



Fibrinogen and Fibronectin Binding Activity and Immunogenic Nature of Choline Binding Protein M

Davoud AFSHAR¹, *Mohammad Reza POURMAND^{1,2}, Mahmood JEDDI-TEHRANI³, Ali Akbar SABOOR YARAGHI¹, Mohammad AZARSA¹, Fazel SHOKRI⁴

1. Dept. of Pathobiology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran
2. Urology Research Center, Tehran University of Medical Sciences, Tehran, Iran
3. Monoclonal Antibody Research Center, Avicenna Research Institute, ACECR, Tehran, Iran
4. Dept. of Immunology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran

*Corresponding Author: Email: mpourmand@tums.ac.ir

(Received 22 Aug 2016; accepted 25 Oct 2016)

Abstract

Background: Choline-binding proteins (CBPs) are a group of surface-exposed proteins, which play crucial and physiological roles in *Streptococcus pneumoniae*. The novel member of CBPs, choline-binding protein M (CbpM) may have binding activity to plasma proteins. This study aimed to clone and express CbpM and demonstrate its interaction with plasma proteins and patients' sera.

Methods: The total length of *cbpM* gene was cloned in *pET21a* vector and expressed in BL21 expression host. Verification of recombinant protein was evaluated by Western blot using anti-His tag monoclonal antibody. Binding ability of the recombinant protein to plasma proteins and the interaction with patients' sera were assessed by Western blot and ELISA methods.

Results: The *cbpM* gene was successfully cloned into *pET21a* and expressed in BL21 host. Binding activity to fibronectin and fibrinogen and antibody reaction of CbpM to patients' sera was demonstrated by Western blot and ELISA methods, respectively.

Conclusion: CbpM is one of the pneumococcal surface-exposed proteins, which mediates pneumococcal binding to fibronectin and fibrinogen proteins.

Keywords: *Streptococcus pneumoniae*, Choline-binding protein M, Fibrinogen, Fibronectin

Introduction

Streptococcus pneumoniae is the major cause of life-threatening diseases such as pneumonia, otitis media, bacteremia and meningitis worldwide. Despite causing numerous clinical diseases, only a few virulence factors i.e. surface proteins, cell wall and capsule components have so far been described (1).

The cell wall of *S. pneumoniae* is composed of peptidoglycan with teichoic acid attached to N-acetylmuramic acid. Structurally, teichoic acid is composed of N-acetyl-d-galactosaminyl repeats, which commonly carry two phosphocholine resi-

dues. Moreover, there are ribitol phosphate and tetrasaccharide containing d-glucose and 2-acetamido-4-amino-2, 4, 6-trideoxy-d-galactose residues. Pneumococci cannot synthesize choline required for the formation of these structures, and it is therefore considered as an essential growth factor (2). Additionally, choline residues of teichoic acid are thought to have an essential role in pneumococcal biofilm formation (3). Choline binding proteins (CBPs), a family of surface proteins, contain a number of short choline-binding repeats (consisting of 2 to 10 repeats of a

20-amino-acid sequence), which bind to choline in the teichoic and lipoteichoic acids in the pneumococcal cell wall (4).

These surface-exposed proteins play a crucial role in *Pneumococcus* pathogenesis and are therefore desirable as new vaccine candidates instead of pneumococcal polysaccharide vaccines as previously described (5). Currently, about twenty choline-binding proteins have been documented, and this number is constantly increasing. Several members of this protein family have been studied, and some unique and common properties have been characterized for each of them. CbpA, a well-characterized choline binding protein, has an elongated structure with α -helical N-terminal region consisting of three distinct domains (6). This protein is involved in development of invasive disease and has a highly protective nature following vaccination (7, 8).

The results of DNA sequencing showed that the *cbpA* coding locus exists in all tested virulent strains of *S. pneumoniae*. However, the locus encodes CBPs with allelic variation in the anchor region, which results in different sizes and compositions (9). Other CBPs have also been characterized, and there is valuable information about their structure and properties (10). CbpD, CbpG, CbpF and CbpE are important choline binding proteins with enzymatic functions. CbpD, CbpE as well as LytA, B and C may be associated with hydrolase activity. There is obvious homology in their C-terminus where the choline-binding domains are located (11). CbpG is a serine protease with multifunctional properties in different forms. This protein cleaves extracellular matrix of the host in the cell-attached or secreted forms, and shows adhesive properties in the cell-attached form. It is also required for pneumococcal virulence, colonization and invasive disease and may be as an effective vaccine (12). Recently, several choline-binding proteins such as CbpM, CbpK, CbpL and CbpF have also been reported, and their properties have been predicted bioinformatically (13). CbpM is a short 14.4 KDa choline binding protein with 129 amino acids. To the best of our knowledge, there is a limited data about this choline binding protein.

In this study, we attempted to clone and express CbpM and demonstrate its interaction with plasma proteins and patients' sera.

Materials and Methods

Recombinant protein expression and purification

Genomic DNA from overnight-cultured *S. pneumoniae* (ATCC 49619) was extracted using QIAamp DNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. The coding sequence of *cbpM* gene was amplified by PCR using the following specific forward and reverse primers carrying *Nde*-I and *Xho*-I restriction sites (underlined), respectively:

- 1) F-5'- GCGCGCCATATGGTAAAAA-GACGTATAAGGAGAGGG-3'
- 2) R: 5'- GCGCGCCTCGAGACGCACC-CATTCACCATTATCATT-3'.

The resulting PCR product was digested with *Nde*-1 and *Xho*-1 restriction enzymes (Fermentase, Germany) and ligated into a *pET21a* vector (Novagen, USA), which was pre-digested with the same enzymes. The ligation product was transformed into competent *Escherichia coli* DH5 α cells, and recombinant colonies were screened by colony PCR using T7 primers. Finally, recombinant vector was sequenced after extraction to confirm the correct gene composition.

The recombinant protein was expressed in BL21 (DE3) host strain. Briefly, a colony of *E. coli* harboring constructed *pET21a* was cultured on Luria-Bertani (LB) broth containing ampicillin (1 μ g/ml) and incubated at 37 °C for 24 h while shaking. After this step, 200 μ l of overnight culture was transferred into 5 mL of LB broth containing ampicillin (1 μ g/ml) at 37 °C. When OD₆₀₀ reached 0.6, isopropyl- β -D-thiogalactopyranoside (IPTG) (Sigma-Aldric, USA) was added to a final concentration of 1mM, and the cells were grown for 4 hours at 37 °C with continuous shaking. Similar cultures were used as control without the addition of IPTG. The bacterial cultures were transferred to centrifuge bottles and centrifuged at 5000g for 5 min. The pellet and supernatant were transferred to

separate tubes, and the cell pellets were resuspended in 100 µl SDS-PAGE sample buffer and were subjected to 12.5% SDS-PAGE. Purification of recombinant protein was performed using affinity chromatography under denaturing condition. Briefly, after overexpression of BL21 (DE3) in 250 ml LB broth (Merck, Germany) containing ampicillin, the bacterial culture was centrifuged at 5000g for 5 minutes and plate lysed by sonication on ice in solution buffer (300 mM NaCl, 50 mM NaH₂PO₄, pH:8.0). Following centrifugation at 5000g for 5 minutes, supernatant was removed and precipitate solved in 5ml of urea 8M. The Ni-NTA resin (Qiagen, Germany) was packed into a column and the denatured lysate gently loaded. Column was washed three times with washing buffers (urea 8M with different pHs (7, 6.3 and 5.9) and the eluted fractions (urea 8M pH: 4.5) were dialyzed overnight against Phosphate-buffered saline (PBS). Quality of recombinant protein purification was evaluated by 12.5% SDS-PAGE method.

Confirmation of recombinant protein

The purified recombinant protein was electrophoresed on 12.5 % SDS-PAGE and was subsequently transferred to nitrocellulose membrane (Millipore, Bedford, USA). The membrane was blocked with 3% blocking buffer (PBS-1X containing 3% skim milk) at room temperature for 12 h and washed three times with washing buffer (PBS-1X containing 0.05% Tween 20). The nitrocellulose membrane was incubated with His-tag monoclonal antibody conjugated to HRP(Thermo Fisher Scientific Inc., USA) at 1:1000 dilution for 1h at 25°C and was washed three times with washing buffer. Finally, the membrane was treated with 3, 3-diaminobenzidine solution (Sigma-Aldrich, USA) for ~3 min.

Interaction with plasma proteins

In order to determine unambiguously whether CbpM is a plasma binding protein, purified CbpM (10 µg/ml) was electrophoresed on a 12.5% SDS-PAGE gel as described above, transferred to a nitrocellulose membrane (Millipore, Bedford, USA) by electroblotting, and blocked in TBS-T containing 3% (wt/vol) Skim milk

(Merck, Germany). Then, biotinylated fibrinogen and fibronectin were used to probe the membrane (at a concentration of 2 µg/ml in washing buffer). The bound ligands were detected by alkaline phosphatase-conjugated streptavidin (Sigma-Aldrich, USA) at a dilution of 1:1000 and NBT/BCIP solution (Roche, Germany). Pneumococcal Spr1754 and staphylococcal ScaA proteins were used as negative and positive controls, respectively (14).

Reactivity of serum antibody with recombinant CbpM protein by ELISA

Ten serum samples were obtained from patients with pneumococcal pneumonia hospitalized at Loghman-Hakim hospital affiliated to Shahid Beheshti University of Medical Sciences (2014-2015). Recombinant CbpM protein (10 µg/well) was adsorbed in PBS 1x buffer into 96-well polystyrene plates (Greinerbio-one, Frickenhausen, Germany) overnight at 4°C. The plate was then washed three times with washing buffer containing 0.05 Tween 20 and blocked with blocking buffer containing 4% skim milk for 1h at room temperature. The plate was washed three times with washing buffer and sera added into wells at dilutions of 1:10, 1:100 and 1:250 and incubated for 1h at room temperature. After wash step, goat anti-human Ig peroxidase-conjugated (Cytomatin Gene Co, Isfahan, Iran) with a dilution of 1:4000 was added into microplate well and incubated for 30 minutes. Finally, TMB (Cytomatin Gene Co, Isfahan, Iran) as substrate was added into wells and the reaction was stopped after 10 min by the addition of 50 µl of 1N H₂SO₄. Two serum samples obtained from healthy individuals were used as negative controls.

Results

Cloning, expression and purification of cbpM gene

The expected amplicon was obtained after PCR assay, and was successfully ligated in pET21a vector following digestion with *Nde*-1 and *Xho*-1 restriction enzymes. The accuracy of cloned target was confirmed after double digestion and se-

quencing stages. The sequence of *cbpM* was submitted to GenBank (Sequence ID: KJ668600.1), and the result of double digestion is presented in Fig.1.

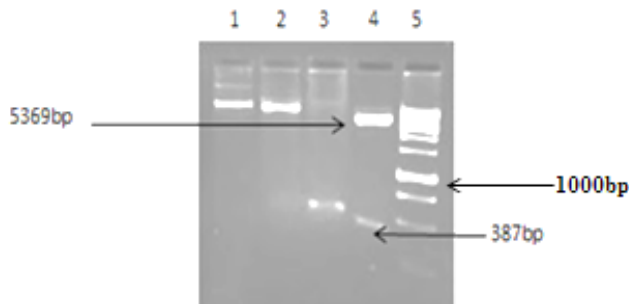


Fig. 1: Electrophoresis of digested recombinant vector and PCR product on agarose gel (1%). Lane 1 and 2 digested vectors with *Xho*-1 and *Nde*-1 restriction enzymes, respectively; lane 3: *cbpM* PCR product (387bp) and lane 4 is double digest of recombinant vector. Lane 5: DNA Marker (1Kb)

As shown in Fig. 2, recombinant protein was successfully expressed in BL21 (DE3) expression host, and the insoluble protein was obtained.

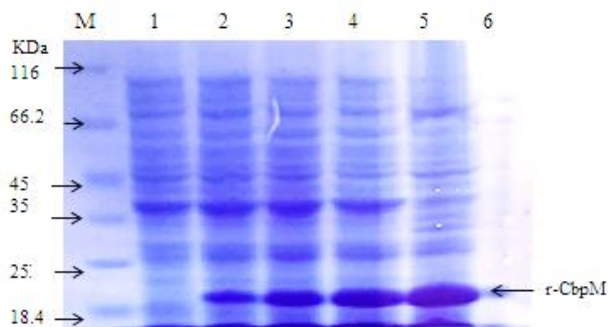


Fig. 2: Identification and analysis of the recombinant protein by SDS-PAGE (12.5%). Lane M: Protein molecular weight marker: 14.4-116 kDa; Lane 1: whole bacteria before induction; Lane 2 to Lane 4: whole bacteria 1, 4 and 8h after induction; Lane 5: Pellet of the sonicated lysate, 6: Supernatant of the sonicated lysate

The best expression condition was obtained at 4h at 37°C after IPTG addition. The recombinant protein was purified with high purity using Ni-

NTA column (Fig. 3). The His-tagged recombinant CbpM protein was confirmed using Anti-His-antibody (Fig. 4).

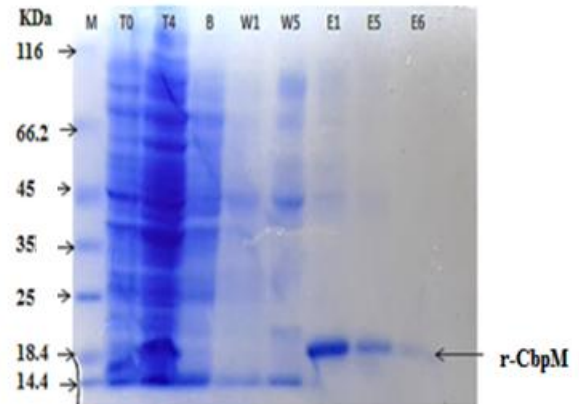


Fig. 3: SDS-PAGE analysis of purified recombinant protein. Lane M: protein molecular weight marker 14.4-116Kda, lane T0: un-induced BL21DE3, lane T4: induced BL21DE3, lane B: binding stage, lane W1: first wash, lane W5: fifth wash, lanes E1, E5 and E6 are elution stages

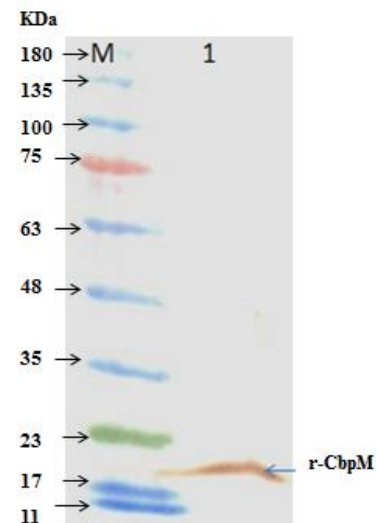


Fig. 4: Western blot analysis of purified recombinant protein CbpM. Lane M: Protein size marker (Sinacolon, Iran); lane 1: Recombinant protein western blotting with His-Tag monoclonal antibody

Interaction of recombinant CbpM protein with fibronectin and fibrinogen

Interaction of recombinant CbpM protein with plasma proteins was evaluated using Western blot and the results showed that this recombinant protein as well as ScaA protein (positive control) has positive reaction with fibronectin and fibrinogen proteins (Fig. 5 and 6).

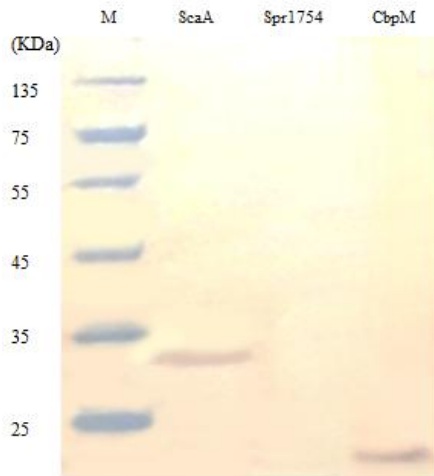


Fig. 5: Analysis of interaction between CbpM and fibronectin proteins. Lane M: stained protein marker (Takapozist, Iran); Spr1754 (36KDa) and ScaA (32KDa) proteins are negative and positive controls, respectively. CbpM has positive interaction with fibrinogen

Reactivity of recombinant CbpM protein with serum antibody from pneumococcal patients

The ELISA results showed that seropositive rate for CbpM antigen was 100% among pneumococcal patients (Fig. 7). The ELISA cut-off value was calculated as the mean +2SD of OD values from control sera, which was 0.004 and so the serum with $OD \geq 0.004$ considered as positive sera.



Fig. 6: Analysis of interaction between CbpM and Fibronectin proteins. Lane M: stained protein marker (Takapozist, Iran); Spr1754 (36KDa) and ScaA (32KDa) proteins are negative and positive controls, respectively. CbpM has positive interaction with fibronectin

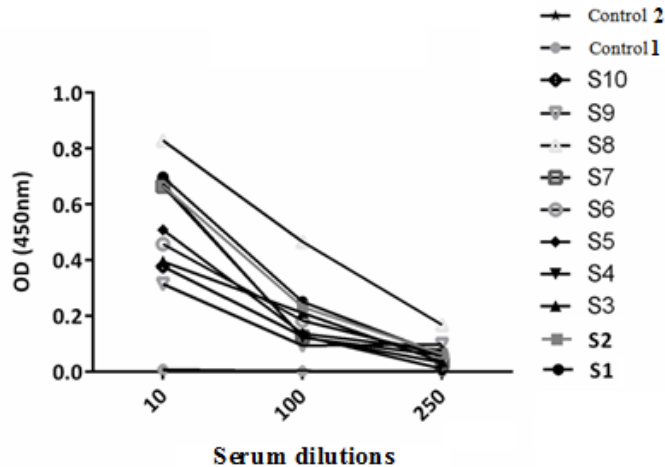


Fig. 7: Anti-CbpM titers in patients' sera with pneumococcal infection. S1toS10 represent patient's sera. Control groups are sera obtained from healthy individuals

Discussion

Choline binding proteins are unique surface-exposed proteins in *S. pneumoniae* that contribute to many physiological functions. In silico analysis of CbpM with 129 amino acid sequence, shows that it is composed of four different domains. Two CW repeats (cell wall binding repeats) each 20 amino acid residues long located in the middle of protein. These repeats are characterized by conserved aromatic and glycine residues found in multiple tandem copies in a number of proteins (15). These repeats are responsible for the specific recognition of choline-containing cell walls in Streptococcus phage Cp-1. The second domain is a region of about 63 amino acids composed of three shorter repeats. These repeats bind with the cell wall because they are choline-binding proteins. The last region is YG repeats that seems to be a Glucan-binding domain. Regarding these information, CbpM lacks a CHAP (cysteine, histidine-dependent amidohydrolase/peptidase) domain, which is associated with many functions mainly in peptidoglycan amidases (16).

A major focus of this study was to determine whether CbpM reacts with the sera obtained from pneumococcal patients and binds to fibrinogen and fibronectin as plasma proteins. As indicated in the results section, CbpM showed a binding activity to fibronectin and fibrinogen. These data were consistent with other publications, which evaluated other surface-exposed proteins of gram-positive bacteria (17). The molecular interaction between plasma proteins and streptococci has been recognized from long time ago. The main purpose of such studies has been the principle that pathogenic streptococci bind to some plasma proteins, especially fibrinogen. There are some publications about bacterial fibrinogen binding proteins. However, most of these studies have focused on bacterial proteins other than pneumococcal proteins. Shannon et al showed that *Staphylococcus aureus* produces an extracellular fibrinogen binding protein, which binds to platelets and inhibits platelet aggregation. This interaction is a function of Efb binding

to fibrinogen, which is naturally found on the surface of activated platelets (18).

In the present study, CbpM also showed a binding property to fibronectin. This is an abundant glycoprotein found in human serum and extracellular matrix, which is involved in cell adhesion processes. Fibronectin binding ability is a cardinal property of streptococci and involved in streptococcal attachment to epithelial cells (19). This protein was also associated with the invasion to human epithelial cells by group A streptococci (20). To date, two Fibronectin-binding proteins, PavA and SP0082 have been characterized in *S. pneumoniae* strain TIGR4 (21). PavA is one of the pneumococcal adhesions for fibronectin; however, Holmes et al. reported that 50% of fibronectin-binding activity is mediated by PavA protein and this activity remains even in pneumococcal PavA-mutants (22), the fact suggesting the presence of another fibronectin-binding molecule such CbpM protein on *S. pneumoniae*. Although there are limited literatures about pneumococcal CBPs interaction with fibronectin, however other plasma protein interactions with pneumococcal surface-exposed proteins were also studied. It has been shown that *S. pneumoniae* adheres and invades host cells through the integrin route by recruitment of a multimeric form of vitronectin (23). Bergmann et al. studied the interaction between pneumococci and vitronectin, and found that this property is lost following proteolytic treatment of the bacterial surface, a fact suggesting the proteinaceous nature of vitronectin-binding factor (24). In line with these findings, elimination of choline-binding proteins from the pneumococcal surface causes decreased interaction between vitronectin and the bacterium (23). However, other factors may be involved in binding of plasma proteins to pneumococcal surface antigens. CbpM interacts with CRP and weakly binds to elastin in a dose-response state (13). From a bioinformatics point of view, CbpM is associated with pneumococcal choline binding proteins and is considered to be a surface-exposed protein. Due to lack of a LPXTG motif, it seems that CbpM is anchored to cell wall with ionic bond (25).

In the present study, CbpM reacted with sera obtained from patients with pneumococcal infections but not normal subjects, the fact that suggests it as a novel pneumococcal vaccine candidate, although a comprehensive study would be needed to reveal its nature.

Conclusion

CbpM showed positive reaction in interaction with plasma proteins and patients sera. Considering the results of this study and bioinformatic analysis, CbpM seems to be involved in pneumococcal attachment to epithelial cells via glucan binding activity. Hence, this recombinant protein needs more evaluation from immunological point of views.

Ethical Considerations

All ethical issues including plagiarism, Informed Consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc. have been completely observed by the author.

Acknowledgements

This study was supported by Tehran University of Medical Sciences under research grant 24744 as part of PhD thesis. We thank all coworkers in the Urology Research Center at Sina Hospital for their technical assistance. The authors declare that there is no conflict of interest.

References

1. Jedrzejewski MJ (2001). Pneumococcal virulence factors: structure and function. *Microbiol Mol Biol Rev*, 65(2):187-207.
2. Yother J, Leopold K, White J et al (1998). Generation and properties of a *Streptococcus pneumoniae* mutant, which does not require choline or analogs for growth. *J Bacteriol*, 180(8):2093-101.
3. Moscoso M, Garcia E, Lopez R (2006). Biofilm formation by *Streptococcus pneumoniae*: role of choline, extracellular DNA, and capsular polysaccharide in microbial accretion. *J Bacteriol*, 188(22):7785-95.
4. Kausmally L, Johnsborg O, Lunde M et al (2005). Choline-binding protein D (CbpD) in *Streptococcus pneumoniae* is essential for competence-induced cell lysis. *J Bacteriol*, 187(13):4338-45.
5. Feldman C, Anderson R (2014). Review: current and new generation pneumococcal vaccines. *J Infect*, 69(4):309-25.
6. Luo R, Mann B, Lewis WS et al (2005). Solution structure of choline binding protein A, the major adhesin of *Streptococcus pneumoniae*. *EMBO J*, 24(1):34-43.
7. Orihuela CJ, Gao G, Francis KP et al (2004). Tissue-specific contributions of pneumococcal virulence factors to pathogenesis. *J Infect Dis*, 190(9):1661-69.
8. Ogunniyi AD, Woodrow MC, Poolman JT et al (2001). Protection against *Streptococcus pneumoniae* elicited by immunization with pneumolysin and CbpA. *Infect Immun*, 69(10):5997-6003.
9. Iannelli F, Oggioni MR, Pozzi G (2002). Allelic variation in the highly polymorphic locus *pspC* of *Streptococcus pneumoniae*. *Gene*, 284(1):63-71.
10. Gosink KK, Mann ER, Guglielmo C et al (2000). Role of novel choline binding proteins in virulence of *Streptococcus pneumoniae*. *Infect Immun*, 68 (10):5690-95.
11. Hammerschmidt S (2006). Adherence molecules of pathogenic pneumococci. *Curr Opin Microbiol*, 9(1):12-20.
12. Mann B, Orihuela C, Antikainen J et al (2006). Multifunctional role of choline binding protein G in pneumococcal pathogenesis. *Infect Immun*, 74 (2):821-29.
13. Frolet C, Beniazza M, Roux L et al (2010). New adhesin functions of surface-exposed pneumococcal proteins. *BMC Microbiol*, 10(1):190.
14. Ramina Mahboobi, Davoud Afshar, Mohammad Reza Pourmand, Rahil Mashhadi (2016). Autolytic activity and plasma binding study of Aap, a novel minor autolysin of *Streptococcus pneumoniae*. *Acta Med Iran*, 54(3):196-200.
15. Marchler-Bauer A, Derbyshire MK, Gonzales NR et al (2015). CDD: NCBI's conserved domain database. *Nucleic Acids Res*, 43:D222-26.

16. Bateman A, Rawlings ND (2003). The CHAP domain: a large family of amidases including GSP amidase and peptidoglycan hydrolases. *Trends Biochem Sci*, 28(5):234-37.
17. Mitchell J, Tristan A, Foster TJ (2004). Characterization of the fibrinogen-binding surface protein Fbl of *Staphylococcus lugdunensis*. *Microbiology*, 150(11):3831-41.
18. Shannon O, Flock JI (2004). Extracellular fibrinogen binding protein, Efb, from *Staphylococcus aureus* binds to platelets and inhibits platelet aggregation. *Thromb Haemost*, 91(4):779-89.
19. Hasty D, Ofek I, Courtney H, Doyle R (1992). Multiple adhesins of streptococci. *Infect Immun*, 60(6):2147-52.
20. Molinari G, Rohde M, Guzmán CA, Chhatwal GS (2000). Two distinct pathways for the invasion of *Streptococcus pyogenes* in non-phagocytic cells. *Cell Microbiol*, 2(2):145-54.
21. Bumbaca D, Littlejohn JE, Nayakanti H, Rigden DJ, Galperin MY, Jedrzejewski MJ (2004). Sequence analysis and characterization of a novel fibronectin-binding repeat domain from the surface of *Streptococcus pneumoniae*. *Omicron*, 8(4):341-56.
22. Holmes AR, McNab R, Millsap KW, Rohde M, Hammerschmidt S, Mawdsley JL, et al (2001). The pavA gene of *Streptococcus pneumoniae* encodes a fibronectin-binding protein that is essential for virulence. *Mol Microbiol*, 41(6):1395-408.
23. Voss S, Hallstrom T, Saleh M et al (2013). The choline-binding protein PspC of *Streptococcus pneumoniae* interacts with the C-terminal heparin-binding domain of vitronectin. *J Biol Chem*, 288(22):15614-27.
24. Bergmann S, Lang A, Rohde M et al (2009). Integrin-linked kinase is required for vitronectin-mediated internalization of *Streptococcus pneumoniae* by host cells. *J Cell Sci*, 122(2):256-67.
25. Kelley LA, Sternberg MJ (2009). Sternberg MJ Protein structure prediction on the Web: a case study using the Phyre server. *Nat Protoc*, 4(3):363-71.