Study the Prophylactic Role of Anti-Type IV Pili (fimbriae) Antibody Against Pulmonary Infection Caused by *P.aeruginosa in vivo* (mice)

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Abstract:

   This study was aimed to investigate the effect of anti- type 4 pili antibody in prevention pulmonary infections caused by *P. aeruginosa in vivo.* This was achieved by Evaluation of biofilm formation by the microtiter plate method to select *P.aeruginosa* isolate with highest biofilm formation capacity, Extraction and the partial purification of type IV pili from the selected isolate, then Preparation of type IV pili antibodies by rabbit immunization. The lung histological sections of non immunized mice were severly damaged ,while the damage were markedly decrease in the lung of immunized mice with anti-type 4 pili antibody.

Keywords: Anti-type IV pili (fimbriae) antibody, *P.aeruginosa*, cystic fibrosis

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دراسة الدور الوقائي للمصل المضاد لخمل النمط الرابع في الوقاية من الالتهاب الرئوي من قبل بكتريا *P.aeruginosa*   داخل جسم الكائن الحي (الفأر)

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الخلاصة:

تهدف الدراسة الى معرفة تاثير المصل المضاد لخمل النمط الرابع في الوقاية من الالتهاب الرئوي من قبل بكتريا *P.aeruginosa* وذلك بأختبار قابلية العزلات على تكوين الغشاء الحيوي باستعمال تقنية التصبيغ بالبنفسج البلوري في اطباق المعايرة الدقيقة واختيار العزلة الامثل المكونة للغشاء الحيوي الاسمك من بين جميع

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العزلات لاستخلاص بروتين خمل النمط الرابع ,تم استخلاص بروتين الخمل من العزلة الامثل وتنقيته جزئيا و
استخدامه في تمنيع الحيوانات المختبرية (الارانب) للحصول على المصل المضاد لبروتين الخمل . اظهرت
نتائج المقاطع النسجية لرئة الفئران حدوث ضرر كبير بالنسيج الرئوي للفئران الغير ممنعة بينما انخفض الضرر
بشكل كبير في النسيج الرئوي للفئران الممنعة للاجسام المضادة لخمل النمط الرابع.

Introduction:

Pseudomonas aeruginosa is an important opportunistic pathogen which infected immunocompromised patients and accounts for 25% of the gram-negative bacteria isolated from hospital environments. *P.aeruginosa* primarily associated with the lung pathology and morbidity of cystic fibrosis patients [1].

Cystic fibrosis (CF) is a genetic disease, which is caused by a defect in the transmembrane conductance regulator (CFTR) protein that functions as a chloride channel. Dysfunction of the CFTR protein results in salty sweat, pancreatic insufficiency, intestinal obstruction, male infertility and severe pulmonary disease. In most patients with CF life expectancy is limited due to a progressive loss of functional lung tissue [2]. Colonization of the lung of CF individuals with *P.aeruginosa* represents a significant negative milestone in the progression of this disease. Once colonized, patients are subject to the damaging effects of various secreted virulence factors and to the inflammatory response of the host immune system [3].

Bacterial adhesion to the surface is the first step towards the formation of mature biofilm .this step is dependent on surface component on the bacterial cell play a significant role in the establishment of infection by pathogens [4].

A key component of colonization of *P.aeruginosa* is the adhesion of Type IV pili (T4P) to the surface of epithelial cells [3].T4P are hair like appendages on the bacterial cell surface which composed of pilin polymers arranged in a helical structure with five subunits perturn . The portion of the pilin protein responsible for cell binding is located near the C terminus on the tip of pili which interacts with receptors on epithelial cells [5].   Furthermore, studies have shown that both pili-mediated adherence and twitching motility are critical to *P. aeruginosa* virulence. In an infant mouse model of lung infection, piliated strains of *P. aeruginosa* caused more severe and diffuse pneumonia than corresponding non-piliated mutants;[6].Therefore, pili seem to be legitimate targets in developing anti-pseudomonal immunotherapy. In order to interfere with bacterial adhesion, anti-pilin antibodies will recognize residues that are normally located at the C-terminal loop of pilin [7,8].

These mean, Generating antibodies against type IV pili of *P.aeruginosa* may be useful in reducing or eliminating colonization the lung of CF patients, Therefore we carried out this study to investigate the role of the prepared anti-type IV pili antibodies in blocking the adherence of T4P *in vivo* to find out if this will affect the biofilm formation and the importance of type IV pili to *P.aeruginosa* biofilm formation will proved.

Materials and Methods:

Collection of samples: One hundred and twenty three specimens of sputum were collected in sterilized containers from patients suffering from cystic fibrosis from the Educational Baghdad Hospital and Alyarmok Teaching Hospital.

Bacterial isolation and identification: The collected specimens were streaked directly on C for 24 hr. The pale non lactose fermentor colonies were °MacConkey agar then incubated at 37 selected then a single colony was inoculated on Blood agar for the activation and detection of bacterial ability to lyses red blood cells. The isolated colonies were inoculated on Cetrimide 0.03% C for 24 hr. Then a single colony from colonies that grow on Cetrimide °medium, incubated at 37 media was inoculated on King A agar and King B agar to determine their ability to produce pigments. Identification achieved by biochemical tests and re-confirmation with API 20 E system.

Detection of bacterial ability to Adhesion and production of slime layer: Adhesion test was achieved by congo red agar method according to Freeman *et al.*, [9] a positive result was indicated by black colonies with a dry crystalline consistency. Non-slime producers usually remained pink.

Biofilm assay and selection the co-efficient isolate: The ability of the *P.aeruginosa* isolates to produce biofilm were evaluated by using crystal violet staining technique in polystyrene microtiter plates and then O.D. was determined at 630 nm The O.D. represented the degree of the biofilm thickness according to Tang *et al.*, [10] .

Extraction and partial purification of type 4 fimbriae from the co-efficient isolate: This procedure was done according to Huang *et al.*, [11]; the bacteria were subculture in Luria-bertani broth for 48 hr. intervals three to four times and then the final broth which resulted from the subculturing used as inoculum for the 500 ml of Luria-bertani broth which was grown at 37C° for (48-72) hr. The bacteria were collected by centrifugation at 11,000xg for 30 min at 4 C°, pellet suspended in 100ml Phosphate Buffer Saline pH (7).The suspension were heated at 65C° for 3hrs and then homogenized in a blender for 20min at peripheral temperature, and then cell pellet removed by centrifugation at 8000xg for 30 min and supernatant was collected. Sodium azide and sodium deoxycholate were added to supernatant to afinal concentration of 0.1% and the suspention was kept at 4C° overnight. The resultant supernatant from the previous steps were undergoes to detection by agglutination test.

Antisera preparation: anti- type 4 fimbriae antisera were prepared according to Thankavel *et al.*, [12]. Two wild type males rabbits (weight 2-2.5 Kg) were used, the rabbits were divided into:

- Control: injected with PBS pH 7.2
- Test: injected with partially purified type 4 fimbriae.

Each rabbit was injected first with 1 ml Freund's complete adjuvant (olive oil) and then with 1 ml of partially purified type 4 fimbriae 1 ml amounts into two sites, namely subscapularly , subcutaneous at one site and intramuscularly into the hind leg. After two weeks, a booster injection was given. One week after the second injection, the animals were bled by cardiac puncture, and the serum was obtained by centrifugation at 2000 xg for 10 min, and stored at -20°C.

The role of anti-type 4 fimbriae antisera in the prevention of biofilm formation by *P. aeruginosa* in vivo experiment: Five females mice were used (age 8-12 weeks, weight 20-25 gm) the mices were divided into three groups:

- **Control negative:** Included one mice injected with 0.5 ml PBS pH 7.2.
- **Control positive:** two mice injected with 0.5 ml of bacterial cell suspension 1×10^8 cell/ml viable which previously prepared with macferland 0.5 [13].
- **Test:** included two mice injected with 0.5 ml of 1\10 dilution of rabbit serum treated with bacterial cell suspension 1×10^8 cell/ml (immunized group). Each mice were injected intraperitoneally seven days, the period between one injection and the other was one day.

Preparation of histopathological sections of mice lungs:

The survive mices were killed, and dissected taking the lung for study of histological changes. The histological sections were made according to Humson [14]. The lung of each mice were fixed by 10% formalin (10 ml formalin + 90 ml 0.9% NaCl) , then washed by tap water for several min., passing through a serial concentrations of alcohol (50%, 70%, 80%, 90% and 100%) for 2 hr. in each concentration , then cleared by xylol, saturated with paraffin at 60°C for 3 hr., embedded in pure paraffin; the blocks were then cut into sections with 5 µm in thickness by using Microtome. Haematoxylin and Eosin stains were used.

Results and discussion:

Bacterial isolation and identification:

One hundred and twenty three specimens of sputum were collected from patients suffering from cystic fibrosis (CF). 50 isolates of different bacteria were obtained. the results of *P. aeruginosa* isolation revealed that 6.5 % (8 isolates).the identification were achieved by the biochemical tests table 1 and confirmed by API 20E.

Table 1- The biochemical tests and their results for *P. aeruginosa*

Id	Biochemical tests	Results
1	Gram stain	–
2	Growth Citramide agar medium	+
3	Blood agar medium	β-hemolysis
4	Growth at 42C°	Growth
5	Growth at 4C°	No growth
6	Pigment production	Pyocyanine (blue-green)
7	Oxidase	+
8	Catalase	Babbles (+)
9	Indole production	–
10	Methyle red test	–
11	Vogas-proskauer	–
12	Citrate utilization	+
13	Motility	+
14	Urease production	+
15	Kliglar iron agar (KIA)	A/A, No gas , No H$_2$S

Adhesion test: All the isolates of *P. aeruginosa* were tested for their ability to produce slime layer by the Congo red agar method choose the isolates with a greater ability for adhesion. Results in this study show that most isolates gave positive results which appeared as black colonies with a dry crystalline consistency on Congo red agar, and the isolates P (3) and P (4) were selected as high slime layer producers figure-1.


Figure 1- *P. aeruginosa* isolate (4) in Congo red method, showing the black colonies with a dry crystalline consistency.

Biofilm assay:

Detection the ability of *P.aeruginosa* isolates to produce biofilm were evaluated by using pre-sterilized 96-well polystyrene microtiter plates and then absorbance was determined at 630 nm in an ELISA reader for determination of the degree of biofilm formation for studied strains that adhered on the surface of the microtiter wells as shown in figure-2.

Absorbance values were represented the degree of the biofilm thickness that formed by the studied strains on the surface of the microtiter well. The isolates P (3) and P (4) that selected from the Congo red method were assayed for the production of biofilm. The result indicated that the isolate P (3) and P (4) were produce strong biofilm and there is no significant differences observed in the readings of optical density at 630nm between the two isolates as shown in the table 2.
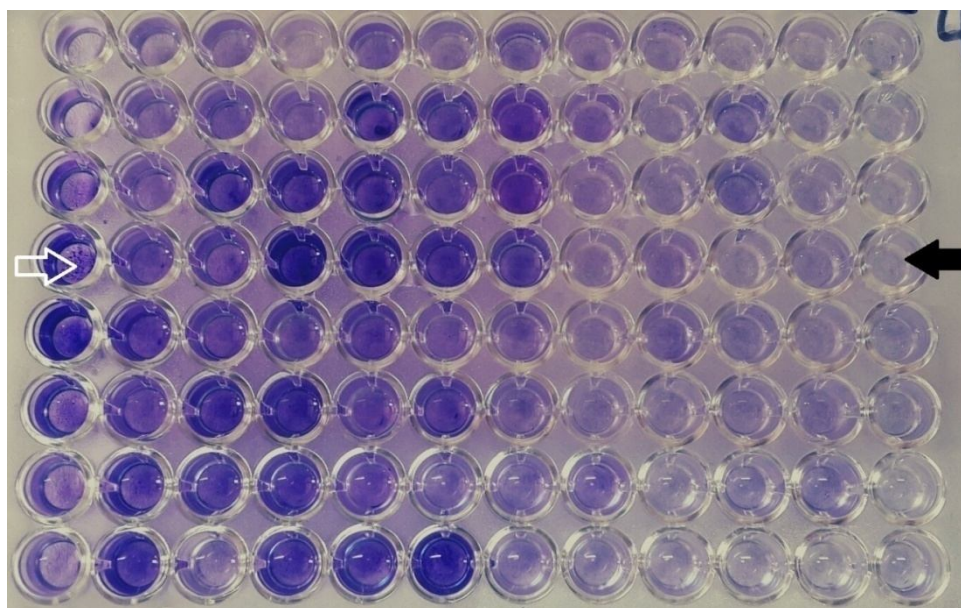
Figure 2- Biofilm formation in microtiter plate method by *P.aeruginosa*
(➡) Control   ,   (⇨)Test

Table 2- Absorbance for P.aeruginosa biofilm at 630 nm

Isolate code	Absorption (OD) ± SD
P(3)	**0.851 ± 0.221**
P(4)	**1.125 ± 0.073**

P-value: 0.114   SD (standard deviation)

Extraction and partial purification of type 4 fimbriae from the co-efficient isolate:

The *P.aeruginosa* isolate P (4) which recorded with the higher ability of biofilm formation was selected for the type 4 fimbriae extraction. According to Huang *et.al*.,[11] Heat extraction and mechanical shearing resulted in the release of the Fimbriae from the bacterial cell, While the defimbriated bacteria were removed by centrifugation.The type 4 fimbriae was partially purified

from the other bacterial component present in the supernatant which result from the earlier treatment by ultracentrifugation (48,000xg for 3hrs).

Studying the Histopathological Changes in the lungs of Non-immunized and Immunized Mice: The histological sections of the untreated control negative mice that injected with 0.5 ml PBS show no evidence of inflammation in the parenchyma or airways figure -3.
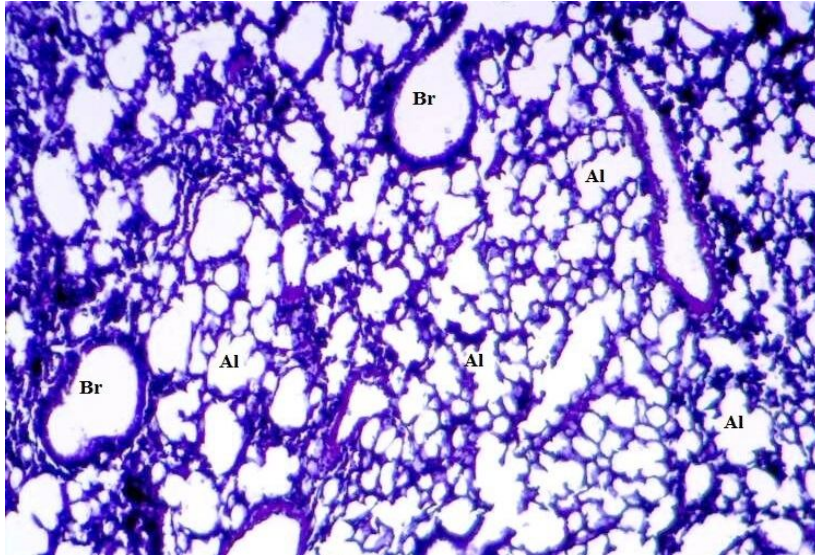

Figure 3- lung section of control negative mice (injected intraperitonially with phosphate buffer saline) showing normal appearance of lung tissue which consist of Alveoli (Al) and Bronchial (Br)(X200)(H & E).

The lung is severely damaged in most sections of the lung of mice that have not been immunized which infected with *P. aeruginosa* (control positive group), histological abnormalities included increased in the thickness of alveoli septa with inflammatory cells (neutrophiles and lymphocytes) infiltration near the small bronchus, sever blood congestion and large population of macrophage has been congregated to the alveolar wall. Emphysema or the destruction of the alveoli walls has also seen as shown in figure- (4A, 4B) and figure-5.
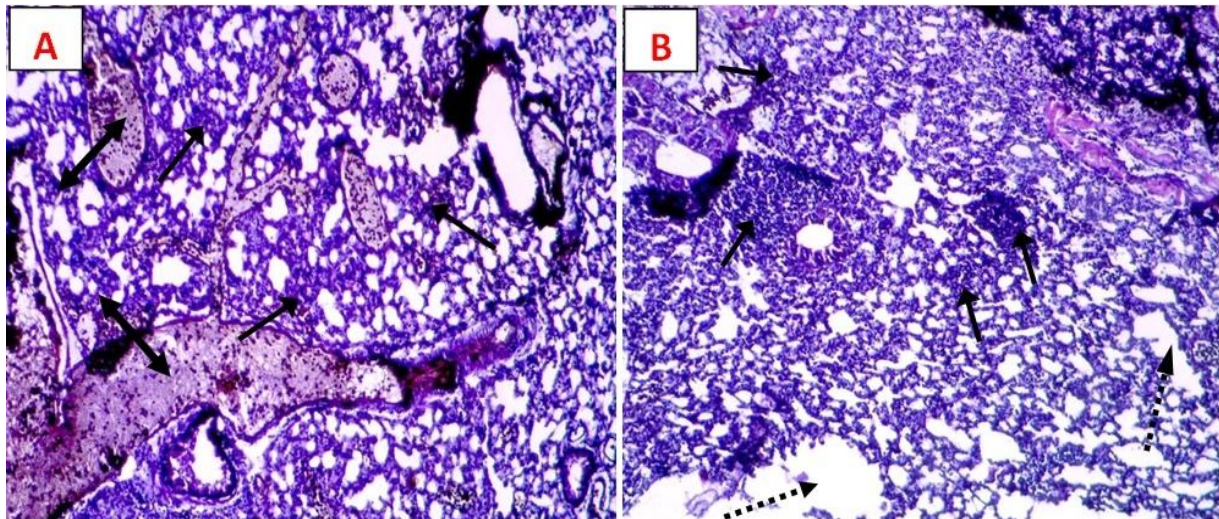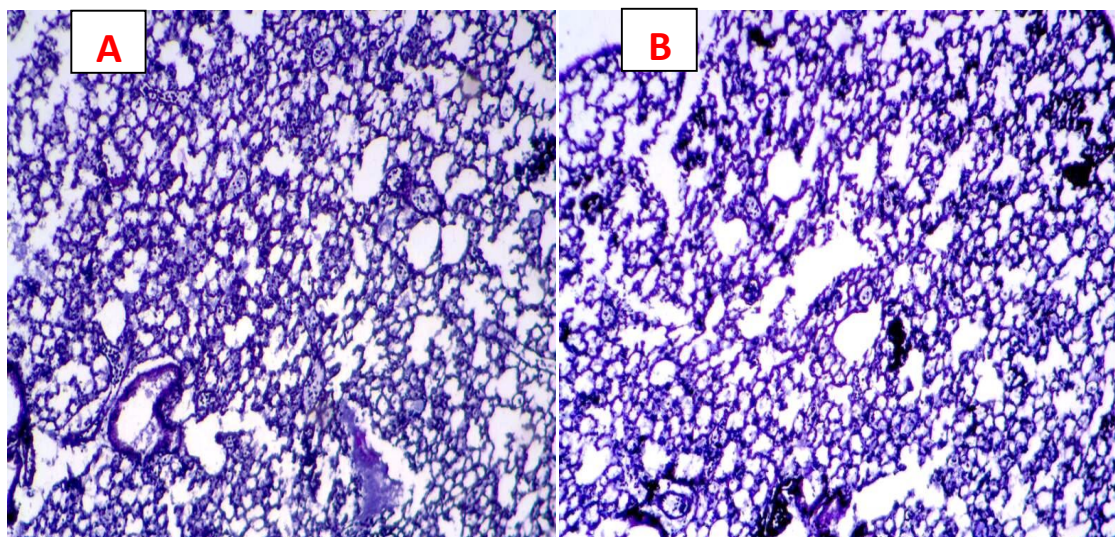
Figure 4- (A) and (B) Lung sections of the non immunized group of mice show large scale obliteration of normal lung architecture by increase the thickness of alveolar walls due to the heavily infiltration of neutrophils (⟶), sever blood congestion and many of macrophages appeared as dark brown cells (▪▪▪▪▪▶).the destruction of alveoli wall (⟵⟶). (X100) (H & E).
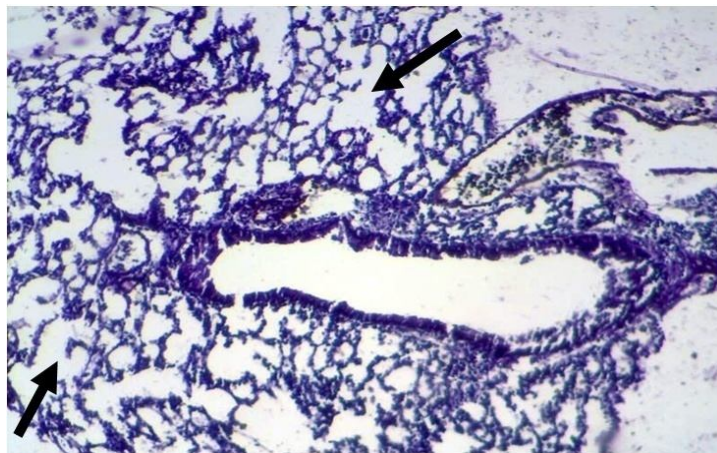
Figure 5- Lung sections of the non immunized group of mice image from a neighboring field showing typical appearance of the destruction of the alveoli walls (X200) (H & E).

The aboviouse histological changes were markedly decreased in extent and severity when evaluated in immunized mice. In immunized mice (test group) that injected with 0.5 ml of 1\10 dilution of rabbit serum treated with *P. aeruginosa* suspension, the lung sections show decrease in the thickness of the alveoli walls , Decrease the ratio of congestion and inflammation compared with non immunized group figure- (6 A,6B).

Figure 6-A and B Lung sections of the immunized group of mice with decreasing in the thickness of alveoli wall and congestion in compare with none immunized.

According to Hogg and Senior [15] Neutrophil-dominated airway inflammation has been implicated as a key feature of airway remodeling and bronchiectasis. This process is mediated by the production of reactive oxygen species and metallo–proteases, which are associated with lung fibrosis. Neutrophils possess several highly related proteases, which are contained within specific granules. These proteases are critical for neutrophil Responses against infection. However, large amounts of these enzymes escape from neutrophils in death and during phagocytosis. The protease burden overwhelms existing antiprotease defenses, injures the respiratory

The destruction of the lung parenchyma is commonly referred to as pulmonary emphysema, which defined by Snider *et al.* [16] as "an abnormal permanent enlargement of the air space distal to the terminal bronchioles, accompanied by destruction of the alveolar walls, and without obvious fibrosis". The reason behinend of pulmonary emphysema has not been fully established, but the prevailing concept is that lung inflammation caused bacterial products leads to an imbalance of proteases and anti-proteases [17]. It's important to mention that emphysema is characterized by air flow limitation that is not fully reversible [18].

Type IV pili of *P.aeruginosa* involved in bacterial adherence, biofilm formation and mediating twitching motility. Using purified pili protein or pilin peptides conjugated to carrier proteins as vaccine candidates showed efficacy in mice [19,20].

Lee *et al.*, [21] reported that Anti-adhesin antibodies that recognize a receptor-binding motif inhibit both pilus/fimbrial mediated adherence of *P.aeruginosa* and *Candida albicans* respectively to asialo-GM1 receptors and human buccal epithelial cell surface receptors.

Cachia *et al.*, [22] shown that the C-terminal region of the pilin monomer contains the epithelial cell binding domain .Antibodies to this region of the pilin molecule are able to block and prevent the infection process. Ohama *et al.,* [20] suggested that intratracheal pili protein immunization is effective against respiratory tract infection caused by *P. aeruginosa* in mice. Horzempa *et al.*, [23] showed that

Immunization with a *P.aeruginosa* 1244 strian pilin provided protection in a murine model for respiratory infections and a burn model.

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