

QUANTITATIVE AND QUALITATIVE ASSAYS OF BACTERIAL BIOFILM PRODUCED BY *Pseudomonas aeruginosa* AND *Klebsiella* spp.



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ABSTRACT

The aim of this study was to detect biofilm formation by study isolates of *Pseudomonas aeruginosa* and *Klebsiella* spp. qualitatively and quantitatively. Twenty-five isolates were taken from patients admitted to Ramadi General Hospital were included in this study. Qualitative biofilm formation assays (tube method and Foley-catheter assay) and quantitative assay by spectrophotometric method with ELISA reader were achieved under two experimental conditions.

In tube method, the result showed that out of 8 (32%) isolates of *Pseudomonas aeruginosa* and 17 (68%) isolates of *Klebsiella* species, the biofilm were produced in 7 (87.5%), and 14(82.35%) respectively. while, in foley catheter method, the biofilm were produced on the surfaces of the catheters in all pseudomonal isolates (100%), and 15(88.23%)of klebsiella isolates respectively. In the spectrophotometric method, the results showed that out of 17 isolates of *Klebsiella* spp., all isolates were produced biofilm strongly in the glucose supplemented media while 15 (88.23%) of them were produced biofilm strongly in the glucose non-supplemented media and 2 (11.76%) isolates were weak biofilm producers in the glucose non-supplemented media. With regard to *Pseudomonas aeruginosa*, the results showed that all pseudomonal isolates, which submitted to this study, were produced biofilm strongly in the glucose supplemented and non-supplemented brain heart infusion broth. The study concluded that all isolates of *Pseudomonas aeruginosa* and *Klebsiella* spp. were produced biofilm qualitatively by both techniques. Further, the spectrophotometric method was an accurate method for detection the bacterial adherence to the surface of Microtiter plates. Further more, biofilm production was not affected by this factor in both of *Pseudomonas aeruginosa* and *Klebsiella* spp.

Introduction:-

It is now well documented that microbial biofilm is defined as structured communities of microbial species embedded in a biopolymer matrix on either biotic or a biotic substrate. Studies have revealed that the adherent bacteria, growing in consortia known as biofilms, are present in virtually all natural and pathogenic ecosystems^(1, 2).

Most importantly, the biofilm is characterized by its resistance to biocides, antibiotic chemotherapy, and clearance by humoral or cellular host defense mechanisms^(3, 4, 5).

Pseudomonas aeruginosa is a gram-negative bacterium, opportunistic human pathogen that causes chronic infections in immunocompromised individuals. These infections are hard to treat, partly due to the high intrinsic resistance of the bacterium to clinically used antibiotics and partly due to the

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formation of antibiotic-tolerant biofilms. The three most common ways of growing bacteria *in vitro* are as planktonic cultures, colonies on agar plates, and biofilms in continuous-flow systems. Biofilms are known to express genes different from those of planktonic cells, and biofilm cells are generally believed to closely resemble planktonic cells in stationary phase⁶. This study has been undertaken *in vitro*, to detect biofilm formation by locally isolates of *Pseudomonas aeruginosa* and *Klebsiella* spp. qualitatively by tube adhesion method and foley catheter assay. Further, Quantitative determination of bacterial adhesion on the surfaces of microtiter plate by spectrophotometric assay with ELISA reader under two experimental conditions

Patients and Methods:

Twenty-five isolates were taken from patients admitted to Ramadi General Hospital, in Ramadi during the period from April to August 2006. Out of 25 patients, 17 (68%) were male and 8(32%) were female with male to female ratio 1: 2.1. The age of the patients was varying between 13 years and 60 years old with mean (31.3 Y). The clinical data regarding the distribution of isolates, type of specimens and type of infection are presented in table 1. All study isolates were well bacteriologically identified and confirmed by biochemical tests⁷. Bacteria were stored in brain heart infusion broth (BHI) medium containing 20% glycerol. Before each experiment, one aliquot was thawed quickly at 37 C° and subcultured on blood agar plate's at 37C° for 24 hr⁷.

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 C° 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 tube⁸.

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 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 (9,10).

Quantitative biofilm formation assays:

Spectrophotometric method

Working cultures were prepared by inoculation on Columbia agar supplemented with 5% blood and incubated aerobically at 37 C° 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. After that, poured into the wells of plastic microplates¹¹.

The 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 C° for 24hr. following incubation, the content of each well was

aspirated, and each well was washed three times with 300 μ L of sterile distilled water. The remaining attached bacteria were fixed with 200 μ L of methanol per well, and after 15 min the plates were emptied and left to air dry. After that the plates were stained for 5 min with 160 μ L 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 resolubilized with 160 μ L of 33 % (v/v) glacial acetic acid per well. The optical density (OD) of each well was measured at 570 nm^(8, 11) by ELISA reader.

Results:

Under the field of biofilm production, particularly qualitative biofilm assay by tube method, the results showed that out of 8 (32%) isolates of *Pseudomonas aeruginosa*, 17 (68%) isolates of *Klebsiella* species, the biofilm were produced on the inner lining of the tubes in 7 (87.5%), and 14(82.35%) respectively. while this phenomenon was not observed in 1 (12.5%), and 3(17.64%) isolates of *Pseudomonas aeruginosa* and *Klebsiella* spp. respectively (table 2-A).

Further, in the other qualitative assay (foley catheter method) the study revealed that out of 8 (32%) isolates of *Pseudomonas aeruginosa*, 17 (68%) isolates of *Klebsiella* species, the biofilm were produced on the surfaces of the catheters in all pseudomonal isolates (100%), and 15(88.23%) of *klebsiella* species respectively. On the other hand this phenomenon was not observed in Two (11.76%) of *Klebsiella* spp. (table 2-B).

In the quantitative biofilm formation assay, spectrophotometric method was achieved under two set of experimental conditions (with and without glucose). The results showed that out of 17 isolates of

Klebsiella spp., all isolates were produced biofilm strongly (OD was more than 0.25) in the glucose supplemented media while 15 (88.23%) of them were produced biofilm strongly in the glucose non-supplemented media and 2 (11.76%) isolates were weak biofilm producers in the glucose non-supplemented media. 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 *Klebsiella* (0.712 \pm 0.30) and (0.54 \pm 0.12) respectively (P value = 0.07) (table 3).

With regard to *Pseudomonas aeruginosa*, the results showed that all pseudomonal isolates, which submitted to this study, were produced biofilm strongly in the glucose supplemented and non-supplemented brain heart infusion broth. 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 *Pseudomonas aeruginosa* (1.11 \pm 0.68) and (0.58 \pm 0.02) respectively (P value = 0.12) (table 4).

The isolates were classified according to biofilm production according to the criteria laid down by Christensen¹² as following: non-biofilm producers less than 0.125, weak biofilm producer between 0.125-0.25 and strong biofilm producers more than 0.25

Discussion:

It is well realized that bacterial adhesion to biomaterial surfaces is the essential step in the pathogenesis of prosthetic device related infections¹³. Microorganisms attach to surfaces and develop into biofilms. Biofilm-associated cells can be differentiated from their suspended counterparts by generation of an extracellular polymeric substance (EPS) matrix, reduced growth rates, and the regulation of specific genes. Cells may also communicate via quorum

sensing, which may in turn affect biofilm processes such as detachment¹⁴. The modern-day acute infections can often be resolved effectively with antibiotics (except for cases caused by antibiotic-resistant strains)¹⁵.

As the biofilm spreads and develops, it obstructs the flow of urine through the catheter especially in infection caused by *Proteus* spp., causing either incontinence due to leakage of urine around the catheter or retention of urine in the bladder. In the later case, painful distension of the bladder and reflux of infected urine to the kidney can culminate in episode of pyelonephritis, septicaemia and endotoxic shock¹⁵. Thus, our study has been undertaken and designed to shed a light on the biofilm phenomenon formed on the surfaces of indwelling bladder catheter in more than direction: formation, most reliable qualitative and quantitative detection techniques *in vitro* and the most accurate biofilm susceptibility testing in a trail to help the clinicians for make a precise decision regarding treatment of such infections.

Under the field of biofilm production, particularly qualitative biofilm assay by tube method, the results showed that out of 8 (32 %) isolates of *Pseudomonas aeruginosa*, 17 (68%) isolates of *Klebsiella* species, biofilm were produced on the inner lining of the tubes in 7 (87.5%), and 14(82.35%) respectively. Further, in the other qualitative assay (foley catheter method) our study revealed that out of 8 (32 %) isolates of *Pseudomonas aeruginosa*, 17 (68%) isolates of *Klebsiella* species, biofilm were produced on the surfaces of the catheters in all pseudomonal isolates (100%), and 15 (88.23%) respectively. the results are in agreement with those obtained from the study laid down by Christensen and associates¹². Mathur and associates¹⁶ who observed good correlation between the results of qualitative tube

assay and spectrophotometric (tissue culture plate) 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^(12,16).

In order to enable easier study of bacterial attachment and colonization, a variety of experimental, direct and indirect, observation methods have been developed. Microtiter plate assay (spectrophotometric method) is the most frequently used techniques for quantifying biofilm formation¹⁷. Microtiter plate procedure is an indirect method for estimation of bacteria *in situ*, it has the advantage of enabling researchers to rapidly analyze adhesion of multiple bacterial strains or growth conditions within each experiment, easy technique and used widely for antimicrobial agents susceptibility of biofilm. In the quantitative biofilm formation assay, spectrophotometric method was achieved under two set of experimental conditions (with and without glucose). the results showed that out of 17 isolates of *Klebsiella* spp., all isolates were produced biofilm strongly (OD was more than 0.25) in the glucose supplemented media while 15 (88.23%) of them were produced biofilm strongly in the glucose supplemented media and 2 (11.76%) isolates were weak biofilm producers in the glucose non-supplemented media. 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 *Klebsiella* (0.712 ±0.30) and (0.54 ±0.12) respectively (P value = 0.07). With regard to *Pseudomonas*

aeruginosa, Our results showed that all pseudomonal isolates which were submitted to this study were produced biofilm strongly in the glucose supplemented and non-supplemented media. 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 *Pseudomonas aeruginosa* (1.11 ± 0.68) and (0.58 ± 0.02) respectively (P value = 0.12).

Conclusions:The study concluded that all studied isolates of *Pseudomonas aeruginosa* and *Klebsiella* spp. were produced biofilm qualitatively by both of tube methods and Foley catheter methods. Further, the quantitative spectrophotometric method with ELISA reader was an accurate method for detection the bacterial adherence to the surface of Microtiter plates. Further more, under two set of experimental conditions, in the glucose supplemented and non-supplemented media, biofilm production was not affected by this factor in both of *Pseudomonas aeruginosa* and *Klebsiella* spp.

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Table 1. Distribution of clinical isolates of *Klebsiella* spp. and *Pseudomonas* spp. according to the type of specimens and infection.

Study isolates no (%)	Type of specimen	Type of infection
<i>Klebsiella</i> spp. 17 (68%)		
12(70.58%)	Catheterize d urine	Catheterize d Urinary tract infection
3(17.64%)	Wound swab	Diabetic foot infection
1(5.88%)	Wound swab	Burn infection
1(5.88%)	Ear swab	Otitis media
<i>Pseudomonas aeruginosa</i> 8 (32%)		
5(62.5%)	Catheterize d urine	Catheterize d Urinary tract infection
1(12.5%)	Wound swab	Diabetic foot infection
2(25%)	Wound swab	Burn infection

Table 2. Qualitative biofilm formation by tube and foley -catheter method among isolates of *Pseudomonas aeruginosa* and *Klebsiella spp.*

A- tube method			
Bacterial isolates	No. (%)	Positive no. (%)	Negative no.(%)
<i>Pseudomonas spp</i>	8 (32%)	7 (87.5%)	1 (12.5%)
<i>Klebsiellae spp</i>	17 (68%)	14 (82.35%)	3(17.64%)
B- Detection of biofilm on Foly-catheter			
Bacterial isolates	No.(%)	Positive (%)	Negative (%)
<i>Pseudomonas spp</i>	8 (32%)	8 (100%)	.
<i>Klebsiellae spp</i>	17 (68%)	15 (88.23%)	2(11.76%)

Table 3. Spectrophometric assay of bioflim formation among isolates of *Klebsiellae spp* under two experimental conditions.

Without glucose	With glucose	Experimental condition
0.54 ±0.12	0.712 ±0.30	O.D ₅₇₀ Mean±SD
17 (68%)	17 (68%)	No. (%) biofilm producer
15 (88.23%)	17 (100%)	No. (%) Strong producer isolates
2 (11.76%)	-	No. (%) weak producer isolates
-	-	No. (%) Non- producer isolates
0.07		P Value

Table 4. Spectrophometric assay of biofilm formation among isolates of *Pseudomonas aeruginosa* under two experimental conditions.

Without glucose	With glucose	Experimental condition
0.58±0.02	1.11 ±0.68	O.D ₅₇₀ m Mean±SD
8 (100%)	8 (100%)	No. biofilm producer
8 (100%)	8 (100%)	No. (%) Strong producer isolates
-	-	No. (%) weak producer isolates
-	-	No. (%) Non- producer isolates
0.121		P Value

القياس الكمي والنوعي لتكوين الغشاء الحيوي من قبل بكتريا الكلبسيلا وبكتريا الزوائف الزنجارية

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الخلاصة:

أن الهدف من هذه الدراسة هو لتحديد تكوين الغشاء الحيوي في عزلات الدراسة من الزوائف الزنجارية وبكتريا الكلبسيلا كميًا ونوعيًا. تم عزل ٢٥ عزلة من المرضى الداخليين إلى مستشفى الرمادي العام في محافظة الانبار. تم قياس تكوين الغشاء الحيوي (لللبايوفيلم) نوعيًا بطريقتي الأنايب وأنبوب قسطرة المثانة التي أنجزت تحت طرفين تجريبيين. أظهرت النتائج في طريقة الأنايب بأنه من مجموع ٨ (٣٢%) عزلات من الزوائف الزنجارية و ١٧ (٦٨%) عزلة في الكلبسيلا أنتج الغشاء الحيوي بواقع ٧ (٨٧,٥%) و ١٤ (٨٢,٣٥%) عزلة على التوالي. أما في طريقة أنبوب قسطرة المثانة فأنتج هذا الغشاء في جميع عزلات الزوائف الزنجارية (١٠٠%) و ١٥ (٨٨,٢٣%) عزلة من الكلبسيلا على التوالي. في طريقة المطياف الضوئي أظهرت النتائج أن جميع العزلات الكلبسيلا كانت العزلات منتجة قوية في الوسط المغذي المعزز بالكلوكوز بينما ١٥ عزلة (٨٨,٢٣%) كانت منتجة قوية للبايوفلم في الوسط المغذي الخالي من الكلوكوز وأظهرت عزلتان (١١.٧٦%) ضعفًا في الإنتاج في الوسط المغذي الخالي من الكلوكوز. أظهرت النتائج إن جميع العزلات من الزوائف الزنجارية التي خضعت للدراسة كانت منتجة قوية للبايوفلم في الوسطين الحاوي على الكلوكوز والغير مجهز بالكلوكوز. تستنتج الدراسة أن عزلات التي خضعت للدراسة من الزوائف الزنجارية والكلبسيلا كانت منتجة لتكوين البايوفلم بطريقتي الأنايب وأنبوب قسطرة المثانة كما إن الطريقة الكمية (المطياف الضوئي) كانت دقيقة في تحديد الالتصاق البكتيري.