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Evaluation of Outer Membrane Proteins of *Pseudomonas aeruginosa* as a Protective Agent in Mice Model

[1]Mohammad Kazem Sharifi-Yyazdi, [2]Farhad Esmaily, [3]Farhad Vaezzadeh and [4]Hossien Dargahi
[1]Department of Medical Laboratory Sciences, Faculty of Para medicine,
Tehran Medical Sciences, Tehran, Iran
[2]Department of Microbiology, Razi Vaccine and Serum Research Institute, Karaj, Iran
[3]Department of Pathobiology, School of Public Health,
Tehran University of Medical Sciences, Tehran, Iran
[4]Department of Health Care Management, Faculty of Para medicine,
Tehran University of Medical Sciences, Tehran, Iran

Abstract: The crude Outer Membrane Protein (OMP) from a strain of *P. aeruginosa* isolated from burn patient was purified by two different methods. One procedure involved separation of Sodium Dodecyle Sulphate (SDS) and Triton X-100, where as the other involved using lysozyme enzyme. Both methods showed very similar polypeptide pattern and the major peptide band with molecular weight of 37 KD was common in both procedures. The protein estimation of OMP extracted by lysosyme was 3 mg mL^{-1} compared to 5.5 mg mL^{-1} extracted by Triton-X100 method. The latter was chosen to examine for the immunogenicity study in a mice model. The efficacy of immunization with OMP and challenge with homologous strain in mice showed a very good protection compared to control mice injected with saline. The passive haemoagglutination test (PHA) in mice, injected with OMP showed increased level of antibody after the second injection and stayed constant after repeated injection. The results of this study showed that the crude OMP extracted from *P. aeruginosa* induced a significant protection in mice against *Pseudomonas* infections and could be used as a vaccine candidate.

Key words: *P. aeruginosa*, outer membrane protein, vaccine, cystic fibrosis, septic shock

INTRODUCTION

P. aeruginosa is an opportunistic pathogen which infects people with defective immune system duo to severe burns, cystic fibrosis, immunosuppressive or cancer therapy (Burns *et al*., 2001; Cryz *et al*., 1984). More than 90% of cystic fibrosis patients die of lung damage resulting from recurrent lung infection with *P. aeruginosa* (Edgeworth *et al*., 1999). In patients with severe burn wounds or immunosupperressive therapy, the regional colonization with *P. aeruginosa* leads to systemic infection, causing septic shock (Velasco *et al*., 1997). *P. aeruginosa* is naturally resistant to many antibiotic, because of the existence of drug efflux components in cytoplasmic membrane (Gales *et al*., 2001; Poole and Srikumar, 2001). These characteristics to (give rise difficulties) in treating *Pseudomonas* infections and need for an effective vaccine has been urgently requested. Several attempts have been made to develop effective and safe vaccine against *P. aeruginosa*. Lipopolysaccaride (LPS) and capsular polysaccharide (CPS), two major cell surface components are highly immunogenic and antibodies raised against them are protective against *Pseudomonas* infections, but their toxicity limits use as vaccine (Cryz *et al*., 1984). Other cell surface components, Outer Membrane Proteins (OMPs), have been the target for vaccine development. In this study use of crude OMPs, extracted from strain of *P. aeruginosa* as a vaccine candidate for the protection activity in mice was investigated.

MATERIALS AND METHODS

Bacterial strain and growth conditions: The strain of *Pseudomonas aeruginosa* used in this study was isolated from a burn patient. The medium used was tryptone soy broth (oxoid). Overnight cultures grown with shaking at

Corresponding Author: Mksharifiy-Yazdi, Department of Medical Laboratory Sciences, Faculty of Para Medicine,
Tehran University of Medical Sciences, Tehran, Iran
Tel: +9821-88964009 Fax: +9821-88964009

37°C harvested and washed three times in sterile Phosphate-Buffered Saline (PBS) and resuspendend in PBS prior to use.

Preparation of OMP by SDS method: The crude OMP was prepared with a few modifications as described previously (Van Celder *et al*., 1994). Briefly the bacterial suspension was transferred into sonicating tube. The cells were sonicated in sonicating tube located in a flask of ice crashed for 10 min with 10 sec intervals between each minute. The suspension was centrifuged at 10000 g for 30 min at 4°C and the supernatant was collected and centrifuged again for 1 h. The clear pellet was suspended in 4 mL of 10 mM Herpes buffer containing 2% triton X-100. Two milliliter of 2% SDS was added and was left at 56°C for 15 min, the suspension was centrifuged in the same manner for 1 h. The sediment which consisted of protein complex and peptidoglycan was suspended in mL of distilled water and dialyzed against 2 mL of 5 M NaCl containing 2% SDS and it was left overnight at 37°C for 10 min. The dialysate was centrifuged at 5000 g at 4°C for 10 min, the supernatant was dialyzed three times against distilled water for 48 h at room temperature, thus SDS and NaCl was separated from protein complex.

Preparation of OMP by lysosyme method: The method of preparation of OMP by Lysosyme method was similar to the method described for SDS method with the exception that Lysosyme was used instead of Triton X-100 and SDS.

Protein concentration determination: Protein concentration was determined according to Lowry method (Lowry *et al*., 1951), with bovine serum albumin as the standard and folinciocalteus phenol reagent.

Electrophoresis: Using sodium dodecyle-polyacrylamide gel electrophoresis (SDS-PAGE), the polypeptide patterns of crude OMP were determined as described previously (Laemmli, 1970). To determine the molecular weights of the protein components, high and low molecular weight markers were used.

Determination of *P. aeuroginosa* LD 100 in mice: The LD100 of *P. aeruginosa* was determined as described previously (Dixon, 1991). Fifteen mice were set in three group of 5 and injected with 0.5 mL suspension of *P. aeruginosa* (between 10^7 to 10^9 cells). The injected mice were observed for 3 day.

Immunization and challenge: A total of 30 mice (six to eight week of female BALB/c) were used for immunization and challenge. To assess the immunogenicity of crude OMP two groups of mice each compromising of 15 mice were set.

Group 1 (5 g of OMP suspended in 5 mL of PBS) were injected intraperitoneally (ip) in nine days interval. Group 2 as a control received saline only. Some of the mice injected with OMP were bled after 11 and 16 days. Both groups were challenged after 18 days.

Passive haemoagglutination assay (PHA): The PHA was performed as previously described (Kuusi *et al*., 1981).

RESULTS

The SDS-PAGE results: The pattern of OMP prepared by both methods were similar and a peptide band with molecular weight of 37 kDa, which is the major OMP band in *P. aeruginosa* strains was observed. The rest of the bands were almost similar with different densities (Fig. 1).

Protein concentration determination: Protein concentration of OMP extracted by Triton X-100-SDS was 5.5 mg mL^{-1} as by lysozyme method was 3 mg mL^{-1}.

The results of LD 100 in mice: The LD 100 injected with *P. aeruginosa* was 6×10^9 cfu mL^{-1}.

Immunization and challenge: The results of immunization and challenge of mice are shown in Table 1.

Results of Passive Haemoagglutination Assay (PHA): The PHA test in mice injected with OMP extracted by Triton X-100- SDS showed agglutination in serum dilution of 1/2, 1/4, after 11 days and 1/2, 1/4, 1/8 after 16 days of first injection. The titer remained steady afterward.

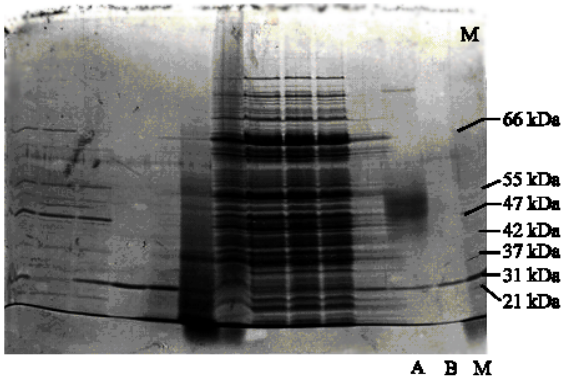

Fig. 1: Comparison between polypeptide patterns of OMP of *P. aeruginosa* extracted by different methods A = Trion X-100 and SDS method, B = Lysozyme method, M = Marker

Table 1: Immunization schedule of mice with OMP, challenge with homologous strain

Indexes	Antigen OMP	Control
No. of mice	15.0	15.0
No. of injection	2.0	2.0
Dose of injection (μL)	50.0	50.0
Days of intervals	9.0	9.0
Amount of challenge (mL)	0.2	0.2
Challenge dose cfu mL^{-1}	6×10^9	6×10^9
No. of dead	0/15	15/15

DISCUSSION

The major OMP in several gram-negative bacteria are immunologically important components because they are exposed on the cell surface and this feature makes them attractive as potential vaccine candidates *P. aeruginosa* is opportunistic pathogens that can causes severe to life threatening infections in compromised patients; therefore it can be added to the list of organisms for which the potential of OMP as a vaccine can be considered. In this study we have showed, that crude OMPs of *P. aeuroginosa* elicit an antibody response in mice and it is capable of protecting mice against *P. aeruginosa* infections. other research worker also showed that OMP are useful candidate for vaccine in number of gram-negative bacteria (Mathers *et al*., 1999; Roy *et al*., 1994). We used two different methods of lysozome and Triton X-100 and SDS for the preparation of OMP. In comparison the triton X-100 and SDS produced much higher amount of protein (7 mg mL^{-1}) compared to lysozome method (4 mg mL^{-1}). The polypeptide pattern of OMP prepared by both methods were similar and peptide bands with molecular weight of 37 kDa, which is the major bands in *P. aeuroginosa* was seen in both methods.

In our protective study mice immunized with OMP and challenged with LD 100 from homologous strain, showed very good protection compared to the control mice injected with saline. In passive heamoagglutination assay (PHA) the level of antibody was increased after second injection with OMP and remained constant after further injection. Our results is in correlation with previous finding (Zaidi *et al*., 2006; Park *et al*., 1997) and provides sufficient evidence that OMP has the potential for the protection against *P. aeuroginosa*.

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