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Efficacy of Immunization with Outer Membrane Proteins for Induction of Pulmonary Clearance of Nontypeable *Haemophilus Influenzae* in a Rat Respiratory Model

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ABSTRACT

Three strains of nontypeable *Haemophilus influenzae* namely NTHi-I , NTHi-II and NTHi-III were isolated from the sputum of patients with bronchitis and identified by biochemical, serological and electron microscopy. The polypeptide patterns of isolates were compared and found to have similar sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) polypeptide patterns, although some of the bands were specific in some strains. A similar comparison was made on extracted outer membrane proteins (OMPs) on the above mentioned strains, using Triton X-100 and sodium dodecyle sulphate (SDS). It was found that the polypeptides with molecular weights of 70, 42, 33 and 27 KDa were identified as P1, P2, P4 and P5 respectivly. The protein estimation of crude OMPs from the three strains were calculated, and OPM-I prepared from NTHi–I showed the highest amount of protein and was chosen for its immunogenicity in a rat respiratory model.

The efficacy of immunization with OMP was determined by enhancement of pulmonary clearance of live bacteria in the rat lung. A significant protective immune response induced by OMP was observed by enhanced respiratory clearance of nontypeable H. influenzae following mucosal immunization.

Key words: Nontypeable *Haemophilus influenzae*; Outer membrane protein; Pulmonary clearance

INTRODUCTION

Unencapsulated or nontypeable *Haemophilus influenzae* (NTHi) is a common cause of wide range of human diseases including tracheobronchitis, pneumonia, epiglotitis, and otitis media.[1-3]

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Unlike *Heamophilus influenzae* type b for which a highly successful vaccine is available, there is no vaccine available that can protect the occurrence of these NTHi infections. Outer membrane proteins (OMPs) and lipooligosaccharide (LOS) are major surface antigens of NTHi and considered as a potential vaccine candidates.

The search for an effective vaccine has focused on components of the outer membrane proteins, and most vaccine development efforts have been directed towards

integral outer membrane proteins component. Several outer membrane proteins such as P1, P2, P4 and P6 have been investigated, and P6 has been proposed as a likely candidate for effective vaccine against NTHi infections.[4-7] In this study attempt was made to extract and purify the crude OMP from NTHi and to assess the immunogenicity and protective activity of the extracted NTHi OMP by clearance of NTHi from the rat respiratory tract.

MATERIALS AND METHODS

Bacterial Strains and Growth

Three strains of NTHi namely NTHi-I, NTHi-II and NTHi-III were isolated from sputum of patients with bronchitis. The isolates were grown on different culture media, and identified by biochemical tests according to standard method.[8] The NTHi ATCC 860295 was used as positive control strain. The biotypes of isolated strains were determined using indole, ornithine, urease, oxidase and catalase tests.

Electron Microscopy

The electron microscopy was used for the confirmation of lack of capsules (unencapsulated) of strains, by the electron microscopy unit of Razi institute.

Preparation of OMP by SDS Method

The bacteria were grown overnight at 37°C in 5% CO2 on Levintal agar, purified colonies were transferred into brain heart infusion (BHI) broth, supplemented with 50 ml of defibrinated horse blood per liter of agar at 37°C in 5% CO2. 70 mg wet weight culture were obtained from each isolates. The OMP were prepared with a few modifications as described previously [9] and designated as OMP-I, OMP-II, and OMP-III respectively.

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 10000g for 30 min at 4°C and the supernatant was collected and centrifuged again for 1 h. The clear pellet was suspended in 4ml of 10 mM Hepes buffer containing 2% triton X-100. 2ml 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 1 ml of distilled water and dialyzed against 2ml of 5M NaCl containing 2% SDS and it was left overnight at 37°C. The dialysate was centrifuged at 5000 g at 4°C for 10 min; the supernatant was dialyzed three times against distilled water for 48h at room temperature, thus SDS and NaCl was separated from protein complex.

Protein Concentration Determination

Protein concentrations of various strains of NTHi were determined according to lowry method [10] with bovine serum albumin as the standard and folin phenol reagent.

Electrophoresis

Using sodium dodecyle sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), the polypeptide patterns of the three isolates and the crude OMP extracts, were determined as described previously.[11] To determine the molecular weights of the protein components, high and low molecular weight markers were used.

Immunization for Pulmonary Challenge

Immunization was performed as described previously.[12] Briefly, the male rats aged between 8 and 10 weeks were sedated with halothane given intraperitoneally (ip), and the small intestine was exposed to enable inoculation of intestinal Peyer's patches with 1:1 mixture of immunogen emulsified with incomplete Freund's adjuvant (IFA), and phosphate buffered saline (PBS). The immunogen protein was prepared by emulsifying 200 and 800 μg/ml of protein respectively. Control groups included, negative control in which rats receiving 1FA and PBS only and positive control immunized with killed bacteria of the homologous NTHi strain.

The bacteria were killed in 1% (w/v) paraformaldehyde in PBS and incubated at 37°C for 2 hours. The bacteria were washed four times in PBS and the concentration was adjusted to a bacterial equivalent to 2×10^{10} per ml. Killing was verified by plating onto agar and overnight culture, the bacteria were then emulsified in a 1:1 with IFA so that each animal received approximately 5×10^{8} bacteria. Fourteen days post immunization, rats received a boost, delivered intratracheally to the lungs, of 15 μg of OMP-I in PBS. A live NTHi challenge was delivered 21 days postimmunization intratracheally (pulmonary challenge) as described previously.[12] For the challenge each rat

received approximately 5×10^8 CFU. Viability count was confirmed by concurrent overnight culture onto chocolate blood agar.

RESULTS

Electron Microscopy

The electron micrograph isolates of NTHi for the confirmation of lack of capsules (unencapsulated) are shown in figure 1.

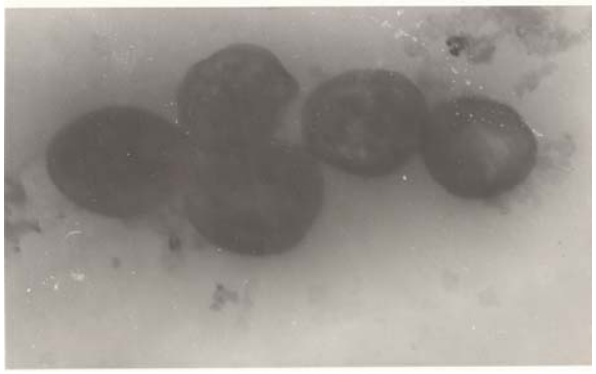

Figure 1. Elelctron micrograph of an unencapsulated strain of NTHi-I Satellitism phenomenon

The need of NTHi to V factor for its growth, was provided by using S. aureus to produce V factor figure 2.

Figure. 2. Satellites phenomenon of NTHi using *S. aureus*

Results of Serological Tests

Agglutination

Two sera of polyvalent anti-serum against NTHi and monospecific anti-serum against biotype II were used. The results showed all the isolates were unencapsulated and of biotype II.

The biotype of isolates were confirmed by indole (+), ornithine, (-)urease(+), oxidase(+) and catalase (+).

The SDS-PAGE Results

The comparison between polypeptide patterns of isolated strains showed similarity, although the densities and molecular weights of some of the bands were specific. A polypeptide with 48 KDa was seen only in standard strain, while bands with molecular weights of 75, 78 and 72 KDa were seen in NTHi-I, NTHi-II and NTHi-III respectively, figure 3.

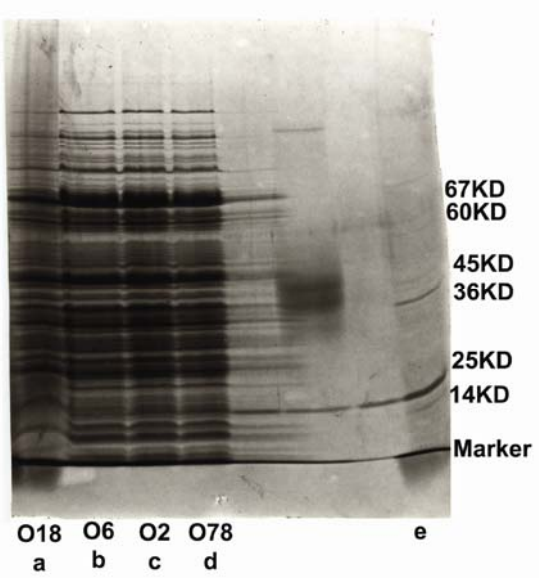

Figure 3. Comparison between polypeptide patterns of three strains of NTHi-I, NTHi-II, NTHi-III and strain 860295

Polypeptide patterns of OMPs extracted from all strains showed bands with molecular weights of 70, 42, 33 and 27 which were common in all strains as P1, P2, P4 and P5 respectively, figure 4.

Protein Concentration Determination

Protein concentration of OMPs for all stains were determined as follow, 1.5, 0.9 and 1.2 mg. for OMP-I, OMP-2 and OMP-3 respectively. OMP-I with the highest amount (1.5) mg were chosen for the in-vitro studies.

Clearance of NTHI after Immunization with OMP-I

4 hours post-challenge, bacterial clearance in bronchoalveolar lavage (BAL) fluid and lung homogenates were compared with those for the non-immune rats. The results showed that immunization with

15 μg of OMP-1 per rat resulted in a significantly enhanced clearance of NTHi-I from the BAL fluid and lung homogenates (P<0.001) (table 1).

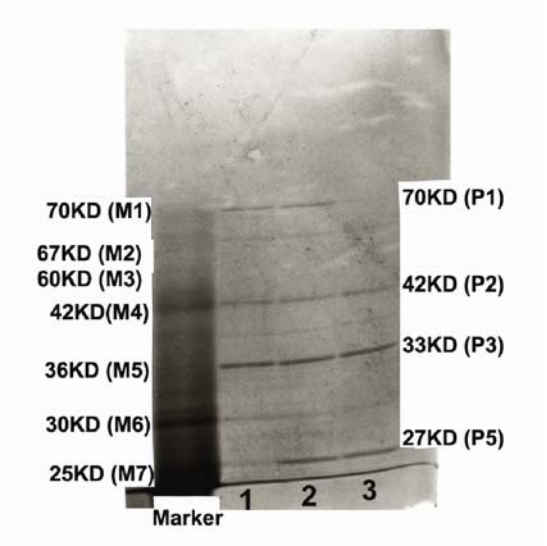

Figure 4. Comparison between OMPs extracted from three strains of NTHi-I, NTHi-II, NTHi-III, on tracks 1, 2, 3.

Table 1. Pulmonary clearance of NTHi-I following mucosal immunization with OMP-1 and challenging with homologous NTHi-I strains

	Mean NTHi-I recovered 4 hours post challenge (log^{10} CFU) ± SEM (% clearance)	
Rat group	**BAL**	**Lung**
Nonimmune (negative control)	6.52 ± 1.304	6.22 ± 1.30
Immunized with killed bacteria (positive control)	5.45 ± 1.140	5.26 ± 1.14
Immunized with OMP-I	4.5 ± 0.212	4.48 ± 1.7

DISCUSSION

Many factors contribute to both pathogenesis of bacterial bronchitis and the host's ability to resolve these infections. It has been shown by many laboratories, that the greatest likelihood for the successful development of a nontypeable *Haemophilus influenzae* directed vaccine relies upon the careful selection of conserved and protective immunogenes.[13] Ideally the vaccine must induce a balanced and broadly protective immune response in very young children,.Because of the increased incidence of antibiotic resistant NTHi in recent years[14] have been the thought for the development of an effective vaccine against this bacterium and considered as an important goal for public heath.[15] For this reason we isolated three strains of NTHi namely NTHi-I, NTHi-II and NTHi-III from children with bronchitis, and the comparison was made between the polypeptide bands of these strains. It showed similarity between polypeptide bands, although the existence and the density of some bands were variable e.g: three bands with molecular weights of 75, 78, and 72 KDa only existed in NTHi-I, NTHi-II and NTHi-III, respectively.

We extracted OMPs from all strains and were compared by SDS-PAGE. The polypeptide patterns of all OMPs were very similar and bands with 27, 33, 42 and 70 KDa were identified as P1, P2, P4 and P5 respectively, which were common in all strains. The protein estimation of crude OMPs were calculated and was found that the protein of OMP-I extracted from NTHi-I had the highest amount and was chosen for in-vitro studies. By using mono specific antibody, and indole (+), ornithine (-), urease (+), oxidase (+) and catalase (+) test the isolates were classified as biotype II. This type is the most common cause of bronchitis in children and adults.[16]

There is currently considerable interest in OMP of nontypeable *H. influenzae* as a vaccine candidate against NTHi. The protection of major outer membrane proteins of NTHi such as P1, P2, P4, P5 and particulary P6 have been shown to enhance pulmonary clearance in rat and chinchilla upon direct challenge, suggesting as a suitable candidate for vaccine.[17-19] In this study, we have demonstrated that mucosal immunization with major OMP of nontypeable *H. influenzae* in rat, resulted in significant pulmonary clearance in bronchoalveolar lavage (BAL) and lung (P<0.001). Our results is in correlation with previous findings,[20] and provides sufficient evidence that OMP has the potential for the protection against NTHi infections.

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