



# The Open Microbiology Journal

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## RESEARCH ARTICLE

### High Titer of Antibody Against Pneumococcal IgA1 Protease in Healthy Individuals

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#### Abstract:

##### Background and Objectives:

Considering rising antibiotic resistance in various strains of *Streptococcus pneumoniae*, there is a need to find new immunogenic candidates for developing pneumococcal vaccines. Immunoglobulin A1 (IgA1) protease is one of the virulence factors playing an important role in the pathogenesis of *S. pneumoniae* infections. In the present study, we aimed to evaluate the titer of antibody against pneumococcal recombinant IgA1 protease in the serum of healthy humans.

##### Materials and Methods:

A part of the IgA1 protease gene (705 bp) from *S. pneumoniae* ATCC 49619 was amplified by PCR and then digested using restriction enzymes and ligated by the pET28a expression vector. The recombinant protein was expressed in *E. coli* BL21 strain. Affinity chromatography was used to purify the protein. The titer of antibody against the recombinant protease was determined in healthy individuals in three age groups of <2, 2-40, and > 40 years using indirect Enzyme-Linked Immunosorbent Assay (ELISA).

##### Results:

The expression and purification of the IgA1 recombinant protease were successful. The concentration of the purified protein was determined as 1.013 mg/ml using the NanoDrop method. The titer of anti-recombinant IgA1 protease antibody (20, 40, 80 and 160) showed a significant correlation with age (p-value<0.05). According to our results, the antibody titer was desirable, especially in individuals over two years old.

##### Conclusion:

In the present study, desirable antibody titers against the pneumococcal recombinant IgA1 protease were seen in the three groups' serum of healthy individuals. However, a significant correlation was not totally observed among groups.

**Keywords:** IgA1 protease, *Streptococcus pneumoniae*, Vaccine candidate, IgA1 protease, NanoDrop method, Pneumococcal surface protein A (PspA).

#### Article History

Received: March 12, 2020

Revised: July 16, 2020

Accepted: July 18, 2020

## 1. INTRODUCTION

*Streptococcus pneumoniae* is a Gram-positive and extracellular invasive bacterium colonizing mucosal surfaces of upper airways in carriers and patients [1, 2]. The bacterium is an important cause of global morbidity and mortality, inflicting more deaths than any other bacterial infection. It causes mild respiratory tract infections such as otitis media and sinusitis to more severe diseases such as pneumonia, septicemia and meningitis [3, 4]. The main virulence factors involved in the

pathogenesis of pneumococcal infections include Capsular Polysaccharide (CPS), Pneumolysin (Ply), Pneumococcal surface protein A (PspA), Choline-binding protein A (CbpA), CbpE, autolysin LytA, Immunoglobulin A1 (IgA1) protease, ZmpB, ZmpC, lipopeptides, lipoteichoic acid, and peptidoglycan fragments [5].

Over the past decades, the resistance of *S. pneumoniae* to various antimicrobial agents such as penicillins has increased the successful treatment of the infection [6]. The best approach to reduce the mortality rate and treatment costs of *S. pneumoniae* infections seems to be designing an appropriate vaccine to prevent the disease. The bacterial polysaccharide

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capsule, which has been used to develop pneumococcal vaccines, is one of the most important antigenic determinants of this bacterium. There are currently two types of vaccines available for the disease: polysaccharide and conjugate vaccines. The polysaccharide vaccine, known as Pneumovax 23, consists of purified capsular antigens derived from 23 bacterial serotypes. The recent vaccine, however, is not effective in younger children. Unlike the Pneumovax 23, conjugate vaccines such as Prevnar13 and Synflorix promote high immunity in all age groups [7, 8].

Nowadays, in addition to encapsulated bacteria, non-encapsulated strains of *S. pneumoniae* are frequently isolated from many patients with pneumococcal infections. Therefore, current vaccines, which are generally based on capsular antigens, are not effective against the newly emerged infections [9]. For this reason, researchers have been investigating other proteins of the bacterium to develop new vaccines with the ability to induce high protection against all pneumococcal serotypes.

The IgA1 protease, as one of the most important pneumococcal proteins, facilitates the entry and colonization of the bacterium by destroying the mucosal IgA antibody [10]. The IgA1 protease of *S. pneumoniae*, as a metalloproteinase with a molecular weight of 215 KDa, breaks down the IgA1 at the hinge region (between Pro-227 and Thr-228) within the heavy chain of the antibody. IgA1 constitutes more than 90% of the IgA in the human airways. The cleavage of antigen-bound IgA1 creates Fab fragments on the bacterial surface that inhibits phagocytosis, and consequently, the bacterial cells are not effectively neutralized [11]. Indeed, the decrease in pneumococcal colonization following the use of the capsular conjugate vaccine may be due to its induction of high IgG levels that are not targeted by the IgA1 protease [12, 13]. However, the main antibody-mediated defense in airways is related to the IgA class, and its opsonization role has been proven [14]. IgA1 protease is a conserved protein in *S. pneumoniae* serotypes and plays a main role in the control of the host immune response, it may be useful to apply in pneumococcal vaccines [15]. In the present study, the pneumococcal truncated IgA1 protease gene was cloned, expressed, and purified and then assessed the titer of its corresponding antibody in the serum of healthy volunteers.

## 2. METHODS

### 2.1. DNA Extraction, PCR and Cloning of Recombinant Protease Gene

Genomic DNA was extracted from *S. pneumoniae* ATCC 49619 strain using a commercial DNA extraction Kit (FAVORGEN) according to the manufacturer's instructions. Polymerase chain reaction (PCR) was carried out using 0.5  $\mu$ M of forward primer (CCCA CAT ATG AAC GGA ACT GCA ACA AAA C), 0.5  $\mu$ M of reverse primer (CCCA CTC GAG ACC ATC AAC ATG AAC TTG TT), 25 ng template DNA and 12.5  $\mu$ l of PCR-mastermix (2X). PCR product (705 bp) was purified using a DNA Gel Recovery Kit (PCR purification kit, GTP, Iran) and then double-digested by *Nde*-1 and *Xho*-1 restriction enzymes (Thermo Fisher Scientific, U.S) by a mixture containing 2  $\mu$ l buffer O (10X), 3  $\mu$ l *Nde*-1, 3  $\mu$ l *Xho*-1

and 1  $\mu$ g purified amplicon. The digested amplicons were ligated to the (predigested with *Nde*-1 and *Xho*-1 restriction enzymes) PET28a vector using T4 ligase enzyme (Takara, Japan). The ligation reaction was carried out for 16 h at 4°C, and the ligated product was transformed into *E. coli* DH5- $\alpha$  strain using the chemical CaCl<sub>2</sub> transformation method [16]. The transformation suspension was cultured in LB agar containing kanamycin (1  $\mu$ g/ml) and incubated at 37°C for 24 h. Plasmid extraction was performed using a plasmid extraction kit (Bio Basic, Canada) according to the manufacturer's instructions.

### 2.2. Recombinant Protein Expression, Purification and Confirmation

Recombinant PET28a (705 bp) protein was transformed into *E. coli* BL21 based on the chemical CaCl<sub>2</sub> method, as described in the section above. The transformed bacterial cells were cultured in LB agar (Merck, Germany) containing kanamycin (1  $\mu$ g/ml) and incubated at 37°C for 24 h. The next day, a single colony was cultured in LB broth containing kanamycin (1  $\mu$ g/ml) and incubated at 37°C for 24 h. A 200  $\mu$ l volume of the bacteria grown overnight was sub-cultured in LB broth supplemented with kanamycin (1  $\mu$ g/ml) and incubated at 37°C to reach the desired optical density (OD: 0.6 at 600 nm). In order to induce the expression of the recombinant protein, 1 mM Isopropyl  $\beta$ -D-thiogalactopyranoside (IPTG) was added to the culture of the transformed bacteria, and after 4 hours, the bacterial cell membranes were destroyed by sonication. The recombinant protein was purified by Ni-NTA affinity chromatography, and its concentration determined by reading ODs at 280 nm and 260 nm using a Nanodrop device and the following formula:

$$\text{Protein concentration (mg/ml): } (1.55 \times \text{OD}_{280}) - (0.76 \times \text{OD}_{260})$$

The quality of the purified IgA protease recombinant protein was assessed by 10% SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE). Briefly, five  $\mu$ g of IgA protease recombinant protein was loaded into well of polyacrylamide gel and electrophoresed at 150V for 3 h. The gel was stained with coomassie brilliant blue for 30 min and destained with the destaining solution (100 ml deionized water, 100 ml methanol and 100 ml acetic acid). To confirm IgA protease recombinant protein, it was electrophoresed in 10% polyacrylamide gel and then transferred into polyvinylidene difluoride (PVDF) membrane. The membrane was blocked in blocking solution (Phosphate-Buffered Saline (PBS) containing 0.05% Tween 20 and 5% skimmed milk) at 4°C overnight and after washing with wash buffer (PBS containing 0.05% Tween 20), the membrane incubated with monoclonal anti-His tagged antibody conjugated to Horseradish Peroxidase (HRP) for 1 h. The membrane was washed 3 times with wash buffer and incubated with 3, 3'-Diaminobenzidine (DAB) substrate for 3 min.

### 2.3. Detection of Antibodies Against IgA1 Protease Recombinant Protein

Serum samples were obtained from sixty healthy people aged from <2, 2 to 40 and more than 40 years old. Antibodies against truncated-IgA1 protease were detected by indirect

ELISA assay. One hundred  $\mu\text{l}$  of truncated-IgA1 protease recombinant protein (10  $\mu\text{g}/\text{mL}$ ) was added into 96-well polystyrene microplates (Cytomatin, Isfahan) and incubated at  $4^{\circ}\text{C}$  for 12 h. The wells were washed 3 times with washing buffer and blocked with 5% skim milk at  $25^{\circ}\text{C}$  for 1 h. The wells were washed 3 times with washing buffer, 100  $\mu\text{l}$  from each serum at dilution of 1:10 to 1:160 added into the wells, and incubated at  $25^{\circ}\text{C}$  for 1h. The wells were washed 3 times with washing buffer, and 100  $\mu\text{l}$  anti-human IgG conjugated with peroxidase (Cytomatin, Isfahan, Iran) was added into the wells and incubated at  $25^{\circ}\text{C}$  for 30 min. The wells were again washed 3 times with washing buffer and 100  $\mu\text{l}$  3,3',5,5'-Tetramethylbenzidine (TMB) (Cytomatin Gene Co, Isfahan, Iran) substrate added into the wells. After 15 min, the reaction was terminated by 100  $\mu\text{l}$  of 1N  $\text{H}_2\text{SO}_4$  and optical density (OD) of samples was measured at 450-630 nm.

#### 2.4. Statistical Analysis

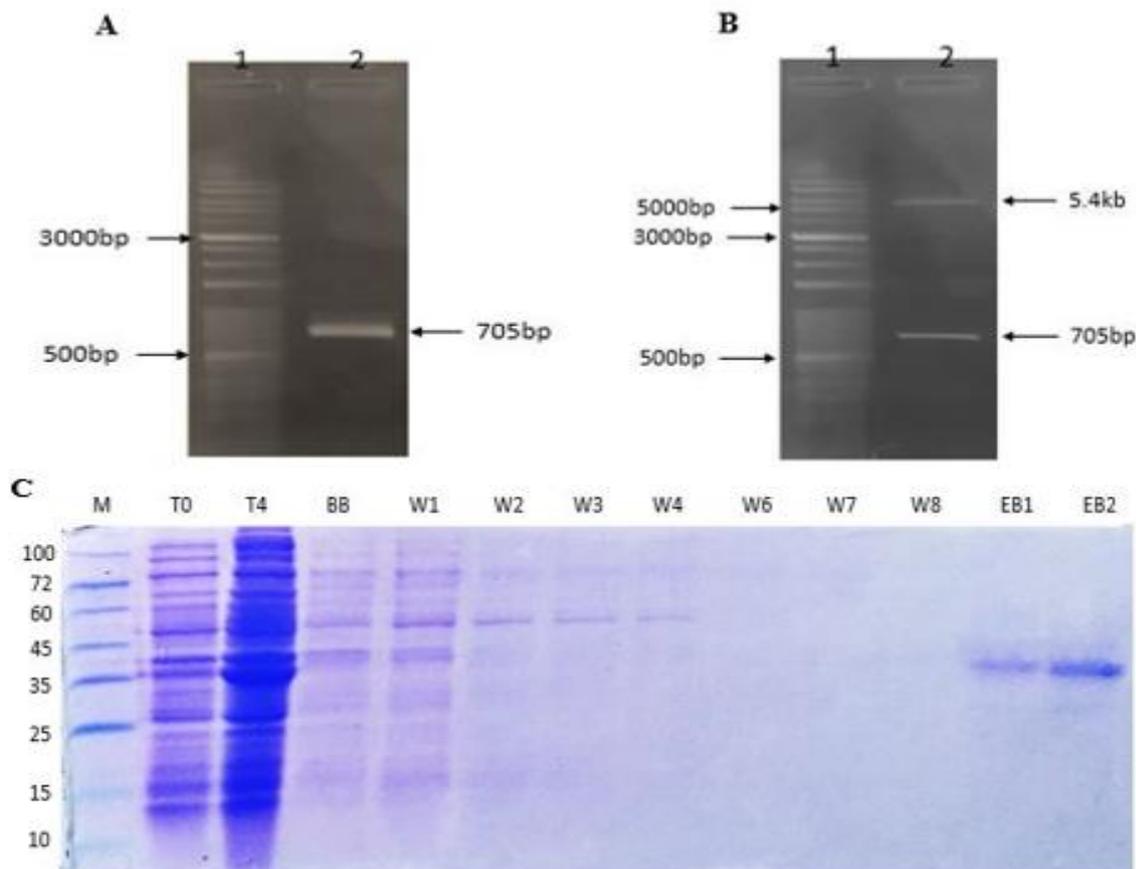
The data was analyzed by SPSS version 16.0 software

(SPSS, Inc., Chicago, IL). The participants were categorized into three age groups of  $<2$ , 2-40, and  $>40$  years. One-way ANOVA (Tukey) test was used to compare the antibody titer among the three groups. A p-value less than 0.05 was regarded as statistically significant.

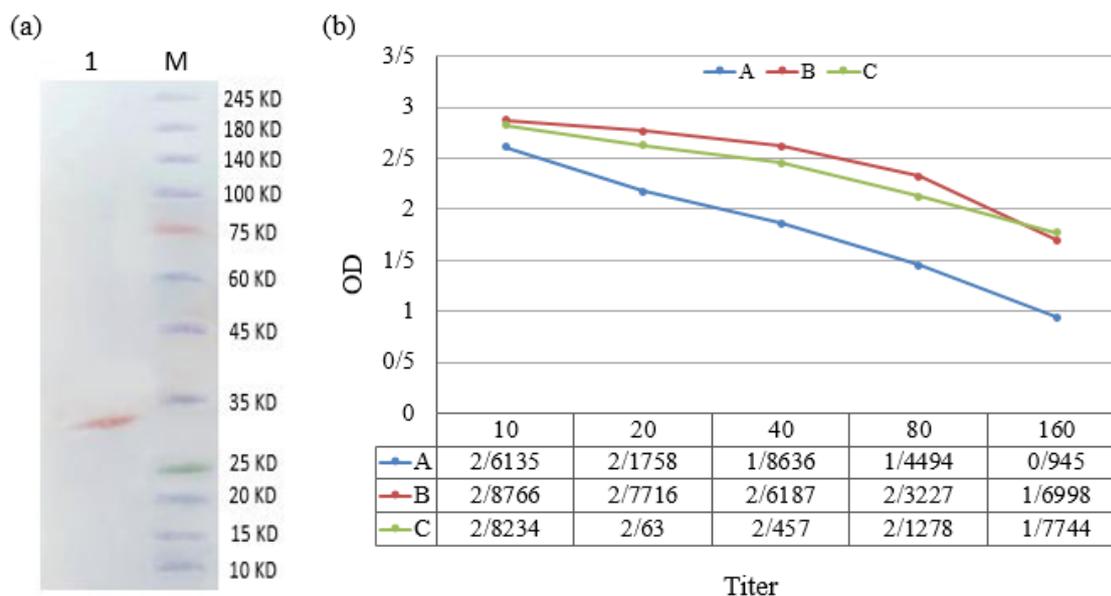
### 3. RESULTS

#### 3.1. IgA1 Protease Gene Cloning, Purification and Confirmation of the Recombinant Protein

The expected PCR-product band was observed in agarose gel electrophoresis, as shown in Fig. (1A). The cloning of the IgA1 protease gene was confirmed by double-digestion of the recombinant vector by *Nde* I and *Xho* I restriction enzymes (Fig. 1B), and the recombinant vector was optimally expressed in 1 mM concentration of IPTG after 4h induction. Purification of protein was successfully yielded 1.013 mg/ml truncated IgA protease with high purity (Fig. 1C). The purified IgA protease was confirmed by western blot analysis (Fig. 2a).



**Fig. (1).** Electrophoresis of PCR products on 1% agarose gel. **A)** IgA1 protease gene was amplified using Taq DNA Polymerase enzyme. Lane 1: DNA marker (1 Kbp), lane 2: PCR product; IgA1 protease (705 bp). **B)** Electrophoresis of the recombinant plasmid digested with *Xho*I and *Nde*I restriction enzymes. Lane 1: 1 Kbp DNA marker, 2: PCR product; vector without IgA protease (705 bp). **C)** Electrophoresis of recombinant IgA1 protease. M: protein marker, T0: BL21 bacteria containing recombinant pET28a without IPTG. T4: BL21 bacteria containing recombinant pET28a with IPTG (after 4 hours of incubation). EB1-EB2: Expressed recombinant protein.



**Fig. (2).** (a). Western blot analysis of IgA1 protease recombinant protein. M: protein marker, 1: the IgA1 protease recombinant protein with ~28KDa MW. (b). Antibody responses in three age groups (A: <2 years, B: 2-40 years, C: >40 years). The axis X shows titers 10, 20, 40 and 160 and axis Y are serum samples' ODs.

### 3.2. Serological Analysis

Comparison of anti-recombinant IgA1 protease antibody titers among three groups showed a significant response of anti-recombinant IgA1 protease antibody between individuals >2 years and those who have <2 years old ( $P < 0.05$ ). A significant highest antibody titers were observed in the group with 2-40-years old while the lowest titers belonged to children <2 years old ( $P < 0.05$ ). One-way ANOVA (Tukey) test revealed a significant difference in the averages of antibody titers (20, 40, 80 and 160) between the groups of <2 and >2 years old ( $P < 0.05$ ) (Fig. 2b).

### 4. DISCUSSION

IgA1 protease, which cleaves human IgA1, is one of the most important virulence factors produced by *S. pneumoniae* and several other invasive mucosal pathogens (e.g., *Neisseria* species and *Haemophilus influenzae*) [17]. Given the importance of streptococcal infections and the lack of an effective vaccine against all strains of *S. pneumoniae* [9], we here cloned, expressed, and purified a part of the pneumococcal IgA1 protease to assess its immunogenicity. For this purpose, antibody titer against the truncated recombinant IgA1 protease was determined in the serum of healthy individuals from three age groups. Our findings showed an appropriate level of the anti-recombinant IgA1 protease antibody in healthy individuals, especially those aged 2-40 years old.

In a study by Romanello *et al.*, the researchers cloned, expressed, and purified three segments of the *S. pneumoniae* IgA1 protease to investigate their function and immunogenicity. The results of the recent study showed a replacement of alanine with glutamate in the HEXXH motif (1605-1609)

within the conserved domain of IgA protease. Considering the identification of antibodies against these segments of the IgA protease, it would be possible to develop vaccines against *S. pneumoniae* using inactive mutant IgA protease peptides [18]. In another study by Janoff *et al.*, it was shown that the IgA protease could prevent removing of *S. pneumoniae* bacteria by human IgA *in vivo* [19]. The recent study supports the applicability of IgA protease as a potential candidate for designing vaccines against *S. pneumoniae*.

In the present study, the IgA1 proteolytic domain (corresponding to the 1465 to 2616 nucleotides of the corresponding gene) was cloned, expressed, and purified as a recombinant truncated protein. The serological analysis revealed a satisfactory antibody titer against this protease in healthy individuals, especially in those aged 2 to 40 years old. Probably, the suitable antibody titers against IgA protease in middle-age groups resulted from exposure to other bacterial colonization in the oral cavity that produces IgA protease and stimulates mucosal immunity. A study by Poulsen *et al.* showed that there is a 75 to 79% sequence similarity of IgA1 proteases between *S. pneumoniae* and *S. oralis* and *S. mitis* [20].

There was a significant association between the titer of anti-pneumococcal IgA1 protease antibody and age. In this regard, statistical analysis showed significant differences in antibody titers (20, 40, 80 and 160) between the two age groups of <2 and 2-40 years. The possible explanation for the lower antibody titers in the age group of <2 years may be incomplete colonization of *S. pneumoniae* in young children and lack of maternal antibodies against IgA protease. Likewise, a reduction in the antibody titers in people over 40 years old can be related to the weakening of the immune system with age.

## CONCLUSION

In the present study, desirable antibody titers against the pneumococcal recombinant IgA1 protease were seen in the three groups' serum of healthy individuals. However, a significant correlation was not totally observed among groups. A high titer of antibody against pneumococcal recombinant IgA1 protease in healthy groups may be due to sustained stimulation of the immune system by IgA1 proteases produced by bacteria naturally found in the oral cavity.

## ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

## HUMAN AND ANIMAL RIGHTS

No animal or human were the basis of this research.

## CONSENT FOR PUBLICATION

Not applicable.

## AVAILABILITY OF DATA AND MATERIALS

Not applicable.

## FUNDING

None.

## CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

## ACKNOWLEDGEMENTS

Declared none.

## REFERENCES

- [1] Mitchell AM, Mitchell TJ. *Streptococcus pneumoniae*: Virulence factors and variation Clin Microbiol Infect 2010; 16(5): 411-8. [http://dx.doi.org/10.1111/j.1469-0691.2010.03183.x] [PMID: 20132250]
- [2] Jedrzejewski MJ. Pneumococcal virulence factors: Structure and function. Microbiol Mol Biol Rev 2001; 65(2): 187-207. [http://dx.doi.org/10.1128/MMBR.65.2.187-207.2001] [PMID: 11381099]
- [3] Henriques-Normark B, Tuomanen EI. The pneumococcus: Epidemiology, microbiology, and pathogenesis. Cold Spring Harb Perspect Med 2013; 3(7):a010215 [http://dx.doi.org/10.1101/cshperspect.a010215] [PMID: 23818515]
- [4] Keller LE, Robinson DA, McDaniel LS. Nonencapsulated *Streptococcus pneumoniae*: Emergence and pathogenesis. MBio 2016; 7(2): e01792-15. [http://dx.doi.org/10.1128/mBio.01792-15] [PMID: 27006456]
- [5] Weiser JN, Ferreira DM, Paton JC. *Streptococcus pneumoniae*: Transmission, colonization and invasion. Nat Rev Microbiol 2018; 16(6): 355-67. [http://dx.doi.org/10.1038/s41579-018-0001-8] [PMID: 29599457]
- [6] Koliou MG, Andreou K, Lamnisos D, et al. Risk factors for carriage of *Streptococcus pneumoniae* in children BMC Pediatr 2018; 18(1): 144. [http://dx.doi.org/10.1186/s12887-018-1119-6] [PMID: 29699525]
- [7] Kaplonek P, Khan N, Reppe K, et al. Improving vaccines against *Streptococcus pneumoniae* using synthetic glycans. Proc Natl Acad Sci USA 2018; 115(52): 13353-8. [http://dx.doi.org/10.1073/pnas.1811862115] [PMID: 30530654]
- [8] Kyaw MH, Lynfield R, Schaffner W, et al. Effect of introduction of the pneumococcal conjugate vaccine on drug-resistant *Streptococcus pneumoniae* N Engl J Med 2006; 354(14): 1455-63. [http://dx.doi.org/10.1056/NEJMoa051642] [PMID: 16598044]
- [9] Langereis JD, de Jonge MI. Non-encapsulated *Streptococcus pneumoniae*, vaccination as a measure to interfere with horizontal gene transfer Virulence 2017; 8(6): 637-9. [http://dx.doi.org/10.1080/21505594.2017.1309492] [PMID: 28328284]
- [10] Poulsen K, Reinholdt J, Kilian M. Characterization of the *Streptococcus pneumoniae* immunoglobulin A1 protease gene (iga) and its translation product Infect Immun 1996; 64(10): 3957-66. [http://dx.doi.org/10.1128/IAI.64.10.3957-3966.1996] [PMID: 8926055]
- [11] Vidarsson G, Overbeeke N, Stemerding AM, et al. Working mechanism of immunoglobulin A1 (IgA1) protease: Cleavage of IgA1 antibody to *Neisseria meningitidis* PorA requires de novo synthesis of IgA1 Protease. Infect Immun 2005; 73(10): 6721-6. [http://dx.doi.org/10.1128/IAI.73.10.6721-6726.2005] [PMID: 16177349]
- [12] Batten MR, Senior BW, Kilian M, Woof JM. Amino acid sequence requirements in the hinge of human immunoglobulin A1 (IgA1) for cleavage by streptococcal IgA1 proteases. Infect Immun 2003; 71(3): 1462-9. [http://dx.doi.org/10.1128/IAI.71.3.1462-1469.2003] [PMID: 12595464]
- [13] 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. [http://dx.doi.org/10.1038/nrmicro1871] [PMID: 18340341]
- [14] Breedveld A, van Egmond M. IgA and FcαRI: Pathological roles and therapeutic opportunities. Front Immunol 2019; 10: 553. [http://dx.doi.org/10.3389/fimmu.2019.00553] [PMID: 30984170]
- [15] Chi YC, Rahkola JT, Kendrick AA, et al. *Streptococcus pneumoniae* IgA1 protease: A metalloprotease that can catalyze in a split manner *in vitro* Protein Sci 2017; 26(3): 600-10. [http://dx.doi.org/10.1002/pro.3110] [PMID: 28028839]
- [16] Panja S, Saha S, Jana B, Basu T. Role of membrane potential on artificial transformation of *E. coli* with plasmid DNA J Biotechnol 2006; 127(1): 14-20. [http://dx.doi.org/10.1016/j.jbiotec.2006.06.008] [PMID: 16876281]
- [17] Wani JH, Gilbert JV, Plaut AG, Weiser JN. Identification, cloning, and sequencing of the immunoglobulin A1 protease gene of *Streptococcus pneumoniae* Infect Immun 1996; 64(10): 3967-74. [http://dx.doi.org/10.1128/IAI.64.10.3967-3974.1996] [PMID: 8926056]
- [18] Romanello V, Marcacci M, Dal Molin F, et al. Cloning, expression, purification, and characterization of *Streptococcus pneumoniae* IgA1 protease Protein Expr Purif 2006; 45(1): 142-9. [http://dx.doi.org/10.1016/j.pep.2005.07.015] [PMID: 16146695]
- [19] Janoff EN, Rubins JB, Fasching C, et al. Pneumococcal IgA1 protease subverts specific protection by human IgA1. Mucosal Immunol 2014; 7(2): 249-56. [http://dx.doi.org/10.1038/mi.2013.41] [PMID: 23820749]
- [20] Poulsen K, Reinholdt J, Jespersgaard C, et al. A comprehensive genetic study of streptococcal immunoglobulin A1 proteases: Evidence for recombination within and between species. Infect Immun 1998; 66(1): 181-90. [http://dx.doi.org/10.1128/IAI.66.1.181-190.1998] [PMID: 9423856]