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The protective effect of flagellin A and B as candidate vaccine against Pseudomonas aeruginosa respiratory infections

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***Abstract*---**Background: Multi drug resistance (MDR) *P. aeruginosa* consider the main cause of morbidity in hospitalize patients suffering from chronic airway infections such as chronic pulmonary disease, pneumonia and cystic fibrosis (CF). Also, the rapidly development of antibiotic resistance to the last generations of a broad spectrum antibiotic were detected for many *P. aeruginosa* isolates. Accordingly the vaccines have the potential to prevent and treatment such infections. Aim: The present study aimed to investigate the active immunization using flagellin to provide the protection against MDR *P. aeruginosa* clinical isolates in the fatal respiratory acute infection in animal model. Methods: Flagellin a and flagellin b purified partially from local isolates of *P. aeruginosa* were used for animal immunization. After that the animals were immunized intranasally after sedative the animal by applying 10 µl of flagellin a (4.8 µg), or flagellin b (4.8 µg*l*) on each nostril for 100 mg weight of rat at weekly intervals. After week from the 6th dose, the animal were exposed to $2*10^7$ CFU of *P. aeruginosa* direct into each nostril (intranasally). Then the blood samples were collected from all animals by heart puncture under anesthesia to detect the Th17 cells using a specific markers by a flow cytometry technique. Results: The flagellin b show a good effect in the elevation of total Th17 (p value 0.008) and Th17 subset from Th cells (p value 0.003) when compare the immunized groups before and after infection, while show a decrease of these cells in the flagellin a-immunized group and non-immunized group. Conclusion: We can conclude from the present study that flagellin b considered a good candidate vaccine to eradicate MDR *P. aeruginosa* respiratory infection

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due its improvement of the immune responses against this bacterium via enhance the cellular immune response especially Th17 cells.

Keywords---*Pseudomonas aeruginosa*, Flagellin, Th17, vaccine, CD161.

Introduction

Pseudomonas and other critical group in MDR bacteria such as (*Acinetobacter* and some enterobacteriaceae) is consider a threat in hospital infection (nosocomial infection) and among the immunocompromised patients as those have a blood catheter. They can developed into severe septicemia or pneumonia leading to death ("Who Publishes List of Bacteria for which New Antibiotics are Urgently Needed," 2017).

P. aeruginosa is consider one of the main opportunistic pathogen that cause infections in immunocompromised patients as those with malignant diseases or infected with HIV (2). Also increase the morbidity after infection by this bacteria in those suffer from chronic airway infection as chronic obstructive pulmonary disease (COPD), bronchiectasis or ventilator associated pneumonia (VAP) (3,4).

From the major threat of *P. aeruginosa* is its ability to adapt and acquire resistance to antibiotics in addition to its intrinsic mechanisms of resistance such as reduction of the inner and outer membrane permeability, Beta-lactamases Class C and Modifications in the membrane proteins (5).

Many studies show that the increase of multidrug resistance (MDR) *P. aeruginosa* infection especially among hospitalize patients (6,7) . Also the rapidly development of antibiotic resistance to the last generations of a broad spectrum antibiotic were detected for many *P. aeruginosa* isolates (8).

World Health Organization (WHO) include *P. aeruginosa* strain that resist carbapenems that consider one of "critical" group of pathogens that needed a new antibiotics become urgently (9,10). Also a recent study performed in Iraq showed that 12.4% of isolates are resistant to Imipenem (11)

P. aeruginosa may develop several strategies to circumvent the host's immune response equipped with several virulence factors and antibiotic resistance machinery, which play important roles in the bacterium pathogenesis and drug resistance (12,13).

P. aeruginosa possesses several virulence factors one of them is the flagella that composed from polymerization of small subunit called flagellin, its major structural protein, and attached to a transmembrane motor complex. Flagellin is classified into two distinct serotypes a and b and it consider a good pathogen-associated molecular pattern (14).

Materials and Methods

The aim of this study was to investigate the active immunization using flagellin (as a candidate vaccine) to provide the protection against MDR *P. aeruginosa* clinical isolates in the respiratory acute infection in animal model. Flagellin a and b were isolated and purified form *P. aeruginosa* local clinical isolates (15).
The protocol of study was reviewed and approved by a local committee of ethics at faculty of Science/ University of Kufa, Iraq.

Laboratory Animals:

In this study, six groups of albino female adult rats were conducted at the animal house in faculty of Science / University of Kufa / Al-Najaf province, Iraq; each group contained five animals that were distributed as follows:

1st group (A1): it includes treated animals with flagellin a only.
2nd group (A2): it includes treated animals with flagellin a and then infected with MDR *P. aeruginosa*.
3rd group (B1): it includes treated animals with flagellin b only.
4th group (B2): it includes treated animals with flagellin b and then infected with MDR *P. aeruginosa* .
5th group (C1): it includes non-treated animals that infected with MDR *P. aeruginosa* .
6th group (C2): it includes normal animals without immunization or infection.

Animals Immunization:

The animals were immunized (intranasally) after using sedative I.P. by mixture of Ketamine (40 mg for each Kg) and Diazepam and Diazepam (5 mg for each Kg) (16) and then immunized by placing applying 10 μl of flagellin a (4.8 μg /10 μl), or flagellin b (4.8 μg) on each nostril for 100mg weight of rat at weekly intervals. After 42 days from the beginning of infection the rats were exposed to $2*10^7$ CFU of *P. aeruginosa* strains directly into each nostril. The blood were taken after 24 h of infections for immunological studies using flow cytometry techniques.

Markers used in Flow-Cytometry techniques:

The flow cytometry was performed to count the immunological cells differentially by using anti-rat CD161 antibody (BioLegend Company) as a markers specific for Th17 (17) using 3 leaser 8 color FACSCanto™ II flow cytometer device. The procedure of flow cytometry was performed according to company instruction.

Results

Analysis The percentage of Th17 Lymphocytes:

Recently show that a many cells subset from T lymphocytes that produce IL-17 cytokine that play many role in the regulation of immune response through the promoting of epithelial cells in the lung to produce antimicrobial peptide or by mediating as a pro-inflammatory cytokine that recruit the phagocytic cells (neutrophil and microphage) to the lung to enhance its bacterial phagocytosis and clearance. (18–20).

In the peripheral blood, the CD4+ cells that secret IL-17 that have CD161 marker on its surface that consider a hallmark to Th17 cells. These cells secrete two type of IL-17 (IL-17 A and F) and consider a novel subtype from the t helper cells(21). Also there is other cells that secret IL-17 and have CD161 on its surface but it subtype from CD8+ T cells(22). So that use CD161 marker as indicator for Th17 In this study, the result's graphics of flow cytometry are clear in (Figures 1 and 2).

Analysis The percentage of total Th17 (CD3+CD161+) Lymphocytes:

The investigation of CD3+CD161+ cells comprise the total Th17 rather it subtype from T helper or cytotoxic T cells. When compare among study groups, noted that a significant increase of these cells in the immunized groups with flagellin b when compare before (group B1) and after infection (group B2), the p-value is (0.008). While there is no any significant difference between the immunized groups with flagellin a before (group A1) and after infection (group A2), the P value is (0.1). Also when compare between the non-immunized groups C1 (normal animals) and C2 (non-immunized + infected group) there is no any significant difference between them (p-value 0.21) as shown in (Table 1).

On the other hand, when compare among infected groups was showed that a significant difference between the flagellin b-immunized group (B2) when compare with the non-immunized group (C2) or with the flagellin a-immunized groups (A2), the p-value is 0.001 and 0.003 respectively. While there is no any significant difference between the group immunized by flagellin a (A2) and the non-immunized group (C2), the p-value is (0.79) as shown in (Table 1).
These results indicate that the flagellin b was elevate the Th-17 that important in the eradication of *P. aeruginosa* infection more than flagellin a.

Analysis of total Th17 subset of Th (CD3+CD4+CD161+) Lymphocytes:

The analysis of Th17 subset from T helper cells was achieved by the investigation of cells that carry CD3+CD4+CD161+ markers on its surface.
After the comparison among the study groups, the results showed that the significant elevation of CD3+CD4+CD161+ cells in the flagellin b-immunized group (B2) after infection when compare with the group before infection (B1) the P-value is (0.003). Also when compare among groups after infection were showed that the flagellin b-immunized group (B2) is highly significant elevate these cells comparing with the non-immunized group (C2) or with the flagellin a-immunized group (A2), the P-value is <0.001 and 0.001 respectively, as shown in (Table 2).
These results are support with the above results of total Th17 that the flagellin b consider a good immunogen rather than flagellin a that enhance the elevation of Th17 especially that the subset from T helper cells.

Analysis of total Th17 subset of CTL (CD3+ CD4-CD161+) Lymphocytes:

To complete the investigation of Th17, the Th17 subset from CTL was calculated by subtract CD3+CD4+CD161+ cells from the CD3+CD4+CD161+ cells.
When compare among the immunized groups before and after infection showed that there is no any significant differences between flagellin b-immunized groups

(B1 and B2) or between flagellin a-immunized groups (A1 and A2), the p value 0.174 and 0.376 respectively. Also when compare between the non-immunized groups before infection (C1) and after infection (C2) there is no significant difference between them (p value 0.447) as shown in (3).
Also when compare among immunized and non-immunized groups before or after infection there is no any significant difference among them that clear in (Table 3).

Discussion

Th17 cells play a critical role in the specific and non-specific immune response against bacteria (23–26). The indirect role of Th17 cells to protect against infection is the recruitment of phagocytic cells to the site of infection (27). These cells were shown to increase in 8 hour post-acute respiratory infection with *P. aeruginosa* and elevate the production of IL-23 production from these cells (28). This cytokine (IL-23) promotes memory CD4+ cells differentiation to Th17 cells. Also the neutrophil is consider the major cells in the lung in *P. aeruginosa* infection(18,29). So the protection of Th17 against this bacteria is achieved by neutrophil recruitment to the lung (30).
As clear from the above the results of current research in thee analysis of total Th17, Th17 subset from T helper and Th17 subset from CTL that flagellin b is a good in activation of Th17 in the immunized groups rather than flagellin a especially the total Th17 cells subset from Th cells rather than Th subset from CTL that compatible with the results of (18,29,30)
Th17 cells especially those subset from Th cells rather than those subset from CTL is necessary in the eradication of *P. aeruginosa* infection in the lungs, it was achieved by rapid requirement of neutrophil in the lung.

Conclusion

Flagellin b is a good candidate vaccine to eradicate MDR *P. aeruginosa* respiratory infection rather than flagellin a because it was show a significant increase of total Th17 and Th17 subset from Th cells after *P. aeruginosa* respiratory infection in the immunized groups.

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Interest Conflicts
None interest conflicts.

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Table (1): The mean and mean difference of CD3+CD161+ lymphocytes (total Th17) among the experimental groups

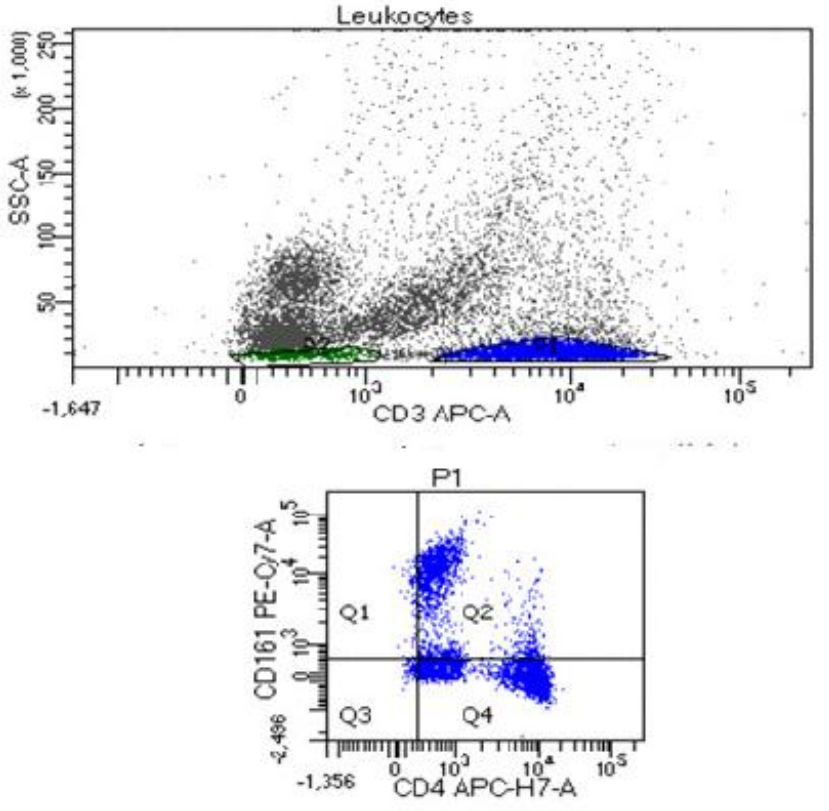
	Mean of (CD3+CD161+) Lymphocytes ± std. Error		Mean Difference	p-value
Group B1	0.42 ±	0.017	- 0.29	0.008*
Group B2	0.71 ±	0.081		
Group A1	0.48 ±	0.129	0.13	0.28
Group A2	0.35 ±	0.1		
Control C1	0.45 ±	0.06	0.13	0.21
Control C2	0.32 ±	0.04		
Multiple Comparisons between groups				
	Mean Difference		**p-value**	
B1 & A1	- 0.06		0.58	
B1 & C1	- 0.03		0.75	
A1 & C1	0.03		0.78	
B2 & A2	0.36		0.003*	
B2 & C2	0.39		0.001*	
C2 & A2	0.03		0.798	
***significant less than 0.05**				

Table (2): The mean and mean difference of CD3+CD4+CD161+ lymphocytes (Th17 subset from Th) among the experimental groups

	Mean of (CD3+ CD4+CD161+) Lymphocytes ± std. Error		Mean Difference	p-value
Group B1	0.38	0.01	- 0.32	0.003*
Group B2	0.7	0.08		
Group A1	0.48	0.13	0.18	0.136
Group A2	0.3	0.08		
Control C1	0.37	0.05	0.05	0.619
Control C2	0.32	0.04		
Multiple Comparisons between groups				
	Mean Difference		**p-value**	
B1 & A1	- 0.09		0.37	
B1 & C1	0.012		0.9	
A1 & C1	0.11		0.3	
B2 & A2	0.4		0.001*	
B2 & C2	0.38		< 0.001*	
C2 & A2	0.02		0.834	
***significant less than 0.05**				

Table (3): The mean and mean difference of CD3+ CD4-CD161+ lymphocytes (Th17 subset from CTL) among the experimental groups

	Mean Rank of (CD3+ CD4-CD161+) Lymphocytes	**p-value**
Group B1	6.38	0.174
Group B2	3.90	
Group A1	2.83	0.376
Group A2	4.17	
Control C1	5.75	0.447
Control C2	4.40	
Multiple Comparisons between groups		
	p-value	
B1 & A1	0.212	
B1 & C1	0.663	
A1 & C1	0.471	
B2 & A2	0.365	
B2 & C2	0.237	
C2 & A2	0.273	
***significant less than 0.05**		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200
150
100
50
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0
10³
10⁴
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CD3 APC-A
P1
CD161 PE-Cy7-A
Q1
Q2
Q3
Q4
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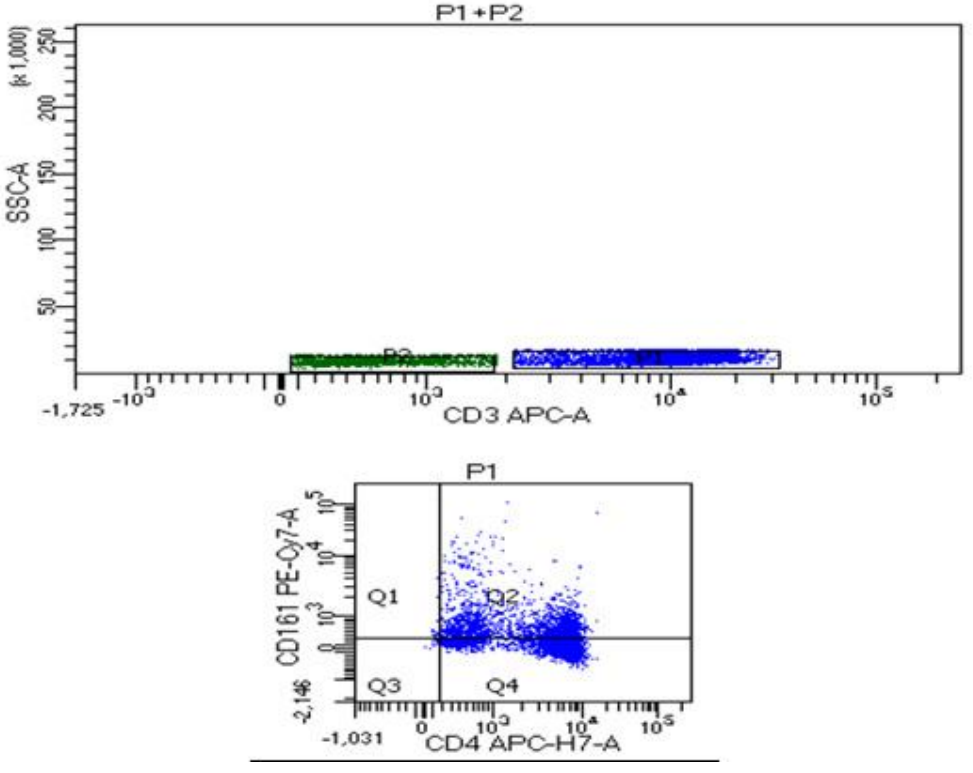
-1,356
CD4 APC-H7-A

Figure (1)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10³
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10⁵
CD3 APC-A
P1
CD161 PE-Cy7-A
Q1
Q2
Q3
Q4
-2,146
-1,031
CD4 APC-H7-A

Figure (2)

Figures Legends:

Figure 1: The Results of flow cytometry for animal immunized by flagellin b before infection (group B1). It illustrates the CD3 positive and negative cells (the blue is T lymphocytes and the green is B lymphocytes). Also, it shows the CD4+ cells (Th cells) and the CD161 positive and negative cells (Th17) that subset from Th cells.

Figure 2: The Results of flow cytometry for animal immunized by flagellin b after infection (group B2). It is showing the CD3 positive and negative cells (the blue is T lymphocytes and the green is B lymphocytes). Also, it shows the CD4+ cells (Th cells) and the CD161 positive and negative cells (Th17) that subset from Th cells.