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Immunogenicity of slime layer of *Pseudomonas aeruginosa* in experimental models

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Abstract

Nasal swabs were taken from 136 calves with clinical signs of respiratory manifestation aged from 1-6 month and subjected for bacteriological study. *P. aeruginosa* was isolated with an incidence of 10.29%. The isolated strains tested for its ability for serum resistance, the results obtained revealed that all strains were able to survive and grow chicken and human serum with a percentage of 100%. Trials for preparation of vaccine were done from slime layer of *P. aeruginosa* and tested for its immunogenicity in mice by I/P injection of sera collected from immunized rabbits with different doses (10 mg, 100 mg and 1000 mg). The results revealed that the best protection 75% fate at 1/10 concentration. On the other hand, the same prepared antigen was tested by passive protection test through injection of serum of immunized rabbits with different dilution (1/10, 1/100, 1/1000) in mice. The results revealed that the best protection rate was 100% at 1/10 concentration. With regard to ELISA which was used to detect and measure the antibody titer of humeral response of immunized mice with prepared alcohol precipitated slime antigen of *P. aeruginosa.* The results showed increase in the antibody titer of immunized mice comparing to control non immunized mice, by I/P injection of mice with 2.5 x 10^8 (0.2 ml) of the viable *P. aeruginosa* strain. From 80-100 % of mice were died within 24-48 hours. Pathological picture of dead mice was carried out.

Introduction

In the recent year, there is a great interest directed to the role of opportunistic gram negative psychotropic bacteria in animals, particular attention has been directed to Pseudomonas infection. *Pseudomonas aeruginosa* is found a worldwide, it can be found on skin, mucous membrane and in external environmental condition surrounding the animals such as in external sewage, air, grasses, hay, faeces and beading materials **(14; 20; 22).**

It is necessary to secure urgent measures for restriction of spread of *Ps. aeruginosa*. The importance of *Ps. aeruginosa* is that it causes different diseases and capable of secreting many extracellular products such as lipopolysaccharides (LPS), fibrinolysin, haemolysin, exotoxin and enterotoxin. These products have a major role in the virulence of pathogenic

strains of *Ps. aeruginosa* (4; 6). Prolonged administration of broad spectrum antibiotics suppressing the normal flora, general immuno deficiency, immunosuppressive therapy and chronic diseases (**7**; **26**).

The slime layer is a glucoprotein. It has antiphagocytic, lethal and neutropenic activities in human and animals increase the risk of infection (**18**; **30**).

Recent researches have been focused on resistance to multiple classes of antibiotics, particularly resistance that arises following treatment with single antimicrobial agents. This may be due, in some circumstances, to synergy between enhanced production of beta lactames and diminished outer membrane permeability (**21**), for that much attention has been directed.

The objective of this work is planned for studying the immunogenicity of slime layer of *Pseudomonas aeruginosa* in experimental model by using different immunological assay.

Material and Methods

Sampling:

A total of 136 nasal swabs collected from diseased calves suffering from respiratory manifestation. All samples collected under complete aseptic conditions and transferred with minimum delay to the laboratory in ice box.

Isolation of Bacteria:

Swabs of nasal samples were inoculated into sterile nutrient broth and incubated at 37oC for 24 h. then, a loop full from each tubes was streaked into 5% blood agar, trypticase soya agar and XLD medium and incubated at 37°C for 24 hr., then examined for bacterial growth (**8**), biochemical testes for further identification (**13**) and detection of some virulence factors (**22**)

Detection of virulence factor:

Serum resistance:

This test was carried out as described by (**3**). Chicken blood was obtained from apparently healthy chicken and allowed to clot for 2 hours at room temperature, the serum was decanted polled and filtered through membrane filter 0.45 mm, neutrient broth culture of each *P. aeroginosa*,

was incubated at 37 C for 24 hours and centrifuged at 1500 xg for 15 min. The pellet was resuspended in an equal volume of phosphate buffered saline (PH 7.4) two tests were carried out. The first to detect the survival and the second to observe the growth of the organism in serum.

a- Survival of *P. aeroginosa* in serum:

By using a straight wire (10^4CFU /ml) the bacterial suspension was incubated in a well of haemagglutination tray containing 150 u of serum. The inoculated serum was incubated at 37° C and after 0,1,3,6, hours 20 ml was plated onto tryptose medium. After incubation the number of colonies was counted. Isolates of *P. aeroginosa* were classified as serum resistance when the number of colonies in the 1, 3, 6 hours was more than in zero samples (**3**).

b- Growth of *P.aeroginosa* in serum:

P. aeroginosa strains were tested for their ability to grow in serum for 18 hours by using this modified test (**19**).

For each tested *P.aeroginosa* strain, in a covered haemagglutination tray 30 ml of solution of 2.5 % glucose containing 0.25 % bromothymol blue were pipetted into two wells, then left to dry at 37°C over-night. To one of the well, 120 ml of the chicken serum were added and to the other well a similar volume of 1% peptone water (PH 7.4) were added. Both wells were inoculated using a straight wire with 10^4 CFU /ml with bacterial suspension, and then the plates incubated at 18 hours at 37° C. Bacterial growth indicated by the development of yellow color with bacterial pellet in the bottom of the well. No growth was indicated by blue color. The same procedure was conducted by using human serum.

Preparation of immunogen:

Alcohol precipitated fraction (A.P.F) antigen from slime layer of *P. aeruginosa* (**2**). Pure colonies of *P. aeruginosa* were grown on slants of brain heart infusion agar (BHI) over night at 37oC for 18 hours, the growing colonies were harvested from the slant and incubated into brain heart infusion broth after incubation at 37°C for 6 hours, 5 ml of this culture were transferred to brain heart infusion agar in Roux culture bottle and incubated overnight at 37°C, the bacteria were harvested by gentle agitation with 5 ml of (0.15 µ. Nacl solution per bottle), then centrifuged at 2700 g for 1 hours

to remove debris of cell, then solution of sodium acetate 10% w/v and glacial acetic acid (1% w / v) was added to this supernatant which contain the crude slime. The slime was precipitated by adding equal value of alcohol by drops and 4°C by centrifugation 21.000 gm for 1 hour, the slime precipitated by alcohol was collected. The precipitated slime was washed once with distilled water, then dissolved in 0.15 μ Nacl and left for 72 hr. at 4°C. The solution was afterward dialyzed for 48 hr. against distilled water lyophilized and stored at 4°C.

Experimental models:

A total of 60 Swiss albino mice with an average weight of 18-20 gm were used and kept under observation for three days in experiment to be sure that they are free from pathogens.

Protocol of immunization:

The protective capacity of the prepared antigen was tested in mice, by injection 0.1 of prepared slime antigen I.P to mice with different dilution (10 mg, 100 mg and 1000 mg), 8 mice for each dilution and the other two mice used as a control. 1 week after the immunized mice as well as the control one were challenged with 0.2 ml of the same isolated strains and the surviving mice was counted 24hr., after word.

Preparation of challenged dose:

Ps aeruginosa was grown on nutrient agar for 24 hr. at 37°C , then harvested from the plates, then resuspended in saline to reach a concentrate of 2x 10^9 organisms / ml from this suspension 0.2 ml injected into immunized one and un immunized mice (control).

Passive protection test:

A rabbit weight 2-3 kg was injected with an antigen of *Ps aeruginosa* in complete freun adjuvant for a serious of four intramuscular injections. Animals were bled, a week after the last inoculation, then the sera were separated and 0.2 ml of serum were injected intraproteneal IP 4 hours before the challenge in experimental animals with different dilution 1/10, 1/100; 1/1000.

Enzyme linked immuno sorbant assay (ELISA):

The adapted technique (27) was carried out to determine antibody titre present in serum of immunized and non immunized experimental model.

Results

Episodes of respiratory disordered in calves aged from 1-6 month reported in El-Giza and Kafr-El-.Sheik province. Bacteriological examination of nasal swabs revealed an isolation of *P. aeruginosa* with an incidence of 10-29%, all isolated purified strains tested for determination of serum resistance revealed that all strains were able to survive and grow in serum of chicken and human with a percentage of 100%

Protection of mice:-

The result of protection of mice against infection with *P.aeurignosa* after immunization with prepared alcohol precipitated slime layer antigen with different doses revealed that the best protection obtained when mouse immunized with 100 mg of prepared antigen as shown in table 1

Table (1): Immunization of mice with different doses of prepared antigen

Immunized dose/ml	Death / Total		Survival / Total	
	No.	%	No.	%
10 mg	6/8	75.0	2/8	25%
50 mg	5/8	62.5	3/8	37.5%
100 mg	2/8	25.0	6/8	75%
Control for each group	2/2	100	0	0

The unimmunized mice (control) died within 24h.

Passive mouse protection test-

The I/P injection of sera collected from immunized rabbits with different dilution (1/10, 1/100, 1/1000) revealed that the best protection obtained at 1/10 concentration. The survive mice counted illustrated in table (2).

Table (2): Immunization of mice with sera with different dilution

Sera/dilute.	Death/total		Survival/total	
	No	%	No	%
1/10	0/8	100	8/8	100
1/100	3/8	37.5	5/8	62.5
1/1000	6/8	75	2/8	25
Control /unimmunized.	2/2	100	0	0

ELISA:

As for implementation of the Eliza for detecting and measurement the antibody titer of humeral response of immunized mice with prepared alcohol precipitated slime layer antigen of *P. aeruginosa* revealed an increase in the antibody titer of immunized mice comparing to control unimmunized mice.

Mortality test:

The I/P injection of mice by 2.5 x 10^8 (0.2 ml) of the viable *P. aeruginosa* strain revealed 80-100 % of mice within 24-48 hours, were died, the pathological picture of dead mice revealed the production of hemorrhagic lesion on the lung, intestine with necrosis in the liver.

Discussion

Respiratory diseases are the most frequently causes of morbidity and mortality in calves (**12**) it is a complex syndrome involving environmental, bacterial, fungal and viral infections. They generally developed when the immune system is comprised (**Roy, 1990**). *P.aeruginosa* considers to be one of the most pathogenic microorganism present in young calves aged from 1-6 month (**11; 28**).

The bacteriological examination of nasal samples collected from diseased calves' revealed isolation of *P. aeruginosa* with an incidence of 10.29% in young calves aged from (1-6 month). These results appeared higher than 9.75% (**1**) and 5.8% (**10**) at young aged calves. This may be attributed to maternal antibody level which is likely to have considerable waned (**5**). It is clear that P. *aeruginosa* was isolated as a sole causative agent only not mixed with other microorganisms attributed to the production of pyocyanin pigment that exhibits inhibitory bacteria due to its antibacterial action (7)

The expression of the virulence factors of *P.aeruginosa* is highly regulated often by environmental and host conditions this suggestion was supported by a previous study (**29**). *P.aeruginosa* ubiquitous environmental bacteria capable of producing several virulence factors among these factors serum resistance, the isolated strains were positive for serum resistant and this agreed with others (**25**). More virulent strains were resistant of

bactericidal effect of both human and chicken serum (**16**). The antigen prepared appear to be important in conferring the property of microbial resistance to phagocytes, the antibodies to these surface antigen may act on concert to protect against initiation and spread of organism through the blood stream (**17**). In recent years more attention was given to finding means for enhancing local and systemic host defense against virulence factors of gram negative organisms, *P.aeruginosa* consider to be one of these microorganisms which resist most of the antibiotic used. This study showed that protection against *P.aeruginosa* induced by using alcohol precipitated fraction of slime layer antigen in which the antibody to slime layer were probably the most important fraction in immunity to *P.aeruginosa* infection in mice, this agreed with that recorded previously (**2**).

Concerning to passive mice protection test which carried out to estimate the protective capacity of alcohol precipitated fraction of slime layer antibody in challenge with *P.aeruginosa* isolate the result revealed 100% protection with dilution of 1/10 and these results agreed to certain extent with that reported by others (**24**).

This attributed to that slime is a glycolipoprotein that has antiphagocytic lethal neuropenic activity in mice equally important to the antiphogocytic activity of slime polysaccharide in its ability to induce neuropenic .it is clear that preexistence of neuropenic in human and animal increase the risk of infection with *P.aeruginosa*, then if slime glucolipoprotein has the ability to prolonged an aggravate a neuropenic episode it has an important role in pathogenesis of disease due to pathogenic *P.aeruginosa* .thus passive protection with potent antibody preparation may be a more realistic form of prophylaxis in view of most problem of an impaired antibody response after active immunization. Rabbits immunized with prepared antigen showed titre ranged from 1/640.1/2500. These results were similar to those reported before (**15**)

The prepared antigen from slime layer of *P.aeruginosa* appears to be important in conferring the property of microbial resistance to the phagocytosis. Antibodies to this antigen may act as in concert to protect against initiation of infection and to limit the proliferation and spread of organism through the blood stream (**31**).

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الحماية المناعية للغلاف اللزج لميكروب السودوموناس اريجينوزا في النماذج التجريبية

عزة فراج
قسم البكتيريولوجي – معهد صحة الحيوان – الدقي

تم تجميع عدد ١٣٦ مسحة انفية من عجول مصابة باعراض تنفسية يبلغ متوسط اعمارهم تتراوح بين ١-٦ أشهر وتم عزل ميكروب السودوموناس الاخضر من مسحات الأنف التي جمعت بنسبة مئوية ١٠-٢٩ ٪ ، تم اختبار قدرة السلالات المعزوله لالمصل المقاومة وكشفت النتائج عن قدرة المعزولات على البقاء والنمو في المصل من الدجاج والبشريه مع نسبة مئويه ١٠٠ ٪. تم اعداد لقاح من طبقة الغلاف اللزج واختبار لقدرتها لحمايه الفئران حيث تم حقن الامصال التي جمعت من مختلف تحصين الفئران بجرعات مختلفة (١٠ سم ، ١٠٠ سم ، ١٠٠٠٠ سم) وكشفت النتائج ان افضل وسيلة للحمايه التي حصل عليها مع التركيز ١٠ مجم وحمايه ٧٥ ٪. ومن ناحية اخرى ، تم اعدادا لقاح تم اختبارها لحمايه سلبية الاختبار عن طريق الحقن من المصل للتطعيم الارانب مع مختلف تخفيف (١ / ١٠ ، ١ / ١٠٠ ، ١ / ١٠٠٠) في الفئران وكشفت النتائج أن أفضل وسيلة للحمايه التي حصل عليها في ١ / ١٠ تركيز مع حمايه ١٠٠ ٪.

ايضا تم استخدام اختبار الاليزا للكشف عن الاجسام المضاده والقياس العياري للاستجابة للتحصين وكشفت النتائج عن زيادة في الاجسام المضادة العيار الحجمي للفئران المحصنة مقارنة للسيطره على الفئران غير المطعمين. تم دراسة تاثير الميكروب المحقونة على نسبة الوفيات غى الحيوانات التى لم تحصن باستخدام الفاكسين المحضر فقد تبين ان الحيوامات المحقونة فقد توفيت فى فترة تتراوح من يوم الى يومين بنسبة ٨٠-١٠٠ ٪ من الفئران في غضون ٢٤-٤٨ ساعة ، الصورة المرضيه للفئران تم تسجيلها.