16 *The 7th Inter. Conf."Plant & Microbial Biotech. & their Role in the Development of the Society"* pp.197 - 204 (2017)

The Immunoprotective Efficacy of Exopolysaccharides Produced from Different Strains of *Pseudomonas syringae* against Human Pathogenic *Pseudomonas aeruginosa*

Wagih El-Shouny#, Mohammed El-Anwar Osman, Ragdah Talat* and Heba El-Zahaby

*Botany Department, Faculty of Science and *Microbiology Department, Faculty of Medicine, Tanta University, Tanta, 31527, Egypt.*

EXOPOLYSACCHARIDES (EPS) produced from *Pseudomonas syringae* pv. *tomato*, *P. syringae* davson 973 and *P. syringae* pv. *coriandricola* 908 were studied**.** Mice immunized (i.p.) with bacterial EPS preparations from three strains (divided into 3 days) and in the fifth day, challenged (s.c.) with 1 ml of human pathogen *P. aeruginosa* 3 to each mouse, all groups of mice showed an increase in total leukocyte counts as well as neutrophil and monocyte numbers if compared with saline control group. Studing the active immunization by purified bacterial EPS on the counts of *P. aeruginosa* in lungs, spleens and livers of challenged mice revealed the protection of organs against bacteremia. Thus, the immunoprotective characters of the three purified EPSs could be concluded and recommended for prophylactic applications.

Keywords: Exopolysaccharides, *P. aeruginosa,* Mice, Immunization, Bacteremia.

Introduction

Humans are frequently infected by opportunistic pathogens that take advantage of their compromised immunological status to cause persistent and chronic infections. The Gram-negative bacterium *Pseudomonas aeruginosa* is one of those recurrent human pathogens. *P. aeruginosa* remains one of the most important pathogens in nosocomial infections, and it is often associated with skin, urinary tract and respiratory tract infections (El-Shouny, 2000 and Lavoie et al., 2011). Respiratory tract infections are of major relevance in cystic fibrosis patients given that *P. aeruginosa* deeply affects their pulmonary function, causing life-threatening infections (Hauser et al., 2011)**.** One of the better-known adaptive resistance mechanisms of *P. aeruginosa* to evade either the host immune response and drug therapy is its ability to form biofilms. The *Pseudomonas aeruginosa* biofilm is an extremely stable capsule-like structure constituted primarily of polysaccharides, proteins, and DNA, in which PsI exopolysaccharide seems to be a key player for biofilm matrix stability (Ma et al., 2009). Quorum sensing signals promote the formation of *P. aeruginosa* biofilms, which minimizes the entry of antimicrobial compounds inside bacterial cells and hinders the recognition of pathogen-associated molecular patterns (PAMPs) by the host immune system (Alhede et al., 2014). .Consequently, current treatments against *P. aeruginosa* fail to resolve infections before tissue deterioration occurs. To address this concern, more efficient alternatives to abolish *P. aeruginosa* infections have produced promising but not definitive results. Accordingly, several candidate *P. aeruginosa* vaccines have been developed by targeting outer membrane proteins (Opr), lipopolysaccharides (LPS), polysaccharides (PS) as reported by Horn et al. (2010), Kamei et al. (2011), Campodonico et al. (2011) and Hilker et al. (2015). *Pseudomonas aeruginosa* exopolysaccharide (Psl) is a serotype-independent, antibody-accessible antigen that is prevalent among both nonmucoid and mucoid clinical isolates. Interestingly, monoclonal antibody (mAbs) that bound to one particular Psl epitope mediated potent opsonophagocytic killing of *P. aeruginosa in vitro*, inhibited bacterial attachment to cultured lung epithelial cells and provided potent prophylactic protection

#Corresponding author emails: elshounyw@yahoo.com,wageh.elshoni@science.tanta.edu.eg.
DOI: 10.21608/ejbo.2017.971.1082

in multiple animal models of *P. aeruginosa* infection. So that, Psl is a novel protective antigen and that antibodies targeting Psl might be an effective measure for prophylaxis against *P. aeruginosa* infections in high-risk patients (Hraiech et al., 2015; Emmanuelle et al., 2017 and Alessio et al., 2017). This paper concerns with the enhancement of the immune response in mice induced by exopolysaccharides produced by different strains of *Pseudomonas syringae* against human pathogenic *Pseudomonas aeruginosa*.

Materials and Methods

Three strains of Gram-ve bacteria of *Pseudomonas syringae* (*P. syringae* pv. *tomato*, *P. syringae* pv. *coriandericola* 908, *P. syringae* davson 973 were studied for their alginate (EPS) protective efficacy against human pathogenic *Pseudomonas aeruginosa*.

Isolation of exopolysaccharides

Preparation of inoculum

For inoculum preparation, two successive pre-cultures were grown. The growth was measured turbidemetrically at 660 nm in order to standardize the quantity of the bacterial cells used for inoculation throughout the experiments. The first pre-culture was prepared by suspending aloop of bacteria in about 30 ml of KW- medium (Keen & Williams, 1971).

Production of exopolysaccharides

250 ml Eelenmeyer flasks were filled with 100ml of autoclaved medium and inoculated by 2% portion of the pre-culture of the experimental bacterium. The flasks were incubated at 37°C for 1 day in rotary shaker (120 rpm). At the end of the incubation period, the bacteria were pelleted by centrifugation and re-centrifugation at 6000 rpm. The clear supernatant was used for further investigations since it contained the crude Bacterial EPS**.**

Partial purification of exopolysaccharides

The culture supernatant was treated by the addition of trichloroacetic acid (TCA) 5% for removing protein according to Khalil (2002) and stored at freezer overnight. The supernatant was mixed with three volumes of 95% of ethanol, stirred vigorously overnight at 4°C. The resultant precipitate was recovered by centrifugation at 3000 rpm for 20 min (Wu et al., 2008). Exopolysaccharides were estimated by phenol sulphuric assay (Dubois et al., 1956).

Blood counts

Total white blood cell counts

It was done according to Kruse et al. (1973) Firstly, red blood cells were removed by lyses in diluted acid to prevent the erroneous counting of red blood cells as small lymphocytes. Total white blood cell counts were determined by addition of 0.01 % gentian violet in 3 % acetic acid (Turk`s solution) to facilitates counting since it stain the white blood cells.

Procedure

The appropriate dilutions of cell suspension prior to counting were made, the optimal concentration of cells was (50—100 cells per large square) after dilution in the counting solution, Pasteur pipette with finger control were used to lead the cell suspension flow under the cover slip until the grid area is just full, then all of the cells contained in each of the four large squares were counted, finally, the average number of the cells per large square must be determined by the following equation :

$$\text{Cells / ml} = \frac{\text{N X 4000 X 10}}{\text{16 X 16}}$$

These were the number of cells per 4000 ml

N :- Total number of leucocytes counted in the 16 large square

The leucocytes count in all large squares = 16

Number of dilution = 10

Differential white blood cell counts

A blood film was treated by using Leishman stain, and the number of each type of leukocytes per field was recorded.

Mice immunization

Adult male mice, 6 to 8 weeks of age (weigh, 15 to 25 g) were housed on the basis of study requirements. Mice were divided into 12 groups (6 animals per cage), out of them, three groups were taken as control groups.Three of the EPS obtained from the three *P.syringae* pathovars were subjected to test on mice.

Mice lethal challenge

The bacterial challenge dose of *Pseudomonas aeurginosa* which caused > 90% mortality within 2 days was determined to be the lethal dose. The bacterial challenge for the subsequent experiments was prepared as follows; *P. aeurginosa* was grown overnight (18 -24 h) on nutrient agar, the bacterial cells were collected and suspended in sterile

phosphate- buffered solution (PBS). Bacterial cells were suspended to an optical density of 0.3 at 660 nm., corresponding to 10^8CFU/ml as lethal dose, immediately before injection. Mice were challenged by administering intraperitoneal (i.p.) one ml of bacterial suspension. Non-immunized control mice were challenged with one ml of PBS (Fattom et al., 1996).

Active immunization

Mice were immunized subcutaneouly (s.c) in first, second and third days with 1 ml of *P. syringae* EPS (divide into 3 doses). Animals were challenged intraperitoneal (i.p.) in fifth day with doses of *P. aeurginosa* $10^7$and 10^8CFU/ml). A serum sample was obtained from each mouse after 5 days of the last immunization and the mice of control group injected s.c with PBS were also bled concurrently and challenged (Fattom et al., 1996), blood samples were also collected from mice after bleeding for blood count (total and differential blood counts).

Mice were randomly selected and removed from cages at designated time points after challenge, selected mice were weighted, bled and evaluated for livers, spleens and lunges. Organs were excited, weighted, washed, with 70 % ethyl alcohol to eliminate surface-attached organisms, then washed with sterile PBS, and homogenized in 1 ml PBS. 0.1ml of this suspensions were cultured on King`B agar plates and bacteria were counted after overnight incubation and expressed as CFU per organ (Fattom et al., 1996).

Results

Mice lethal challenge

In Table 1, groups of non-immunized mice were injected intraperitoneally (i.p.) with different doses of virulent human pathogen *P. aeruginosa* ranged from 10^5 to 10^{10} CFU/ mouse. It recorded that the bacterial challenge caused more than 75% mice mortality within two days of injection was 10^8 CFU/ mouse and sub-lethal dose was 10^7CFU/ mouse. These doses were used for challenge in subsequent experiments.

TABLE 1. Effect of challenge doses of *P. aeruginosa* on mortality of non-immunized mice.

Bacterial challenge i.p. CFU/mouse	Mortality of mice in (days)						
	1	2	3	4	5	6	7
10^5	0/4	0/4	0/4	0/4	0/4	0/4	0/4
10^6	2/4	3/4	4/4	4/4	4/4	4/4	4/4
10^7	3/4	3/4	2/4	2/4	4/4	4/4	4/4
10^8	4/4	4/4	4/4	4/4	4/4	4/4	4/4
10^9	4/4	4/4	4/4	4/4	4/4	4/4	4/4
10^{10}	4/4	4/4	4/4	4/4	4/4	4/4	4/4

-Numerator indicates the cumulative number of mice that dead and denominator indicates the number of mice used.

Effect of bacterial challenge on leukocyte count

Leukocyte counts of mice blood which infected only with *P. aeruginosa*, mice which immunized by bacterial EPS preparations and mice which immunized with bacterial EPS preparations and infected with *P. aeruginosa* 3 were presented in Table 2. The results revealed that all groups of mice recorded an increase in total leukocyte counts as well as neutrophil and monocyte numbers if compared with saline control group.

Bacteremia

Data present in Table 3 show the effect of (ip) active immunization by purified bacterial EPS on the counts of *P. aeruginosa* in lungs, spleens and livers of challenged mice. The data revealed the protection of organs against bacteremia except group 7 and group 8 which recorded bacterial cells in their lungs, spleen and liver. Group 7 immunized with 1ml *P. syringae* pv. *tomato* EPS and challenged with 1ml of lethal dose (10^8 CFU/ mouse) recorded in lung 6 x 10^3 CFU, in spleen 11 x 10^2 CFU and in liver (two lobes) was 34 x10^2 CFU. Group 8 immunized also with 1ml *P. syringae* pv. *tomato* EPS and challenged with 1ml of sub-lethal dose (10^7 CFU/ mouse) recorded in lung 5 x 10^3 CFU, in spleen was 9 x 10^2 CFU and in liver (two lobes) was 32 x 10^2 CFU. If compared with control groups group 2 and group 3 which recorded bacterial counts more than group 7 and group 8.

TABLE 2. Leukocytic count of mice blood that infected with *P. aeruginosa* after immunization by bacterial EPS preparation.

Group No.	Immunizing EPS	Differential leukocyte count %					AS
		Lymphocyte	Monocyte	Eosinophil	Neutrophil	Basophil	
1	Control (saline)	37	5	1	50	1	4175
2	Control with L.D	14	18	2	65	1	5000
3	Control with S.L.D	16	19	3	61	1	5100
4	EPS from *P. syringae* pv. *tomato*	14	17	2	66	1	5254
5	EPS from *P. syringae* davson 973	18	22	2	57	1	5451
6	EPS from *P. syringae* pv.*coriandericola* 908	20	18	1	60	1	5336
7	EPS from *P. syringae* pv. *tomato* with L.D	12	21	1	65	1	5210
8	EPS from *P. syringae* pv. *tomato* with S.L.D	13	20	2	65	1	5560
9	EPS from *P. syringae* davson 973 with L.D	19	20	2	58	1	6900
10	EPS from *P. syringae* davson 973 with S.L.D	16	16	2	65	1	7200
11	EPS from *P. syringae* pv.*coriandericola* 908 with L.D	20	18	1	60	1	5750
12	EPS from *P. syringae* pv.*coriandericola* 908 with S.L.D	20	20	4	55	1	5960

TABLE 3. Effect of active immunization (ip) by purified bacterial EPS on the counts of *P. aeruginosa* in lungs, spleens and livers of challenged mice.

Group No.	Immunizing EPS	CFU /ml 1ml of challenge bacteria	CFU / organ		
			Lung	Spleen	Liver
1	Control with saline	Saline	0	0	0
2	Control with L.D	10^8	12×10^3	19×10^2	52×10^2
3	Control with S.L.D	10^7	9×10^3	16×10^2	39×10^2
4	EPS from *P. syringae* pv. *tomato*	----	0	0	0
5	EPS from *P. syringae* davson 973	----	0	0	0
6	EPS from *P. syringae* pv. *coriandericola* 908	----	0	0	0
7	EPS from *P. syringae* pv. *tomato*	10^8	6×10^3	11×10^2	34×10^2
8	EPS from *P. syringae* pv. *tomato*	10^7	5×10^3	9×10^2	32×10^2
9	EPS from *P. syringae* davson 973	10^8	0	0	0
10	EPS from *P. syringae* davson 973	10^7	0	0	0
11	EPS from *P. syringae* pv. *coriandericola* 908	10^8	0	0	0
12	EPS from *P. syringae* pv. *coriandericola* 908	10^7	0	0	0

Discussion

In Table 1, mice infection was accomplished by intraperitonial injection of the determined lethal dose of *P. aeurginosa* 10^8 CFU/ mouse). At these dose more than 85% of non-immunized mice were died within two days of injection and also recorded decreasing of mice weight. These results indicated the presence of bacterial infection which causes death. Similar results were obtained by Fattom et al. (1996) found that a dose of 2 x 10^5 CFU of *S. aureus* type 5 CP per mouse administrated intraperitoneally (i.p.) with 5% hog mucin was found to cause 80 to 100% mortality in BALB/c mice within 2-5 days. While mice actively immunized with the monovalent type 5 CP-*P. aeruginosa* exoprotein. showed a survival rate of 73% compared with 13% in phosphate-buffered saline-immunized animals. Similar results were found by El-Shouny (2000) who reported that the mortality rate reached >75 % within 2 days in mice after (i.p.) injection with 10^9 CFU/mouse with *P. aeruginosa*. Also**,** Allam & Elslik (2011) found that the use of lipopolysaccharide (LPS), exopolysaccharide (EPS) and mutant strain as subunit vaccines against intraperitoneal (I/P), challenge of *Serratia marcescens* W225 in mouse model referring to *E. coli* as standard, that elicit the secretions of IgM, which have protection role against lethal doses challenge, or their toxicity in mice through the follow up of liver enzymes, urea and weight. The results indicated that capsular or EPS and mutant strain (*S. marcescens* W1765) antigens (vaccines) confer the highest protective immunization.

As shown in Table 2, the total white blood cells and differential count included neutrophils and monocytes were increased due to the bacterial challenge and lymphocytes were decreased due to the bacterial infection. In accordance with their results, Santos et al. (2008) reported that the decrease in lymphocyte counts was observed in parasitized animals with Gram-negative bacteria: *Citrobacter divergens, Burkholderia cepacia, Stenotrophomonas maltophilia*, *Proteus vulgaris, Enterobacter sakazakii*, *Enterobacter ammnigenus*, *Pseudomonas aeruginosa, Pantoea* sp. and *Providencia rettgeri*.

The obtained results from Table 3 revealed reduction of bacteremia as response of active immunization with 1 ml of intraperitoneal (i.p) injection of purified EPS on three successive doses before bacterial challenge with 1ml *P. aeruginosa* (s.c). Group of mice immunized with EPS from *P. syringae* pv. *tomato* was dead when challenged with lethal dose (10^8 CFU/ml) but when challenged with sub-lethal dose (10^7 CFU/ ml), the bacterial cells recorded in their lungs, spleens and livers were $5x10^3$, 9 $x10^2$ and $32x10^2$ CFU/organ, respectively, when compared with control groups which recorded higher bacterial counts. However, other groups of mice were immunized with EPS from *P. syringae* davson 973 and *P. syringae* pv. *coriandricola* 908 had no bacterial cells recorded in their lungs, spleens and livers, after bacterial challenge with lethal dose and sub-lethal dose, respectively, These data suggested that the dose of *Pseudomonas syringae* pv. *tomato* EPS did not exhibit immunization but the doses" of *P. syringae* davson 973 and *P. syringae* pv. *coriandricola* 908 "EPS in successive doses enhanced the immune response to overcome the bacterial challenge and preventing seeding of organs with bacteria. This was in agreement with Hatcher et al. (2015) who found that the immunogenicity of the polyvalent O-polysaccharide-toxin A conjugate vaccine reflected lower frequency of *P. aeruginosa* in sputum/throat cultures and preservation of lung function. Also, Gamboa et al. (2009) found that when animals (red deer) received vaccine (alginate composite microspheres containing a non-immunogenic, eggshell-precursor protein of the parasite *Fasciola hepatica*) exhibited significant reduction in bacterial counts in their spleens. El-Shouny (2000) proved the immunoprotective efficacy of *Pseudomonas syringae* pv. *coriandricola* exopolysac chride against *Pseudomonas aeurginosa* infection in mice. Finally, Laroussarie et al. (2015) suggested that *Pseudomonas aeruginosa* exopolysaccharide (Ps l) is a serotype-independent, antibody-accessible antigen that is prevalent among both nonmucoid and mucoid clinical isolates. Interestingly, monoclonal antibody (mAbs) that bound to one particular Ps l epitope mediated potent opsonophagocytic killing of *P. aeruginosa in vitro*, inhibited bacterial attachment to cultured lung epithelial cells and provided potent prophylactic protection in multiple animal models of *P. aeruginosa* infection. So that, Ps l is a novel protective antigen and that antibodies targeting Ps l might be an effective measure for prophylaxis against *P. aeruginosa* infections in high-risk patients (Laroussarie et al. (2015); Lei et al. (2016) and Van et al. (2017).

It could be concluded that the three tested EPSs were proven to be immunoprotective and prophylactic agents against *P. aeruginosa* infection in mice.

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*(Received 1*9/4/2017*;*
accepted 8/10/2017 *)*

كفاءه الحمايه المناعيه لعديدات التسكر الخارجيه المنتجه من سلالات مختلفه من سيدومونس سرنجي ضد بكتريه سيدومونس اريجنوزا التي تصيب الانسان

وجيه عبد الفتاح الشوني، محمد انور عثمان، رغده طلعت* و هبه الذهبي
قسم النبات - كليه العلوم و*قسم الميكروبيولوجي – كليه الطب – جامعه طنطا – طنطا - مصر.

تم دراسه السكريات عديده التسكر الخارجيه المنتجه من بكتريا السيدومونس سرنحي – طوماطو سيدوموناس سرنجي دافزون 973 و سيدوموناس سرنجي كورانيدروكولا 908 .

تم تحصين الفئران بتجضيرات السكريات عديده التسكر من الثلاث سلالات البكتريه عن طريق الحقن البريتوني (على مدي ثلاث ايام) ، وفي اليوم الخامس تمت العدوي ببكتيره سيدومونس اريجنوزا 3بحقن 1ملي لكل فأر. وقد اظهرت كل مجموعات الفئران زياده في العد الكلي لخلايا الدم البيضاء والنيتروفيل والمونوسيت مقارنه بالمجموعه الظابطه. وقد اوضح التحصين النشط بالسكريات العديده المنقاه حمايه للرئتين والطحال والكبد في الفئران المصابه ببكتريه سيدوموناس اريجنوزا وعلى ذلك فقد تم اثبات الصفات المتعلقه بالحمايه المناعيه للثلاث انواع من السكريات العديده المنقاه , ويمكن التوصيه باستحدامتها في التطبيقات الوقائيه.