Study of some immunization effects againest attenuated *Pseudomonas aeroginosa* in local rabbits

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Abstract

The study was carried out on the 12 local rabbits, divided into four groups, the first one was injected by a stock solution of attenuated *Pseudomonas aeroginosa* (0.1ml 26×10^{-4}, the second and third group injected by1/2 and 1/4 dilution respectively, while the last fourth group injected by normal saline and considered as control group. Our results showed significant variations in hypersensitivity test of of immunized group in comparison with the control group. The results of hepatomegaly and spleenomegaly showed valid decrease in first dilution immunized group. Significant enlargement of lung and kidney were found in control group while minimum weight recorded second dilution immunized group. There was significant increase of IgG level of immunized group in compared with control group. The level of complement (C3 &C4) showed significant increase in C4 of immunized group in comparison with the control group.

دراسة بعض مظاهر التمنيع ضد الإصابة بـ*Pseudomonas aeroginosa* في الأرانب المحلية

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الخلاصة

أجريت هذه الدراسة على 12 أرنبا محليا وزعت في أربع مجاميع حقنت المجموعة الأولى بمحلول البكتيريا المضعفة وحقنت المجاميع الثانية والثالثة بالتخافيف 2/1، 4/1 على التوالي في حين حقنت المجموعة الأخيرة بالمحلول الفسلجي واعتبرت كمجموعة سيطرة. أظهرت النتائج فروقا معنوية في اختبار فرط الحساسية للمجاميع الممنعة بالمقارنة مع مجموعة السيطرة، كما أظهرت نتائج تضخم الكبد والطحال انخفاضا واضحا في مجموعة التخفيف الأولى، وارتفاعا معنويا في تضخم الرئة والكلية في مجموعة السيطرة وسجل اقل الأوزان في المجموعة الممنعة بالتخفيف الأول والثاني. كذلك أظهرت النتائج ارتفاعا معنويا في تركيز الأجسام المضادة نوع وارتفاعا في مستوى تركيز 4 في مصل الأرانب الممنعة مقارنة مع مجموعة السيطرة.

Introduction

Pseudomonas aeruginosa is a Gram-negative, aerobic rod, belonging to the bacterial family *Pseudomonadaceae*. (1) The family includes *Xanthomonas*, which together with *Pseudomonas*, comprise the informal group of bacteria known as Pseudomonads (2), These bacteria are common inhabitants of soil and water, They occur regularly on the surfaces of plants and occasionally on the surfaces of animals. The pseudomonads are better known to microbiologists as pathogens of plants rather

than animals, Since *P. aeruginosa* can live in both inanimate and human environments, it has been characterized as a "ubiquitous" microorganism.(3). *Pseudomonas aerogionsa* is a major cause of nosocomal and community acquired chronic infection and has high level of innate antimicrobial resistance, This has led researchers to investigate vaccine and immunotherapeutic approaches to prevent and treat *P. aeruginosa* infection (4). *P. aeruginosa* groups tend to form biofilms, which are complex bacterial communities that adhere to a variety of surfaces, including metals, plastics, medical implant materials, and tissue, biofilms are characterized by "attached for survival" because once they are formed, they are very difficult to destroy. Depending on their locations, biofilms can either be beneficial and detrimental to the environment, for instance, the biofilms found on rocks and pebbles underwater of lakes and ponds are an important food source for many aquatic organisms; on the contrary, those that developed on the interiors of water pipes might cause clogging and corrosions (5,6). *P. aeruginosa* produces two extracellular protein toxins, Exoenzyme S and Exotoxin A in addition to Lipopolysaccharide of outer membrane, purified Exotoxin A is highly lethal for animals including primates(1). This exoproduct is responsible for direct tissue destruction in lung infection (7) It causes urinary tract infections, respiratory system infections, dermatitis, soft tissue infections, bacteremia, bone and joint infections, gastrointestinal infections and a variety of systemic infections (8). *P. aeruginosa* produces several extracellular products that after colonization can cause extensive tissue damage, bloodstream invasion, and dissemination, In vivo studies have shown that mutants defective in the production of exotoxin A, exoenzyme S, elastase, or alkaline protease are essential for maximum virulence of *P. aeruginosa;* however, the relative contribution of a given factor may vary with the type of infection (9). The our research point toward studying possibility of vaccines productions to prevent infection by *P. aeruginosa*.

Materials and Methods

1.**Vaccine:** Attenuated live cells of *Pseudomonas aeruginosa* was prepared as described by (10) and used for experimental animal vaccination , bacterial count was done by method mentioned by Tomasiewicz (11).

2.**Rabbits:** apparently healthy, local rabbits were used. A total of 12 animals were divided to four groups, reared in separated cages and fed green food. Animals in first group were immunized with 0.1ml stock solution of attenuated bacteria 26×10^{-4} by intradermal injection. The second and third groups immunized with ½ and ¼ dilution of stock solution respectively, While the last fourth group injected with 0.1ml of normal saline and was considered as control group.

3.**Hypersensitivity reaction:**

A. The procedure was made as described by Bacharach (12) et al. After first dose of the vaccine, hypersensitivity reaction was evaluated by measuring increase in the thickness of skin at the site of injection and length of redness area as well as increase in the temperature.
B. **Second dose:** After 14 days, a second dose was given to each animal in the same amount and concentration of first dose and the reaction of hyper sensitivity was read in the first 24hrs, 48hrs, 72hrs and 96hrs respectively.
C. **Challenge dose:** After 28 days challenge dose 0.06ml of stock solution was given intranasal to all animals in all groups.

- **Weighing of Liver, spleen and lung:** After 3days the liver, spleen and lung of each animal of all groups were weighed.

- **Differential white blood cells counts :** Differential WBCs count evaluated as described by Al Dragee et al (13).
- **Statistical Analysis:** The results were analyzed by using Complete Randomized Design for identifying of the effect of different treatment in different cases and using of least significant differences among median of treatments to identifying significant differences according to the Franey et al (14).

Results

The results of our study showed the effect of three different concentration of the thickness of the skin in comparison with control group. maximum significant thickness increase was present after 24hrs in all three groups as compared with the control. Additionally, significant increase of stock solution and first dilution as compared with the second concentration . Also there was significant increase in thickness after 24hrs and 48hrs as compared with 72hrs, 96hrs and the thickness before injection in stock solution group. Table (1) summarizes effects of time and concentration on thickness of skin in all tested groups.

Table (1) Mean of rabbits skin thickness evaluated by millimetres before and after intradermal injection by attenuated *Pseudomonas aeruginosa*

	Before	After 24hrs	After48hrs	After 72hrs	After 96hrs
Stock	1.33 0.33± b	3.33 0.88± Aa	3.16 0.72± Aa	2.00 0.57± Ab	2.00 0.57± Ab
1st dilution ½	1.33 0.33± b	3.33 0.88± Aa	2.00 0.57± Bb	1.66 0.33± ABb	1.66 0.33± B
2nd dilution ¼	1.33 0.33±	2.50 0.29± B	1.33 0.33± C	1.33 0.33± B	1.33 0.33± B
Control	1.33 0.33± a	1.33 0.33± Ca	1.33 0.33± Ca	1.33 0.33± Ba	1.33 0.33± Ba

Different capital letters refer to significant variation between different groups.
Different small letters refer to significant variation between different periods.

The results showed significant presence of redness area after 24hrs of injection by all three different concentration in comparison with animals state before injection. The control group did not show any redness area after injection with normal saline. The static differences and mean of diameters were explained in Table(2).

Table (2) Diameters mean of redness area evaluated by centimeters before and after intradermal injection by attenuated *Pseudomonas aeruginosa*.

	Before injection	After 24hrs	After 48hrs	After 72hrs	
Stock	Zero Ac	15.00 1.52± Aa	9.33 0.33± Ab	Zero Ac	Zero Ac
1st dilution	Zero Ab	7.33 3.69± Ba	4.30 2.18± Ba	Zero Ab	Zero Ab
2nd dilution	Zero Ab	4.00 3.98± Ca	3.33 3.31± Bab	Zero Ab	Zero Ab
Control	Zero Aa	Zero Da	Zero Ca	Zero Aa	Zero Aa

Different capital letters refer to significant variation between different groups.
Different small letters refer to significant variation between different periods.

The immunized animals showed slight increase in temperature, while control group kept normal level of temperature. The difference among group did not reach the level of significance (Table 3).

Table (3) Temperature (Cº) of rabbits before and after intradermal injection by attenuated *Pseudomonas aerugenosa*

	Before injection	After 24hrs	After48hrs	After 72hrs	After 96hrs
Stock solution	37.76 0.27±	37.96 0.27±	37.17 0.61±	37.40 0.32±	33.40 0.32±
1st dilution	37.80 0.15±	37.87 0.29±	37.44 0.29±	37.30 0.27±	37.3 0.27±
2nd dilution	37.60 0.21±	38.2 0.1±2	38.16 0.38±	38.1 0.38±	38.1 0.38±
Control	37.63 0.2±4	37.63 0.24±	37.63 0.24±	37.63 0.24±	37.63 0.24±

After 14 days, the animals retreated with same first dose, the results of thickness revealed significant increase skin thickness of stock solution group after 24hrs and 48hrs as compared to thickness of skin after 72hrs as well as in compared with control group which re-injected with same dose of normal saline. The differences among all groups illustrated in (Table 4).

Table (43) Means of rabbits skin thickness evaluated by millimetres before and after i/d 2nd injection by attenuated *Pseudomonas aerugenosa*

	After 24hrs	After48hrs	After72hrs
Stock	3.18 0.73± Aa	2.63 0.41± aab	2.01 0.47± Ab
1st dilution	2.17 0.38± B	1.42 0.23± B	1.32 0.19 B
2nd dilution	2.29 0.41± Ba	1.78 0.22± Bab	1.28 0.06± Bb
Control	1.33 0.33± C	1.33 0.33± B	1.33 0.33± B

Different capital letters refer to significant variation between different groups.
Different small letters refer to significant variation between different periods.

The mean of diameters of rednessarea revealed significant increase of stock solution group in comparision to first dilution and second dilution as well as control group. Both first and second dilution shwed significant increase in redness area as compared with control group. Also significant increase in diameter of stock solution group after 24hrs to state of animals after 72hrs. The correlation among groups and significance variation levels illustrated in Table (5).

Table (5) Diameter means of redness area evaluated by centimetersbefore and after intradermal injection by attenuated *Pseudomonas aeruginosa*

	After 24hrs	After48hrs	After72hrs
Stock	11.49 0.56± Aa	8.10 0.80± Aab	Zero Ab
1st dilution	5.79 0.15± B	5.35 0.31± AB	Zero
2nd dilution	5.44 2.32± B	1.67 1.66± Bc	Zero
Control	Zero C	Zero C	Zero

Different capital letters refer to significant variation between different groups.
Different small letters refer to significant variation between different periods.

The animals in all groups injected intranasal by challenge dose of Pseudomonas aeruginosa. After 3 days weighining of immune organs showed that enlargement of stock solution injected group and control group as compared with 2nd group and 1st dilution group that give minimum liver weight. The spleen did not show high differences (Table 6, Fig.1 and Fig. 2) Other important organ lung and kidney were weighed the result showed significant enlargement of lung in case of stock solution group, first diluton and control group as compared to second dilution group (minimum level of lung weight). The mean of kidney weight results shwed enlargement of kidney in case of stock solution group, first dilution and control group as compared with second dilution (minimum weight of kidney) as explained in table (Table 6).

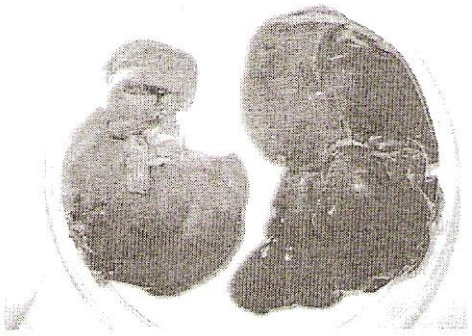

Fig. (1) The difference between liver size of immunized group (left side) and control group (right side)

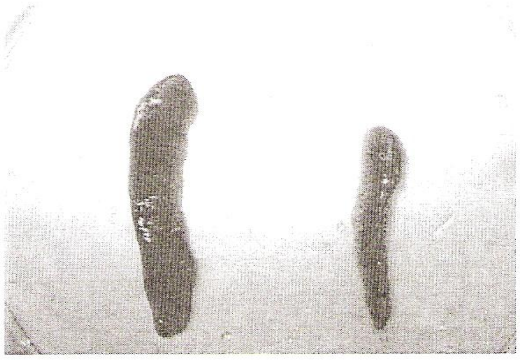

Fig. (2) The difference between spleen size of immunized group (right side) and control group (left side)

Table (6) The percent ratio of weight of immune organ to weight of animal show the difference between subjected animals and control group

	Stock solution	1st dilution	2nd dilution	Control
Liver	3.56 0.020	2.35 0.840	2.68 0.070	3.11 0.780
Spleen	0.043 0.003	0.036 0.003	0.043 0.007	0.043 0.009
Lung	0.553 0.098 AB	0.646 0.018 A	0.386 0.072 B	0.766 0.049 A
Kidney	0.996 0.022 B	1.106 0.029 A	0.743 0.009 C	0.966 0.083 B

Different capital letters refer to significant variation between different groups.

Table (7) The mean of total and differential WBC count of rabbits in different groups

	Stock	1st dilution	2nd dilution	Control
WBC (10^3/mm^3)	49.33 0.66	48.00 1.15	54.67 5.67	54.67 5.67
N(%)	60.33 3.65	52.30 2.32	53.66 3.17	59.66 5.87
E(%)	4.33 0.33	4.66 2.59	4.00 Zero	3.33 1.67
M(%)	8.33 4.31	16.66 2.02	18.33 2.01	17.66 8.37
L(%)	24.00 Zero	26.00 1.99	22.33 0.33	19.00 3.77
B(%)	Zero	Zero	Zero	Zero

The total IgA, IgG, C3 and C4 were investigated the results showed significant increase in IgG level and C4 as compared to control group (Table 8) and (Table 9).

Table (8) The differences between concentration of immunoglobulins (IgA and IgG) in both treated and control group

	IgA	IgG
Treatment group	185.66 12.01±	765.33* 51.31±
Control group	=125.66$\bar{X}$ 12.06±	=504.33$\bar{X}$ 73.79±

***significant increase**

Table (9) The differences between concentration of complement component (C3, C4) in both treated and control group

	C3	C4
Treatment group	144.66 10.40	42.5* 5.5
Control group	120.33 14.57	11.50 0.5

***significant increase**

Discussions

All animal groups injected by attenuated *Pseudomonas aerugenosa* assessed by delayed hypersensitivity test exhibited specific immunological response. Our results showed significant increase in thickness of skin as well as sensitive area that appear red, the highest mean was present in stock solution after 24and 48hrs that reach to

(3.33mm While redness area 15cm), (3.16mm While redness area 9.33mm) respectively, also there was slight significant increase in temperature. The results are in agreement with those obtained by (15,16) who used antigen of Entameba hisyolotytica to induce skin hypersensitivity reaction. The second dose after 14 days demonstrated significant increase in thickness of skin and highest mean was present in first group which injected by stock solution (3.18mm) after 24hrs. The same group revealed highest mean in redness (11.49mm) after 24hrs. The thickness of skin may be due to aggregation of T-cells and releasing of cytokines that attract cells & other inflammatory cells at site of reaction, while the redness explained by increase blood vessels permeability (17).

Our finding showed increase in liver weight of control & stock solution groups this finding is in agreement with that obtained by (18), this result may refer to effectiveness of 1st dilution to increase resistance against infection.

Our results showed that the subjected animals to doses of bacteria will stimulate immune system in response to largest dose of bacteria even at specific site of infection, this result is in agreement with (18,19) results who found that serum obtained from vaccinated rabbits was able to confer temporary protection to mice against challenge with homologus or hetrologous strain of Pseudomonas.

The immunological parameters showed siginificant increase in level of IgG. The result is in agreement with (21) and in agreement with (22) who reported significant increase of 24.6% of IgG antibodies against P.aeruginosa in patients with cystic fibrosis (CF) in human.

Our results show non significant increase in level of secretory IgA, however, Herbert et al (19) found increase level of IgG& IgA this may be down to Herbert study rely on secretion, while our study deal with serum immunoglobulins.

C3 has no significant increase this result is in agreement with those obtained by other investigators (23). *P.aeruginosa* evade human complement attack by binding the human plasma regulator factor H and factor H-related protein-1(FHR-) to its surface. Similarly factor H bound to intact P. Aeruginosa showed complement regulatory activity and mediated C3b degeradation.

The significant increase in the level of C4 recorded in this study was similar to those ontained by Shaker (14) who eported significant increase of C4 in case of immunization against Entamoeba histolotytica in New Zealand rabbits and this may be due to presence of antigen- antibody complex that lead to complement activation.

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