the practical points of bacterial biofilm in more details and in several directions, firstly *In Vitro*, To detect biofilm formation by some uropathogens isolated from patients with indwelling bladder catheters qualitatively and quantitatively by spectrophotometric assay with ELISA reader. Further, in terms of MICs and sub-MICs, to determine antimicrobial susceptibility test for logarithmic phase planktonic cells of study isolates of *Pseudomonas aeruginosa*. Further more, in term of BICs and MBECs, to detect biofilm antimicrobial susceptibility test for biofilm producer isolates of *Pseudomonas aeruginosa* to selected antimicrobial agents.

Patients Study

A total of seventy five (75) catheter urine specimens belonged to sixty four (64) patients admitted to Department of Urology in Ramadi General Hospital was studied during the period from May to September 2007. In some cases, two urine catheter specimens obtained for patient's follow up. Catheter specimens of urine (CSU) were obtained by withdrawing a sample with a syringe and needle from the catheter tube (junction area between catheter and collection tube). The duration for catheter for most patients was more than 7 days (Mims *et al.*, 1993).

Firstly, urine specimen was cultured immediately by semiquantitative culture technique. In this technique, under aseptic conditions a standardized (fixed known volume) inoculum of uncentrifuged urine were streaked on the sectors of nutrient, blood and MacConkey agar plates and incubated at 37°C for 24 hours, if no growth was detected, plates were re-incubated for another 24 hours before reported as negative cultures. After incubation, the total number of colonies per ml was counted. Afterthat, the specimen was examined macroscopically and microscopically. All microbiological investigations were also confirmed by using biochemical tests (Baron *et al.*, 1994).

Biofilm Study

-Qualitative assay of biofilm formation:

A- Tube method: (Adhesion assay):

Glycocalyx production was determined as described by (Yassien and Khardori, 2001); (Mathur *et al.*, 2006) as the following:

1. Two to three colonies of study isolate were inoculated into 5 ml of brain heart infusion broth supplemented with 2% glucose in plastic conical tubes.
2. Cultures were incubated at 37°C for 18-24 hours.
3. The contents were aspirated; one tube was examined unstained and other stained with safranin or crystal violet.
4. Slime positivity was judged by the presence of visible stained or unstained film lining the wall of the tube.

B- Formation of biofilm on catheter:

An overnight culture of tested bacteria (10µl) in brain heart infusion broth was inoculated into 500µl of the same medium and injected into clear Foley catheters (the silicon type Foley catheter was used). The catheters were capped at both ends and incubated at 37°C overnight. Then catheters were rinsed with phosphate buffer saline. After drying at room temperature for 15 min, 700 µl crystal violet (1%) was added to the catheters for 20 min, then the stained biofilm rinsed several times with BPS and allowed to dry at room temperature before examination (Mireles *et al.*, 2001); (Arif, 2006).

Quantitative assay of biofilm formation (Microtiter plate assay)

Adhesion and biofilm formation was determined by using a spectrophotometric method described by (Yassien and Khardori, 2001); (Stepanovic *et al.*, 2003) as the following:

1. Working cultures were prepared by inoculation study isolate on Columbia agar supplemented with 5% blood and incubated aerobically at 35°C for 24 hr.
2. These cultures were used to prepare standardized bacterial suspension in sterile normal saline adjusted to a 0.5 McFarland turbidity standard to reach 10⁸ CFU/ml.
3. The suspensions obtained were inoculated into a brain heart infusion broth with glucose (glucose supplemented medium) and without glucose.
4. 200 µL of standardized cultures were added to each well of sterile polystyrene Microtiter plate and incubated at 37°C for 18 hr.
5. Following incubation, the content of each well was aspirated, and each well was washed 3-4 times with sterile distilled water.
6. The remaining attached bacteria were fixed with 200 µL of methanol per well.
7. After 15 minutes, the plate wells were emptied and left to air dry.
8. Afterwards the plates were stained for 5 min with 160 µL per well of crystal violet (0.25 %).
9. Excess stain was rinsed off by placing the Microtiter plate under the running tap water.
10. The plates were air dried and the dye which was bound to the adherent cells was resolubilized with 160 µL of 33 % glacial acetic acid per well.

11. Finally the optical density (OD) of each well was measured at 570 and 630 nm by using Stat Fax 3200 Elisa Reader.

The isolates were classified according to biofilm production depending on the criteria laid down by Christensen *et al.*, (1985) as following: Strong producer more than 0.240; weak producer between 0.125-0.250 while non-producer less than 0.120.

Antimicrobial susceptibility for planktonic cells

Broth macrodilution method (MIC method):

Three antimicrobial agents used for planktonic and biofilm study belonged to the following groups: Ciprofloxacin (flouoroquinolones), Amikacin (aminoglycosides), and Cefotaxime (third generation cephalosporins). Seven study isolates of *Pseudomonas aeruginosa* were included in this part of study. The bacterial standardization was performed according to 0.5 McFarland turbidity standards (Miles and Amyes, 1996). Different antibiotic concentrations (0.5-256 µg/ml) were prepared in 5 ml of Mueller-Hinton broth and transferred to sterile capped tubes. At least 4-5 morphologically similar colonies were inoculated into Mueller-Hinton broth and incubated at 37°C until the viable turbidity was equal to the 0.5 McFarland, (about 10⁸ cfu/ml). After that, the suspension was diluted 1:100 and certain volumes transferred to the tubes containing antibiotic dilutions, to reach a final cell concentration of (about 10⁵ cfu/ml). Negative controls were represented by two tubes; one of them contained broth only and the other contained broth plus microorganism. Then the tubes were incubated overnight at 37°C. The result of minimal inhibitory concentration (MIC) was interpreted as the lowest concentration of antimicrobial agents which inhibits visible bacterial growth after overnight incubation (Ferraro *et al.*, 2000).

Biofilm antimicrobial susceptibility test

Formation of biofilm producer-isolates of *Pseudomonas aeruginosa* on catheter segments (*In vitro* model):

The method used for bacterial biofilm formation on catheter segments was described by (Ishida *et al.*, 1998). Briefly, the tested bacteria incubated in brain heart infusion broth overnight at 37°C. Then 10 µL of overnight culture was added to 500 µL of sterile media in which catheter segments (1cm²) was inoculated, and subsequently incubated overnight at 37°C. After that washing for the segments was achieved by sterile media (3-4) times to remove weakly attached bacteria. Then segments were resuspended with sterile media and vortexed vigorously for 2 min which was considered as controls.

Bactericidal activity of antibacterials against biofilm forming sessile cells

To test the bactericidal activity of selected antibiotics against the sessile cells, the catheter segments was incubated with the organism as described above, were taken out, washed gently with sterile media or saline and subsequently transferred to saline containing a given antibiotic with distinct concentrations (10X, 50X, 100X, 500X and 1000X) at which X represent the minimal inhibitory concentration of mentioned antimicrobial agents against planktonic cells which was previously detected. After that the tubes were incubated for 24 hr. at 37°C (Arif, 2006).

Extraction and quantification of biofilm bacteria

After exposure of tested organism to the desired concentrations of antibiotic, they were transferred to 10 ml of fresh brain heart infusion broth and stirred vigorously with a vortex mixer for 2 min. for dispersion sessile or adherent cells. Then the suspension was diluted and plated on nutrient agar plates for bacterial colony counting and compared with original bacterial count before exposing to antimicrobial agents (Ishida *et al.*, 1998).

Detection of biofilm inhibitory concentrations (BICs) and minimal biofilm eradication concentration (MBECs):

After incubation the tubes for 24 hr. at 37°C, the biofilm inhibitory concentration was detected and defined as the lowest concentrations of antimicrobial agents which inhibits bacterial biofilm growth on a surface of catheter. It was represented by the clearance of broth medium consisting (1 cm) catheters and the required concentrations of antimicrobial agents (Ceri *et al.*, 2006). After plating the diluted suspension into agar plates and counting the number of bacterial colonies, minimal biofilm eradication concentration (MBEC) was determined. MBEC was defined as the lowest concentration of antibiotic or biocide capable of killing biofilm producer bacteria. It was represented by disappearing of colonies of biofilm producer organisms on the culture plates (Ceri *et al.*, 2006).

RESULTS

Out of 75 catheter urine specimen, 61 (81.4%) were culture positive, 7 (9.3%) sterile urine while the other 7 (9.3%) were defined as sterile Pyuria. Among 61 (81.4%) culture positive cases, the yielded microbial causative agents were distributed as the followings: *Escherichia coli* 17 (27.9%), *Klebsiella* spp. 13 (21.3%), *Pseudomonas aeruginosa* 12 (19.7%), Staphylococci 9 (14.8%) divided into 4 (44.4%) *Staphylococcus aureus* and 5 (55.6%) *Staphylococcus epidermidis*, *Proteus mirabilis* 7 (11.5%), *Enterobacter* spp. 1 (1.6), in addition to *Candida* spp which was found as a mixed form with the above mentioned bacteria in 2 (3.3%) cases.

Under the field of biofilm production, particularly qualitative biofilm assay, tube method, our result showed that out of 17 (27.9 %) isolates of *Escherichia coli*, 13 (21.3 %) isolates of *Klebsiella* species, 12 (19.7 %) isolates of *Pseudomonas aeruginosa*, and 7 (11.5 %) *Proteus mirabilis*, biofilm were produced on the inner lining of the tubes in 11 (64.7 %), 9 (69.2 %), 12 (100 %), and 7 (100 %) respectively. On the other hand this phenomenon was not observed in 6 (35.3 %), 4 (30.8 %) and 1 (100 %) of *Escherichia coli*, *Klebsiella* spp. and *Enterobacter* spp. respectively.

Regarding formation of biofilm on catheters, After incubation of the catheter which was previously inoculated with an over night culture of *P. aeruginosa*, a clear biofilm covering the catheter lumen was detected after staining with 1% crystal violet.

Further, in the other qualitative assay (foley catheter method) our study revealed that out of 17 isolates of *Escherichia coli*, 13 isolates of *Klebsiella* species, 12 isolates of *Pseudomonas aeruginosa*, and 7 *Proteus mirabilis*, biofilm were produced on the surface of the catheters in 11 (64.7%), 11 (84.6 %), 12 (100%), 7 (100%) respectively. Furthermore, biofilm production was not observed in 6 (35.3%), 2 (15.4%) and 1 (100 %) of *Escherichia coli*, *Klebsiella* spp. and *Enterobacter* spp. respectively.

Quantitative biofilm production (Spectrophotometric assay)

In the quantitative biofilm formation assay, spectrophotometric method was achieved under two set of experimental conditions (with and without glucose).

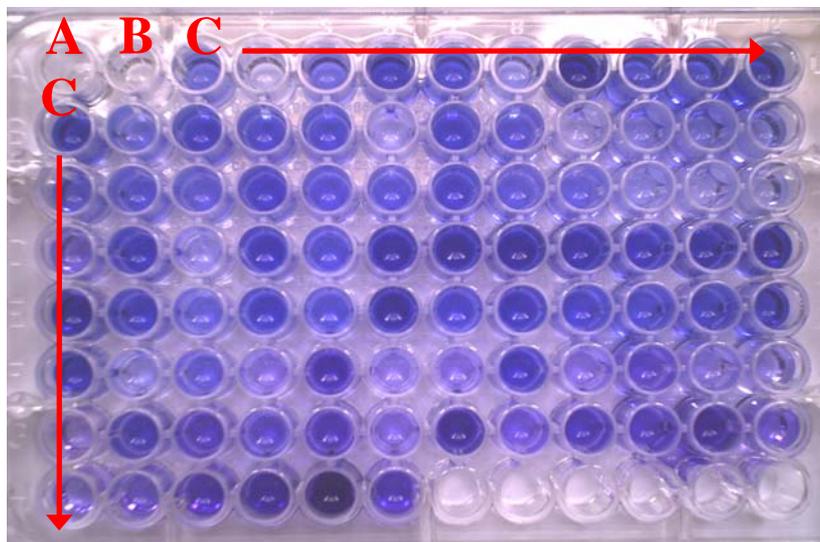


Fig. 1: Biofilm formation on microtiterplate wells (A: Air blank (empty), B: control (brain heart infusion only), C: study isolates).

Our results showed that out of 17 isolates of *Escherichia coli*, 12 (70.6%) were biofilm producers. All of these isolates were strong biofilm producers (OD was more than 0.25) with the presence of glucose in the experiment. Further, with the absence of glucose 12 (70.6%) out of 17 isolates of *Escherichia coli* produced biofilm weakly. Nine (75%) of these were strong biofilm producers while 3 (25%) of them produced biofilm weakly (OD was between 0.125 and 0.25). On the other hand no significant differences observed in the readings of optical density at 570 nm with the presence and absence of glucose among isolates of *Escherichia coli* (0.366 ± 0.335) and (0.351 ± 0.282) respectively (P value = 0.886) (Fig. 2).

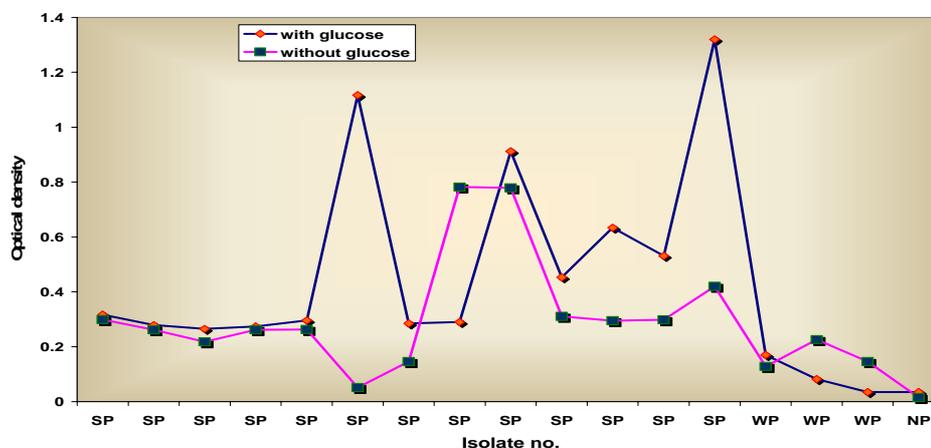


Fig. 2: Effect of glucose on the production of biofilm by spectrophotometric assay among strong and weak biofilm producer isolates of *Escherichia coli*. (SP:- Strong biofilm producer, WP:- Weak producer, NP:- Non biofilm producer).

Also, our study revealed that out of 13 isolates of *Klebsiella* spp., 12 (92.3%) of them were biofilm producers in the glucose supplemented media. Of these, 10 (83.3%) *Klebsiella* isolates were produced biofilm strongly, while 2 (16.7%) isolates weakly produced biofilm. On the other hand in the absence of glucose, 11 (84.6%) out of 13 isolates produced biofilm of them 7 (63.6%) strong biofilm producers and 4 (36.4%) weak biofilm producers. Also, no significant differences were observed in the readings of optical density at 570 nm with the presence and absence of glucose among isolates of *Klebsiella* spp. (0.746 ± 0.564) and (0.598 ± 0.538) respectively (P value = 0.502) (Fig. 3).

Further, our results appeared that all study isolates of *Pseudomonas aeruginosa*, 12 (100%) were produced biofilm in the glucose supplemented media. Of these, 11 (91.7%) isolates were produced biofilm strongly while 1 (8.3%) isolates produced biofilm weakly. On the other hand, in the absence of glucose, 10 (83.3%) out of 12 isolates were produced biofilm of them 7 (70%) were strong biofilm producers and 3 (30%) were weak biofilm producers. Also, there is statistically significant differences in the reading of optical density at 570 nm with the presence and absence of glucose among isolates of *Pseudomonas aeruginosa* (0.78 ± 0.62) and (0.36 ± 0.22) respectively (P value = 0.037) (Fig. 4).

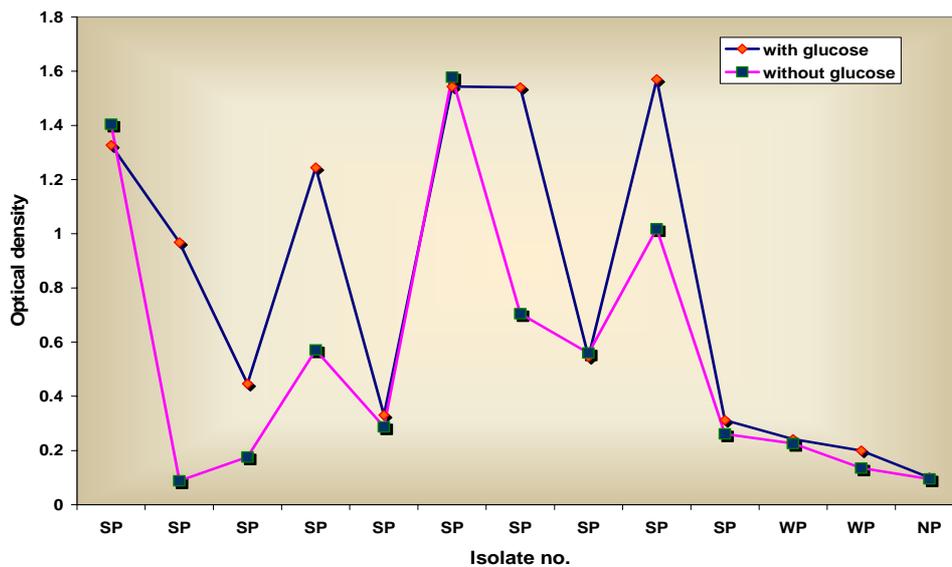


Fig. 3: Effect of glucose on the production of biofilm by spectrophotometric assay among strong and weak biofilm producer isolates of *Klebsiella* spp. (SP:- Strong biofilm producer, WP:- Weak producer, NP:- Non biofilm producer).

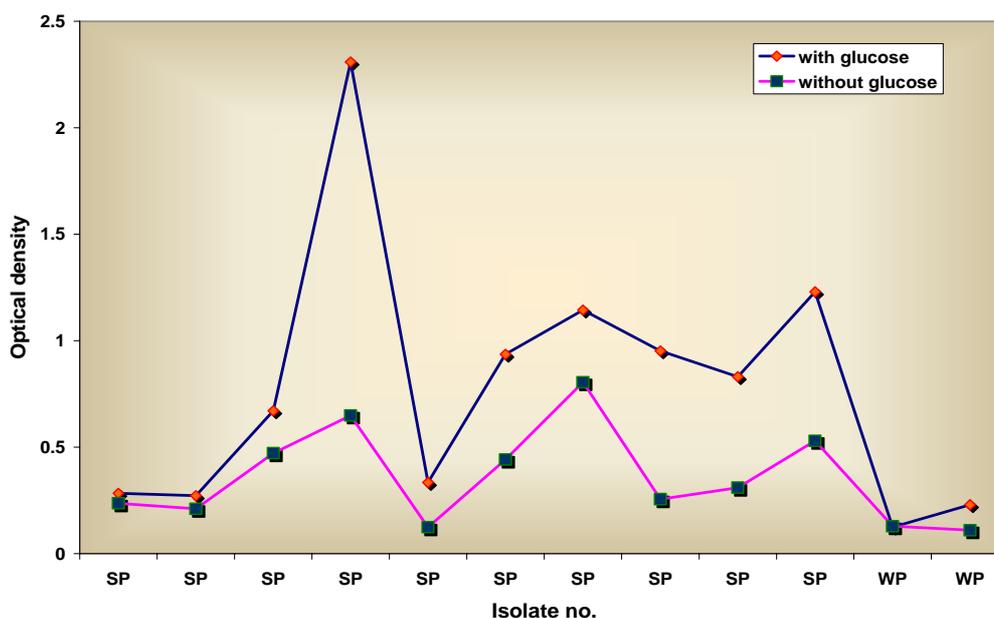


Fig. 4: Effect of glucose on the production of biofilm by spectrophotometric assay among strong and weak biofilm producer isolates of *Pseudomonas aeruginosa*. (SP:-Strong biofilm producer, WP:-Weak producer, NP:-Non biofilm producer).

Also, our study revealed that all study isolates of *Proteus mirabilis* produced biofilm in the glucose supplemented and non-supplemented media, of these 6 (85.7%) produced biofilm strongly while 1 (14.3%) isolates were weakly biofilm producers under two mentioned conditions respectively. Further, the readings means of optical density at 570 nm with the presence and absence of glucose among isolates of *Proteus mirabilis* were 1.233 ± 0.511 and 0.754 ± 0.363 respectively (P value = 0.067) (Fig. 5).

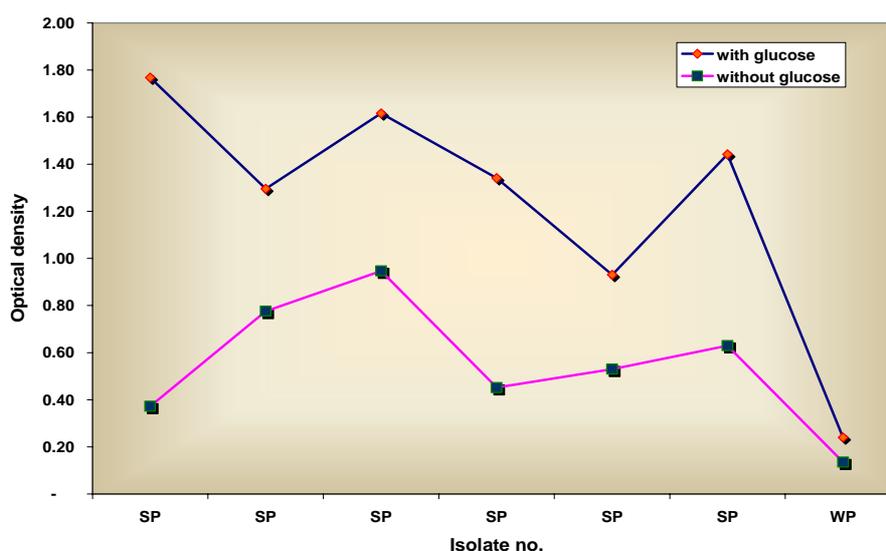


Fig. 5: Effect of glucose on the production of biofilm by spectrophotometric assay among strong and weak biofilm producer isolates of *Proteus mirabilis*. (SP:- Strong biofilm producer, WP:-Weak producer, NP:-Non biofilm producer).

Antimicrobial susceptibility test for planktonic cells

-Broth macrodilution technique:

In this part of study, our result showed that minimal inhibitory concentrations were 26.29 ± 10.03 , 118.86 ± 24.19 and 10.29 ± 3.90 for ciprofloxacin, cefotaxime and amikacin respectively against logarithmic phase planktonic cells of *Pseudomonas aeruginosa* while the sub-minimal inhibitory concentrations were 13.14 ± 5.01 , 59.43 ± 12.09 and 5.14 ± 1.95 for the same antimicrobial agents respectively.

Biofilm antimicrobial susceptibility test

Antimicrobial susceptibility test for sessile cell of *Pseudomonas aeruginosa* was achieved by detecting the biofilm inhibitory concentration (BIC) and minimal biofilm eradication concentration (MBEC). Concentrations of 1, 10, 50, 100 and 500 X MIC were chosen to determine the biofilm inhibitory concentrations (BICs) and minimum biofilm eradication concentrations (MBECs).

With regard to ciprofloxacin, the BIC for N4, N6, N10, N12, N15, N26 and N30 study isolates were 320 (10 X MIC), 320 (10 X MIC), 1600 (50 X MIC), 320 (10 X MIC), 320 (10 X MIC), 1600 (50 X MIC), 320 (10 X MIC) µg/ml respectively. These isolates has been shown clearance of broth (inhibition of biofilm) and reduced the viable count of bacterial biofilm from 38×10^5 , 30×10^5 , 79×10^5 , 113×10^5 , 160×10^4 , 85×10^5 , and 67×10^5 to 45×10^2 , 37×10^2 , 72 , 96 , 33×10^2 , 34×10^2 and 63×10^2 respectively. On the other hand, the concentrations of 1600 (50 X MIC), 1600 (50 X MIC), 3200 (100 X MIC), 1600 (50 X MIC), 1600 (50 X MIC), 3200 (100 X MIC), 1600 (50 X MIC) µg/ml were enough to eradicate pseudomonal biofilm from the catheters for the above mentioned isolates respectively with high significant difference, MIC mean (26.29 ± 10.028) and MBEC mean (1942.86 ± 907.11) with P value < 0.0001 (Fig. 6).

Third generation cephalosporins, cefotaxime inhibited pseudomonal biofilm at the concentrations range between 50-100 folds higher than MIC at the following inhibitory concentrations: 12800, 12800, 6400, 6400, 3200, 1600, and 1600 µg/ml for the above mentioned isolates of *Pseudomonas aeruginosa* respectively. (Fig. 7).

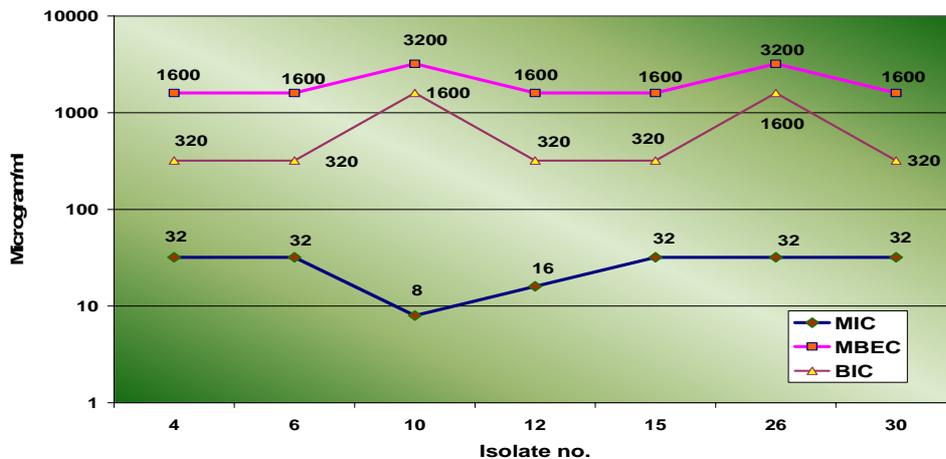


Fig. 6: Comparison of susceptibility parameters for ciprofloxacin against logarithmic phase planktonic cells and sessile (adherent) cells of seven study isolates of *Pseudomonas aeruginosa*.

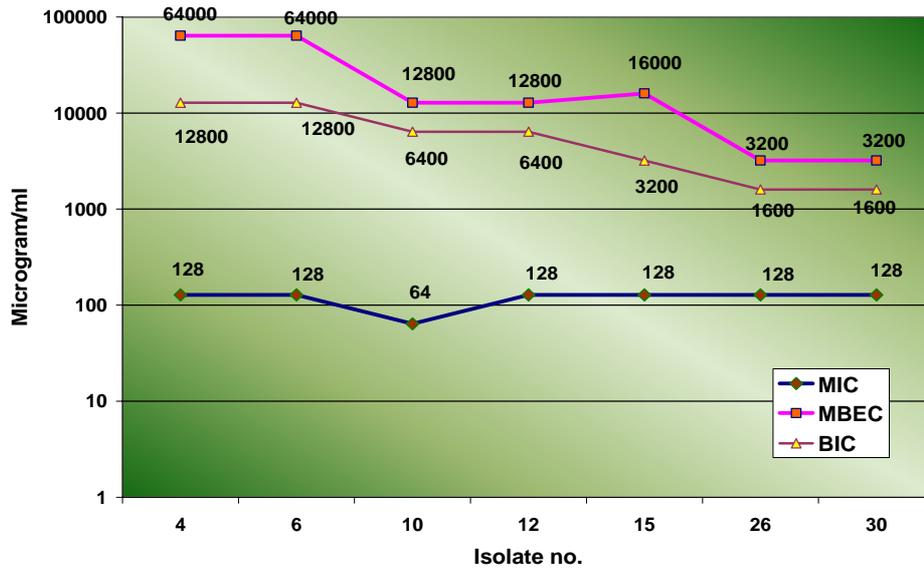


Fig. 7: Comparison of susceptibility parameters for cefotaxime against logarithmic phase planktonic cells and sessile (adherent) cells of Seven study isolates of *Pseudomonas aeruginosa*.

Also, our results showed that amikacin, inhibit pseudomonal biofilm at the following inhibitory concentrations: 800, 1600, 800, 80, 400, 80, and 400 $\mu\text{g/ml}$ for the above mentioned isolates of *Pseudomonas aeruginosa* respectively and reduced the viable counts for these isolates markedly as observed in table 4.10, but these concentrations were not enough to eradicate bacterial biofilm from the catheters. On the other hand, the MBECs for amikacin against these study isolates were 1600, 8000, 3200, 400, 800, 400, and 800 $\mu\text{g/ml}$ respectively and no any colony appeared on the culture counting plate. Statistically, there is high significant difference (P value < 0.0001) with MICs mean (10.29 ± 3.90) and MBEC mean (628.57 ± 213.8) as represented in Fig. 8.

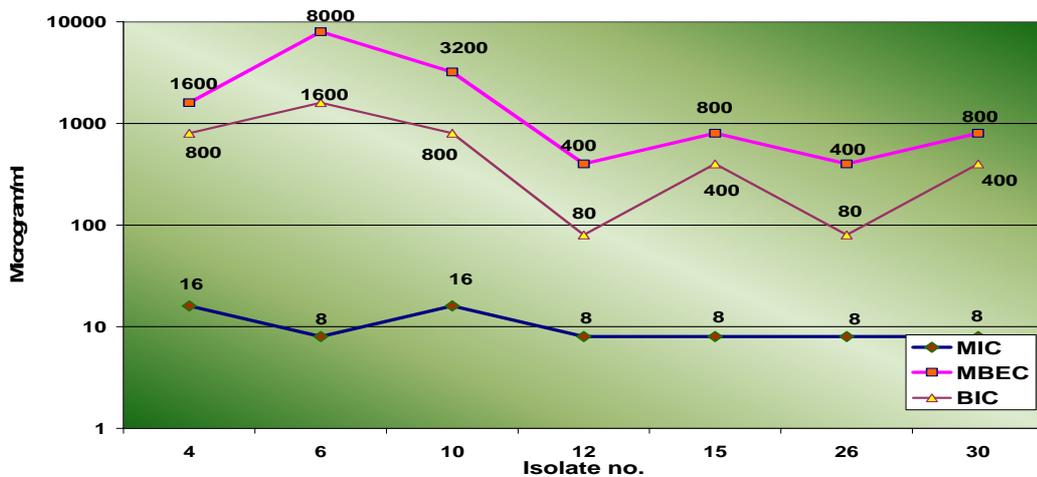


Fig. 8: Comparison of susceptibility parameters for amikacin against logarithmic phase planktonic cells and sessile (adherent) cells of seven study isolates of *Pseudomonas aeruginosa*.

DISCUSSION

It is remarkable that the biofilm (adherence) mode of growth of bacteria on the surfaces of biomaterials has been well documented as one of the important causes of prosthetic device related infections that are refractory of antimicrobial therapy (Yassien and Khardori, 2001). Indwelling bladder catheters are the most commonly deployed prosthetic medical devices (Darouiche, 2001). Indwelling catheter constitutes a convenient means of managing bladder dysfunction; unfortunately, they also provide access for bacteria from contaminated external environment into a vulnerable body cavity. Implanted prosthetic devices constitute particularly attractive surfaces for bacterial colonization, as they have none of the protective mechanisms of health tissue surfaces. Unfortunately, the care of many patients undergoing long-term catheterization is frequently complicated by microbial infection. These microbial causes colonize the catheter, forming surface biofilm communities (Jones *et al.*, 2005).

In the bacteriological part of this study, our study showed that *Escherichia coli* was the most common microorganism isolated from patients with indwelling urinary catheters followed by *Klebsiella* spp., *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Proteus mirabilis*, *Enterobacter aerogenes* and *Candida* species respectively. On the other hand, (Talpur and co-workers, 2004) found that catheterization not only increased bacteriuria, but they also found that 92% of prostatic tissues of catheterized patients were positive for bacterial cultures, while only 28% of prostatic tissues of non-catheterized patients showed positive bacterial cultures. The high rates of bacteriuria associated with catheterizations might be due to the presence of catheter bypasses normal host defenses allowing continuous access of microorganisms into the urinary bladder and markedly increasing the frequency of UTI.

Under the field of biofilm production, particularly qualitative adhesion assay, tube method, our result showed that out of 17 (27.9 %) isolates of *Escherichia coli*, 13 (21.3 %) isolates of *Klebsiella* species, 12 (19.7 %) isolates of *Pseudomonas aeruginosa*, and 7 (11.5 %) *Proteus mirabilis*, biofilm were produced on the inner lining of the tubes in 11 (64.7 %), 9 (69.2 %), 12 (100 %), and 7 (100 %) respectively. Our results are in agreement with those obtained from the study laid down by (Mathur *et al.*, 2006) who observed good correlation between the results of qualitative tube assay and spectrophotometric assay regarding strongly biofilm producing isolates. The same authors concluded that it was difficult to discriminate between weak and biofilm negative isolates due to the variability in observed results by different observers. Consequently, high variability was observed and classification in biofilm positive and negative was difficult by tube method. Thus, tube test cannot be recommended as general screening test to identify biofilm-producing isolate.

In the quantitative biofilm formation assay, spectrophotometric method with ELISA reader was achieved under two set of experimental conditions (with and without glucose) and the crystal violet stain was dependent in this technique. In order to enable easier study of bacterial attachment and colonization, microtiter plate assay (spectrophotometric method) is the most frequently used techniques for quantifying biofilm formation (Stepanovic *et al.*, 2000 ; Manzon *et al.*, 2001). In this technique, our results showed that among 17 isolates of *Escherichia coli*, 12 (70.6%) isolates were biofilm producers in the presence and absence of glucose. no significant differences observed in the readings of optical density at 570 nm in two conditions (0.366 ± 0.335) and (0.351 ± 0.282) respectively (P value = 0.886). Also,

among 13 isolates of *Klebsiella* spp, 12 (92.3%) and 11 (84.6%) isolates of this bacterium were produced biofilm in the glucose supplemented and non-supplemented media respectively. At other meaning, regarding these bacteria, adhesion process was not affected by the presence or absence of glucose in the culture media. (Anderson and colleagues, 2003) demonstrated that uropathogenic *E. coli* can form biofilms, albeit unusual ones. These authors showed that, while infecting the bladder umbrella cells, *E. coli* can invade into the cells and form biofilms intracellularly, consisting of small, coccoid-like cells. Protected by the mammalian cellular membrane, *E. coli* are sequestered from the phagocytic action of neutrophils. Intracellular *E. coli* may also be protected from antibiotic therapy. Eventually, however, the infected cell will apoptose and slough into the lumen of the bladder. During this process, the uropathogenic *E. coli* adds another twist – it changes morphology from small, almost coccoid cells to long, filamentous cells. This finding of intracellular biofilms, as well as being a description of a novel biofilm mechanism, also explains the persistence and recurrence of urinary tract infections (Dodge *et al.*, 2006).

Also, it is well realized that the model of biofilm resistance is based on the persister's survival. An initial treatment with antibiotics kills planktonic cells and the majority of biofilm cells. The immune system kills planktonic persisters, but the biofilm persisters are protected from immune defense mechanisms by the extracellular matrix. After the antibiotic concentration drops, persisters resurrect the biofilm and the infection relapses (Vuong *et al.*, 2004); (Lewis *et al.*, 2006).

Antimicrobial susceptibility test for sessile cell of *Pseudomonas aeruginosa* was achieved by detecting the minimal biofilm eradication concentration (MBEC). The calculation of different concentrations of antimicrobial agents used was based on the minimal inhibitory concentrations obtained by broth macrodilution technique achieved against logarithmic phase planktonic cells of *Pseudomonas aeruginosa*. Our result revealed that the biofilm cells were required 50-100 times the MIC values for ciprofloxacin obtained for the same isolates in the logarithmic phase of planktonic cells with MIC mean (26.29 ± 10.028) $\mu\text{g/ml}$ and MBEC mean (1942.86 ± 907.11) $\mu\text{g/ml}$ with high significant difference (P value < 0.0001). (Yassien and Khardori, 2001) documented that the high concentrations of flouoroquinolones were used to treat the performed biofilms because 1) these concentrations would be expected to reach the biofilms when therapeutic doses of flouoroquinolones (ciprofloxacin is one of the flouoroquinolones) are infused through the vascular catheters and, 2) using such high concentrations would minimize the exposure of very large inoculum of bacteria in the biofilms to sub inhibitory concentrations of the flouoroquinolones. This effect is concentration dependent. On the other hand, Lewis and associates, (2006) documented that resistance of biofilms to killing by antimicrobials is dramatic, 100-1000 fold above the MIC, and occurs for all antimicrobials tested and in all species examined. This means that all pathogens potentially harbor an essentially perfect multi-drug resistance mechanism, but only "choose" to express it when growing as biofilm.

It is well documented that the mechanisms of ciprofloxacin effect in the biofilm include: (1) electrostatic interference with the adhesion of bacteria and/or glycocalyx to the substratum, (2) activation or release of enzymes to disrupt the exopolysaccharide (glycocalyx) in the biofilm and, (3) inhibition of the formation of new glycocalyx. Irrespective of the mechanisms involved, the observed effects of the ciprofloxacin may

improve the pharmacodynamics of the antibacterial agents used to treat prosthetic device related infections (Yassien and Khardori, 2001).

Also, with regard to cefotaxime, our result revealed that the biofilm cells were required 50-100 times the MIC values obtained against study isolates in logarithmic phase planktonic cells. (Mah and co-workers, 2003) explained the genetic basis for *Pseudomonas aeruginosa* biofilm antibiotic resistance by identifying a chromosomal locus in his study called 'ndvB' which is required for the synthesis of periplasmic glucans. These periplasmic glucans interact physically with antimicrobials and the formed glucose polymers may prevent antimicrobials from reaching their sites of action by sequestering these antimicrobial agents in the periplasm. Further, the same researchers indicated that biofilm themselves are not simply a diffusion barrier to these antimicrobials, but rather that bacteria within these microbial communities employ distinct mechanisms to resist the action of antimicrobial agents.

Also, with regard to amikacin, our result revealed that the biofilm cells were required 10-50 fold greater than MICs observed for study isolates of *Pseudomonas aeruginosa* in logarithmic phase planktonic cells. (Pascual, 2003) observed that MICs of amikacin against *Pseudomonas aeruginosa* increased in the presence of siliconized latex, a biomaterial commonly used to make urinary tract catheters. This effect was not strain-dependent and was similar for different branches of catheters. In fact, this phenomenon was induced by substances eluted from the biomaterials (Pascual, 2003).

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