Role of Pili and Polysaccharide in adherence of *Pseudomonas aeruginosa* to mammalian epithelial cells

دورالاهداب وعديد السكريد بالتصاق بكتريا *Pseudomonas aeruginosa* بالخلايا الطلائية

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Abstract

Adherence capability of *Pseudomonas aeruginosa* isolated from Iraqi patients was tested in accordance to their capability to adhere to epithelial cells. A range of adherence ability was found: The highest capacity with mean number of adhering bacteria of (13.5) was noticed. A sparse content or alginate was detected in these isolates analysis of pilin protein isolated from cultures exposed to ciprofloxacin antibiotic at sub MIC, level using polyacrylamide gel electrophoresis indicated that treatment of cells with $^{3}/_{4}$ MIC show highest activity as compared with others. Results of protein profile followed the same trend of that in adhesion experiment which allows hypothesizing that these isolates are piliated, not mucous.

المستخلص

اختبرت قابلية الالتصاق لبكتريا*Pseudomonas aeruginosa* المعزولة من عينات لمرضى عراقيون اعتمادا على قابليتها للاتصاق بالخلايا الطلائية . لوحظ مدى واسع من القدرة على الالتصاق بين العزلات بمتوسط التصاق [13,5] خلية/طلائية.ولتحديد محتوى العزلات من عديد السكريد وجد بانها تحتوى على كمية ضئيلة منه .لكن بتحليل بروتينات الاهداب لمزارع معاملة بالمضاد الحياتي السيبروفلوكاسين بتراكيز مثبطة تحت الحد الادنى وباستخدام ترحيل هلام متعدد الاكريل امايد وجد بان $^{3}/_{4}$ التركيز المثبط تحت الحد الادنى يعطي اعلى فعالية تثبيطية مقارنة مع باقى التراكيز . ظهر بالمقابل بان الخلايا المعرضة له قليلة التهدب مما يدفعنا للاعتقاد بان هذه الخلايا مهدبة غير لزجة.

Introduction:

Adhesion is considered as important step for colonization of pathogens to living tissue and hence facilitates development of diseases. Infections, such as burns, wounds, urinary tract infection, Otitis externa and respiratory tract infections caused by *Pseudomonas aeruginosa* started by adhesion of microbes to cells of selected tissues which then increase the severity of infections and aggressiveness of the pathogen. Accordingly, adhesion is categorized as a major virulence factor concerning this bacteria [1] .

Adherence of *Pseudomonad* to epithelial cells was found associated with the presence of surface components identified as either pili or / and cxopolysaccharide characterized as alginate [2,3] However, other investigators dout the role of pili in adhesion since non-piliated strains of *P. aeruginosa*bind to the same receptors specified for piliated strains. They concluded that pili have indirect role in carrying adhesion molecules and function in correct presentation of the adherent bacteria to receptors on the cells[4].

The aim of this work is to study the role of pili and polysaccharide in adherence of *P. aeruginosa* to human mouth cavity epithelial cells.

Materials and Methods

Sampling and Culture Collection

A hundred swabs samples from burn, wound, sputum operation room and ear were collected, using sterile tubes containing normal saline. From patients of Al-Wassit, Al-Karkh and Baghdad Medical Hospital and cultivated on MacConkey and blood agar plates then incubated over night at 37°C.

Non-fermentative, pale colonies on MacConkey's agar were selected and then streaked on selective media (Cetrimide agar and King A agar)Incubated at 37°C for 24 h., colonies were tested for pigmentation and then sub-cultured on brain heart infusion agar to obtain pure cultures for further diagnosis test.

A number biochemical tests. Related to identification *Pseodomonas aeroginosa*as indicated schematic diagram of identification suggested by[5 , 6] were performed Confirmatory tests were also done using API 20 E micro tubes systems.

Preparation of the Epithelial Cell Suspension:

Epithelial cells were collected from mouth cavity of healthy humans using swab or stick and placed in sterile tubes containing phosphate buffer saline (PBS), the epithelial cells were centrifuged at 1000g for 5 minutes then they were washed three times in PBS at 1000g for 10 min. and suspended in PBS to a concentration of 10^5 cells /ml.

Preparation of the Bacterial suspensions

Ten milliliters of Muller Hinton broth medium was inoculated with bacterial growth. The culture was incubated at 37°Covernight to ($O.D_{600}$ about 0.4) giving 1x 10^9 CFU/ml. Cultures of bacteria were washed twice in PBS at 1000*g* for20 min. and resuspended in PBS.

Adherence test:

A mixture of 0.2 ml of the bacterial suspension, 0.2 ml of the epithelial, cell suspension and 0.1 ml of PBS was incubated at 37°Cfor one hr. Unattached bacteria were removed by centrifugation in PBS at 1000*g*for 10 min. then the final pellet was resuspended in a drop of PBS, dropped onto a glass microscope slide, air dried, fixed with methanol and stained with methylene blue. The numbers of adherent bacteria on 20 epithelial cells were counted by light microscope; Control of epithelial cells was also included.

Purification of Alginate:

Selection of isolate:

Two isolates [13 ,14]considered as mucous -secreting alginate as capsule material were used as control as well as another twelve isolates suspected to be piliated non - mucous were selected for performance of the experiment of separation of alginate.

Purification of Alginate

The method of Learn [7] was used to perform the test. All isolates were grown on MacConkey agar with 5% glycerol and incubated at 25°C for 96 hrs.; confluent growth was scraped off the agar surface and suspended in sterile saline with 2mMEDT'A. The suspension was centrifuged at 12000*g* for 1 hr. and the supernatant was again centrifuged at 12000*g* for 2 hrs., the alginate was precipitated by the

addition of 3 volume of isopropanol to the supernatant fluid and washed twice with deionized distilled water.

Pilin Purification

The methods of [8 , 9] for the isolation of pilin were followed. A loop-full amount of stock culture was streaked on Muller-Hinton agar and incubated overnight at 37°C. Cells were scraped off and placed into a mixture of standard saline citrate solution and 15% sucrose then filtered through a fine mesh sieve to remove agar. The components were stirred overnight at 4°Cand pillin was sheared off in a waving blender pulsed on lowest speed. The mixture was then centrifuged at 8000*g* for 20 min to remove debris; the supernatant was dialyzed against sterile distilled water at 4°C with frequent changes of dialysate for 72 hrs. The pilin protein was precipitated with 50% ammonium sulfate and the solution was allowed to stand overnight at 5°C during the time. The precipitate was pelleted by centrifugation at 8000*g* for I hr., and dissolved in standard saline citrate buffer free sucrose, and this mixture was precipitated with 20 %ammonium sulfate. The precipitate was re-dissolved in standards saline citrate buffer then the solution was dialyzed against sterile distilled water at 4°C for 48 hr. 'The protein obtained was electrophoresed on SDS polyacrylamide gel and stained with coornassie blue R250.

Polyacrylamide gel electrophoresis:

polyacrylamide gel treated with 2.5% SDS as described in [10]. The separating, gel solution was transferred to PAGE tubes and allowed to polymerize. The tubes were submerged in. running buffer (Tris glycine buffer system pH 8.3) and protein samples were loaded on the gel and electrophoresed at 10mAtube for 4 hr. at room temperature.

Acetic acid -water (50*:*10:40v/v) for 2 hr., and then staining solutions of coomassie brilliant blue in methanol -acetic acid -water (40 : 10 : 50 v/v) for 24 hr. The gel was then distained with methanol -acetic acid -water (40:10:50 v/v).

Results and Discussion

Bacterial isolates were isolated from samples of patients suffering from burns, wound, operation –room, sputum and ear infection (10) and identified as *Pseudomonas aerusrinosa* according to the schematic diagram of identification suggested by[5,6] , a conformation of results was also done using API 20 E micro tubes systems.

The adhesion capacity of *P. aerugtnosa* was tested using strains considered as piliated non mucous, and mammalian epithelial cells isolated from mouth cavity. Moreover, the selected strains showed a distribution in resistance as indicated by test of MICs for different antibiotics. Adhesive ability was compared depending on the frequency of distribution of bacteria on epithelial cells and by the mean number of bacteria adhering to [20] epithelial cells as shown in Table (1) and considered as criteria for adhesive capability of cells, as long as adhering bacterial cells visible under light microscope and easy to count.

Table (1): Adherence of *P.aeruginosa* to Epithelial cells From Human-mouth Cavity

Isolates Designation	In Phosphate Buffer Saline				
	No.of Adherent P.aeruginesa To No. of epithelial cells				Mean No. of Adherent P.earuginosa cell ±SD
	0	1-5	6-20	> 20	Mean ±SD
SLS 1	0	6	10	4	11.6±9.6
SLS 2	1	4	8	7	13.5±11.2
SLS 3	0	12	6	2	4.7±4.0
SLS 4	0	5	5	10	11.7±9.7
SLS 5	2	9	4	5	5.1±3.7
SLS 6	3	10	7	0	3.8±3.1
SLS 7	0	6	14	0	9.5±9.0
SLS 8	1	12	5	2	4.2±6.2
SLS 9	0	6	14	0	9.5±11.1
SLS 10	0	3	8	9	9.2±10.6
SLS 11	2	13	5	0	3.5±4.7
SLS 12	0	10	7	3	5.5±7.2

In general all strains adhered to most of the epithelial cells and showed a mean number of adhering bacteria ranging from 3.5 to 13.5 bacteria per cell. However, a variation in adhesive capacity among various strains of *P.aeruginosa* was found. In comparison, only one strain (SLS-2) possessed high adhesive capacity with a mean number of adhering bacteria of 13.5, as shown in Table (1), Moreover, quick scanning of the results indicates a small difference in adhesive capacity as observed among the remaining strains tested. These results agree with [11], who also found a difference in adherence capability of strains of *E. coli* isolated from urinary tract infection. It seems that adhesive capacity of cells is affected by various components of the cell wall as well as types of epithelial cells tested [12]. Surrounding environmental conditions may also contributed to enhancement of adhesion ability [1].

Alginate Separation:

Attempt was also made to determine the role of alginate in adhesion ability of the isolates. Sugar content of exopolysaccharide extract separated from culture was taken as indicator for alginate content Figure (1) and protein content Figure (2).

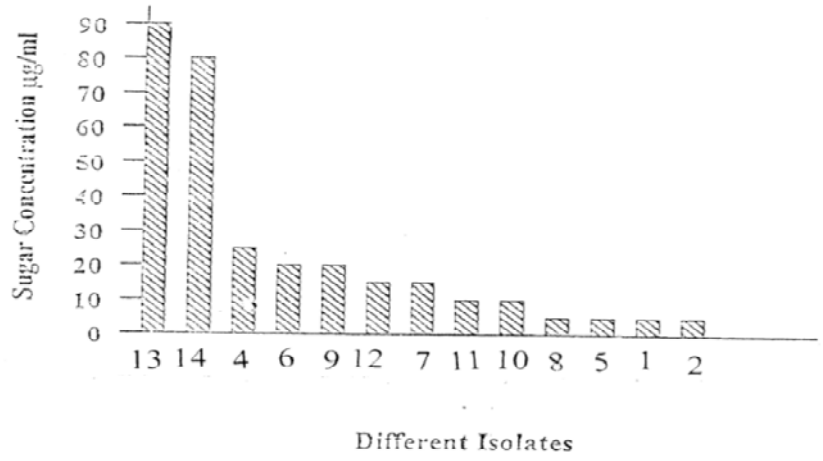

Fig (1): sugar contents of cell wall for selected isolates of *Pseudomonas aeruginosa*

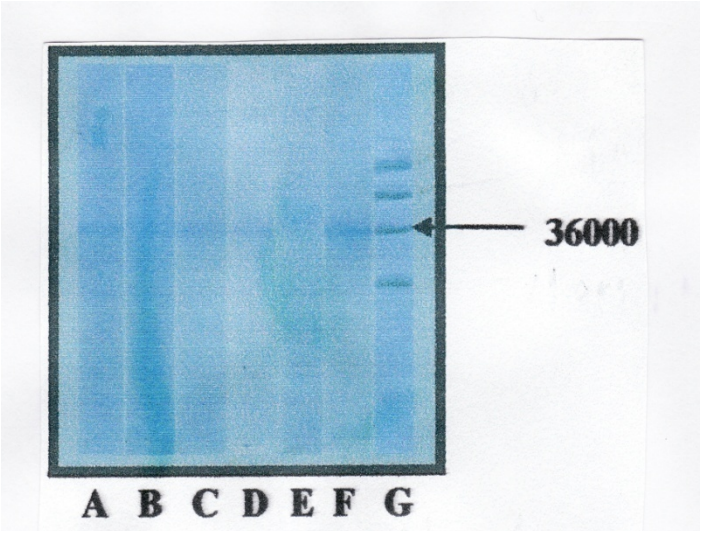

Fig (2): Pillin protein from *Pseudomonas aeruginosa*.SLS-2
A: Pili Protein from *P. aeruginosa* in 0.25 µ.g/ml MIC of Ciprofloxacin,
B: pili Protein from *P. aeruginosa* in 0.3 p.g/ml,
C: Pili Protein from *P. rieruginosa* in 0.5 p.g/ml MIC of Ciprofloxacin,
D: Pili Protein from *P. aeruginosa* in 0.75 p.g/ml MIC of Cefotaxime,
E: Pili Protein from *P. aeruginosa* in 0.75 p.g/ml MIC of Ciprofloxacin
F: Pili Protein from *P. aeruginosa* Free Antibiotics and G: Standard Protein

Results shown in Figure (1) Indicate a difference in amount of sugar contents among isolates selected, the control isolates were considered the highest as long as their content of sugars estimated as 80 and 90 µg / ml respectively and show a sparse content of protein that estimated as less than 1ug protein /ml as shown in Figure (2). Our results agreed with that of [7] who reported that alginate is a non proteinous material composed mainly of polymer of neutral sugars and expected to be poor in its protein content. However, other isolates show sparse contents of sugar as much as 2-20 mg/ml and protein content 100-500 mg / ml. In general, these results indicate that alginate is not the mechanism for adherence for these isolates. It is worth to mention that our isolates are considered as non mucoid piliated strains and are expected to show sparse contents of alginate and high contents of protein which is taken as an indirect indicator for demonstrating the contribution of pili adhesion of these isolates to epithelial cells.

Separation and Purification of Pilin:

In order to demonstrate the role of pili on adhesive capability *P.aeruginosa* in the presence and absence of sub MIC of antibiotics, Pilin protein was separated arid purified for treatments selected in accordance to results of adhesion experiments Show previously.

Results of protein profile for SDS-polyacrylamide gel electrophoresis analysis of control culture as well as cultures exposed to sub MIC levels of ciprofloxacin and cefotaxime are shown in Figure(2) , An alteration in the protein Content was observed with treatment of culture by $^{3}/_{4}$ MIC of ciprofloxacin which was not observed with treatment of cefotaxime. However, pilin production was not effected

with exposure of the cells to $^{1}/_{3}$ and $^{1}/_{4}$ MIC of ciprofloxacin The molecular weight of pilin was also estimated as 32000 as compared with standard proteins .

Comparison of band intensities of different treatments taken as a parameter for quantity determination was also done. The results indicated that intensities of band varies in accordance to exposure to Sub MIC levels of Ciprofloxacin and following the trend of results described in previous experiments. Control cultures show deeper intensity as compared to other treatments. However, no detectable intensity was observed for culture exposed to $^{3}/_{4}$MIC of Ciprofloxacin which was considered as all inhibitor to in pilin synthesis at that concentration causing abolishment of pili and loss of adhesion. However, a faint band was observed with exposure of cells to 3/4 MIC Cefotaxime. Other investigators also reported an effect for Cefotaxime at $^{3}/_{4}$MIC level [8, 7, 14]

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