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# Characterization of *Streptococcus pneumoniae* N-Acetylglucosamine-6-Phosphate Deacetylase as a Novel Diagnostic Marker§

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**The identification of novel diagnostic markers of pathogenic bacteria is essential for improving the accuracy of diagnoses and for developing targeted vaccines. *Streptococcus pneumoniae* is a significant human pathogenic bacterium that causes pneumonia. N-acetylglucosamine-6-phosphate deacetylase (NagA) was identified in a protein mixture secreted by *S. pneumoniae* and its strong immunogenicity was confirmed in an immuno-proteomic assay against the anti-serum of the secreted protein mixture. In this study, recombinant *S. pneumoniae* NagA protein was expressed and purified to analyze its protein characteristics, immunospecificity, and immunogenicity, thereby facilitating its evaluation as a novel diagnostic marker for *S. pneumoniae*. Mass spectrometry analysis showed that *S. pneumoniae* NagA contains four internal disulfide bonds and that it does not undergo post-translational modification. *S. pneumoniae* NagA antibodies successfully detected NagA from different *S. pneumoniae* strains, whereas NagA from other pathogenic bacteria species was not detected. In addition, mice infected with *S. pneumoniae* generated NagA antibodies in an effective manner. These results suggest that NagA has potential as a novel diagnostic marker for *S. pneumoniae* because of its high immunogenicity and immunospecificity.**

***Keywords***: *Streptococcus pneumoniae*, secreted proteins, antiserum, diagnostic marker

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## Introduction

*Streptococcus pneumoniae* is an important human pathogen that resides on the mucosal surface of the upper respiratory tract, which causes bacterial pneumonia, meningitis, and pneumococcal septicemia (Kadioglu *et al*., 2008). Since the completion of the genome sequences of major *S. pneumoniae* strains (Tettelin *et al*., 2001), proteomic investigations have been performed to elucidate the potential application of pathogenic proteins as vaccines and/or diagnostic markers (Morsczeck *et al*., 2008; Choi *et al*., 2010). Cell surface proteins and secreted proteins are considered to be potential candidates for vaccines and diagnostic markers because they are the first proteins that make contact with host cells and many of them are virulent in the host, where they induce immune responses (Sun *et al*., 2011; Gomez-Gascon *et al*., 2012). In a previous study, we identified secreted proteins from *S. pneumoniae* based on a proteomic assay and showed that several secreted proteins were immunogenic antigens (Choi *et al*., 2012). In the present study, N-acetylglucosamine-6-phosphate deacetylase (NagA; EC 3.5.1.25) was selected from the proteins secreted by *S. pneumoniae* and tested as a novel candidate diagnostic marker. NagA is known to be involved with N-acetylglucosamine (GlcNAc) metabolism via the deacetylation of N-acetylglucosamine-6-phosphate (GlcNAc-6-P) to glucosamine-6-phosphate (Gln-6-P) (Yadav *et al*., 2011). However, the potential roles of *S. pneumoniae* NagA as a virulence factor or biomarker have never been studied. In the present study, *S. pneumoniae* NagA was expressed in an *E. coli* system and purified by affinity chromatography. The complete amino acid sequence was determined by tryptic peptide mapping using liquid chromatography-mass spectroscopy (LC-MS) to elucidate the structural characteristics of the recombinant protein. The cellular distributions and antigenic specificity of NagA were determined in *S. pneumoniae* and other bacteria. Finally, the potential of using NagA as a diagnostic marker of *S. pneumoniae* infections was examined.

## Materials and Methods

### Bacterial strains and preparation of the secreted protein

*Streptococcus pneumoniae* BAA-255 strain was used as the standard strain. Three *S. pneumoniae* clinical strains (7633, 6613, and 521), *Klebsiella pneumoniae* ATCC 13883, *Pseudomonas putida* KT2440, and *Acinetobacter baumannii* DU202 were used in this study. The *S. pneumoniae* strains were grown in Todd-Hewitt broth supplemented with 0.5% yeast extract in 5% $CO_2$ at 37°C. Cultures of *S. pneumoniae*

were grown to an optical density (OD) of about 0.9 to prepare the secreted proteins. The supernatants from the *S. pneumoniae*, *K. pneumoniae*, *P. putida*, and *A. baumannii* cultures were isolated by centrifugation at 13,000×*g* for 20 min. The supernatant containing the secreted proteins was saturated with ammonium sulfate (final concentration, 90%) at 4°C for 3 h and precipitated at 23,000×*g* for 20 min. The ammonium sulfate was removed from the precipitated protein samples by dialysis (molecular weight cut-off, 8,000 Da) using over 10 volumes of 50 mM Tris-HCl buffer (pH 7.6).

### Gene cloning, expression, and purification of the NagA protein

*Streptococcus pneumoniae* NagA was cloned by PCR using specific primers (forward, 5′-AGAAGAACATATGCCTA ACTATATTAAAGCGGATCA-3′; reverse, 5′-GAACTCGA GTGCTTGATAACGTTTTACGCCA-3′). The *S. pneumoniae* NagA PCR product was subcloned into the *Nde*I and *Xho*I sites of the pET28a plasmid and transformed into *E. coli* C43(DE3) competent cells. *E. coli* transformants that contained *S. pneumoniae* NagA were pre-cultured in 10 ml LB broth at 37°C and cultured again in 500 ml LB broth to an OD of 0.5–0.6. Next, isopropyl-b-D-thiogalactopyranoside (IPTG, 50 mM) was added to induce the recombinant protein and the cells were cultured overnight. The cultured cells were harvested and disrupted in lysis buffer (50 mM $NaH_2PO_4$, 300 mM NaCl, 10 mM imidazole, pH 8.0) by sonication (Vibra-Cell VC 130, Sonics & Materials, USA). The supernatant was harvested after centrifugation at 13,000 rpm and loaded onto a Ni-NTA column (2 ml) (Qiagen, USA) for protein binding. The column was washed with washing buffer (50 mM $NaH_2PO_4$, 300 mM NaCl, 20 mM imidazole, pH 8.0) and the concentration of imidazole was increased gradually (20 to 100 mM) to elute undesirable proteins. Finally, the over-expressed *S. pneumoniae* NagA was eluted with elution buffer (50 mM $NaH_2PO_4$, 300 mM NaCl, 250 mM imidazole, pH 8.0). The eluted proteins were confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and dialyzed with 20 mM Tris-HCl (pH 8.0) to remove salts, before storage at -80°C until tandem mass spectroscopy (MS/MS) analysis or antibody production.

### Preparation of anti-serum from the rabbit and mouse

Rabbit anti-serum was prepared with technical assistance from Young In Frontier (Seoul, Korea). The purified *S. pneumoniae* NagA protein was mixed with an identical volume of Freund's complete adjuvant (Sigma, USA) and injected under the skin of rabbits. The second and third injections were performed 4 weeks after the previous injections, and the fourth injection was administered 2 weeks later. The same protein samples mixed with Freund's incomplete adjuvant (Sigma) were used as a booster injection 2 weeks after the fourth injection. Blood was collected 1 week after the final injection. To prepare the mouse anti-serum, female mice (C57BL/6J) were infected with different numbers (colony-forming units, CFU) of *S. pneumoniae* BAA-255 ($1\times10^2$ CFU, $1\times10^4$ CFU, $1\times10^6$ CFU, and $1\times10^8$ CFU) and the same volume of PBS as the control. Three injections were applied at intervals of 2 weeks. Two weeks after the third injection, the serum was obtained by retro-orbital bleeding.

### SDS-PAGE and Western blotting

The secreted protein samples were separated by 12% SDS-PAGE (mini-PROTEAN, Bio-Rad, USA). Approximately 10 μg of protein was placed in each lane and the gels were stained with Coomassie Brilliant Blue R-250. Alternatively, the protein bands in the SDS gels were transferred to nitrocellulose membranes (Bio-Rad) for Western blotting. The nitrocellulose membranes were washed with Tris-buffered saline (TBS) after blocking with 5% skim milk in TBS for 1 h and incubated with anti-serum (1:2,000 in 3% skim milk in TBS) for 14 h at 4°C. After washing with TBST (0.5% Tween 20 in TBS), specific IgG binding was visualized by incubation with an anti-rabbit-IgG peroxidase conjugate (1:2,000 in 3% skim milk in TBS) and developed with a chemiluminescent substrate (Intron Biotechnology, Korea). The chemiluminescent signal was detected by ImageQuant™ LAS 400 mini (GE Healthcare).

### In-gel digestion, peptide mapping, and LC-MS analysis

Trypsin solution (0.1 μg/μl) was added to the purified protein sample (NagA) at an enzyme:protein ratio of 1:20 (w/w).

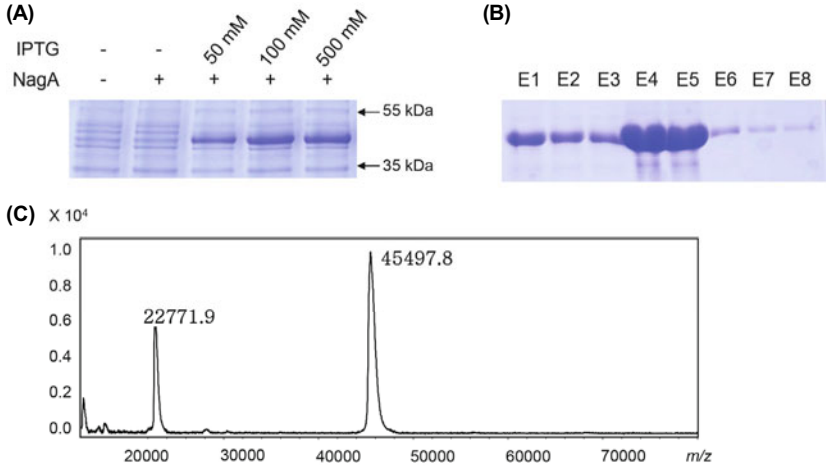


**Fig. 1. Expression and purification of *S. pneumoniae* NagA.** (A) *S. pneumoniae* NagA was strongly expressed in an *E. coli* expression system. (B) NagA protein was purified by affinity chromatography. (C) The molecular weight of the purified NagA protein was measured by MALDI-TOF MS. The theoretical molecular weight of the recombinant NagA protein was calculated as 45.19 kDa and a major peak was observed at a molecular weight of 45.5 kDa.

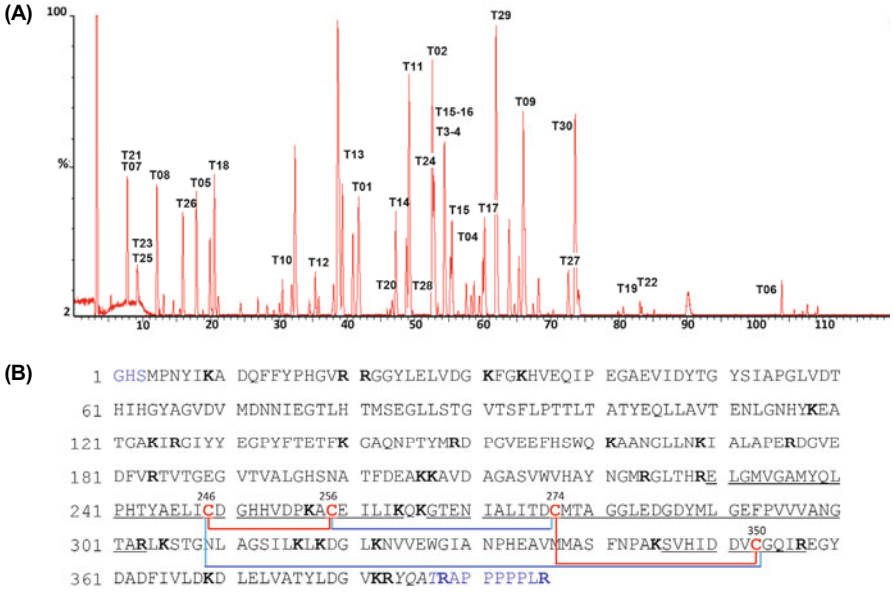


**Fig. 2. Tryptic peptide mapping of NagA.** (A). ESI Q-TOF MS ion chromatogram of NagA. Each peak in the ion chromatogram was identified by MS/MS analysis and the amino acid sequences of the tryptic peptides (T1–T30) are provided in Supplementary data Table S1. (B) Amino acid sequences of recombinant NagA detected by MS analysis. The complete amino acid sequence of NagA was determined by peptide mapping and MS/MS analysis, with the exception of the unidentified peptide ($^{380}$RYQA$^{383}$) indicated with italic letters. The black sequences are from NagA and the blue sequences are the His-tag. The amino acid symbols in bold letters indicate the trypsin digestion sites. Two different types of internal disulfide bonds were detected by the MS analysis: C246-C256/C274-C350 (red lines) and C246-C350/C256-C274 (blue lines).

Enzymatic hydrolysis proceeded for approximately 18 h at 37°C. The reaction was quenched by adding 50 µl of 10% trifluoroacetic acid solution, before storage in a freezer until LC-MS analysis. For peptide mapping and MS/MS analysis, we used an Agilent 1200 series high performance liquid chromatography system (Agilent Technologies, USA) coupled in-line with a Synapt G2 HDMS (Waters, UK) which is an electrospray ionization quadrupole-time-of-flight (ESI Q-TOF) MS. The peptide mixtures were separated using a Vydac 218TP $C_{18}$ polymeric reverse-phase column (internal diameter, 10 mm; length, 250 mm; 5 µm particles with 300 Å pore size) with mobile phases A (0.1% trifluoroacetic acid in $H_2O$) and B (0.1% trifluoroacetic acid in acetonitrile). A sample volume of 100 µl was injected. The gradient (acetonitrile) started with 0% buffer B for 3 min, then increased from 0% to 40% buffer B at 95 min, from 40% to 60% buffer B at 15 min, and was followed by 0% buffer B for 7 min at a flow rate of 1 ml/min, and the signals were detected at 214 nm. The column effluent was divided via a T-union between the UV detector and MS, with a split of 1:12.5. The MS was scanned over a mass-to-charge range (*m/z*) of 200–2,000, with 0.1 sec. per scan. The optimal ionization source working parameters were as follows: capillary voltage, 2 kV; quadrupole ion energy, 5 eV/z; dry temperature, 200°C; nebulizer, 1.2 bar; and dry gas, 6.0 L/min. The MS parameters were controlled by MassLynx. The MS calibration was conducted using sodium iodide (Sigma).

### Molecular weight measurement using matrix-assisted laser desorption/ionization (MALDI) TOF MS

The molecular weights were determined using a method described previously (Lee *et al*., 2012). The protein samples were prepared by mixing equal volumes of protein solution (NagA) and sinapinic acid (10 mg/ml in 50% CAN/0.1% TFA). One microliter of the mixture was spotted onto a MALDI plate and dried in air at room temperature. A MALDI-TOF mass spectrometer (ultrafleXtreme, Bruker, Germany) was used to measure the molecular weights of the protein samples.

## Results

### Expression and purification of NagA

*Streptococcus pneumoniae* NagA was cloned into the pET28a vector and expressed in *E. coli* C43[DE3] cells (Fig. 1A). Next, NagA protein was purified by His-tag chromatography (Fig. 1B). The expressed protein was soluble in the elution buffer and the purification yield was approximately 65.1% (final yield of NagA=11 mg). The theoretical molecular weight of native NagA was estimated as 41.67 kDa. However, the tag sequences (N-terminal, MGSSHHHHHHSSGLVPRGSH; C-terminal, TRAPPPPPLRSGC) were incorporated with NagA so the theoretical molecular weight of the recombinant NagA

**Table 1. Detection of peptide fragments having S-S bridge by MS spectra analysis**

| Peptide fragments having disulfide bond bridge | Theoretical MW[a] | Real mass (m/z) | Retention time (min) |
|---|---|---|---|
| T19-T20 (Cys249-Cys259) | 3909.8930 | 3910.9027 | 80.61 |
| T19- T22 (Cys249-Cys277) | 6921.2200 | n/d[b] | n/d |
| T19- T28 (Cys249-Cys353) | 4462.0970 | 4463.1067 | 79.96 |
| T20-T22 (Cys259-Cys277) | 4586.2050 | 4587.2147 | 85.95 |
| T20-T28 (Cys249-Cys353) | 2127.0820 | n/d | n/d |
| T22-T28 (Cys277-Cys353) | 5138.4090 | 5139.4187 | 84.10 |

[a] Molecular weight; [b] Not detected

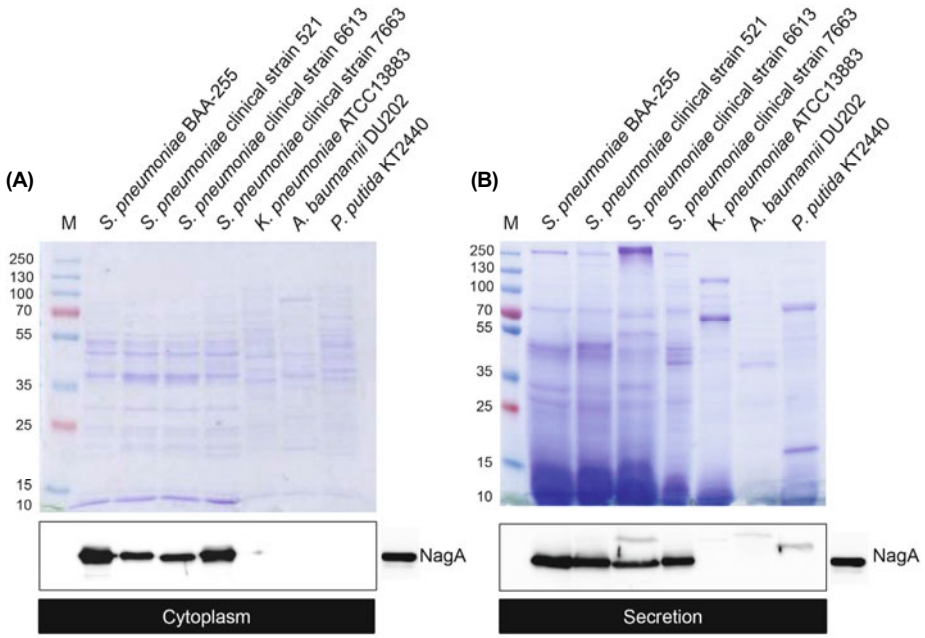


**Fig. 3. Immunospecificity of NagA.** Cytosolic proteins (A) and secreted proteins (B) from various bacterial strains were prepared and loaded on the SDS-PAGE and rabbit anti-serum of *S. pneumoniae* NagA was used as the primary antibody to detect NagA. The upper panels show SDS-PAGE and the lower panels show the results of Western blotting.

protein increased to 45.19 kDa. The MALDI-TOF MS analysis showed that the molecular weight of the recombinant NagA was 45,498 Da (Fig. 1C). Thus, the results showed that the recombinant *S. pneumoniae* NagA protein was expressed successfully by the *E. coli* expression system.

### Complete amino acid sequencing of recombinant NagA by ESI-Q-TOF MS

Next, we performed tryptic peptide mapping and MS/MS analysis to confirm the complete expression of the recombinant *S. pneumoniae* NagA and to analyze its structural characteristics (Fig. 2A). As the result, thirty NagA peptide fragments were detected and there was 99.0% amino acid sequence coverage (Fig. 2B and Supplementary data Table S1). The N-terminal fragment of NagA ($^{1}$MPNYIK$^{6}$) was also detected with the His-tag fragment (GSH). Given that the average sequence coverage of recombinant proteins by peptide mass fingerprinting is 40–70% (Liu *et al*., 2011; Pranchevicius *et al*., 2012), the sequence coverage of NagA was particularly high. The peptide mapping results showed that the recombinant NagA protein was expressed without modification or truncation. Statistically, four cysteine residues of NagA (Cys$^{246}$ of T19, Cys$^{256}$ of T20, Cys$^{274}$ of T22, and Cys$^{350}$ of T28) may contribute to the formation of six internal disulfide bridges. The MS spectra analysis of the tryptic peptides prepared from native recombinant NagA revealed that four disulfide bridges were present (T19-T20, T19-T28, T22-T20, and T22-T28) (Table 1).

### Immunospecificity of *S. pneumoniae* NagA

To determine the immunogenicity and immunospecificity of *S. pneumoniae* NagA, rabbit anti-serum was produced with the recombinant *S. pneumoniae* NagA as an antigen and used for Western blotting. As previously reported, the NagA produced by *S. pneumoniae* BAA-255 was detected in both the secreted protein fraction and the cytosolic protein fraction (Fig. 3). In addition, NagA produced a strong signal in other clinical *S. pneumoniae* stains (Fig. 3). By contrast, the NagA produced by different bacterial species, such as *Klebsiella pneumoniae*, *Pseudomonas putida*, and *Acinetobacter baumannii*, was not detected by the *S. pneumoniae* NagA anti-serum, either in the cytosolic or secreted protein fractions. This suggests that *S. pneumoniae* typically secretes NagA so it can be used as a specific marker protein for the detection of *S. pneumoniae*.

### Detection of NagA from anti-serum of *S. pneumoniae* infected mouse

To demonstrate the potential of *S. pneumoniae* NagA as a diagnostic maker, the presence of NagA antibody was tested in *S. pneumoniae*-infected mice. In this assay, five groups of mice were infected with *S. pneumoniae* on three occa-

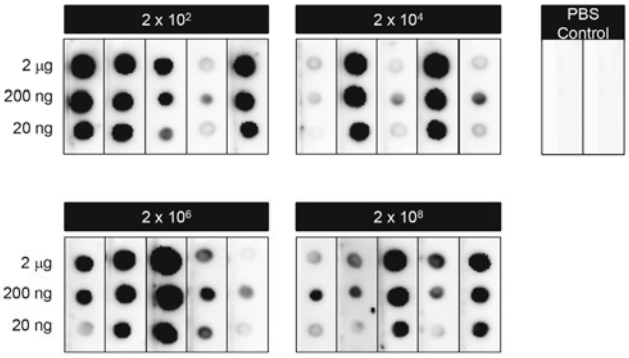


**Fig. 4. Detection of NagA antibody from *S. pneumoniae*-infected mice.** *S. pneumoniae* NagA protein was spotted onto nitrocellulose membranes and detected using anti-serum from mice infected with *S. pneumoniae*. Phosphate buffer was used as control for non-infection of *S. pneumoniae*.

sions at 2-week intervals using $1\times10^2$ CFU, $1\times10^4$ CFU, $1\times10^6$ CFU, and $1\times10^8$ CFU. Two weeks after the final infection, anti-sera were prepared from the infected mice and dot blots were produced (Fig. 4). The dot blots showed that all of the infected mice produced detectable NagA antibody, even the mice infected with very low numbers of *S. pneumoniae*. This strongly suggests that NagA is highly immunogenic, and it may be an excellent diagnostic marker for *S. pneumoniae* infections.

## Discussion

The secretomes of various pathogenic bacteria have been analyzed because of the importance of secreted proteins as potential candidates for protein vaccines (Barbey *et al.*, 2009; Mariappan *et al.*, 2010). Thus, various surface and secreted proteins produced by *S. pneumoniae* have been identified (Sun *et al.*, 2011; Choi *et al.*, 2012; Olaya-Abril *et al.*, 2012). In a previous study, we showed that several proteins (Gsp-781, Sphtra, NagA, PhtD, ZmpB, and Eno) secreted by *S. pneumoniae* were highly immunogenic (Choi *et al.*, 2012). To examine the potential value of one of these secreted proteins as a candidate diagnostic marker for *S. pneumoniae*, the cytosolic protein NagA was expressed in soluble form in an *E. coli* expression system.

The molecular weight measurement by MADLI-TOF-MS demonstrated that the recombinant NagA was expressed without truncation of the C-terminal region or post-translational modification (Fig. 1C). The result suggests that the major immunogenic determinants of *S. pneumoniae* NagA may originate from the secondary or tertiary structures of the amino acid sequence. More detailed information was obtained by tryptic peptide mapping, which identified the presence of four internal disulfide bonds in *S. pneumoniae* NagA (Table 1). This suggests that there may be two types of NagA with different internal disulfide bond configurations: one with T19-20 and T22-28, and another with T19-28 and T20-22. Further research is needed to confirm whether these heterogeneous internal disulfide bonds actually originate from *S. pneumoniae* or if they are produced only by the *E. coli* expression system.

Western blotting using rabbit anti-NagA serum showed that the NagA antibodies detected only *S. pneumoniae* NagA and not NagA from other bacterial species (Fig. 3). This immunospecificity suggests that NagA could be used as a specific marker. To confirm the uniqueness of sequence of *S. pneumoniae* NagA, sequence homology search was performed. *S. pneumoniae* NagA have more than 90% homology with *Streptococcus* stains such as *S. mitis*, *S. oralis*, *S. infantis*, and *S. peroris*. However, NagAs of the used control stains such as *K. pneumoniae*, *A. baumannii*, *P. putida* have only less than 35% homology with *S. pneumoniae* NagA. Other deacetylase (peptdidoglycan GlcNAc deacetylase) from *S. pneumoniae* has a lower sequence homology. This result suggests *S. pneumoniae* NagA can be considered as diagnostic candidate markers of *Streptococcus* stains including *S. pneumoniae*. Because *Streptococcus* stains have different habitats in the body, *S. pneumoniae* NagA can be used for the diagnostic maker for *S. pneumonia*. Major habitat of *S. mitis*, *S. oralis*, *S.infantis*, and *S. peroris* is the oral cavity (Bek-Thomsen *et al.*, 2008). However, *S. pneumoniae* is normally present in nasal cavity, lung, or phlegm. Detection of *Steptococcus* stains from the samples of nasal cavity, lung, or phlegm strongly suggests that detected *Steptococcus* stains will be *S. pneumonia*.

Moreover, observations of the immunogenicity in mice infected with *S. pneumoniae* strongly suggest that secretion of *S. pneumoniae* NagA could be happened in infected mouse and NagA function as a strong immunogenic protein (Fig. 4). In conclusion, NagA has the potential to be a specific and powerful diagnostic marker for the detection and identification of *S. pneumoniae*.

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