Turkish Journal of Immunology - Open Access
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Turk J Immunol 2018; 6(1)8–15

Identification of Hemagglutinin Protein from *Streptococcus pneumoniae* Pili as a Vaccine Candidate by Proteomic Analysis

Proteomic Analiz ile *Streptococcus pneumoniae*'dan Aşı Adayı olarak Hemagglutinin Proteininin Tanımlanması

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Abstract

Introduction: *Streptococcus pneumoniae* is a Gram-positive, bacterial human pathogen that causes a large number of respiratory tract infections, such as pneumonia and sinusitis, as well as invasive diseases such as septicemia and meningitis. Among the surface-exposed virulence factors, the pneumococcal pilus has been shown to play a role in host-cell adhesion. Hemagglutinin activity has been shown to correlate with adhesion ability. The purpose of this study was to identify of *S. pneumoniae* pili proteins with hemagglutinin activity, and to proteonomically analyze such proteins as potential vaccine candidates.

Materials and Methods: The purification of pili was carried out by a pilus cutter method. After SDS-PAGE separation, pili proteins were purified by electroelution and dialysis. After a hemagglutinination assay with mouse erythrocytes, the hemagglutinin protein was identified with Liquid Chromatography/Mass Spectrometry, along with assessments of antigenicity and epitope mapping.

Results: SDS-PAGE showed that pili protein had major bands of molecular weights of 67, 54, 25, and 11 kDa. The 54 kDa pili protein was the hemagglutinin. Computational bioinformatics analysis by Mascot Server revealed this hemagglutinin matched with the Rrgb protein of *S. pneumoniae* (gi: 299856655).

Conclusion: The analysis of its antigenicity, and epitope mapping showed that a novel protein was identified as the pilus backbone (Rrgb) of *Streptococcus pneumoniae,* and has a high antigenicity suitable for further development as a vaccine candidate.

Keywords: *Streptococcus pneumoniae*, hemagglutinin, pili, proteomic analysis

Öz

Giriş: *Streptococcus pneumoniae,* pnömoni ve sinüzit gibi solunum yolu infeksiyonu olduğu kadar septisemi ve menenjit gibi daha ciddi infeksiyonlara da yol açan Gram-pozitif bir bakteridir. Virulansı belirleyen yüzey ile ilişkili öğeleri içinde, pnömokok pilusu önemli bir rol oynar. Hemaglütünin aktivitesinin ise, bakterinin yapışma özelliği ile ilişkili olduğu gösterilmiştir ve bu proteinlerin proteom ile irdelenmesi, oluşturulabilecek bir aşıda kullanılabilmelerini sağlayabilir.

Gereçler ve Yöntemler: Pilusların saflaştırılması, pilusların kesilmesi ile yapılmıştır. Pili proteini SDS-PAGE kullanarak, elektroelusyon ve diyaliz ile saflaştırılmıştır. Fare eritrositleri ile yapılan hemaglütinasyon testinden sonra hemaglütinin proteini, epitop haritalaması ve antijenik özelliği Sıvı Kromatografisi ve Kitle Spektrometresi ile tanımlandı.

Bulgular: SDS-PAGE yöntemi ile pili proteinleri ana bantları 67, 52, 25 ve 11kDA moleküler ağırlığında saptandı. 54 KDa ağırlığındaki pilus proteini hemaglütinin antijeni idi. Mascot sunucusu ile yapılan hesaplamalı biyobilgi analizi bu hemaglutininin *S. pneumoniae*'inin Rrgb proteini ile eşleştiğini gösterdi.

Sonuç: Antijen özelliği ve epitop haritalama *Streptococcus pneumoniae*'dan pilus omurgası (Rrgb) olarak tanımlanan bu yeni proteinin yüksek antijenik özelliğinden dolayı aşı geliştirmede uygun bir aday olabileceğini göstermektedir.

Anahtar Kelimeler: *Streptococcus pneumoniae*, hemagglutinin, pili, proteomik analiz

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Received: Sep 29, 2017
Accepted: Feb 25, 2018

https://doi.org/10.25002/tji.2018.698

Introduction

Streptococcus pneumoniae (pneumococcus) is a gram-positive bacterium that colonizes the human nasopharynx, and causes diseases including pneumonia, otitis media, bacteremia, and meningitis.[1] Pneumococcal pneumonia is a lung infection with high morbidity and mortality in developing countries.[2] According to the World Health Organization (WHO), there are 156 million cases of pneumococcal pneumonia per year, and 2 million infants die annually due to this disease.[3] In 2010, pneumonia was the leading cause of infant death in the world, 30–50% were caused by *S. pneumoniae*.[4]

The manifestation of pneumococcal pneumonia is preceded by attachment of *S. pneumoniae* to the host cell in order to initiate infection. The attachment is mediated by bacterial adhesion proteins, and host cell receptors. Once the bacterium has attached, colonization and replication follow, which initiates host cell responses promoting the destruction of the infected cell.[5] The ability of *S. pneumoniae* to attach is supported by several surface proteins, such as PspC, PsaA, PsrP, PfbB, NanA, PavA, as well as the pili. However, adhesion to the host cell, via adhesins localized on the pili, is the first event of all, and it is followed by the attachment via the other surface proteins.[6,7]

Gram-positive bacteria have a unique, long, thin pili structure, yet their pili are strong and their integrity is maintained by a remarkable sequence of covalent cross-links, both between the individual pili subunits, and within them. The cross-links take the form of isopeptide bonds. Intermolecular linkages are catalyzed by the action of sortase enzymes but internal crosslinks arise through auto-catalytic, intramolecular reactions that occur spontaneously in the pili subunits.[8] These bonds play a critical role in maintaining the integrity of the pili under mechanical or chemical stress so that it can bind to the host cell, and functionally stabilize the surface proteins in order to take part in host cell pathogenesis.[7] Besides that, it has been shown in animal testing that pili of *S. pneumoniae* can increase bacterial colonization, and facilitate the formation of micro-colonies and biofilm.[9]

The colonizing ability of these bacteria is correlated with their ability to hemagglutinate red blood cells. High hemagglutination ability is highly associated with high colonizing ability. Mitra et al.[10] showed that hemagglutinating ability, and colonizing ability were correlated in *Shigella* spp. This correlation is also present with the fimbrial hemagglutinin protein of *Bordetella pertussis* which mediates the attachment of bacteria to the mouse respiratory tract.[11] This hemagglutinin protein has been widely used as one of three components of an acellular vaccine antigen of *B. pertussis* along with pertussis toxin, and pertactin, a *B. pertussis* adhesion protein.[12]

One vaccine that is currently being used against *S. pneumoniae* is the 23-valent pneumococcal polysaccharide vaccine (PPV). It is classified as a vaccine that induces an immune response via the T independent immune response. Hence it cannot be administered to infants younger than 2 years old because their T independent immune response is not yet mature. In this case, the Pneumococcal Polysaccharide Conjugate Vaccine (PCV) can be used. However, its protective ability does not cover all serotypes of *S. pneumoniae*. PCV can give a protection against invasive pneumococcal disease (IPD) from serotypes of *S. pneumoniae* in the vaccine. The serotypes of *S. pneumoniae* may cause different IPD in the different region so the ability to give a protection against IPD depends on the region. The percentages of coverage of PCV7, PCV10, and PCV13 in North America and Europe are 48–82%, 70–84%, and 70–88%, respectively. The emergence of the pneumococcal disease by non-vaccine serotype becomes a serious concern.[13]

To manage that problem, the invention of the more effective vaccine for protecting against all serotypes of *S. pneumoniae* is needed.[14] The pili proteins of *S. pneumoniae,* particularly the hemagglutinin protein can inhibit bacterial attachment to host cells. Therefore, this study aimed to identify hemagglutinin proteins from the pili of *S. pneumoniae* by proteomic analysis as a first step in the development as a vaccine candidate.

Methods

Subject

The bacteria used in this research was *S. pneumoniae* wild type with no process of typing, obtained from Balai Besar Laboratorium Kesehatan Surabaya, East Java, Indonesia.

Culture conditions and isolation of pili protein

Methods from Sumarno et al.[15] were used for *S. pneumoniae* culture, and isolation of pili proteins. *S. pneumoniae* growing on blood agar plates was

inoculated into biphasic brain heart infusion (BHI) medium supplemented with 5% sheep blood, and thiaproline carbonate glutamate (TCG) in order to enrich the growth of *S. pneumoniae* pili. The medium contained 0.02% thioproline, 0.3% $NaHCO_3$, 0.1% monosodium 1-glutamate, 1% bactotryptone, 0.2% yeast extract, 0.5% NaCl, 2% bacto agar, and 1 mM EGTA.

TCA was added into tubes containing bacteria harvested from the media until the concentration reached 3%. The tubes were centrifuged then the supernatant was discarded, and the pellet was resuspended in PBS at pH 7.4. Pili were cut from the bacteria using pili bacterial cutter for 30 seconds at a speed of 5000 rpm, repeated a total of four times (first through fourth cuts). The pili fraction was isolated by centrifugation of the cutting products at 12.000 rpm at 4°C. The supernatants containing the bacterial pili were stored at a temperature of 4°C for further analysis.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The molecular weights (MW) of the protein components of the pili fraction were conducted using SDS-PAGE after a method adapted from Laemmli.[16] Protein samples were heated at 95–100°C for 5 min in a buffer solution containing 5 mM Tris at pH 6.8, 5% 2-mercaptoethanol, 2.5% w/v sodium dodecyl sulfate, 10% v/v glycerol with bromophenol blue tracer color. A portion (12.5%) of a mini slab gel with 4% tracking gel was selected, and the gel was run at 120 mV. MW protein marker (GeneOne protein marker extended 13 bands) was loaded on alongside the samples, and gel was stained with Coomassie Brilliant Blue for visualization. Calculation of the molecular weight of the pili proteins used gel doc.

Pili protein purification

Pili proteins were purified by electroelution. The dominant protein band from the first cut was cut out from the SDS-PAGE gel for further purification. The cut band was placed on cellulose membrane filled with running electrophoresis buffer. Electroelution used a horizontal electrophoresis apparatus at 125 mV for 25 minutes. Dialysis was performed on the product of electroelution with PBS pH 7.4 buffer fluid, as much as 2 liters on 2×24 hours at 4°C.

Hemagglutination assay

Erythrocytes from mice were collected for the hemagglutination assay. Mice blood was taken from the heart, and collected in falcon tubes containing EDTA, which was then homogenized, and sterile 10 mL PBS added. The tubes were subsequently centrifuged at 3500 rpm for 10 minutes at room temperature. The supernatant was discarded, and the pellet resuspended with 1 mL sterile PBS, and centrifuged again for 10 minutes at room temperature. Then, 10 mL sterile PBS was added to 50 µL of the pellet, and homogenized.

The hemagglutination test was conducted in a V-based microplate. The dilutions used were 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, 1/512, and a control, made using a serial dilution method. The control well was not provided with sample suspension. After that, 50 µL of mice erythrocytes were added to all dilution and control wells, the plate shaken, and the presence or absence of agglutination was observed. The result was positive when the control showed a red dot, but treated samples did not show a red dot at the base of the microplate. The test samples used were extracted pili protein using the pili cutter, and purified protein.[17]

Immunization of mice with hemagglutinin protein of *S. pneumoniae* pili

50 µg/50 µL of the hemagglutinin protein of *S. pneumoniae* pili was mixed with equal volume of Complete Freund's adjuvant (Sigma), and then intraperitonel was injected into mice. The second and third injections were performed on days 7 and 14 with incomplete Freund's adjuvant. Seven days after last immunization the blood samples were collected, and its serum contained polyclonal antibody.[18]

Western blotting of hemagglutinin protein of *S. pneumoniae* pili

Gel from SDS-PAGE hemagglutinin protein of *S. pneumoniae* pili was transferred to polyvinylidene diflouride (PVDF) membrane using semidry blotting (Bio-Rad) for 1 h at 100 mA. The membrane was blocked by incubation in 5% nonfat dry milk dissolved in blocking buffer for 1 h at room temperature. After washing with Tween 0.05% three times, the membrane was incubated overnight at 4°C with mice serum which was diluted 1:100 in blocking buffer. After that, the membrane was incubated with alkaline-phosphatase rabbit anti-mouse IgG secondary antibodies at a dilution 1:200 for 2 h at room temperature after washing three times with TBST Nitro-blue tetrazolium-bromo-4-chloro-3 indolyl phosphate was used for color development.[19]

In-gel protein digestion and liquid chromatography-mass spectrometry (LC-MS/MS) analysis

Protein samples were trypsin-digested, and peptides extracted according to standard techniques.[20] Peptides were analyzed by electrospray ionization mass spectrometry using the Agilent 1260 Infinity HPLC system (Agilent) coupled to an Agilent 6540 mass spectrometer. Tryptic peptides were loaded onto a C18 column 300 SB, 5 m (Agilent), and separated by a linear gradient of water/acetonitrile/0.1% formic acid (v/v). Spectra were analyzed to identify proteins of interest using Mascot sequence matching software (Matrix Science) with the MSPnr100 database.

MS data analysis

The Mascot search parameters were a) peptide tolerance (peptide tol): ±0.2; b) MS/MS tol: ±0.2; c) Peptide charge: 2+ 3+ and 4+; d) Mass: Monoisotopic; e) trypsin as the specific enzyme; and f) the database used: MSPnr 100 Q315. The amino acid sequence sample was recovered from Mascot Server (https://sysbio-mascot.wehi.edu.au/mascot). For identifying the protein profile, the amino acid sequence was analyzed by comparing it with the protein data bank sequence from *S. pneumoniae* (taxid: 1313) through BLASTP (http://blast.ncbi.nml.nih.gov/Blast.cgi). All identifications were manually validated based on MW, signal peptide (SignalP4.1: http://www.cbs.dtu.dk/services/SignalP/), and subcellular location. Data reliability was measured by the query coverage, and percent identity of the sample.

Antigenicity and epitope mapping analysis

Protein antigenicity was performed by using Kolaskar and Tongaonkar antigenicity (http://www.iedb.org). A default threshold value was 1.0.[21] Epitope mapping was investigated using Discotope predictions of epitopes from protein structure with a default threshold of -7.7. The Discotope method is a computational method to predict linear B-cell epitopes that can be recognized by antibodies. The similarity between all novel proteins and human proteins was also analyzed by using BLASTP (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Antigenic sites, and epitope mapping were visualized by 3D structure profiles using PyMol software.

Results

Identification of hemagglutinin protein of *S. pneumoniae* pili

S. pneumoniae pili protein was identified by SDS-PAGE. Pili protein profiles were generated from the first to fourth cut of pili proteins as seen in Figure 1.

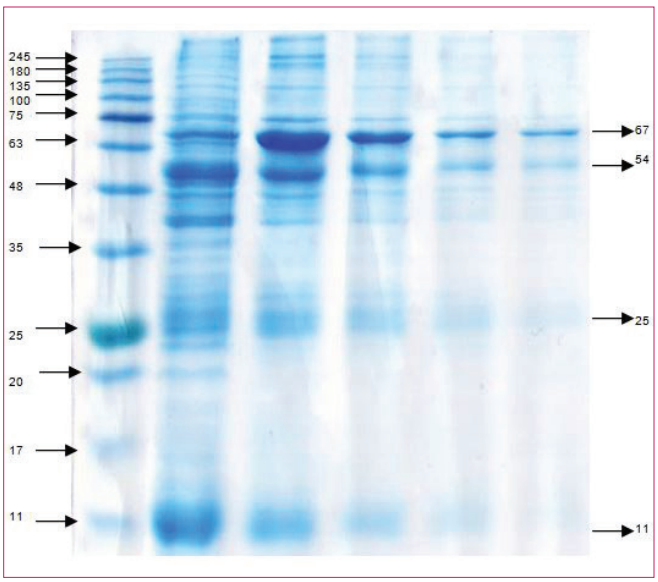

Figure 1. *S. pneumoniae* pili protein profile from SDS-PAGE gel. Annotation: (a) marker protein, (b) whole cell, (c) 1st cut pili, (d) 2nd cut pili, (e) 3rd cut pili, (f) 4th cut pili protein fraction.

The results from the SDS-PAGE gel showed four dominant bands of protein (see right-facing arrows, Figure 1), which had molecular weights of 67 kDa, 54 kDa, 25 kDa and 11 kDa. The first cut of pili protein showed thicker bands than the others, hence it was primarily used to produce pili protein subunits. The dominant protein from the first cut was cut out of the gel, and underwent electroelution and dialysis giving rise to the protein solution tested in the hemagglutination test.

The hemagglutination test showed that the protein with the molecular weight of 54 kDa was the hemagglutinin protein because it was able to agglutinate erythrocyte at up to a 1/16 dilution. This hemagglutinin protein then underwent LC-MS/MS to identify the sequence of amino acid.

Immunogenicity

The result of western blotting showed the 54 kDa of the hemagglutinin protein of *S. pneumoniae* pili is immunogenic protein. Antibody anti-54 kDa of hemagglutinin protein of *S. pneumoniae* pili could recognize 54 kDa of hemagglutinin protein of *S. pneumoniae* pili (Figure 2).

MS analysis of hemagglutinin protein of *S. pneumoniae* pili identification

The MS data suggested that two hypothetical proteins were isolated in the 54 kDa band, both similar to the protein of

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Table 1. List of novel proteins from the 54 kDa protein band of *S. pneumoniae* pili by LC-MS/MS using an in-gel digestion approach

No	Accession number	Protein	Query coverage (%)	Identity (%)	MW (kDa)	Function
1	gi: 299856655	Chain A, Structure of The Pilus Backbone (Rrgb) protein from *Streptococcus pneumoniae*	77	100	48.01	adhesion
2	gi: 761232612	Chain A, The Crystal Structure of Pneumococcal Vaccine Antigen Pcpa	66	63	49.44	None

S. pneumoniae pili (Table 1). The data showed amino acid residue IPFPALAER from the 54 kDa band matched with the chain A structure of the pilus backbone (Rrgb) from *S. pneumoniae* (100% similarity), and the chain A structure of pneumococcal vaccine antigen Pcpa (63% similarity). This protein matched with the MS/MS spectrum that assigned the amino acid sequence IPFPALAER (Figure 3). The proteins were selected based on their similarity to human surface cell proteins selected using BLASTP tools (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Antigenicity and epitope mapping analysis of identified protein as potential candidate vaccine

The selected antigenic proteins of *S. pneumoniae* pili protein that were not similar to human surface cell proteins were analyzed using bioinformatic tools to assess their antigenicity and epitope mapping. Epitope mapping performed using the BepiPred method was used to determine epitopes of selected proteins as the binding portion of the antibody. The antigenicity analysis

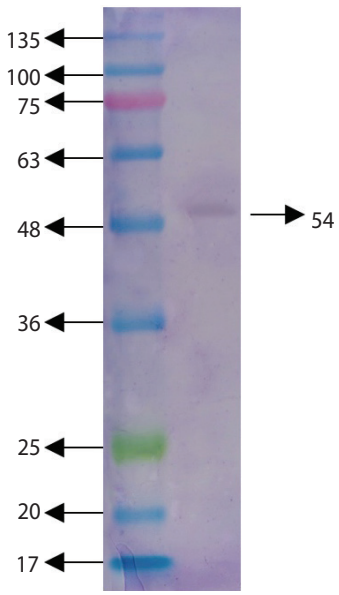

Figure 2. Western blotting using 54 kDa of hemagglutinin protein of *S. pneumoniae* pili detected with antibody anti-54 kDa of hemagglutinin protein of *S. pneumoniae* pili.

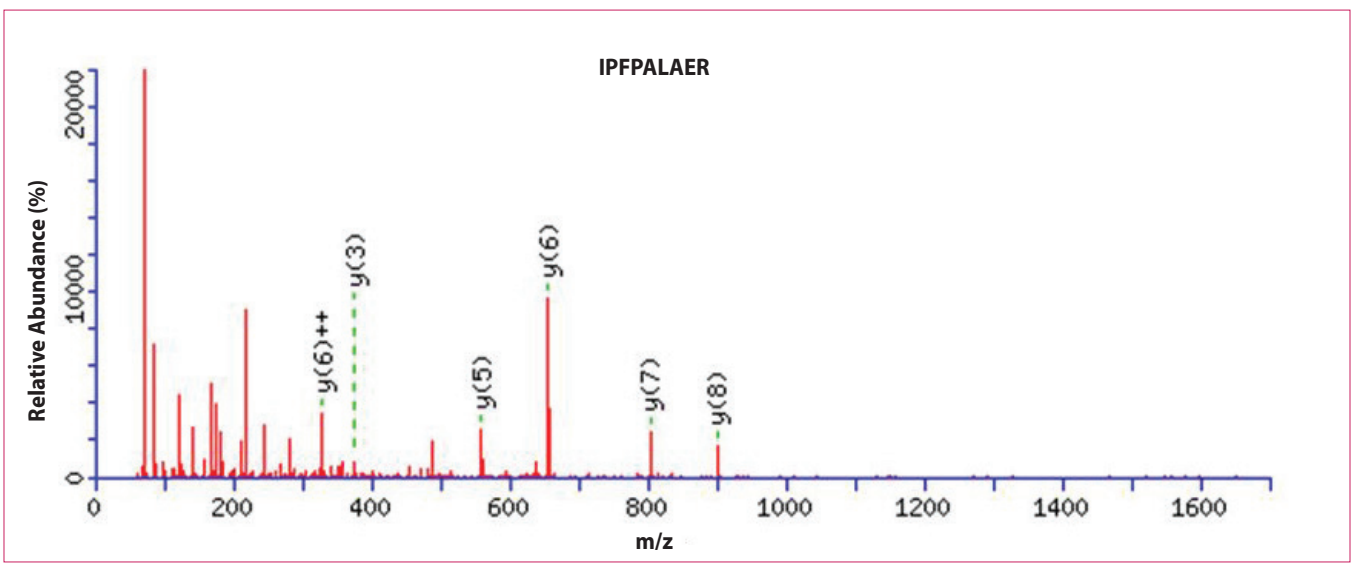

Figure 3. Mass Spectrometry spectrum of peptide sequence IPFPALAER, which matches a novel protein (gi: 299856655) that is designated as the chain A structure of the pilus backbone (Rrgb) protein from *S. pneumoniae*.

Table 2. Antigenic regions of selected proteins as candidates for vaccine against *S. pneumoniae*

Protein identification	Accesion number	Start position	End position	Peptide sequence
Chain A, Structure of The Pilus Backbone (Rrgb) protein from *Streptococcus pneumoniae*	gi: 29985665	188	195	NKPVVDKR
		211	221	GTKIPYVVNTT
		292	307	QITYSATLNSLAVADI
		353	361	KATVQLVNA
		368	373	GAPVEL
		437	443	KKFVKVD
		452	458	AQFVVKK
		464	469	YIAFKS
		486	495	KLDAAVAAYT
		503	510	AQALVDQA
		513	520	EYNVAYKE
		523	529	FGYVEVA
		569	578	IDDVEFVVGA
		598	604	TKVVNKK
		615	623	TIIFAVAGA
		626	634	MGIAVYAYV

Table 3. Epitope regions of selected proteins as candidates for vaccines against *S. pneumoniae*

Protein identification	Accession number	Start position	End position	Peptide sequence	Peptide length
Chain A, Structure of The Pilus Backbone (Rrgb) protein from *Streptococcus pneumoniae*	gi: 299856655	214	236	VNHQVGDVVEYEIVTKIPALANY	23
		255	270	TVKVTVDDVALEAGDY	16
		368	386	TFDLVNAQAGKVVQTVTLT	19
		444	458	PKVVTYGKKFVKVN	14
		504	516	DRAVAAYNALTAQ	13

result showed that selected proteins have a polyantigenic region, and polyepitope regions (Table 2 and 3). These data showed chain A, the structure of the pilus backbone (Rrgb) from *S. pneumoniae* has a high antigenicity and epitope mapping.

Discussion

Hemagglutinating activity is directly correlated with the colonization ability of *Shigella* spp. in a suckling mouse model, thus showing hemagglutination may be used as a reliable indicator of bacterial adherence[10]. Further, a hemagglutinin, 160 kDa surface protein of gram-positive *Staphylococcus saprophyticus* exhibited adhesion to the urothelial cell, so this protein could be characterized as an adhesion.[22] A gram-positive bacteria like *Staphylococcus saprophyticus* has a hemagglutinin protein which plays in an adhesion process, so it is called as the adhesion protein. In human, it plays its role by binding the human fibronectin of ureter. This phenomenon has been examined in vitro. In mice, it plays its role in colonization of kidney, and has been examined in vivo.[23] In another experiment conducted by Conolly et al.[24] *Porphyromonas ginggivalis* has hemagglutinin protein pili which acts in adhesion on the epitel cell of mouth, colonization, and biofilm formation. Thus, the hemagglutinin protein of *S. pneumoniae* pili might also involve in the cell attachment process.

The pili proteins of *S. pneumoniae* were detected by SDS-PAGE as major bands with MWs of 67, 54, 25 and 11 kDa (Figure 1). These major bands were purified by electroelution and dialysis, then tested for their ability to hemagglutinate mice erythrocytes. The pili protein with MW of 54 kDa caused agglutination of erythrocytes of mice up to 1/16 dilution.

S. pneumoniae pili protein of 54 kDa is immunogenic. In the Western blot analysis, IgG was purified from polyclonal antibodies which were prepared against the hemagglutinin protein of pili of 54 kDa. It specifically recognized the similar antigen, indicating that the protein was immunogenic (Figure 2). Another hemagglutinin protein which has immunogenicity is hemagglutinin protein from *B.pertussis* fimbrial. The immunogenicity was found when an immunization of DPT vaccine contained hemagglutinin protein of *B.pertussis* fimbrial induced a higher antibody.[25]

The immunogenicity of a protein depends on several factors such as the degree of protein alienation on the host cell (nonself), the molecular weight of protein,

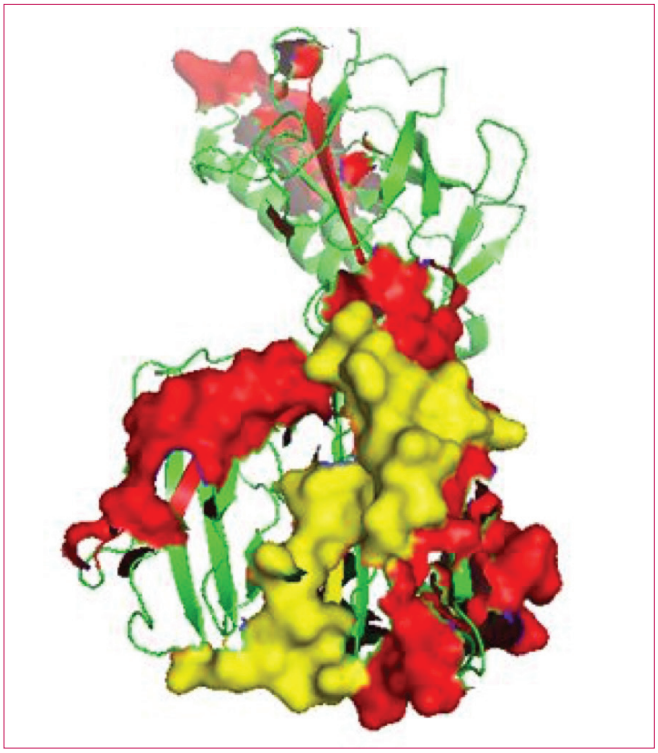

Figure 4. Predicted epitope and antigenic regions of chain A, structure of the pilus backbone (Rrgb) protein from *S. pneumoniae* (gi: 299856655) as a candidate for vaccine against *S. pneumoniae*, as visualized by Pymol.

composition, complexity, and chemical structure. The results of protein similarity of RrgB protein, and the cell surface protein in human by using BLASTP showed that the hemagglutinin protein of *S. pneumoniae* of 54 kDa is a non-self protein. A protein can be developed to be a vaccine candidate if the macromolecule protein is a potent immunogen. A protein with molecular weight over 10 kDa is a strong immunogen. The hemagglutinin protein of pili has molecular weight of 54 kDa, so it is a macromolecule protein, and its immunogenicity has been proven.[26]

The immunogenicity also depends on its structure. Protein which is composed of amino acids with positive charges (lysine, arginine, and histidine) and aromatic amino acid (tyrosine) is the stronger immunogen compared by the other groups. If those amino acids are dominant in composing the protein then the protein becomes a stronger antigen. A complex chemical structure of a protein will make the protein to be a strong immunogen. The sequences of amino acids of RrgB protein which are antigenic contain those proteins in Table 2.[26]

A novel hypothetical hemagglutinin protein from this study (54 kDa) is similar to the chain A, the structure of the pilus backbone (Rrgb) from *S. pneumoniae* (gi: 299856655) (Figure 4), and the chain A, the structure of Pneumococcal vaccine antigen Pcpa (gi: 761232612). Pili of *S. pneumoniae* is composed of a backbone fiber formed by RrgB, to which are joined two minor pilins, RrgA and RrgC. RrgB is composed of four domains, D1-D4.[27] The chain A, the structure of the pilus backbone (Rrgb) from *S. pneumoniae* (gi: 299856655) is the structure of the RrgB D2-D4 domains.[28]

RrgB (gi: 299856655) has high antigenic properties. RrgB has five regions with length 13–23 amino acids as potential antigenic sites, so RrgB has potential to be developed as vaccine candidate based on its antigenic properties, and epitope mapping. This result is supported by the research of Gentile et al.[29], who found that RrgB contains multiple protective epitopes. Immunization of Balb/c mice with RrgB protein elicited high IgG responses, and protected against lethal challenge with the *S. pneumoniae* serotype 4 strain TIGR4.[30] As noted above, hemagglutinin protein is a component of a vaccine for *Bordetella pertussis*[12]. Therefore, Rrgb as novel hemagglutinin protein of pilus backbone has a high antigenicity, and is predicted as a potential candidate for a new generation of pili protein-based vaccines. Further study is needed to prove the immune response to this protein.

Peer-review: Externally peer-reviewed.

Ethics Committee Approval: The experimental protocol was approved by Ethical Clearance from the Research Ethics Committee (Animal Care and Use Committee) of Medicine Faculty, Jember University no : 698/ H25.1.11/KE/2015

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declared that this study has received no financial support.

Author Contributions: DCM designed and performed the experiments, derived the models and analysed the data. KH assisted with SRP measurements. DCM and SS wrote the manuscript in consultation with KH and SRP

References

1. Kim KS. Acute bacterial meningitis in infants and children. Lancet Infect Dis 2010;10:32–42. https://doi.org/10.1016/S1473-3099(09)70306-8
2. Pletz MW, Maus U, Krug N, Welte T, Lode H. Pneumococcal vaccines: mechanism of action, impact on epidemiology and adaption of the species. Int J Antimicrob Agents 2008;32:199–206. https://doi.org/10.1016/j.ijantimicag.2008.01.021
3. Rudan I, Boschi-Pinto C, Biloglav Z, Mulhollang K, Campbell H. Epidemiology and etiology of childhood pneumonia. Bull World Health Organ 2008;86:408–16.
4. Liu L, Johnson HL, Cousens S, Perin J, Scott S, Lawn JE, et al. Global, regional, and national causes of child mortality: an updated systematic analysis for 2010 with time trends since 2000.

Lancet 2012;379:2151–61. https://doi.org/10.1016/S0140-6736(12)60560-1

5. Henriques-Normark B, Tuomanen EI. The Pneumococcus: epidemiology, microbiology, and pathogenesis. Cold Spring Harb Perspect Med 2013;3:a010215. https://doi.org/10.1101/cshperspect.a010215
6. Telford JL, Barocchi MA, Margarit I, Rappuoli R, Grandi G. Pili in Gram posistive pathogen. Nat Rev Microbiol 2006;4:509–19. https://doi.org/10.1038/nrmicro1443
7. Moschioni M, Emolo C, Biagini M, Maccari S, Pansegrau W, Donati C, et al. The two variants of the Streptococcus pneumoniase pilus 1 RrgA adhesin retain the same function and elicit cross-protection in vivo. Infect Immun 2010;78:5033–42. https://doi.org/10.1128/IAI.00601-10
8. Paterson NG, Baker EN. Structure of the full length major pilin from Streptococcus pneumoniae: implications for isopeptide bond formation in Gram positive bacterial pili. PLoS One 2011;6:e22095. https://doi.org/10.1371/journal.pone.0022095
9. Barocchi MA, Ries J, Zogaj X, Hemsley C, Albiger B, Kanth A, et al. A pneumococcal pilus influences virulence and host inflammatory responses. Proc Natl Acad Sci U S A 2006;103:2857–62. https://doi.org/10.1073/pnas.0511017103
10. Mitra S, Saha DR, Pal A, Niyogi SK., Mitra U, Koley H. Hemagglutinating activity is directly correlated with colonization ability of shigellae in suckling mouse model. Can J Microbiol 2012;58:1159–66. https://doi.org/10.1139/w2012-095
11. Melvin JA, Scheller EV, Noël CR, Cotter PA. New insight into filamentous hemagglutinin secretion reveals a role for full-length FhaB in Bordetella virulence. MBio 2015;6:e01189–15. https://doi.org/10.1128/mBio.01189-15
12. Otsuka N, Han HJ, Toyoizumi-Ajikasa H, Nakamura Y, Arakawa Y, Shibayama K, Kamachi K. Prevalence and genetic characterization of pertactin deficient Bordetella pertussis in Japan. PLoS One 2012;7:e31985. https://doi.org/10.1371/journal.pone.0031985
13. Tarahomjoo S. Recent approaches in vaccine development against Streptococcus .pneumoniae. J Mol Microbiol Biotechnol 2014;24:215–27. https://doi.org/10.1159/000365052
14. Ahmed MS, Derbyshine S, Flanagan B, Loh C, McCormick M, Barocchi M, et al. Immune responses to pneumococcal pilus RrgA and RrgB antigens and their relationship with pneumococcal carriage in humans. J Infect 2014;68:562–71. https://doi.org/10.1016/j.jinf.2014.01.013
15. Sumarno RP, Yanuhar U, Winarsih S, Islam S, Santoso S. Detection of molecule adhesion sub unit pili 48 kDa Salmonella Typhi by immunochemistry method using sera patients suffering from typhoid fever. J Basic Appl Sci Res 2012;2:8527–32.
16. Laemmli UK. Cleavage of structural protein during the assembly of the head of bacteriophaga T4. Nature 1970;227:680–5.
17. Li X, Johnson ED, Mobley HL. Requirement of MrpH for mannosa-resistent Proteus-like fimbria-mediated hemaglutination by Proteus mirabilis. Infect Immun 1999;67:2822–33.
18. Eivazi S, Majidi J, Maleki LA, Abdolalizadeh J, Yousefi M, Ahmadi M, et al. Production and Purification of a Polyclonal Antibody Against Purified Mouse IgG2b in Rabbits Towards Designing Mouse Monoclonal Isotyping Kits. Adv Pharm Bull 2015;5:109–13. https://doi.org/10.5681/apb.2015.015
19. Mahmood T, Yang PC. Western blot: technique, theory, and trouble shooting. N Am J Med Sci 2012;4:429–34. https://doi.org/10.4103/1947-2714.100998
20. Bringans S, Eriksen S, Kendrick T, Gopalakrishnakone P, Livk A, Lock R, Lipscombe R. Proteomic analysis of the venom of Heterometrus longimanus (Asian black scorpion). Proteomics 2008;8:1081–96. https://doi.org/10.1002/pmic.200700948
21. Gomase VS, Changbhale SS, Chitlange NR, Sherkane AS. Prediction of antigenic epitopes from Tityus serrulatus venom allergen 5: an aid to antitoxin vaccine. J Toxicol Res 2012;2:20–4.
22. Meyer HG, Wengler-Becker U, Gatermann SG. The hemagglutinin of Staphylococcus saprophyticus is a major adhesin for uroepithelial cells. Infect Immun 1996;64:3893–6.
23. Kline KA, Lewis AL. Gram-Positive Uropathogens, Polymicrobial Urinary Tract Infection, and the Emerging Microbiota of the Urinary Tract. Microbiol Spectr 2016;4. https://doi.org/10.1128/microbiolspec.UTI-0012-2012
24. Connolly E, Millhouse E, Doyle R, Culshaw C, Ramage G, Maron GP. The Porphyromonas gingivalis hemaglutinins HagB and HagC are mayor mediator of adhesion and biofilm formation. Mol Oral Microbiol 2017;32:35–47. https://doi.org/10.1111/omi.12151
25. Hara M, Okada K, Yamaguchi Y, Uno S, Otsuka Y, Shimanoe C, et al. Immunogenicity and safety after booster vaccination of diphtheria, tetanus, and acellular pertussis in young adults: an open randomized controlled trial in Japan. Clin Vaccine Immunol 2013;20:1799–804. https://doi.org/10.1128/CVI.00490-13
26. Farber D. L., Sleasman J. W., Virella G. Medical Immunology, 6th ed. New York: Taylor & Francis Inc.; 2007. pp.35–54.
27. El Mortaji L, Terrasse R, Dessen A, Vernet T, Di Guilmi AM. Stability and assembly of pilus subunit of Streptococcus pneumoniae. J Biol Chem 2010;285:12405–15. https://doi.org/10.1074/jbc.M109.082776
28. Spraggon G, Koesema E, Scarselli M, Malito E, Biagini M, Norais N, et al. Supramolecular organization of the repetitive backbone unit of the Streptococcus pneumoniae pilus. PLoS One 2010;5:e10919. https://doi.org/10.1371/journal.pone.0010919
29. Gentile MA, Melchiorre S, Emolo C, Moschioni M, Gianfaldoni C, Pancotto L, et al. Structural and functional characterization of the Streptococcus pneumoniae RrgB pilus backbone D1 domain. J Biol Chem 2011;286:14588–97. https://doi.org/10.1074/jbc.M110.202739
30. Gianfaldoni C, Censini S, Hilleringmann M, Moschioni M, Facciotti C, Pansegrau W, et al. Streptococcus pneumoniae pilus subunits protect mice against lethal challenge. Infect Immun 2007;75:1059–62. https://doi.org/10.1128/IAI.01400-06