Crystalline biofilm produced by *Proteus mirabilis*: an overview on their formation assays and antimicrobials interaction

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Abstract:

Objective: The aim of this study was to detect biofilm formation by study isolates of *Proteus mirabilis* qualitatively and quantitatively. Furthermore, depending on minimal inhibitory concentration (MICs) value and in term of biofilm inhibitory concentration (BIC) and minimal biofilm eradication concentration (MBEC), biofilm antimicrobial susceptibility test for selected antimicrobial agents against the study isolates was detected.

Patients and methods: Qualitative biofilm formation assays (tube method and Foley-catheter assay) and quantitative assay by spectrophotometric method with ELISA reader were achieved against 15 isolates of *Proteus mirabilis*. Planktonic and biofilm antimicrobial susceptibility tests were performed.

Results: Out of 15 isolates of *Proteus mirabilis*, biofilms were produced in all these isolates (100%) in both of tube and Foley-catheter method. In the spectrophotometric method, our results showed that, all study isolates produced biofilm strongly in the glucose-supplemented media. Our result showed that minimal inhibitory concentrations were 12.5 ±10.1 µg/ml, 46.4 ±23.7 µg/ml and 9.6 ±3.3 µg/ml for ciprofloxacin, piperacillin and amikacin respectively against logarithmic phase planktonic cells of *Proteus mirabilis*. Also, biofilm inhibitory concentrations and minimal biofilm eradication concentrations for selected antimicrobial agents were reached 50-100 X folds higher than MICs to inhibit and eradicate *Proteus mirabilis* biofilm.

Conclusion: It is suggested that biofilm production was affected by the presence of glucose in the culture media. Furthermore, in biofilm antimicrobial susceptibility test, the biofilm producer isolates of *Proteus mirabilis* required 50-100 X folds higher than MIC for the same isolates at planktonic state to inhibit and eradicate bacterial biofilm from the surface of catheters.

Key words: Crystalline biofilm, formation, antimicrobial interaction.

Introduction:

Indwelling bladder catheters are the most commonly deployed prosthetic medical devices[1]**.** Unfortunately, the care of many patients undergoing long term catheterization is frequently complicated by infection with *Proteus mirabilis*[2]**.**

These organisms colonize the catheter, forming surface biofilm communities, and their Urease activity generates ammonia from urea, elevating the pH of the urine and the biofilm. Under these alkaline conditions, crystals of magnesium, ammonium phosphate and calcium phosphate are formed and become trapped in the biofilm[3].

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As the biofilm spreads and develops it obstructs the flow of urine through the catheter, causing either incontinence due to leakage of urine around the catheter or retention of urine in the bladder. In the latter case, painful distension of the bladder and reflux of infected urine to the kidneys can culminate in episodes of pyelonephritis, septicaemia and endotoxic shock[4].

It is well recognized that *Pro. mirabilis* have the ability to transform when it contacts a surface from small swimming bacilli into elongated, high flagellated swarmer cells is accompanied by a substantial increase in the production of Urease[4]. Swarmming in the presence of urine could therefore accelerate the generation of alkaline conditions that cause the deposition of crystalline material on the catheters and the swarmer cells have been shown to move more rapidly over catheter surfaces.. It is possible therefore that swarming facilitates the initiation of infection by mediating the migration of the organism from the peri-urethral skin along the catheter into the bladder[4]. Thus, this study has been undertaken to detect biofilm formation by study isolates of *Proteus mirabilis* qualitatively and quantitatively. Further, depending on MICs value and in term of BICs and MBECs, to detect biofilm antimicrobial susceptibility test for selected antimicrobial agents against the study isolates.

Patients and Methods:

Fifteen catheter urine specimens belonged to Twenty-five patients admitted to Departments of Urology and Surgery in Ramadi General Hospital were studied during the period from August to November 2007. Out of 15 patients, 6 (40%) were male and 9 (60%) were female with male to female ratio 1: 0.667. The age of the patients was between 9 month and 80 years old with mean (55.15 Y).

Full informative history had been taken directl from the patient or his parents or relatives, and the information was arranged in an informative clearly detailed formula sheet. All study isolates were well bacteriologically identified and confirmed by biochemical tests[5]. Bacteria were stored in brain heart infusion broth (BHI) medium containing 20% glycerol. Before each experiment, one aliquot was thawed quickly at 37 Cº and sub cultured on blood agar plates at37Cº for 24 hr. All study isolates were taken from long term catheter

Under aseptic conditions, 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). Catheter urine specimen was transported to the laboratory with minimum delay. Firstly, urine specimen was cultured immediately by semiquantitative culture technique to avoid contamination. In this technique, under aseptic conditions a standardized (fixed known volume) inoculum of uncentrifuged urine and the swabs were streaked on the sectors of nutrient, blood and MacConkey agar plates and incubated at 37ºC for 24 hours, if no growth was detected, plates were re-incubated for another 24 hours before reported as negative cultures. After incubation, regarding urine specimens, the total number of colonies per ml was counted. All the study isolates were bacteriologically identified and confirmed by biochemical tests[5].

Qualitative biofilm formation assays:

Adhesion assay: Tube method

Briefly, two to three colonies of isolates were inoculated into 5 ml of BHI broth in plastic conical tubes in duplicate. Saccharide free basal medium (BHI broth) without glucose that lacks the substrate for polysaccharides was used as a control. Cultures were incubated at 37 Cº for 18-20 hr.

The contents were aspirated, one tube was examined unstained and one each stained with crystal violate and safranin. Slime positivity was judged by the presence of visible

unstained or stained film lining the wall of the tube[6].

Adhesion assay: 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 catheters were capped at both ends and incubated at 37ºC overnight. Then catheters were rinsed with phosphate buffer saline. 700 µl of crystal violet (1%) was added to the catheters for 20 min after drying at room temperature for 15 min. Then the stained biofilm rinsed several times with phosphate buffer saline and allowed to dry at room temperature before examination[7].

Quantitative biofilm formation assays: Spectrophotometric method

Working cultures were prepared by inoculation on Columbia agar supplemented with 5% blood and incubated aerobically at 37 Cº for 24 hr. The cultures were used to prepare bacterial suspension in sterile distilled water adjusted to a 0.5 McFarland standard. The suspensions obtained were inoculated into a brain-heart infusion broth. Then poured into the wells of plastic microplates[9].

The modified microtiter plate test, with some improvements, was employed for the quantification of biofilm. Wells of sterile 96-well flat- bottomed plastic micro plates were filled with 250µL of the BHI broth. Negative control wells contained the broth only. Twenty µL of bacterial suspension was then added to each well. The plates were incubated at 37 Cº for 24hr. following incubation, the content of each well was aspirated, and each well was washed three times with 300µL of sterile distilled water. The remaining attached bacteria were fixed with 200 µL of methanol per well,

and after 15 min the plates were emptied and left to dry air. After that the plates were stained for 5 min with 160 µL per well of crystal violet used for gram stain. Excess stain was rinsed off by placing the microplates under running tap

water. After the plates were air dried, the dye which was bound to the adherent cells was resolublized with 160 µL of 33 %(v/v) glacial acetic acid per well. The optical density (OD) of each well was measured at 570 nm [6,9].

Planktonic antimicrobial susceptibility test:-

In this part of study, antimicrobial susceptibility test for selected antimicrobial agents against logarithmic phase planktonic cells of *Proteus mirabilis.* was achieved. Minimal and sub-minimal inhibitory concentrations for ciprofloxacin, piperacillin and amikacin were detected. This test was achieved according to the criteria laid down by **National Committee For Clinical Laboratory Standard[10].** by using an international quality isolate of *Pseudomonas aeruginosa* American Type Culture Collection (ATCC) 27853.

***Biofilm antimicrobial susceptibility test*:-**

Antimicrobial susceptibility test for sessile cell of *Proteus mirabilis*. was achieved by detecting the biofilm inhibitory concentration (BIC) and 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. Each study isolates was determined quantitatively by counting the cells of *Proteus mirabilis*. After 24 hours incubation of 1 cm catheter segments with $10^{\wedge 6}$ cfu/ml, the control for number of colonies after vortexing of the broth media containing catheter and compare latterly to the total no. of colonies for these isolates after exposuring to desired concentration of antimicrobial agents.

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).

Results:

Under the field of biofilm production, particularly qualitative biofilm assay, tube method, our result showed that out of **15(57.69%)** isolates of *proteus* spp, biofilm was produced on the inner lining of the tubes in 100%. Furthermore, in the other qualitative assay (Foley catheter method) our study

revealed that all isolates of *proteus* spp, biofilm was produced on the surfaces of the catheters.

In the quantitative biofilm formation assay, spectrophotometric method was achieved under two set of experimental conditions (with and without glucose). Our results showed that out of 15(57.69%) isolates of *proteus* spp, all isolates were produced biofilm strongly (OD was more than 0.25) in the glucose supplemented media and absence of glucose. On the other hand no significant differences were observed in the readings of optical density at 570 nm with the presence and absence of glucose among isolates of *proteus* spp, (1.29 ±0.38) and (0.89 ±0.24) respectively (P value = 0.02).

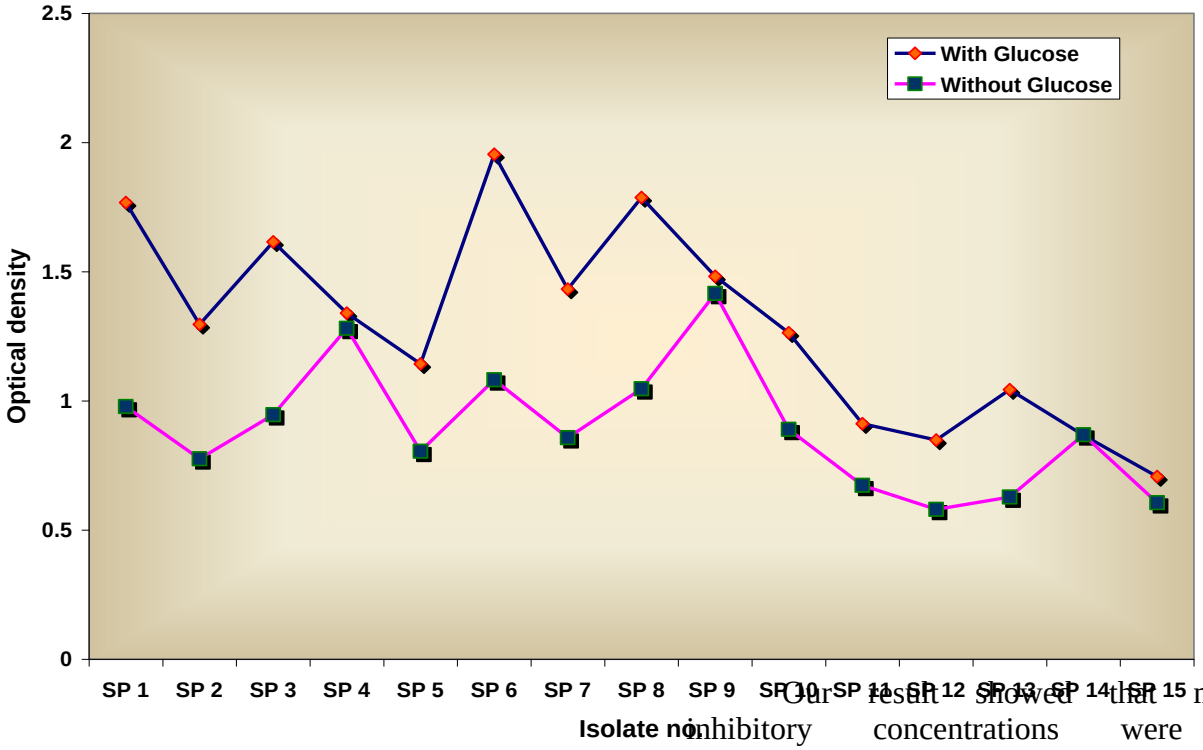

Figure 1: The effect of glucose on the production of biofilm by spectrophotometric assay among strong biofilm producer isolates of Proteus spp.

Our result showed that minimal inhibitory concentrations were 12.5 ±10.1µg/ml, 46.4 ±23.7 µg/ml and 9.6 ±3.3µg/ml for ciprofloxacin, piperacillin and amikacin respectively against logarithmic phase planktonic cells of *Proteus spp*. while the sub-minimal inhibitory concentrations were 6.3 ±5.04µg/ml, 23.2 ±11.8µg/ml and 4.8 ±1.7 µg/ml for the same antimicrobial agents respectively (table 1).

Table 1: The results of minimal and sub-minimal inhibitory concentrations for antimicrobial agents against selected isolates of *Proteus spp*

Isolate no.	Ciprofloxacin		piperecillin		Amikacin	
	MIC µg/ml	Sub MIC µg/ml	MIC µg/ml	Sub MIC µg/ml	MIC µg/ml	Sub MIC µg/ml
SP 1	16	8	64	32	8	4
***SP* 2**	16	8	64	32	8	4
***SP* 3**	1	0.5	64	32	8	4
***SP* 4**	1	0.5	8	4	8	4
***SP* 5**	8	4	64	32	8	4
***SP* 6**	32	16	64	32	16	8
SP 7	32	16	64	32	8	4
***SP* 8**	8	4	8	4	16	8
***SP* 9**	16	8	8	4	8	4
***SP* 10**	16	8	64	32	8	4
***SP* 11**	8	4	64	32	8	4
***SP* 12**	1	0.5	32	16	8	4
***SP* 13**	1	0.5	32	16	16	8
***SP* 14**	16	8	32	16	8	4
***SP* 15**	16	8	64	32	8	4
***Pseudomonas aeruginosa* ATCC 27853**	0.5	0.25	0.25	0.125	1	0.5
Mean ±SD	12.5 ±10.1	6.3 ±5.04	46.4 ±23.7	23.2 ±11.8	9.6 ±3.3	4.8 ±1.7

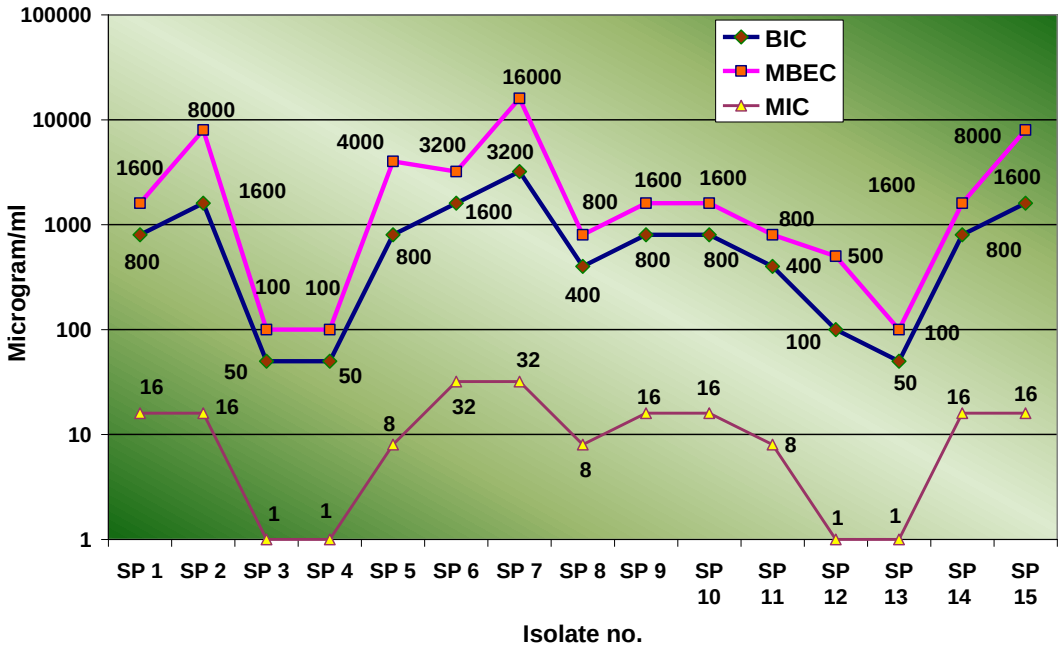

Figure 2. Comparison of susceptibility parameters for ciprofloxacin against logarithmic phase planktonic cells and sessile (adherent) cells of study isolates of *proteus spp.*

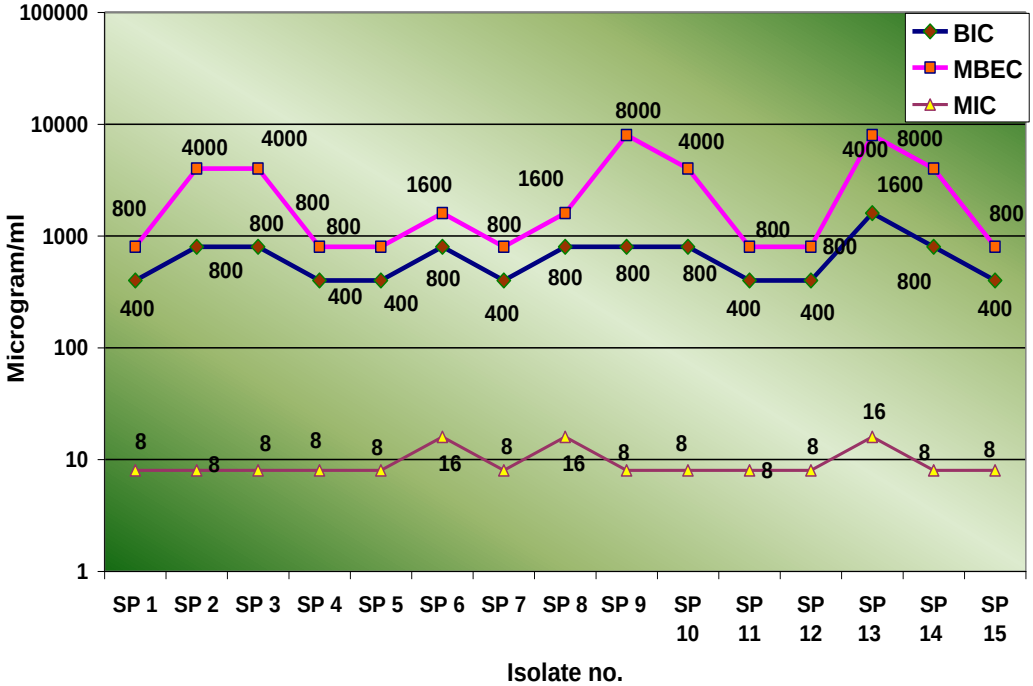

Figure 3. Comparison of susceptibility parameters for amikacin against logarithmic phase planktonic cells and sessile (adherent) cells of study isolates of *proteus spp.*

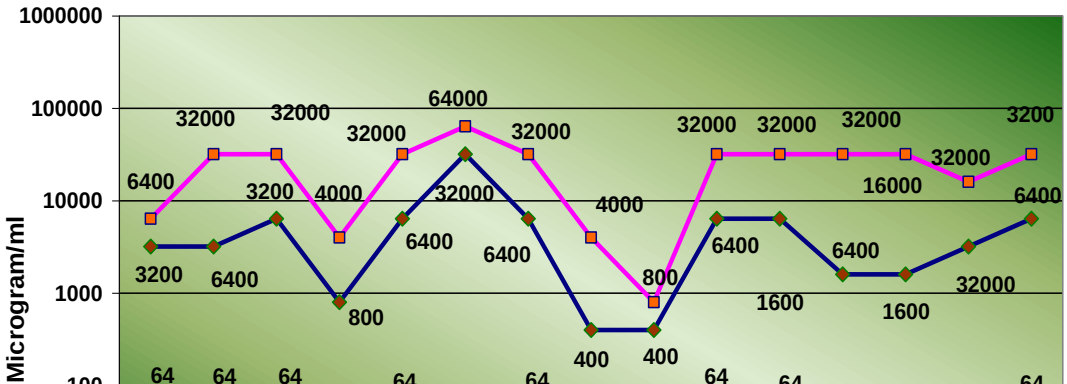

Figure 4. Comparison of susceptibility parameters for piperacillin against logarithmic phase planktonic cells and sessile (adherent) cells of study isolates of *proteus spp.*

Discussion:

Urinary tract infection due to *Proteus mirabilis* is not traditional and mostly reported in individuals with structural abnormalities of the urinary tract and is frequently isolated from the urine of elderly patients undergoing long-term catheterization and women with repeated UTI. Bacteria are attached to the penetrated tissue, resist host defenses and induce change to host tissue. Obviously establishment of *P. mirabilis* in the human urinary tract involves a host-parasitic immunological interaction and failure of host defense mechanisms favors the parasites. To be able to invade and successfully establish on the uroepithelial cells of the host, *P. mirabilis* produces several virulence factors e.g., pore-forming hemolysins, proticine 3, leukocidin, endotoxin, IgA and IgG proteases, urease **,** deaminase, adhesins, polysaccharide capsules**,** Pili/fimbriae[11], peritrichous flagella, the ability to form biofilms[10] and swarming ability.

The above mentioned virulence factors enable the pathogens to overcome the various defense mechanisms of the host. During infections, tones are formed due to the action of urease. This enzyme hydrolyzes urea, which is present in urine at copious amount[4]**,** generating ammonia and carbon dioxide. Ammonia so formed raises the pH, and normal soluble ions precipitate to form stones, usually composed of magnesium ammonium phosphate (struvite) caliculi and calcium phosphate (apatite). Translocation of bacteria from the epithelial surface to into deeper bladder tissues is resisted
by the humoral and cellular mediated immune responses of the host.

Two processes in *P. mirabilis* that are likely to be swarming and biofilm formation. Pathogenic as well as non-pathogenic bacteria prefer to live in colonies. Colonies of bacteria found on the surface of host epithelial tissues are called microcolonies. Colonies of bacteria exhibit high degree of organization and are characterized by distinguishable patterns of development called swarmers. The formation of swarming colonies by *P. mirabilis* is particularly well documented. The swarming process is very critical to the virulence of *P. mirabilis* because the expression of virulence determinants such as urease, hemolysin and the IgA metalloprotease are specific to the swarmer bacteria[11]. Biofilms are the congregated slimy mats of microbial communities that serve as a protective device for bacteria and also increase the ability of bacteria to resist both the actions of antibiotics and host responses. Biofilm formation by *P. mirabilis* is an important factor in the establishment of infections, which leads to a significant challenge to cure the biofilm-associated infections.

Proteus mirabilis is a motile gram-negative bacterium that is commonly associated with complicated urinary tract infections (UTI) in patients with long-term catheterization or with structural or functional abnormalities in the urinary tract. This uropathogen expresses several types of fimbriae that promote colonization of the urinary tract. The mannose-resistant *Proteus*-like (MR/P) fimbria, a surface organelle responsible for mannose-resistant hemagglutination, contributes significantly to colonization and pathogenicity in the CBA mouse model of ascending UTI[12]**.**

With regard to antimicrobial susceptibility test for planktonic cells Our result showed that minimal inhibitory concentrations were 12.5 ±10.1 µg/ml, 46.4 ±23.7 µg/ml and 9.6 ±3.3 µg/ml for ciprofloxacin, piperacillin and amikacin respectively against logarithmic phase planktonic cells of *proteus* spp. while the sub-minimal

inhibitory concentrations were 6.3 ±5.04 µg/ml, 23.2 µg/ml ± 11.8 and 4.8 ±1.7 µg/ml for the same antimicrobial agents respectively. Traditionally, microbiologists have evaluated the efficacy of antimicrobial

agents by measuring the minimal inhibitory concentration (MIC) which is defined as the lowest concentration of antimicrobial agents which inhibits bacterial growth after overnight incubation[13]**.** In virtually all diagnostic laboratories, these measurements are made on freely floating, planktonic laboratory phenotypes. These assays measure only the concentration of chemotherapeutic agents required to inhibit growth or kill planktonic bacteria. For some antibiotics, the concentration required to kill sessile or adherent bacteria may be greater than a thousand times that required to kill planktonic bacteria of exactly the same strain[14]**.** Therefore, the use of typical laboratory planktonic bacteria for selection of chemotherapeutics may be inappropriate under some circumstances[13]**.** In this study, MICs for selected antimicrobial agents (ciprofloxacin, pipericillin and amikacin) against logarithmic phase-planktonic cells were determined by using macrobroth dilution technique with the presence of international standard isolates (*pseudomonas aeruginosa* ATCC 27853). The MICs were 12.5 ±10.1 µg/ml, 46.4 ±23.7 µg/ml and 9.6 ±3.3 µg/ml for the above mentioned antimicrobial agents respectively.

In spite of MIC, assay remains the golden standard test and the best way to select potentially the effective antimicrobial agents. The minimal biofilm eradication concentration (MBEC) was developed for rapid and reproducible antimicrobial susceptibility testing for bacterial biofilm in the anticipation that the MBEC would be more reliable for selection of clinically effective antimicrobials in such infections[13]**.** Thus, our study has been undertaken to explain in one of its purposes which concentrations are required to eradicate proteus biofilm formed on indwelling urinary catheters in term of MBEC based on MICs and sub-MICs for selected antimicrobial agents against logarithmic phase planktonic cells.

Antimicrobial susceptibility test for sessile cell of *proteus* spp. 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 *Proteus mirabilis*. Our result revealed that the biofilm cells required 50-100 times the MIC values for **ciprofloxacin** obtained for the same isolates in the logarithmic phase of planktonic cells with MIC mean (12.5 ±10.1) µg/ml and MBEC mean (3200 ±4378) µg/ml with high significant difference (P value0.05). Yassien and Khardori, (2001)[6] documented that the high concentrations of flouroquinolones were used to treat the performed biofilms because 1) these concentrations would be expected to reach the biofilms when therapeutic doses of flouroquinolones (ciprofloxacin is one of the flouroquinolones) 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 flouroquinolones. This effect is concentration dependent.

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[6].

Also, with regard to piperecillin, our result revealed that the biofilm cells required 50-100 times the MIC values obtained against study isolates in logarithmic phase planktonic cells with MIC mean (46.4± 23.7) µg/ml and MBEC mean (25546±16587.7) µg/ml with high significant difference (P value less than 0.05). Mah, and co-workers, (2003)[15] explained the genetic basis for biofilm antibiotic resistance by identifying a chromosomal locus in his study called 'ndvB' which his 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 50-100 fold greater than MICs observed for study isolates of *proteus spp* in logarithmic phase planktonic cells with MIC mean (9.6 ±3.3) µg/ml and MBEC mean (2720 ±2544.2) µg/ml with high significant difference (P value 0.008). Anderal and associates, (2000)[16] documented that resistance in the biofilm population is probably not genetically encoded or due to the selection of resistant bacterial subpopulations, since the resistance disappears when bacteria are removed from the catheter. Resistance is probably due to the physiological state of the individual cells rather than a function of biofilm formation or slime production. Adherent bacteria grow more slowly than planktonic bacteria as a result of the adherence process rather than of nutrient depletion. It has also been postulated that only the surface of layers of a biofilm are exposed to a lethal dose of the antibiotic, due to a reaction-

diffusion barrier which limits the transportation of the antibiotic to the biofilm.

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