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A Study of response to *Burkholdaria cepacia*

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Abstract:

The current study was aimed to evaluate immune response induced by *Burkholdaria cepacia* antigens to determine whether these antigens could be a useful immunogen for vaccine development against this bacteria. Four successive doses of OMP and killed antigen extracted from *B. cepacia* isolated from sputum of patients with lower respiratory tract infections were injected into rabbits using multisite injection protocol. We found that these antigens stimulate specific mucosal and systemic humoral immunoglobulin titers as well as to the significant increased ($p \leq 0.05$) in the total protein concentrations at serum and mucosal respiratory secretions in addition to the significant increase in the concentrations of IgG, IgA, IgM at rabbits sera. INF-γ concentrations were significant increased at serum and mucosal levels with stimulation of migration inhibition index of leukocytes. These immunogen induced positive skin test tuberculin type delayed hypersensitivity. Thus *B. cepacia* antigens were B and T cells dependent type, and could be a useful immunogen for vaccine development.

Keywords: *Burkholderia cepacia*, OMP, Immune response, Heat killed whole cell antigen.

Introduction:

Burkholderia cepacia has emerged as a significant opportunistic human pathogen causing life-threatening pulmonary infections in immunocompromised and nosocomial patients(1). It causes chronic respiratory infections and septicemia in chronic granulomatous and cystic fibrosis patients (2). *B. cepacia* colonization of cystic fibrosis patients may be asymptomatic which over time can cause the infection to progress in to acute necrotizing pneumonia, or it can be associated with slow deterioration of lung function. Approximately 20% of cystic fibrosis patients colonized with *B. cepacia* can develop cepacia syndrome which is a form of acute necrotizing pneumonia accompanied by sepsis and may often causes progressive respiratory failure ending in death within a few weeks even with proper treatment (3, 4). However, *B. cepacia* has been recently recognized as causing severe infections in normal individuals (1, 4).

Once *B. cepacia* colonization is established, it is rarely eliminated despite strong antibody responsive in serum and sputum(5), and difficult to treat most infections, due to the inherent resistance of these bacteria to multiple antibiotics which is complicated by development of resistance and cross resistance to other antibiotics during therapy (3), the evolution of intracellular and chronic infection stages in the host, and the ability to form biofilms (6). And over the seriousness of the problem, *B. cepacia* can be transmitted between cystic fibrosis patients, and between non cystic fibrosis patient to another cystic fibrosis patient in and outside hospitals, or may be acquired from the environment outside hospital(1,7). Moreover, contamination of disinfectants, medical devices, and pharmaceutical formulations with *B. cepacia* has led to several outbreaks among cystic fibrosis cystic fibrosis and noncystic fibrosis populations(8). For this, the threat posed by *B. cepacia* has led to growing interest in vaccines development for the prevention and control of *B. cepacia* infections.

Various virulence factors such as LPS core antigen, exopolysaccharides, proteases, elastase, and flagella antigens have been suggested as candidate for vaccine development

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against *B. cepacia* infections. However, none of these are considered to be protective (9). Outer membrane proteins (OMPs) of gram negative bacteria are virulence factors that involved in bacterial pathogenesis and able to induce both humoral and cytotoxic responses (10). OMPs of *Burkholderia* cepacia compex are major surface antigens that candidate as potential protective epitopes for development of vaccine, and provided cross-protection among *Burkholderia* members(11). In addition, 18 immunogenic proteins from *B. cepacia* culture supernatant reacted with mice antibodies that raised against *B. cepacia* inactivated whole bacteria, OMP, and culture filtrate antigen and thus suggested to be potential candidates vaccine against *B. cepacia* infections(12). So this study was aimed to examine if OMP and killed antigen extracted from *B. cepacia* could be a useful immunogen for vaccine development by determining the humeral and cellular immune response induced at systemic and mucosal levels via injection of these antigens in rabbits.

Material and Methods

1- Isolation of *B. cepacia* :

B. cepacia was isolated from eight sputum specimens collected from patients suffering from lower respiratory tract infections. Primary identification was performed by culturing on MacConkey's agar and incubated aerobically, non-lactose fermenting bacteria were subcultured for purification. Suspected bacterial isolates which their cells are bacillary, Gram negative, and negative to oxidase, catalase and lysine decarboxlyse further identified by the biochemical test **(table 1)** according to (13, 14), then additional confirmed by Vitek2 Compact system.

2- Extraction of Outer Membrane Protein (OMP) from *B. cepacia*:

OMP was extracted from *B. cepacia* according to (15) as following: we prepared pure culture of this bacteria on brain heart infusion agar, using loopful we transmit (2-4) colony of this bacteria to test tube contain 10 ml of Brain Heart Infusion Broth . The suspension tubes incubated in shaker at 37° for (18-24) hours at 100 rpm\ min. Then we centrifuged 10 ml of bacterial suspension at 10000 rpm\ min for 10 mints at 4°. Then add 1.5 ml (238.31 N) of 2Hydroxyethylpiperazine-N2-Ethane sulfonic acid ($C_8H_{18}N_2O_4S$) to the sedimentation. Centrifuged the suspension again at 8000 rpm\ min for 2 min at 4° then we used the precipitation immediately or keep it at freezing for later using .

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Table 1: Characterization of *B. cepacia*.

Character	Result
Gram stain	**negative**
Shape	**bacillary**
O2 need	**Obligate aerobic**
Glucose oxidation	+
Catalase	+
Oxidase	-
Lysine decarboxylase	+
Ornithine decarboxylase	+
Citrate	+
Gelatine liquefaction	+
TSI	**K/K**
H2S production	-
Smell	**Dirt like odour**
Urease	+
Indole	-
MR	-
VP	-

3- Preperation of Killed antigen from *B. cepacia*:

Heat killed antigen of *B. cepacia* was prepared from 24 hour brain heart infusion agar plate culture. Then was added 6 ml of normal saline to the plat's scrape , The collected solution centrifuged at 4000 rpm\ mint for 5 mints. Double wash were placed with normal saline then compared with standard opacimeter (WHO) to obtain the concentration equals to 10 IU\ml . The suspension tubes were used after placing them in a water path at 60° for 30 mints to kill the bacteria and obtaining the antigen that was used for the immunization of rabbits after exanimate for the sterility test (16, 17).

4- Animals:

Three groups, each of three rabbits *O. canniculus* were elected, adapted to laboratory conditions and housed under Adlibitum standardized conditions, two served as test and other as control group (18).

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5- Immunization protocol:

Four successive doses of OMP and killed antigen extracted from *B. cepacia* each one about 1 ml (each dose about 1 mg/Kg) distributed into 0.25 ml for four sites (High muscle, subcutaneous , right and left neck region) via multisite injection protocol into tested rabbits through four weeks followed by one week then bled by cardiac puncture. Control animals received sterile normal saline in same protocol (19, 20).

6- Mucosal samples:

Respiratory mucosal samples were collected from all tested rabbits after being sacrificed by chloroform as described by (21) as follows:

1- Trachea and bronchi were removed and opened using sterile and clean scissor, then

washed with normal saline.

2-The mucosa and sub mucosa were scrapped by sterile surgical scalpel and then they were placed in another sterile Petri dish containing 5 ml of normal saline.

3-By sterile Pasteur pipette the suspension was transferred to sterile plastic test tube.

4-The suspension was centrifuged at 4000 rpm for 20 min. Supernatant was divided in two test tube.

5-The supernatant in test tube one was stored at -25C until used for mucosal cytokines measurements.

6-While the supernatant in the second tube was used for separation of mucosal Immunoglobulins.

7-Blood Samples:

Coagulated blood were collected for the serological tests (22).

8- Serological tests:

Agglutination test was performed by tube agglutination test, serum and mucosal total protein concentrations were measured according to colorimetric method using readily prepared solutions provided by Biolabo company, France and Randox – Laboratories Ltd , UK. Company , the concentrations of IgG , IgA , IgM were measured using single radial immunodiffusion assay. INF-γ cytokine was assayed using ELISA kit (provided from Immuno. tech. A. Beckman, Coulter Company) as in standard curve that explained in (Figure 1) . Capillary leukocytes migration inhibition test, as well as skin delayed type hypersensitivity were done respectively (22, 23).

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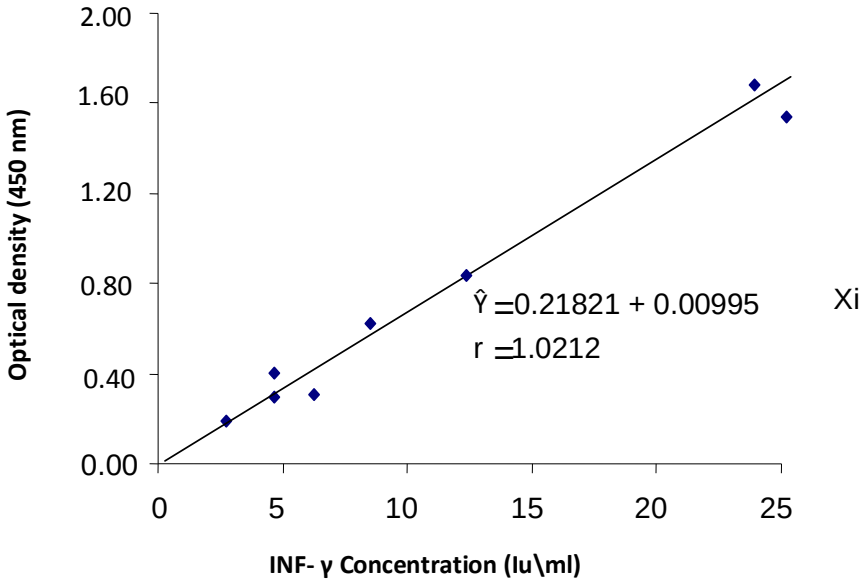

Figure (1) Standard curve of INF-γ

Results and Discussion:

Susceptible populations in need for *Burkholderia* vaccines to acquire prophylactic protection against their infections, and reduce the incidence of chronic infections in the immunocompromised host. As *B. cepacia* exist in the soil, and pathogenic human isolates are not necessarily distinct from environmental ones (24), every susceptible individual subjected to the risk of acquiring these infections through routine daily activities, so the need for a vaccine become crucial (9). The immune response was studied at the end of the immunization period after injection of OMP and killed antigen extracted from *B. cepacia* via multisite injection protocol into tested rabbits and we found that these antigens stimulate specific humoral systemic and mucosal antibody titers (Table 2). It played an important role in the significant increase ($p \leq 0.05$) of the total protein concentrations (Table 3) which correlate with the increase in the number of stimulating B cells responsible for antibodies production (25).

Table (2) Lapin systemic and mucosal antibody titers specific for OMP and killed antigen extracted from *B. cepacia*

Samples	Mean antibodies titer for OMP	Mean antibodies titer for Killed antigen
Serum	**2560**	**1280**
Mucosal		
Trachea	**128**	**128**
Bronchi	**256**	**128**
Serum control	**10**	10
Mucosal control	1	1

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These results similar to the results of (26), they found that the immunization with a *B. multivorans* OMP preparation can induce specific systemic and mucosal immune responses in the respiratory tract, which in turn promoted the clearance of *B. multivorans* and reduced lung inflammatory damage after bacterial challenge. Specific IgA antibodies induced by mucosal immunization are likely to play an effective role in the adaptive immune system, inhibiting adhesion and colonization of bacterial pathogens. This pointed out that IgA is the first line of defense in the mucosal compartment (27).

Table (3) Total protein concentrations at serum and mucosal secretions of rabbits immunized with OMP and killed antigen extracted from *B. cepacia*

Samples	Total protein concentrations (mg/dL) for OMP M±S.D.	P - value	Total protein concentrations (mg/dL) for Killed antigen M±S.D.	P - value
Serum	10.65±0.35	0.000[a]	9.4±0.45	0.000[a]
Mucosal				
Trachea	0.44±0.02	0.000[a]	0.14±0.01	0.000[a]
Bronchi	0.51±0.02	0.000[a]	0.37±0.02	0.000[a]
Serum control	5.73±0.04	0.003[b]	5.73±0.04	0.001[b]
Mucosal control	0.23±0.01	0.000[c]	0.23±0.01	0.000[a]

We also noted significant increased ($p \leq 0.05$) in the concentrations of IgG , IgA and IgM antibodies in serum of immunized rabbits (Table 4) in comparison with control group and this results agreement with (26) that they found the antibody response was specific to *B. cepacia* antigens. In addition, serum IgG antibody titter against *B. cepacia* outer membrane protein were significantly higher in cystic fibrosis patients infected with *B. cepacia* (28).

Serum analysis showed robust IgG and mucosal secretory IgA immune responses in vaccinated versus control mice, also suggested that the 17 kDa OmpA-like protein of *Burkholderia cenocepacia* shows potential for future vaccine development and provides a rational basis for vaccines using recombinant OMP mixed with nanoemulsion as an adjuvant (11).

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Table (4) Immunoglobulines concentrations titers at rabbits sera immunized with OMP and killed antigen extracted from *B. cepacia*

Immunoglobulines concentrations (mg\dl)	Concentrations (mg\dl) for OMP M±S.D.	P- value	Concentrations (mg\dl) for Killed antigen M±S.D.	P- value
IgG Concentrations	2269.7 ±49.45	0.000[a]	1673.4±32.65	0.000[a]
Serum control of IgG	1156.2±25.1	0.000[b]	1156.2±25.1	0.000[b]
IgA Concentrations	497.25±11.8	0.000[a]	392.5±8.55	0.000[a]
Serum control of IgA	171.4±5.3	0.000[b]	171.4±5.3	0.000[b]
IgM Concentrations	495.49±5.5	0.000[a]	474.58±7.5	0.000[a]
Serum control of IgM	230.12 ±3.4	0.000[b]	230.12 ±3.4	0.000[b]

Heat killed whole cell antigens unable to replicate in the host, thus induce a limited range of immune responses. However, The inability to replicate is major advantage for these antigens; making them as an extremely safe prophylactic vaccine appropriate for immunization of immunocompromised individuals. These antigens also stimulate humoral immunity to produce neutralizing antibodies, while do not induce strong cellular-mediated immunity because these antigens are not processed by an endogenous pathway and presented to the T cells by class I MHC. Hence, multiple doses of antigen and adjuvants are required for long lasting protection (25). Thus the cellular systemic and mucosal immune responses were represented by significant increased ($p \leq 0.05$) in the concentrations of cytokine especially INF-γ (Table 5). Hence, their epitopes can be of T dependent type through the activation of Th1 (29).

Table (5) Concentrations of INF-γ in rabbits immunized with OMP and killed antigen extracted from *B. cepacia*

Samples	Concentrations of INF-γ (Iu\ml) for OMP M±S.D.	P - value	Concentrations of INF-γ (Iu\ml) for Killed antigen M±S.D.	P - value
Serum	21.83±2.08	0.000[a]	15.78±1.21	0.000[a]
Mucosal				
Trachae	20.35±1.75	0.010[b]	9.23±0.32	0.010[b]
Bronci	23.81±2.11	0.000[a]	11.76±1.43	0.000[c]
control	7.95±1.45	0.000[c]	7.95±1.45	0.000[d]

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B. cepacia is an invasive pathogen capable of adhering and colonizing prior to entering cells (12). In addition, It is facultative intracellular pathogen which can survive in respiratory epithelial cells, neutrophils, macrophages and lead to evasion of humeral immune response (9, 30, 31). So, the complicated nature of this bacterium, confers major challenges for designing effective and safe vaccines and highlights the need to induce cell-mediated immunity to combat infection. In current study, OMP and killed antigen extracted from *B. cepacia* induced both humoral and cellular immune responses as well as release of T cell dependent immunogen that induce significant increased ($p \leq 0.05$) in migration inhibition of leukocytes invitro (Table 6). Also play a role in release of an allergen inducing delayed type hypersensitivity response (Table 7). Hence, their epitopes can be of T dependent type through the activation of Th1 and Th2 (32).

Table(6) Leucocytes Migration Inhibition test (LIF) of rabbits immunized with OMP and killed antigen extracted from *B. cepacia*

Samples	LIF of OMP M±S.D.	P - value	LIF of Killed antigen M±S.D.	P - value
Serum	0.39±0.01	0.000[a]	0.33±0.01	0.000[a]
Mucosal				
Trachae	0.42± 0.04	0.000[b]	0.39±0.02	0.000[a]
bronchi	0.49±0.05	0.000[b]	0.41±0.03	0.000[b]
control	0.92±0.00	0.000[c]	0.92±0.00	0.000[c]

Table(7) Skin test of rabbits immunizsd with OMP and killed antigen extracted from *B. cepacia*

Animal group	Skin test \ Hours					
	6	18	24	48	72	96
Group 1: rabbits immunized with OMP	**E**	**E**	**E**	**EI**	**EIN** 11mm	**EIN** 11mm
Group 2 : rabbits immunized with killed antigen	**-**	**E**	**E**	**EI**	**EIN** 6 mm	**EI**
Group 3: Control rabbits immunized with N.S.	**-**	**-**	**-**	**-**	**-**	**-**

N- Necrosis, I- Induration, E- Erythema.

Conclusion

From all of the above we can conclude that serum and respiratory compartment is being induction compartment of mucosal immune system . OMP and killed antigen extracted from

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B. cepacia could be a useful immunogen for development of a vaccine based on their ability to induce both humoral and cellular immune responses at mucosal and systemic levels.

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