ORIGINAL ARTICLE
Iran J Allergy Asthma Immunol
In press.

CbpM and CbpG of *Streptococcus Pneumoniae* Elicit a High Protection in Mice Challenged with a Serotype 19F pneumococcus

Hamid Kazemian[1], Davoud Afshar[1], Ernesto Garcia[2], Mohammad Reza Pourmand[1], Mahmood Jeddi-Tehrani[3], Farzaneh Aminharati[1], Fazel Shokri[4], and Mohammad Hossein Yazdi[5]

[1] *Department of Pathobiology, School of Public Health and Urology Research Center Tehran University of Medical Sciences, Tehran, Iran*
[2] *Centro de Investigación Biomédica en Red de Enfermedades Respiratorias, Madrid, Spain*
[3] *Monoclonal Antibody Research Center, Avicenna Research Institute, Academic Center for Education, Culture and Research (ACECR), Tehran, Iran*
[4] *Department of Immunology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran*
[5] *Department of Pharmaceutical Biotechnology, Faculty of Pharmacy and Biotechnology Research Center, Tehran University of Medical Sciences, Tehran, Iran*

Received: 29 October 2017; Received in revised form: 25 June 2018; Accepted: 29 June 2018

ABSTRACT

Among many pneumococcal antigens, choline-binding proteins (CPBs) display a high immunogenicity in animal models. This study aims to determine the immunogenicity of CbpM, CbpG and CbpL proteins of *Streptococcus pneumoniae* in a mice model.

The genes were cloned into *p*ET21a expression vector and the recombinant proteins were produced. Mice were immunized with the purified recombinant proteins. Subsequently, the mice were challenged with *S. pneumoniae* ATCC 49619 (2×10^6 CFU) and their survival and bacterial clearances were followed 24 hours after infection. The antibody responses of the mice were determined by ELISA assay. The opsonophagocytosis assay was performed using rabbit's sera. Passive immunization was carried out using two doses of anti-CbPs antibodies. Finally, these mice were experimentally infected with virulent bacteria and the protective effects of two doses of 10 and 100 μg/mL by monitoring the survival rate and bacterial clearance were determined at 2, 3 and 7 days after bacterial challenge.

The mice actively immunized with CbpM, CbpG and CbpL recombinant proteins showed survival rate of 100%, 85% and 75%, respectively. The survival rates among passively immunized mice groups which received100 μg/mL dose of anti-CbpM, anti-CbpG and anti- CbpL were 50%, 50% and 25%, respectively. The rates of opsonization with rabbit's antibodies against CbpM, CbpG, and CbpL at 100 μg/mL doses was 45.6%, 14.7% and 82.3%, and at 10 μg/mL was 12.9%, 12.2% and 9.35%, respectively.

Our findings suggest that the recombinant proteins particularly CbpM and CbpG can protect the mice against pneumococcus19F serotype and effectively induce a protective antibody response. Thus, CbpG and CbpM proteins might be used as suitable vaccine candidate in pneumococcal vaccine formulations.

Keywords: Choline-binding proteins; Pneumococcal vaccines; Streptococcus pneumoniae

Correspondence Author: Mohammad Reza Pourmand, PhD. Department of Pathobiology, School of Public Health and Urology Research Center, Tehran University of Medical Sciences, Tehran, Iran, Postal Code: 14155-6446, Tel: (+98 21) 8895 4910, Fax: (+98 21) 6646 2267, E-mail: mpourmand@tums.ac.ir

Published by Tehran University of Medical Sciences (http://ijaai.tums.ac.ir)

INTRODUCTION

Since the introduction of the first generation of pneumococcal polysaccharide vaccine in the late 1960s, attempts to develop effective and affordable vaccines is continuing. The efforts led to the development of conjugated vaccines in 2000 which displayed effective in the prevention of pneumococcal infections among all age groups. This has brought a notable reduction in the occurrence of infections due to *Streptococcus pneumoniae*.[1] The conjugated vaccines known as Prevnar (Wyeth Pharmaceuticals Inc, USA) have been effective in reducing of pneumococcal illnesses among immunized patients. The addition of antigens from 1, 3, 5, 6A, 7F and 19A serotypes led to the development of Prevnar 13 vaccine that has been licensed for adults.[2]

The Prevnar 13 vaccine contains antigens from13 serotype polysaccharides that are most commonly isolated from patients with pneumococcal infections. To date, up to 98 distinct pneumococcal serotypes have been identified as responsible for pneumococcal infections.[3] Thus, although developing a polysaccharide-based vaccine that covers antigens from all serotypes could potentially be the best option to protect against all infections caused by *S. pneumoniae*, this approach is neither economically nor technically feasible.[4] Recently, it has been found that serotype replacement following pneumococcal polysaccharide vaccination is challenging.[5] This event limits the usage of the vaccine and also increases the chance of nasopharyngeal carriages of serotypes that cannot be prevented by the vaccine.[6] The current vaccine non-preventable serotypes can cause pneumococcal diseases in both susceptible and immunized individuals. Besides, the serotype replacement remains a concern to the pneumococcal conjugate vaccines. For example, the use of PCV-7 conjugate vaccine that lacks the serotype 19A antigen was found to be associated with an increased carriage rate of serotype 19A.[7] Thus, the efficacy of PCV-7 conjugate vaccine is likely decrease, may be lead to increase in pneumococcal diseases.[8]

The protective effects of the current pneumococcal vaccines which are made of capsule polysaccharides remain controversial and incidence of pneumococcal diseases is increasing due to the non-encapsulated *S. pneumoniae*. Some reports have shown that the capsule based pneumococcal vaccines do not induce protective response against non-encapsulated strains.[9] This is why there is an urgent need for investigating some novel vaccine candidate against *S. pneumoniae* infection. Nowadays, the pneumococcal surface-exposed proteins have been proposed as new candidates for a pneumococcal vaccine.[10] These antigens do not have the limitations described above. Additionally, they should not be concerned by serotype replacement. Choline-binding proteins (Cbps) are surface-exposed proteins and some of them, such as CbpA, PspA or LytB, have demonstrated immunogenic potential in a mice models.[11] However, there is limited information about immunogenicity of other Cbps[12] such as CbpL (SPD_0579), CbpG (SPD_0356) and CbpM (SPD_1248).[13] CbpL is non-covalently attached to the phosphorylcholine of pneumococcal teichoic acids. This protein interacts with collagen, elastin and C-reactive protein suggesting a role in adhesion and immune evasion during the pneumococcal infection.[14] CbpG is an adhesin with putative serine protease activity involved in virulence in both mucosal colonization and sepsis.[15] No structural characterization has been reported about CbpM.[15] Therefore, this study aims to investigate the immunogenic features of CbpM, CbpG and CbpL recombinant proteins in a mouse model of pneumococcal infection.

MATERIALS AND METHODS

Characterization of Recombinant Proteins

Genomic DNA was extracted from exponential cultures of *S. pneumoniae* ATCC 49619 (serotype 19F; used as international reference for pneumococcal susceptibility testing) using QIAamp DNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions.[16] PCR amplification of *cbpG*, *cbpL* and *cbpM* genes was carried out using specific forward and reverse primers (Table 1).

The amplified products of *cbpG*, *cbpL* and *cbpM* were digested with the appropriate restriction enzymes (Fermentase, Germany), and ligated to pET21a vector (Novagen, USA) previously digested with the corresponding enzymes. The ligation products were transformed into competent *Escherichia coli* DH5α cells. Then, the recombinant vectors were purified using the QIAprep spin miniprep kit (QIAgen, USA). All the recombinant plasmids were sequenced to confirm the correct gene composition. Furthermore, the recombinant proteins were expressed in BL21 (DE3) which is ideal for use with bacteriophage T7 promoter-based expression systems.

Table 1. Oligonucleotide primer sets for gene amplification

Primers	Oligonucleotides (5' → 3')[a]	Restriction Enzyme	Amplicon size(bp)
cbpM-F	GCGCGC**CATATG**GTAAAAAGACGTATAAGGAGAGGG	*Nde*I	387
cbpM-R	GCGCGC**CTCGAG**ACGCACCCATTCACCATTATCATT	*Xho*I	
cbpG-F	GCGCGC**CATATG**AATGTAGAAGGGAGGGGAAGTGCG	*Nde*I	816
cbpG-R	GCGCGC**GAATTC**AATCCACTCACCAGATGAAGCGAAGTT	*Eco*RI	
cbpL-F	GCGCGC**GAATTC**ATGGAAGAAAACATCCATTTTTCGA	*Eco*RI	552
cbpL-R	GCGCGC**CTCGAG**ATGATCAATGGCATATTGAGCTG	*Xho*I	

[a] Artificial restriction sites have been underlined.

A single colony of *E. coli* harboring the corresponding recombinant plasmid was grown in LB broth containing ampicillin (100 µg/mL) overnight at 37°C with shaking. Afterwards, 200 µl of the culture were transferred into 5 mL of LB broth containing ampicillin (100 µg/mL) at 37°C. When the optical density at 600 nm (OD_{600}) reached 0.6, isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma-Aldrich, Germany) was added to a final concentration of 1mM, and cells were incubated for 4 h at 37°C with continuous shaking. Similar cultures were used as control without the addition of IPTG. Cultures were centrifuged at 5000×*g* for 5 min, and cells were resuspended in 100µl sample buffer and subjected to 12.5% SDS-PAGE.

This protocol was separately used for purification of CbpG, CbpL and CbpM recombinant proteins. The purification process was carried out by affinity chromatography. After overexpression of the recombinant proteins (see above), bacteria were centrifuged at 5000×*g* for 5 min, resuspended in solution buffer (300 mM NaCl, 50 mM NaH_2PO_4, pH8.0) and lysed by sonication on ice. The lysate was centrifuged (5000× g for 5 min), the supernatant was discarded, and the precipitate was dissolved in 5 mL of 8 M urea and loaded into Ni-NTA resin (Qiagen, Germany) column. The column was washed three times using washing buffers (i.e., 8 M urea pHs 7, 6.3 and 5.9). Finally, elution buffer (i.e., 8 M urea at pH: 4.5) was used to wash the column. The quality of protein purification process was evaluated by 12.5% SDS-PAGE.

Western blot was carried out using His-tag monoclonal antibody (anti-CbpL, anti-CbpM and anti-CbpG) conjugated to horseradish peroxidase (HRP) (Thermo Fisher Scientific Inc., USA). The recombinant proteins were electrophoresed in 12.5% SDS-polyacrylamide gels and transferred into PVDF membranes. Blots were blocked in PBS containing 3% skim milk and 0.05% Tween 20 at room temperature for 12h. Then, the blots were washed three times with PBS containing 0.05% Tween 20, and incubated with the His-tag monoclonal antibody conjugated to HRP (at 1:1000 dilution) for 1 h at 25°C. Finally, after washing three times with PBS containing 0.05% Tween 20, the blots were treated with 3, 3'-diaminobenzidine solution (Sigma-Aldrich, USA) for about 3 min.

Immunization of Mice with CbpM, CbpL and CbpG Recombinant Proteins

Six to eight weeks old female BALB/c mice were obtained from the Pasteur Institute of Iran and maintained in a pathogen-free facility. All experiments were conducted in accordance with animal protocols approved by the Institutional Animal Care and Use Committee. The mice were divided into 8 groups (4 mice in each group including CbpM, CbpG, CbpL, PBS, Adjuvant, CbpM+ adjuvant, CbpG+ adjuvant and CbpL+adjuvant groups. The mice in the CbpM, CbpG, CbpL groups were immunized with 30 µg of CbpM, CbpG, or CbpL recombinant antigens, respectively. The mice in the PBS and adjuvant groups received PBS and Freund's adjuvant (Razi Vaccine and serum Research Institute; Karaj, Iran), respectively. The groups of mice CbpM+ adjuvant, CbpG+ adjuvant and CbpL+ adjuvant were immunized with adjuvant +the 30 µg of the corresponding proteins. All eight groups of mice were subcutaneously immunized4 times at 14 days intervals and checked for antibody titers using blood samples obtained from each mouse-tail before each immunization.

Cytokine Assay

The mice were sacrificed two weeks after the last immunization according to the guidelines of animal ethics (IR.TUMS.SPH.REC.1396.2067). Then, their

spleens were obtained through abdominal incision. Red blood cells of spleen were removed by Ammonium-Chloride-Potassium buffer (NH4Cl (0, 8024 mg), KHCO3 (0, 1001 mg), EDTA (0.372 mg) 100 mL, pH 7.2) treatment and then, cells were washed and suspended in RPMI medium.

Approximately 2×10^6 cells which obtained from spleen cells were again suspended in RPMI medium and after 3 h stimulated by 20 µg/mL recombinant proteins. Finally, the samples were cultured in RPMI medium supplemented with fetal bovine serum (FBS) 10% for 72 h at 37°C under 5% CO_2 in a humidified incubator. After 72 h, supernatants from cell cultures were collected and maintained at -70°C until use. The supernatants were analyzed for IL-4 and IFN-Gamma cytokines using a commercial enzyme linked immunosorbent assay (eBioscience, San Diego, CA) according to the manufacturer's recommendations.

Antibody Titration

Mice serum samples were evaluated for antibody titers using an enzyme-linked immunosorbent assay (ELISA). The recombinant proteins (10 µg/mL) were first adsorbed in PBS into 96-well polystyrene plates (Greinerbio-one, Fricken-hausen, Germany) overnight at 4°C.The wells were washed three times with PBS containing 0.05 Tween20 and blocked with PBS containing 4% skim milk for 1 h at 25°C. Then, wells were washed three times with PBS containing 0.05% Tween20, pooled sera was added into the wells at dilution of 1:100 to 1:12800, and incubated for 1h at 25°C. Following this step, peroxidase-conjugated goat anti-mouse IgG (Cytomatin Gene Co, Isfahan, Iran)(diluted 1:2000) was added into the wells and incubated for 30 min at 25°C. TMB substrate solution (Cytomatin Gene Co, Isfahan, Iran) was added into the wells and the reaction was terminated after 10 min by adding 50 µl of 1N H_2SO_4.

Respiratory Burst Assay

The potential of hyper-immune sera in opsonophagocytosis of pneumococcus serotype 19F were assayed using flow cytometry. Two 1:100 and 1:400 dilutions from the hyper-immune sera of rabbits immunized with CbpM, CbpG and CbpL recombinant antigens were prepared. Then, 20 µL of the sera were heated at 56°C for 30 min to inactivate the complement components. Twenty microliters of pneumococcal suspension containing 10^3 colony-forming units (CFU) were added to each sample and incubated for 20 min at 37°C. The suspensions were mixed with 15 µl of rabbit serum obtained from the Razi Vaccine and Serum Research Institute of Iran as exogenous complement source and incubated again at 37°C for 10 min. After this step, 50 µL of heparinized rabbit blood was added to the suspension and incubated for 5 min. Subsequently, 5 µL of dihydrorhodamine 123(Sigma-Aldrich, USA) was added and the mixture incubated for 5 min at 37°C with Shaking. Finally, cell lysis was performed using the RBC lysing reagent (DAKO, Glostrup, Denmark) and the samples were analyzed by flow cytometry with gating the neutrophils as target cells. All samples were tested in duplicate.

Mice Challenge

Two weeks after the last protein injection the immunized mice and control groups were intraperitoneally challenged with *S. pneumoniae* ATCC 49619 (2×10^6 CFU). Twenty-four hours after infection, a blood sample was obtained from the tail vein of the mice and diluted serially into PBS, and then plated on blood agar plates to detect *S. pneumoniae* in blood samples. . This procedure was followed until 7 d after infection. Moreover, mice were checked daily for mortality until 14 d after infection. Then, the survivors were sacrificed for cytokine assay as mentioned above.

Passive Immunization

In order to evaluate the effectiveness of passive immunotherapy at first rabbits were used to obtain the corresponding antibodies for using in mice. In this regard, three male rabbits with weight of 2–3kg were purchased from the Pasteur Institute of Iran. All rabbits were subcutaneously immunized with Freund's adjuvant along with 50 µg CbpG, CbpM and CbpL recombinant proteins, 10 times at14 days intervals. Before each immunization the rabbits were checked for antibody titers using blood obtained by phlebotomizing the marginal ear vein of each rabbit. Antibody purification was carried out using Sepharose 4B protein A affinity column chromatography (GE Healthcare Life Science, Inc., USA).

To explore the efficacy of antibodies against CbpM, CbpL and CbpG in the protection of non-immune mice from pneumococcal sepsis, female BALB/c, 6–7 week old mice were employed. For each antibody, two doses of 10 and 100 µg/mL were used. Then, equal volumes

of bacterial suspension (2×10^6CFU) and two different antibody doses were purified from rabbit sera were administered intraperitoneally to four mice in each group. The CFU was counted and mixed at 1, 2, 3 and 7 days after bacterial challenge. The mortality rate of the mice in each group was also recorded.

Statistical Analyses

The Kruskal–Wallis test was employed to compare significant differences among different groups of mice. The groups with significant differences were further analyzed by post hoc tests. The significance level was decided at $p< 0.05$. All the statistical tests were done using the IBM SPSS Statistics for Windows, version 22 (IBM Corp., Armonk, N.Y., USA).

RESULTS

Protein Purification and Western Blot

Recombinant proteins expressed as inclusion body were solubilized and subsequently purified by Ni-NTA chromatography. Western blot analysis using anti-Histag antibody revealed positive bands with the recombinant proteins (Figures 1, 2).

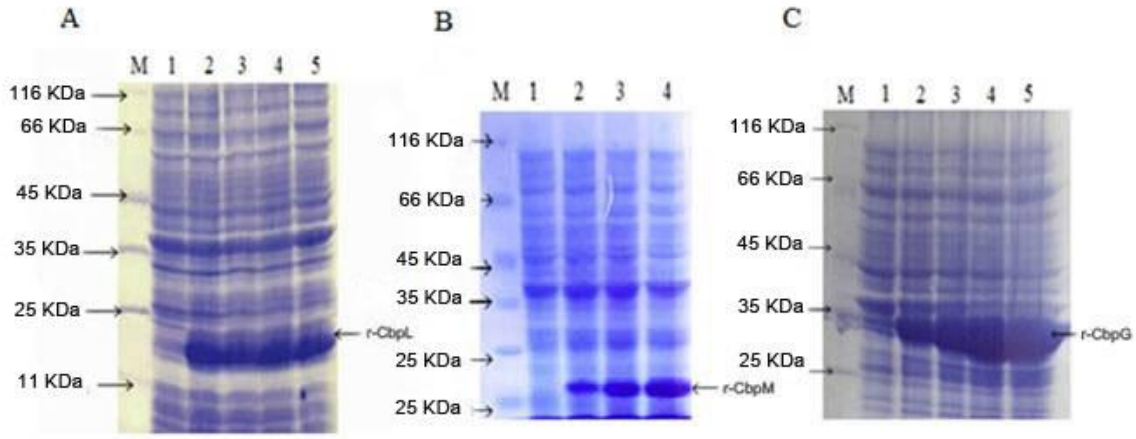

Figure 1. SDS-PAGE analysis of choline-binding proteins L (CbpL) (A), CbpM (B) and CbpG (C) recombinant proteins expression. Lane M: Protein molecular weight marker: 14.4-116 kDa; Lane 1: whole bacteria before induction; Lane 2 to Lane 4(5): whole bacteria 1-4h after induction by IPTG (isopropyl-β-D-thiogalactopyranoside).

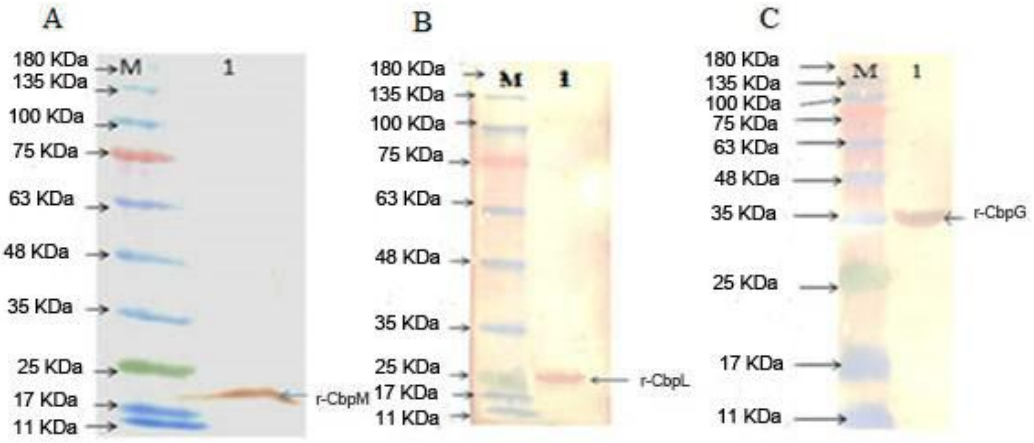

Figure 2. Western blot analysis of purified choline-binding proteins MCbpM (A), CbpL(B) and CbpG (C) recombinant proteins. Lane M: Protein size marker; lane 1: recombinant proteins revealed with His-Tag monoclonal antibody.

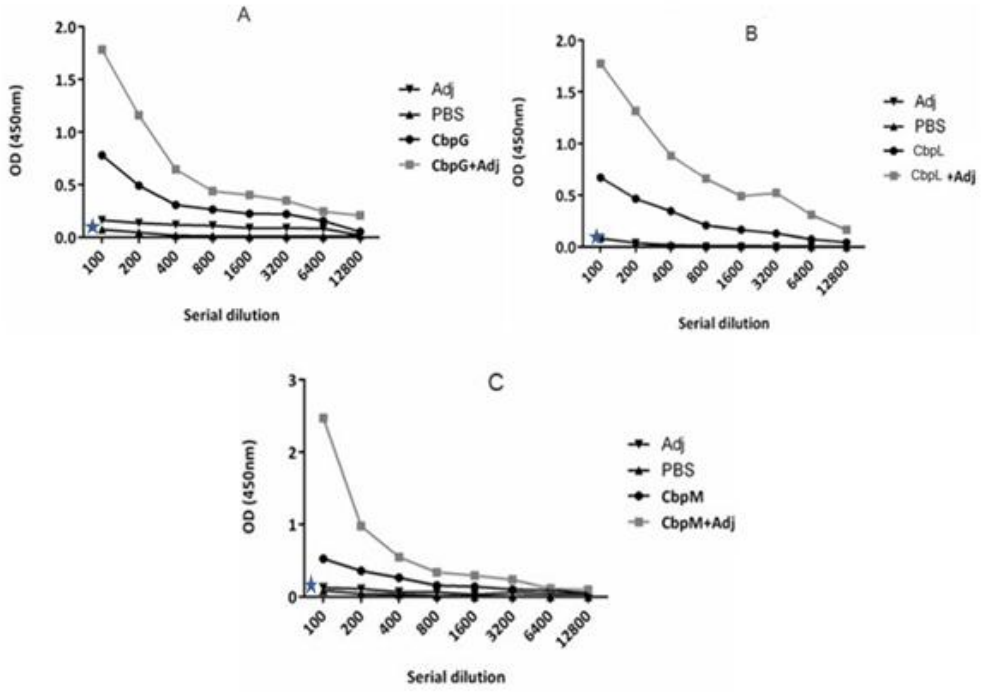

Figure 3. Titration of specific antibodies in sera of immunized mice. Serum samples of each mice group were collected 2 week after last immunization and pooled. The sera were serially diluted on recombinant protein coated plates. Different groups of mice each consisting of 4 mice were administrated with either the recombinant antigens choline-binding proteins G (CbpG) (A), CbpL (B) or CbpM (C) alone or in combination with Freund's adjuvant (+ Adj). Mice were injected with PBS or adjuvant (Adj) alone served as controls. The titers of specific antibodies were significantly high in mice *(p<0.05)*
***: Significant difference was observed between control group and recombinant antigens (*p*<0.05).**

Mice Immune Response to Antigens

Sera collected from immunized mice after the last booster injection were titrated in all groups of mice by antigen based ELISA. The highest titer of antibodies was obtained from the mice were 2.47µg/mL, 1.78 µg/mL and 1.77µg/ml for CbpM+adj, CbpL+adj, and CbpG+adj, respectively. Our results demonstrated that all three recombinant antigens induced a significant antibody response in mice when compared with the PBS and adjuvant alone control groups. The titers of specific antibodies were significantly higher in mice immunized with the combination of antigens and adjuvant compared to those immunized with the recombinant antigens or adjuvant alone (p<0.05) (Figure 3).

Survival Rate and Bacterial Clearance in Immunized Mice

BALB/c mice immunized with the recombinant CbpM (plus adjuvant) showed a statistically significant improvement (p<0.05) in survival rate after bacterial challenge with pneumococcus serotype 19F compared to the other mice groups (Figure 4). The clearance rates from pneumococcal bacteremia also indicated that immunization with CbpM, CbpG and CbpL recombinant proteins resulted in bacterial clearance within 96h after the challenge, although mice immunized with CbpM have been cleared at 48 h after infection (Figure 5). The comparison of immunized groups with each other and control groups showed a significant difference at 24 h with control groups (p<0.05), while no significant differences were seen among immunized groups (p>0.05).

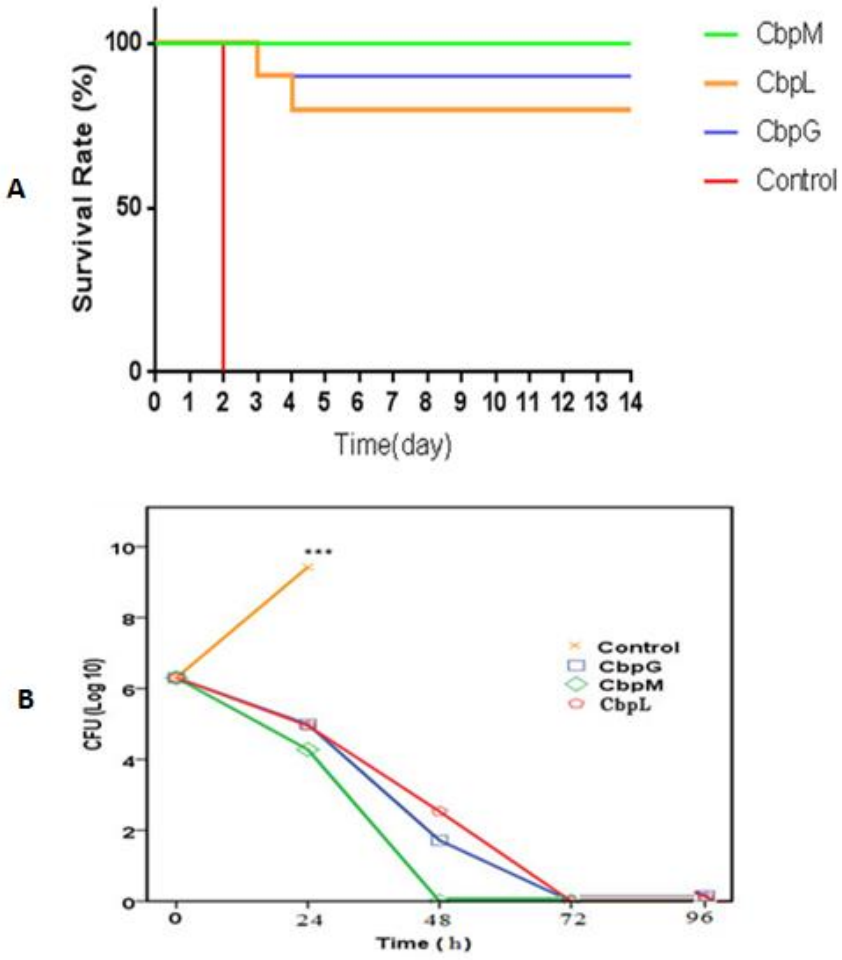

Figure 4. Survival rates (A) and Clearance rate(B) of mice challenged with a serotype 19F *S. pneumoniae* strain. The mice were intraperitoneally infected with 2×10^6 CFU of *Streptococcuspneumoniae* and CFUs in the blood were determined 24, 48, 72, and 96 h after challenge. Survival rate and bacterial clearance of each group was checked for 14 d and 4 d, respectively. There were statistically significant differences ($p<0.05$) between immunized mice and control groups

Cytokine Assay

A significant amount of IFN-Gamma cytokine was produced by the spleen of immunized mice whereas the IL-4 cytokine level was very low or negligible. There were statistically significant differences ($p<0.05$) between the groups immunized with antigens plus adjuvant and the groups that received only the antigens. The levels of IFN-Gamma in the challenged mice did not show statistically significant difference ($p>0.05$) with mice groups that were not challenged by pneumococci (Figure 6).

Phagocytic Activity of Recombinant Proteins

The anti-CbpG sera from hyper-immune rabbits showed statistically significantly higher respiratory burst ($p<0.001$) (82.3%) at a dilution of 1:100 than the CbpM (45.6%) and CbpL (12.2%) ($p>0.05$) sera groups. Again, a statistically significant difference ($p<0.001$) in respiratory burst potential at a dilution of 1:400 was observed among the anti-CbpL (9.35%), anti-CbpG (12.9%), anti-CbpM (14.7%) sera and control (normal serum) (<1%) groups (Figure 7).

The survival rate of the mouse group that received

100 µg/mL dose of anti-CbpM antibody was 50%, whereas that in the low dose category was 25%. The survival rate of the group that received anti-CbpG antibody was similar to that which received 100 µg/mL dose of anti-CbpM antibody. However, the survival rates of the group that received 10 µg/mL of anti CbpL antibody and another group that received 100 µg/mL dose of the same antibody were low (25%) (Figures 8 and 9).

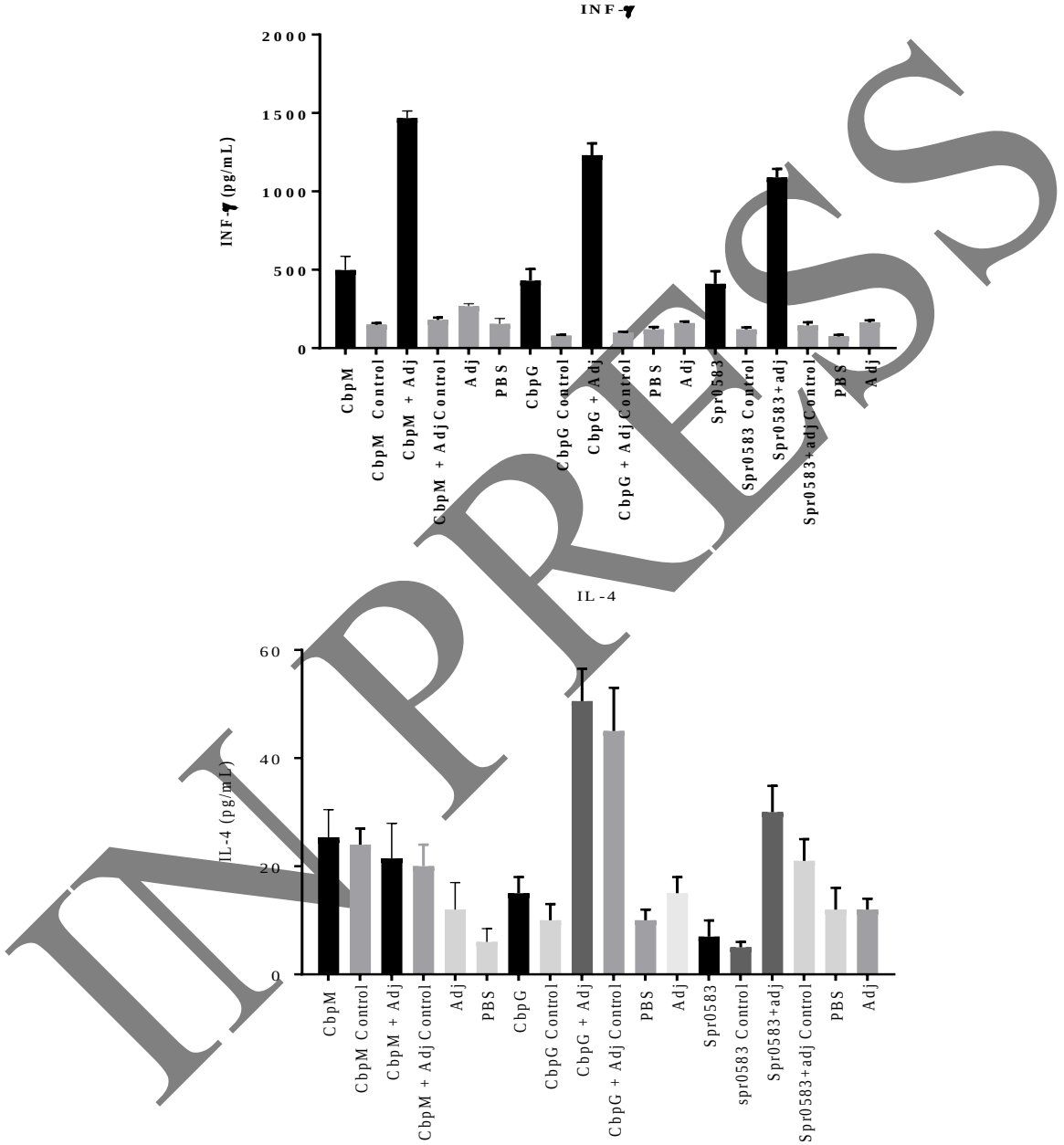

Figure 6. Levels of IL-4 and IFN-Gamma cytokines (pg/mL) in mice groups. Control groups did not stimulates by antigens. IL-4 cytokine level was very low. The levels of IFN-Gamma in the challenged mice did not show statistically significant difference (p>0.05) with mice groups that were not challenged by pneumococci
***: Significant difference was observed between control group and anti-CbpM (Choline-binding proteins M), anti-CbpG and anti-CbpL groups 1 d after pneumococcal challenge (p<0.05)**

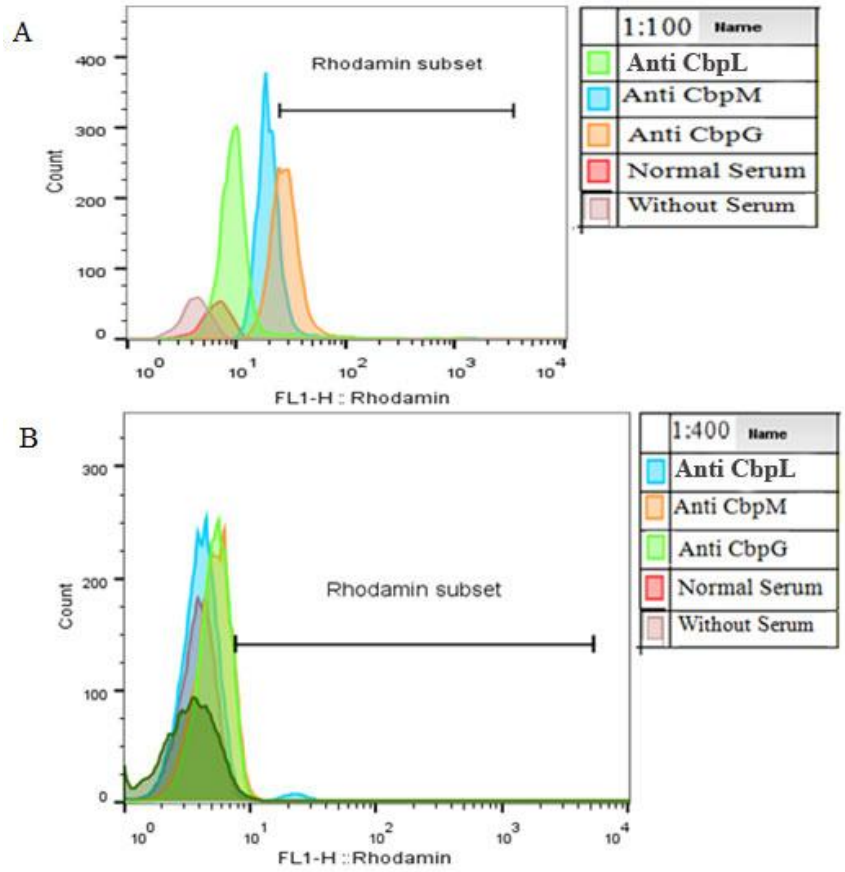

Figure 7. Flow cytometry analysis of opsonophagocytosis. (A) The assay with titer of 1:100 and (B) 1:400.
Passive immunity and pneumococcal challenge of BALB/c mice

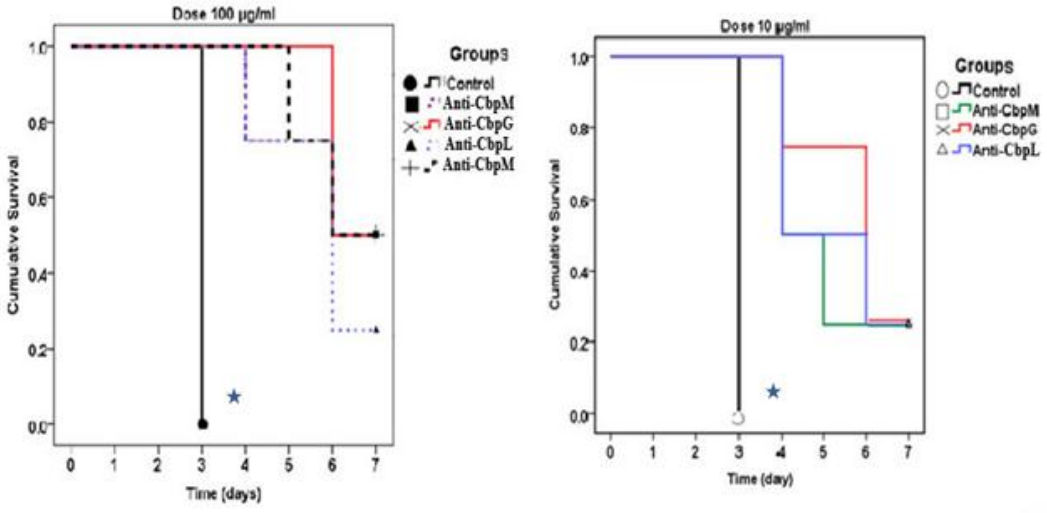

Figure 8. The Kaplan-Meier curve (cumulative survival) of BALB/c mice challenged with *S. pneumoniae*. Survival curves at 100 µg/mL (left panel) or 10 µg/mL (right panel). The cumulative survival rate was plotted against time.
***: Significant difference was observed between control group and other groups (p<0.05).**

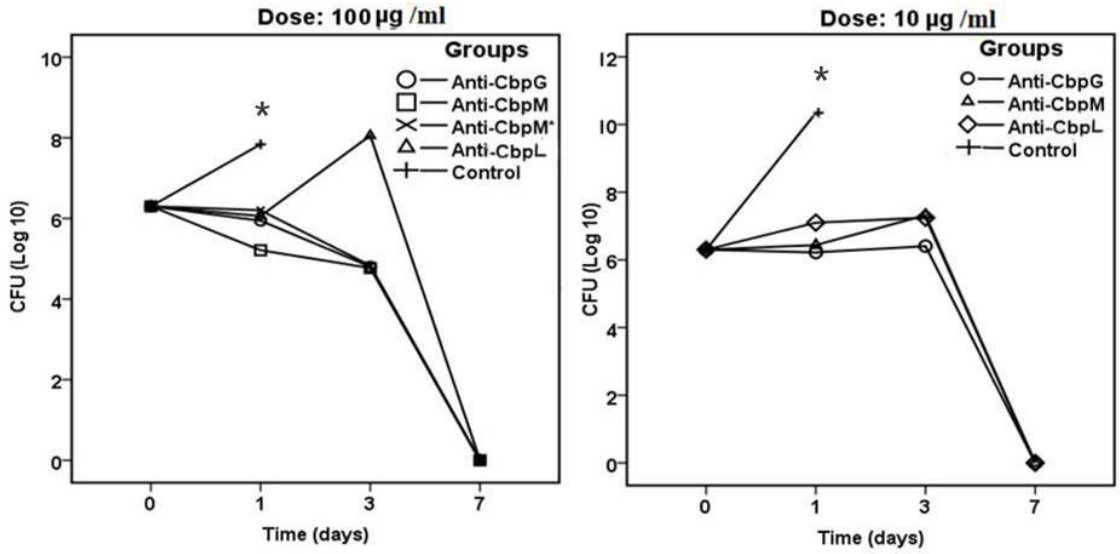

Figure 9. Pneumococcal clearance of mice receiving high (left) and low (right) doses of polyclonal antibodies. *: Significant difference was observed between control group and anti-CbpM (choline-binding proteins), anti-CbpG and anti-CbpL groups 1 d after pneumococcal challenge ($p<0.05$).

DISCUSSION

S. pneumoniae is an important cause of pneumonia, meningitis and otitis media. These infections resulted from the capacity of the bacteria to produce a polysaccharide capsule and various virulence factors that facilitate the spreading from the upper respiratory tract to the lower regions of the lungs.[17] Despite availability of three commercial pneumococcal vaccines, a meta-analysis revealed that the rate of the polysaccharide vaccine efficacy is low, especially among the elderly.[18] The pneumococcus has many proteins located on its cell wall that play major physiological and structural roles. These proteins include Cbps such as the pneumococcal surface proteins A (PspA and C also named CbpA) and hyaluronate lyase.[1] It has been reported that Cbps have high immunogenicity and protectivity in compared to other pneumococcal antigens.[19]

Our findings indicated that the proteins CbpG and CbpM provide high protection in mice challenged with a serotype 19Fpneumococcus.These results may indicate that Cbps are involved in early steps of the pneumococcal pathogenesis. Thus, in the absence of these proteins, pneumococci may not be able either to colonize or to attach to epithelia.[20] Another study reported that CbpA is an essential factor in the adherence and colonization process of pneumococci. Besides, the adherence to the nasopharynx epithelium was enhanced when it was accompanied by the PspA protein.[21]

The clearance test after pneumococcal challenge in our study showed that CbpM-immunized mice become pneumococcal-free pneumococci sooner than those immunized with either CbpG or CbpL. This indicated that there were high immunogenicity and surface-exposing features. The high immunogenicity could have been established for most of the CBPs but there was no such a report in previous studies. We found that CbpM protein bound to fibrinogen in our previous study,[12] which is an important protein in bacterial pathogenesis [3]. The high clearance activity of CbpM may be associated with the high immunogenicity, although this area requires further investigation. It has been shown that the Cbps bring hydrophobic and electrostatic features to the pneumococcal surface. This may facilitate adherence of the cocci to host cells via nonspecific interactions.[22] Thus, the existence of CbpM and other Cbps on the cell wall of the pneumococci may explain the high protectivity of the CbpM and other Cbps proteins. Furthermore, the high survival rate due to immunization of mice with Cbps has formerly

been reported. Consistent with our findings, CbpA and derivative pneumolysin (PdB) proteins are reported to have protected mice against the highly virulent pneumococcus D39 strain.[23] The production level of IFN-Gamma in the mice immunized by Cbps was higher than the IL-4. The high level production of IFN-Gamma might be due to the stimulation of Th-1 lymphocytes. The high level of antibody and Cbps functions could justify the fast clearance of pneumococci from mice blood. The high respiratory burst in results suggests that the opsonophagocytic activity of sera is affected by specific antibody concentration.

In this study, the CbpL protein, similar to the CbpM and CbpG, resulted in high level of antibody production. However, regarding to the results of opsonophagocytosis which have shown low level of opsonic activity, it is proposed that these proteins may not be adequately exposed to the antibodies. Other cause may be related to different in opsonic capacity of antibody subtypes. Hence, the IgM antibodies would be more effective than IgG antibodies in opsonizing bacteria.[24] The low level of passive immunity detected in the anti-CbpL groups had low survival rate and this was correlated with low opsonic activity of the sera. The passive immunity due to polyclonal antibodies indicates that the defense against pneumococci was obtained by humoral immunities. The effective of passive protection was also reported against pneumococcal pneumonia using sera from mice immunized with surface protein A (PspA).[25] These findings indicate that the passive immunity against pneumococcal infections could be an additional therapeutic tool for pneumococcal infections. Although, this research was carefully prepared, there were some limitations; because of the time limit, effects of cellular immunity, T cytokine and also bronchial challenge test did not evaluate and it would be better if it was done in a longer time.

Our findings demonstrated that recombinant proteins protect mice against challenge with a serotype 19F pneumococcal strain. Opsonophagocytosis assay shows adequate response of rabbit antibodies against CbpM and CbpG in high titers. According to our findings, choline binding proteins show efficient immunogenicity but due to the key role of humoral immunity in defense against pneumococcal infections and based on opsonophagocytosis findings, it seems antibodies against CbpG and CbpM effectively opsonize pneumococcus. To conclude, CbpG and CbpM can be used as suitable vaccine candidates, however; CbpL protein cannot appropriately stimulate immune system.

ACKNOWLEDGEMENTS

The TUMS has financially supported this study as part of PhD thesis and is recognized by the research grant Ref. No. 24744.We thank all the staffs of the Pathobiology department at TUMS for their technical assistance.

REFERENCES

1. Grabenstein J, Klugman K. A century of pneumococcal vaccination research in humans. Clin Microbiol Infect 2012; 18(5):15-24.
2. Gruber WC, Scott DA, Emini EA. Development and clinical evaluation of Prevnar 13, a 13-valentpneumocococcal CRM197 conjugate vaccine. Ann N Y Acad Sci 2012; 1263(1):15-26.
3. Guo B, Zhao X, Shi Y, Zhu D, Zhang Y. Pathogenic implication of a fibrinogen-binding protein of Staphylococcus epidermidis in a rat model of intravascular-catheter-associated infection. Infect Immun 2007; 75(6):2991-5.
4. Hausdorff WP, Brueggemann AB, Hackell JG, Scott AJ. Pneumococcal serotype epidemiology 2008;Washington, DC: ASM Press.
5. Weinberger DM, Malley R, Lipsitch M. Serotype replacement in disease after pneumococcal vaccination. *Lancet* 2011; 378(9807):1962-73.
6. Lipsitch M. Bacterial vaccines and serotype replacement: lessons from Haemophilus influenzae and prospects for *Streptococcus pneumoniae*. Emerg Infect Dis 1999; 5(3):336-45.
7. Hanage WP, Finkelstein JA, Huang SS, Pelton SI, Stevenson AE, Kleinman K, et al. Evidence that pneumococcal serotype replacement in Massachusetts following conjugate vaccination is now complete. Epidemics 2010; 2(2):80-4.
8. Aurelio B, Jesús C, Alberto GS, Luis T, José ANA, Fátima I, et al. Effectiveness of the 7-Valent Pneumococcal Conjugate Vaccine: A Population-Based Case-Control Study. Clin Infect Dis 2007; 44(11):1436–41.
9. Keller LE, Luo X, Thornton JA, Seo KS, Moon BY, Robinson DA, et al. Immunization with pneumococcal

surface protein K of nonencapsulated *Streptococcus pneumoniae* provides protection in a mouse model of colonization. Clin Vaccine Immunol 2015; 22(11):1146-53.

10. Darrieux M, Goulart C, Briles D, Leite LC. Current status and perspectives on protein-based pneumococcal vaccines. Crit Rev Microbiol 2015; 41(2):190-200.
11. Chen A, Mann B, Gao G, Heath R, King J, Maissoneuve J, et al. Multivalent pneumococcal protein vaccines comprising pneumolysoid with epitopes/fragments of CbpA and/or PspA elicit strong and broad protection. Clin Vaccine Immunol 2015; 22(10):1079-89.
12. Afshar D, Pourmand MR, Jeddi-Tehrani M, Saboor Yaraghi AA, Azarsa M, Shokri F. Fibrinogen and Fibronectin Binding Activity and Immunogenic Nature of Choline Binding Protein M. Iran J Public Health 2016; 45(12):1610-7.
13. Jedrzejas MJ. Pneumococcal virulence factors: structure and function. Microbiol Mol Biol Rev 2001; 65(2):187-207.
14. Gutierrez-Fernandez J, Saleh M, Alcorlo M, Gómez-Mejía A, Pantoja-Uceda D, Treviño MA, et al. Modular Architecture and Unique Teichoic Acid Recognition Features of Choline-Binding Protein L (CbpL) Contributing to Pneumococcal Pathogenesis. Sci Rep 2016; 6:38094.
15. Brown J, Hammerschmidt S, Orihuela C. *Streptococcus Pneumoniae*: Molecular Mechanisms of Host-Pathogen Interactions. Academic Press, 2015.
16. Long JP, Tong HH, DeMaria TF. Immunization with native or recombinant Streptococcus pneumoniae neuraminidase affords protection in the chinchilla otitis media model. Infect Immun 2004; 72(7):4309-13.
17. Kadioglu A, Weiser JN, Paton JC, Andrew PW. The role of *Streptococcus pneumoniae* virulence factors in host respiratory colonization and disease. Nat Rev Microbiol 2008; 6(4):288-301.
18. Mangtani P, Cutts F, Hall AJ. Efficacy of polysaccharide pneumococcal vaccine in adults in more developed countries: the state of the evidence. Lancet Infect Dis 2003; 3(2):71-8.
19. Ogunniyi AD, Grabowicz M, Briles DE, Cook J, Paton JC. Development of a vaccine against invasive pneumococcal disease based on combinations of virulence proteins of *Streptococcus pneumoniae*. Infect Immun 2007; 75(1):350-7.
20. Gosink KK, Mann ER, Guglielmo C, Tuomanen EI, Masure HR. Role of novel choline binding proteins in virulence of *Streptococcus pneumoniae*. Infect Immun 2000; 68(10):5690-5.
21. Balachandran P, Brooks-Walter A, Virolainen-Julkunen A, Hollingshead SK, Briles DE. Role of pneumococcal surface protein C in nasopharyngeal carriage and pneumonia and its ability to elicit protection against carriage of *Streptococcus pneumoniae*. Infect Immun 2002; 70(5):2526-34.
22. Swiatlo E, Champlin FR, Holman SC, Wilson WW, Watt JM. Contribution of choline-binding proteins to cell surface properties of *Streptococcus pneumoniae*. Infect Immun 2002; 70(1):412-5.
23. Ogunniyi AD, Woodrow MC, Poolman JT, Paton JC. Protection against *Streptococcus pneumoniae* elicited by immunization with pneumolysin and CbpA. Infect Immun 2001; 69(10):5997-6003.
24. Park S, Nahm MH. Older adults have a low capacity to opsonize pneumococci due to low IgM antibody response to pneumococcal vaccinations. Infect Immun 2011; 79(1):314-20.
25. Arulanandam BP, Lynch JM, Brile DE, hollingshead S, Metzgar DW. Intranasal vaccination with pneumococcal surface protein A and interleukin-12 augments antibody-mediated opsonization and protective immunity against *Streptococcus pneumoniae* infection. Infect Immun 2001; 69(11):6718-24.