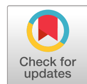
Original article

CLINICAL AND EXPERIMENTAL VACCINE RESEARCH



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Autolysin (lytA) recombinant protein: a potential target for developing vaccines against pneumococcal infections

Davoud Afshar[1], Farzaneh Rafiee[1], Mozhgan Kheirandish[1], Solmaz Ohadian Moghadam[2], Mohammad Azarsa[3]

[1]Department of Microbiology and Virology, School of Medicine, Zanjan University of Medical Sciences, Zanjan; [2]Uro-Oncology Research Center, Tehran University of Medical Sciences, Tehran; [3]Department of Microbiology, Khoy University of Medical Sciences, Khoy, Iran

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Corresponding author: Mohammad Azarsa, PhD
Department of Microbiology, Khoy University of Medical Sciences, Valiasr St., Valiasr Sq., Khoy, Iran
Tel: +98-44-36-25-5777; Fax: +98-44-36-25-5777
E-mail: mo.azarsa@yahoo.com; m_Azarsa@khoyums.ac.ir

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Purpose: N-acetylmuramoyl-l-alanine amidase known as lytA, is an immunogenic protein that plays an important role in the pathogenesis of *Streptococcus pneumoniae*. It is highly conserved among *S. pneumoniae* strains and is absent among other *Streptococcus* species. In the present study, the level of antibodies against the lytA recombinant protein was evaluated in healthy individuals' sera.
Materials and Methods: DNA was extracted from *S. pneumoniae* ATCC 49619 to amplify *lyt*A gene by polymerase chain reaction assay. The lytA amplicon and *p*ET28a vector were separately double digested using *Nde*-1 and *Xho*1 restriction enzymes and then ligated together with ligase enzyme. The recombinant plasmid was expressed in *Escherichia coli* BL21 strain and the lytA recombinant protein purified using nickel-nitrilotriacetic acid affinity chromatography. Western blot was carried to detect lytA recombinant protein. Sixty healthy individual's sera (at three age groups: group 1, <2; group 2, 2–40; and group 3, 60–90 years old) were collected and the titers of anti-lytA antibodies were determined.
Results: The *lyt*A gene was highly expressed in *E. coli* BL21 host. The recombinant lytA protein was purified and confirmed by western blotting. Tukey test analysis showed that there were no significant differences among the age groups considering the anti-lytA titer of 10. However, at the anti-lytA titer of 60, significant differences were observed between group 1 vs. group 2 ($p<0.001$); group 1 vs. group 3 ($p=0.003$), and group 2 vs. group 3 ($p=0.024$).
Conclusion: The lytA protein seems to be a highly immunogenic antigen and a potential target for developing vaccines against pneumococcal infections.

Keywords: *Streptococcus pneumoniae*, N-acetylmuramoyl-l-alanine amidase, Antibody, Surface protein, Western blotting

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Introduction

Streptococcus pneumoniae is a leading cause of pneumonia, meningitis, otitis media, sinusitis, and bacteremia among adults and children globally [1]. Peoples with underlying diseases including individuals with diabetes, asthma, chronic obstructive pulmonary disease, cardiovascular disease, acquired immune deficiency syndrome, and sickle cell disease are more susceptible for pneumococcal infections [2]. Pneumococcal invasive diseases have a mortality rate of 5%–35%, which is associated with some indexes including site of infection, comorbidity, patient's age, and pneumococcus serotype [3].

Currently, two types of pneumococcal vaccines, PPSV23 and PCV13, are used to prevent pneumococcal infections in countries with high incidence rates of these infections. The PPSV23 and PCV13 are polysaccharide vaccines used in children and adults of 5 to 64 years old. However, polysaccharide vaccines do not elicit protective immune responses in children <2 years old. As a result, conjugate vaccines have been introduced in order to resolve this limitation [4].

Although all pneumococcal proteins are potentially immunogenic for the immune system of human body, those proteins exposed on the surface of diplococci can be particularly useful for developing vaccines. The surface proteins of pneumococci encompass four main families including typical Gram-positive surface proteins, lipoproteins, choline-binding proteins (CBPs), and non-classical surface proteins [5].

The pneumococcal major autolysin, N-acetylmuramoyl-l-alanine amidase known as lytA, is expressed at both cytoplasmic and surface membrane levels and plays an important role in the pathogenesis of pneumococci infections [6]. This virulence factor binds to the bacterium cell wall by its choline binding domain and subsequently triggers bacterium autolysis.

The lytA protein is immunogenic and highly conserved among *S. pneumoniae* strains and is not observed among other Streptococcus species. Nevertheless, it may have cross-reactivity with lytA similar proteins produced by other species and may cause a high level of antibodies against lytA protein. For this reason, in the present study, we examined the titer of anti-recombinant lytA antibodies in the sera of healthy individuals.

Materials and Methods

Gene cloning and recombinant protein production

Genomic DNA was extracted from exponential cultures of *S. pneumoniae* ATCC 49619 using Genomic DNA Extraction Kit (Favorgen Biotech Corp., Pingtung, Taiwan) according to the manufacturer's instructions. Polymerase chain reaction for *lyt*A gene was carried out using specific forward (CCCACATATGGAAATTAATGTGAGTAAA) and reverse (CCCACTCGAGTTTTACTGTAATCAAGCCAT) primers.

The *lyt*A amplicon and *p*ET28a vector (Novagen Inc., Madison, WI, USA) were separately digested with *Nde*-1 and *Xho*1 restriction enzymes (Fermentase, Burlington, Germany) and ligated together with ligase enzyme. Transformation of ligation product into competent *Escherichia coli* DH5α cells was done using $CaCl_2$ method. Recombinant plasmid was sequenced to confirm the *lyt*A gene sequence. For gene induction, *E. coli* BL21 (DE3) strain containing recombinant plasmid was cultured in Luria-Bertani (LB) broth (with 50 μg/mL kanamycin) overnight at 37°C with shaking. Subsequently, the overnight culture was sub-cultured into 250 mL of LB broth containing kanamycin (50 μg/mL) and incubated at 37°C until its optical density reached 0.6. Plasmid induction was done by adding isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma-Aldrich, St. Louis, MO, USA) (final concentration: 1 mM) and culture were incubated for 4 hours at 37°C with shaking. Cultures were centrifuged and plate was used for recombinant protein purification [7].

Western blot was carried out using His-tag monoclonal antibody conjugated to horseradish peroxidase (HRP; Thermo Fisher Scientific Inc., Waltham, MA, USA). The lytA recombinant protein was electrophoresed in 10% sodium dodecyl sulfate-polyacrylamide gel and transferred into polyvinylidene fluoride (PVDF) membrane. PVDF blocking was done using 3% skim milk at room temperature for 12 hours. Following washing with phosphate-buffered saline (PBS) containing 0.05% Tween 20, the PVDF membrane was incubated at His-tag monoclonal antibody conjugated to HRP for 1 hour at 25°C. After washing with PBS containing 0.05% Tween 20, the blots were treated with 3, 3'-diaminobenzidine solution (Sigma-Aldrich) for 2 minutes.

The enzyme-linked immunosorbent assay

Sixty human sera samples were collected (group 1, 20 samples from neonatal up to 2 years old; group 2, 20 samples from adults with 2–40 years old; and group 3: 20 samples from elderly with 60–90 years old) and evaluated for antibody titers using an enzyme-linked immunosorbent assay. Ten μg/mL of lytA recombinant protein was coated into 96-well polystyrene plates (Greiner, Stonehouse, UK) and incubated overnight at 4°C. Following 3 times washing with PBS containing 0.05 Tween 20, the wells blocked with PBS containing 3% skim milk for 1 hour at 25°C. Then, wells were washed 3 times and sera added into the wells at dilution of 1:10 to 1:160, and incubated for 1 hour at 25°C. Peroxidase-conjugated mouse anti-human immunoglobulin G (IgG; Cytomatin Gene Co., Isfahan, Iran) (1:4,000 dilution) was added into the wells and incubated for 30 minutes at 25°C. About 50 μL TMB (3,3',5,5'-tetramethylbenzidine) substrate solution (Cytomatin Gene Co.) was added into the wells and the reaction was finally stopped after 15 minutes by adding 50 μL of 1N H_2SO_4. Our

study was approved by the ethical committee of Khoy University of Medical Sciences (ETHICAL code no., IR.KHOY.REC.1397.005).

Results

Gene cloning and expression

In the present study, the *lyt*A gene was successfully amplified and its gel electrophoresis is shown at Fig. 1. Following double digestion of *lyt*A gene and pET28a vector with *Xho*1 and *Nde*1 restriction enzymes, the digested insert and vector were ligated successfully.

Recombinant protein expression and purification

The *lyt*A gene was highly expressed in *E. coli* BL21 host and noticeable amount of recombinant protein yielded (approximately 35 kDa) after 4 hours induction with 0.1 mM IPTG. The purification of recombinant protein carried out using nickel-nitrilotriacetic acid affinity chromatography system and highly pure recombinant protein eluted (Fig. 2).

Western blotting

Western blot analysis using anti-His tag antibody confirmed the recombinant lytA recombinant protein, which is presented in Fig. 3.

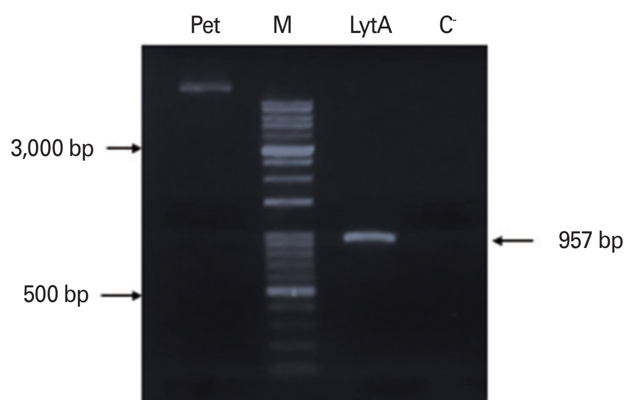

Fig. 1. Gel electrophoresis of extracted plasmid and lytA amplicon. Lane pet, pPET28a; lane M, DNA ladder 100–10,000 bp; lane lytA, lytA amplicon; C, negative control.

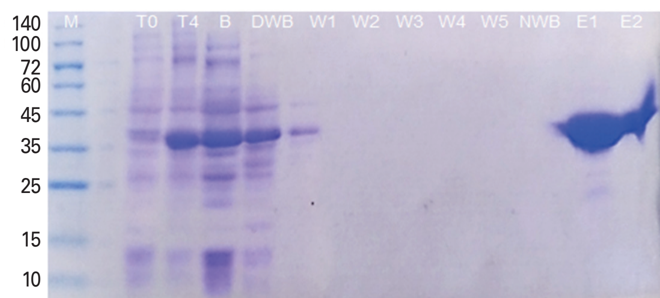

Fig. 2. Polyacrylamide gel electrophoresis of samples collected from protein production and purification. M, protein marker; T0, *E. coli* BL21 before induction; T4, *E. coli* BL21 after 4 hours induction with IPTG (isopropyl-β-D-thiogalactopyranoside); B, unbounded proteins in flow-through fraction from binding step; DWB, flow-through fraction following washing column with denature wash buffer; W1–W5, flow-through fractions following washing column; NWB, flow-through fraction after washing with native wash buffer; E1–E2, sample from eluted proteins. *E. coli*, *Escherichia coli*.

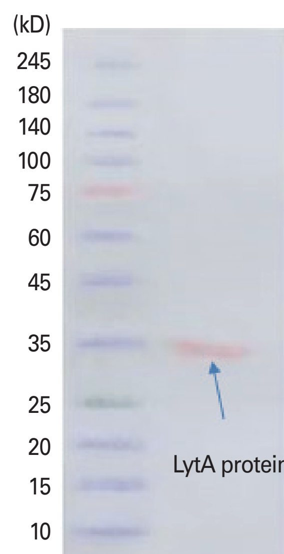

Fig. 3. Western blot analysis of lytA recombinant protein.

Table 1. Compering the sera titers among different age groups

Titer	Age comparison (y)		p-value
10	0–2	2–40	0.087
		40–90	0.360
	2–40	40–90	0.716
20	0–2	2–40	0.000
		40–90	0.002
	2–40	40–90	0.614
40	0–2	2–40	0.000
		40–90	0.000
	2–40	40–90	0.375
80	0–2	2–40	0.000
		40–90	0.000
	2–40	40–90	0.179
160	0–2	2–40	0.000
		40–90	0.003
	2–40	40–90	0.024

Enzyme-linked immunosorbent assay

Five dilutions of human sera ranging from 1:10 to 1:160 were used for determining the titer of anti- recombinant lytA antibody. The results of the Tukey test analysis showed that there was no significant difference among the age groups considering 10 antibody titer. However, significant differences were observed comparing the other sera as shown in Table 1.

Discussion

S. pneumoniae is a common cause of serious infectious diseases such as meningitis, sepsis, and pneumonia. In the recent years, there have been attempts to identify a reliable protein to develop efficient vaccines against pneumococcal diseases [8,9]. Vaccination has been particularly effective in inducing nasopharyngeal immunization and limiting pneumococci colonization [10]. CBPs are a family of pneumococcal surface exposed proteins that have structural and physiological functions.

CBPs are a family of pneumococcal surface proteins with wide-range structural and physiological functions. Antibodies against these proteins are increased during life suggesting these proteins as potential candidates for developing vaccines against pneumococcal infections. In our study, the presence of IgG antibodies against lytA pneumococcal protein, a member of surface-exposed CBPs, was investigated in the sera of 60 healthy individuals at different age groups. There were no significance differences among the assessed age groups (i.e., <2, 2–40, and 60–90 years old) regarding the antibody titer of 1:10. Considering the titer of 1:160, however, significant differences were observed comparing the first versus second ($p<0.05$); first versus third ($p=0.003$), and second vs. third ($p=0.024$) age groups.

The high titer of antibodies against lytA protein can be related to immune responses against the bacteria colonizing in the nasopharynx or other sites and also the detection of the protein by mucosal immune cells [11]. The *lyt*A is a highly conserved gene in *S. pneumoniae* with the highest intra-species similarity being related to the *lyt*A gene of *S. mitis* B6 strain with only 20% dissimilarity in their sequences [12].

Mitis group streptococci are genetically similar species and include *S. mitis, S. pneumoniae, S. australis, S. peroris, S. cristatus, S. gordonii, S. infantis, S. oligofermentans, S. oralis, S. parasanguinis, S. pseudopneumoniae, S. tigurinus,* and *S. sanguinis* [13]. These species are colonized into oral cavity and stimulate mucosal immunity permanently. In a study by Gosink et al. [14], the degree of similarity between choline binding domains of choline binding protein A and other CBPs calculated to be 30% to 60%. The presence of these domains in all of the CBPs can cause cross reaction immunity, justifying the high antibody titers against all CBPs. In another study, the similarity among CBPs has been also reported [15]. On the bacterium surface, it can effectively trigger immune system leading to a high titer of anti-lytA antibody in individuals who have a history of pneumococcal infections or those who are carriers of nasopharyngeal non-capsulated pneumococcal strains [16].

In the present study, the highest antibody titer against lytA protein was observed in the 2–40-year-old age group with decreasing trends in children <2 years and elders >60 years old. This pattern probably reflects the function of immune system against pneumococcal and other infections in lifetime. In fact, this observation may partly explain the higher rates of pneumococcal infections in children and elder [17,18].

In conclusion, the high titer of anti-lytA antibody in midlife may be a result of either stimulation of immune system by non-pneumococcal proteins with similar structures to pneumococcal lytA or prior nasopharyngeal colonization of pneumococci in healthy individuals. Overall, lytA protein presents appropriate immunogenicity properties and beside other pneumococcal immunogenic proteins it can be applied in pneumococcal new vaccines.

ORCID

Davoud Afshar *https://orcid.org/0000-0001-6376-417X*
Farzaneh Rafiee *https://orcid.org/0000-0002-3300-3713*
Mozhgan Kheirandish *https://orcid.org/0000-0003-1159-4599*
Solmaz Ohadian Moghadam *https://orcid.org/0000-0001-9745-7063*
Mohammad Azarsa *https://orcid.org/0000-0002-3220-7597*

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