

The Effect of Intranasal Immunization with *Streptococcus Pilus* Protein on Nasopharyngeal pIgR and IgA Expression in Rats

Sıçanlarda *Streptococcus Pilus* Proteinini ile İntranasal Bağışıklamanın Nazofaringeal pIgR ve IgA İfadesi Üzerine Etkisi

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Abstract

Introduction: *Streptococcus pneumoniae* (*S. pneumoniae*) causes pneumococcal disease, which has high mortality and morbidity in children under two years of age, the elderly and immunocompromised individuals. This disease can be prevented by immunization, but the current vaccine, pili protein vaccine (PPV), is less likely to protect children under the age of two and only protects against the serotypes contained in the vaccine. Hence, a new vaccine is needed to enable full protection. The use of bacterial pili proteins may offer an alternative new vaccine. Therefore, the determination of the ability of such proteins to stimulate mucosal immunity with indicator expression of pIgR and s-IgA is required.

Materials and Methods: Pili were isolated using the pili bacterial cutter method, and used for nasal vaccination to the rats. TGF- β 1, IL-17A, and s-IgA were measured by ELISA while pIgR was examined by immunohistochemistry.

Results: This study demonstrated that intranasal immunization with antigen (54 kDa pili protein) and antigen plus adjuvant significantly increased ($p<0.05$) the expression of TGF- β 1. However, the expression of IL-17A increased significantly ($p<0.05$) only in rats immunized with antigen plus adjuvant. Further analysis demonstrated that intranasal immunization with antigen and antigen plus adjuvant significantly increased ($p<0.05$) expression of pIgR. Expression of sIgA in nasal lavage significantly increased ($p<0.05$) in those rats which had been immunized with pili protein plus adjuvant.

Conclusion: This study showed that the immunization with *S. pneumoniae* pili protein increased expression of pIgR and sIgA which are important mucosal immunity components. Therefore, this protein has potency to be developed as nasal vaccination to prevent *S. pneumoniae* infection.

Keywords: Intranasal immunization, pili protein *S. pneumoniae*, pIgR, sIgA

Öz

Giriş: İnfeksiyonlara yol açan *Streptococcus pneumoniae* (*S. pneumoniae*) iki yaşın altındaki bebeklerde, yaşlı ve immün yetmezliği olan hastalarda yüksek morbidite ve ölüm oranlarına neden olur. Bu hastalık, aşılama ile önenebilir, ancak, pili proteini aşısı, 2 yaşın altındaki çocuklarda daha az koruma sağlar ve sadece aşısındaki serotiplere karşı koruma sağlar. Bu neden ile, tam bir koruma sağlayabilecek yeni bir aşısıya gerek bulunmaktadır. Anlamlı belirteçler olan pIgR ve sIgA salgılanmasını sağlayarak mukozadaki bağışıklığı oluşturabilecek proteinlere gereksinim vardır.

Gereç ve Yöntemler: Pililer, bakterinin pilusunu kesen metod kullanılarak elde edildi ve sıçanların burnundan bağışıklama için kullanıldı. TGF- β 1, IL-17A ve sIgA, ELISA metodu ile, pIgR ise, immünohistokimya ile ölçüldü.

Bulgular: Bu çalışma, 54kDA'luk pili proteini yapılan aşılama ve protein ile birlikte verilen adjuvanın TGF- β 1 ifadesini istatistiksel olarak anlamlı düzeyde ($p<0.05$) artırdığını gösterdi. Bununla birlikte, IL-17A ifadesi sadece antijen ile birlikte adjuvan verilen sıçanlarda yükseldi ($p<0.05$). Buna ek olarak, antijen ve antijen ile birlikte verilen adjuvan, pIgR ifadesini istatistiksel olarak anlamlı düzeyde yükseltti ($p<0.05$). Burun yıkama suyundaki sIgA düzeyi ise pili proteini ile birlikte adjuvan verilen sıçanlarda artmış olarak saptandı ($p<0.05$).

Sonuç: Bu çalışma, *S. pneumoniae*'nin pili proteini yapılan aşılamının, mukoza bağışıklığı için önemli bir unsur olan pIgR ve sIgA düzeylerini artırdığını gösterdi. Bulgularımız bu proteinin, *S. pneumoniae* infeksiyonunu önlemek için kullanılabilirliği yönündedir.

Anahtar Kelimeler: Burun için aşılama, pili proteini, *S. pneumoniae*, pIgR, sIgA

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Introduction

Streptococcus pneumoniae (*S. pneumoniae*) colonizes the human nasopharynx, and can cause several diseases including community-acquired bacterial pneumonia and otitis

media, but can also give rise to severe cases of meningitis and sepsis.^[1] The pneumococcal disease remains one of the major health problems infecting humans worldwide. It is the most important cause of morbidity and mortality in children under five years of age and the elderly. It is estimated that 12.000 to 28.000 deaths are due to pneumococcal disease every year worldwide.^[2]

Pneumococcal disease can be prevented by immunization with Pneumococcal Polysaccharide Vaccine (PPV) 23 Valens. PPV is a subunit vaccine that induces the immune system through the T-independent pathway, so it is not used in children below two years of age, as they have no mature T-independent immune response. The limitation of PPV 23 can be overcome with the use of Pneumococcal Polysaccharide Conjugate Vaccine (PCV). PCV has a high potency (88.4%) in preventing IPD (invasive pneumococcal diseases) in children is caused by the serotype in the vaccine.^[3] However, PCV still has the limitation that it only gives limited protection against the serotype in the vaccine. Hence, a new vaccine that covers all *S. pneumoniae* serotypes needs to be developed. One potential candidate for such a pneumococcal disease vaccine is virulence factor protein.^[4]

S. pneumoniae has several virulence factors, including the polysaccharide capsule, surface proteins, pili, enzymes and the pneumolysin toxin. Virulence factors function as anti-phagocytic compounds and assist in adhesion, invasion, iron transport, protection against oxidative stress, and biofilm formation.^[5] *S. pneumoniae* has a hemagglutinin pilus protein with a molecular weight of 54 kDa, which has high antigenicity. This protein has the potential to be developed as a vaccine material, particularly for intranasal immunization.

Intranasal immunization involves applying the vaccine to the mucosal tissue, which gives the lungs and upper respiratory tract protection against pathogen infection. A nasal mucosa is an appropriate place for vaccination against respiratory tract infection because not only is it where pathogens are often introduced, but it is also where the mucosal immune system is stimulated by nasopharyngeal-associated lymphoid tissue (NALT).^[6]

Intranasal immunization can increase the expression of TGF- β in macrophages and CD4⁺ T cells. *In vivo*, TGF- β plays a role in increasing the production of Secretory IgA (sIgA).^[7] Intranasal immunization also elevates the expression of IL-17A.^[8] Intranasal immunization also

elevates the expression of CCR6 on the surface of CD4⁺ T cells. CCR6 is the marker for a Th17 cell, which expresses many cytokines, one of the most important being IL-17A, which has a role in increasing the expression of Polymeric Immunoglobulin Receptor (pIgR) in basolateral epithelial cells.^[9] Increasing expression of pIgR also increases the expression of s-IgA. IL-17R^{-/-} mice injected with segmented filamentous bacteria showed lower pIgR concentration in their feces as compared with IL-17R^{+/+} mice. This condition was proportional to the concentration of s-IgA in IL-17R^{-/-} mice and these mice had lower s-IgA concentration compared with IL-17R^{+/+} mice.^[10] This study aims to show that *S. pneumoniae* pili protein of 54 kDa can affect pIgR and s-IgA expression in rat nasopharynx.

Materials and Methods

Culture conditions and isolation of pili protein

S. pneumoniae from the Paru Hospital Jember East Java Indonesia was cultured using blood agar. This agar contained biphasic medium brain heart infusion (BHI) supplemented with 5% sheep blood-Thioprolin Carbonate Glutamate (TCG) to enrich the growth of *S. pneumoniae* pili. The medium contained 0.02% thioproline; 0.3% NaHCO₃, 0.1% monosodium 1-glutamate, 1% bacto tryptone; 0.2% yeast extract, 0.5% NaCl, 2% bacto agar and 1 mM β amino-ethyl ether-N, N, N 'n',-tetra acetic acid (EGTA). Harvested bacteria were transferred to TCA until the concentration reached 3%. They were then centrifuged, and the pellet was suspended in PBS pH 7.4. Bacterial pili were cut four times using a bacterial pili cutter for 30 seconds at a speed of 5.000 rpm. The pilus fraction was then centrifuged to isolate cut products at 12.000 rpm at 4°C. The supernatant containing pilus protein was stored at 4°C.^[11]

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Molecular weight measurement by SDS-PAGE was performed by adopting the method of Laemmli. Protein samples were heated in a buffer solution at 95–100°C for 5 minute in 5 mM Tris pH 6.8, 5% 2-mercaptoethanol; 2.5% w/v sodium dodecyl sulfate, and 10% v/v glycerol with bromophenol blue tracer color. 12.5% of a mini slab gel with 4% tracking gel was selected. A voltage of 120 mV was required. Molecular weight protein marker (Gene One protein marker extended 13 bands) were

loaded on the gel and stained with Coomassie brilliant blue. Calculation of pili protein molecular weight was then carried out by used of gel doc.^[12]

Pili protein purification

Pili protein purification was performed using electroelution. Pili proteins were cut at the 54 kDa band. The cut band was inserted into a cellulose membrane filled with running electrophoresis buffer. Electroelution used a horizontal electrophoresis apparatus at 125 mV for 25 minutes. Dialysis was performed on the electroelution product with PBS pH 7.4 buffer fluid, as much as 2 liters for 2 x 24 hours at 4°C.

Immunization

Wistar rats that were 12–16 weeks of age were grouped into four groups of seven. Rats in the control group were immunized with 40 µL PBS; Rats in group 1 were immunized with 40 µL PBS containing adjuvant (2 µg CTB); Rats from group 2 were immunized with 40 µL PBS containing 20 µg Ag (54 kDa *S. pneumoniae* pili protein); Rats from group 3 were immunized with 40 µL PBS containing 20 µg Ag and 2 µg CTB. Vaccines were given intranasally, 20 µL per nostril. All rats were vaccinated on days 0, 7 and 14. A week after the last immunization, the nose wash and nasopharyngeal tissue were isolated from the rats.^[13,14]

Sample isolation

One week after the last immunization, animals were anesthetized with ether and then decapitated. Nasal wash isolation was performed retrograde by inserting 1–2 mL of sterile saline solution through the trachea and drops of this saline solution through the nostril were fed into sterile Eppendorf tubes. The scalp was then cleaned, the lower jaw removed and the nasopharyngeal tissue is taken.^[15]

Measurement of nasal wash TGF-β1 and IL-17A

The concentrations of TGF-β1 and IL-17A were measured using a commercial ELISA kit according to the manufacturer's instructions. Samples were transferred into wells and incubated for 24 hours for the antigen to be adsorbed. The plate was then washed with washing solution, then 100 µL of antibody to IL-17A or TGF-β1 was added according to the measurement objective, then incubated for 1 hour at room temperature. After 1 hour, the plate was washed and 100 µL HRP-Streptavidin solution was added. The plate was then incubated for 45

minutes at room temperature and washed with washing solution. One-hundred µL TMB substrate was then added, the mixture was incubated for 30 minutes and then supplemented with 50 µL stop solution. The OD₄₀₅ of the solution was then taken using an ELISA reader.

Immunohistochemistry for detection of pIgR

Nasopharyngeal tissue was cut using a microtome at a thickness of 6 mm and placed on top of an object glass and covered with 1.5% BSA. The preparation underwent deparaffinization using xylene and was then rehydrated with ethyl alcohol. Then, it was washed with PBS containing BSA (1 mg/mL). Nonspecific binding was blocked with 1% goat serum for 20 min at room temperature. The preparation was stained with the primary antibody of rabbit anti-rat IgR (1:2500) s-IgA for 30 minutes and then incubated with secondary anti-goat anti-rabbit IgA antibody conjugated biotin (1:200) for 30 minutes. Then, it was dyed using avidin-biotin to horse peroxidase and hematoxylin as a contrast. After that, the preparation was dehydrated with alcohol. Furthermore, pIgR expression was observed using a microscope and calculated as a carpenter. Cell expressing pIgR calculated per 100 nasopharyngeal epithelial cells.^[16]

Anti *S. pneumoniae* pili antibody measurement

The antibodies observed were s-IgA of the nasal washing, as assayed by ELISA. The plates were coated with 54 kDa *S. pneumoniae* pili proteins as an adhesion molecule and incubated for 24 hours. After the antigen was adsorbed, plates were washed with PBS containing Tween 0.05% (PBS-T) and blocked with PBS-T containing 5% FBS. Nasal washing was then added and plates were incubated for 2 hours at room temperature. Furthermore, plates were washed with PBS-T and secondary antibodies, which was horseradish peroxidase-labeled goat anti mouse s-IgA specific Abs (Southern Biotechnology Associates, Birmingham, AL, USA). To assay color changes due to antibody-antigen reactions, 2,2'-azino-Bis (3-ethyl benzothi-azoline-6-sulfonic acid in H₂O₂) was added (Moss, Inc., Pasadena, MD, USA). OD₄₀₅ was measured using an ELISA reader.^[13,14]

Ethics

An ethical approval was obtained from the Ethical Committee of the Faculty of Medicine Universitas Jember No. 698/H25.1.11/KE/2015

Statistics

All results are expressed as mean \pm standard deviation (SD). Statistical analysis was performed using one-way ANOVAs with LSD multiple comparison post-tests, with $p < 0.05$ being considered significant.

RESULTS

Intranasal immunization with 54 kDa hemagglutinin protein promotes expression of cytokine TGF- β 1

SDS-PAGE analysis revealed dominant pili proteins of 67, 54, 25 and 11 kDa molecular weight (Fig. 1). Pili proteins with a molecular weight of 54 kDa were cut and purified by electroelution and continued dialysis. The results of purification were then used as an Ag (antigen) to immunize rats.

Wistar rats that were immunized intranasally were subjected to isolation of nasal washing one week after

the second booster. The levels of cytokines TGF- β 1 and IL-17A in the nasal washings were measured. Intranasal immunization of Wistar rats with antigen plus adjuvant induced a significant increase ($p < 0.05$) in TGF- β 1 concentration (3 x compared to controls). Ag-immunized rats also had a significant increase ($p < 0.05$) in TGF- β 1 levels of about 2 x compared to controls, and adjuvant-immunized rats showed slightly decreased expression of TGF- β 1 (Fig. 2A). Measurements of IL-17A show that Wistar rats immunized with antigen plus adjuvant showed a significant increase ($p < 0.05$) in IL-17A expression as compared to other groups. However, the increase in IL-17A was not as high as the expression of TGF- β 1.

Intranasal immunization using Ag plus adjuvant increased expression of pIgR

The expression of nasopharyngeal pIgR after intranasal immunization was assessed. The results showed that pIgR expression of nasopharyngeal tissue of rats immunized by pilus protein plus adjuvant was higher than those of the other groups (Fig. 3). Rats immunized with Ag plus adjuvant had a significantly higher pIgR ($p < 0.05$). Rats immunized with pilus protein showed a 1.5-fold increase in pIgR expression, whereas the rats immunized with adjuvant showed a slightly increased pIgR expression compared to the that of control group (Fig. 4).

Increased expression of sIgA by intranasal immunization with pilus protein plus adjuvant

Rats that were nasally immunized with adjuvant had slightly increased concentrations of sIgA as compared to control group (Fig. 5).

DISCUSSION

Intranasal immunization using the 54 kDa *S. pneumoniae* hemagglutinin pilus protein increased expression of TGF- β 1 (Fig. 2A). This result is similar to those reported by Wang, et al.^[17], which stated that intranasal administration of *Streptococcus* group A bacteria significantly increased expression of TGF- β 1 in NALT, cervical lymphoid node, spleen, and serum. Intranasal immunization of RSV fusion protein formulated with three adjuvant increase TGF- β 1 expression.^[18] TGF- β 1 is a cytokine secreted by various immune cells, including macrophage dendritic cells and CD4 + T cells that play a role in inducing T cell-IgA isotype class switching together with CD40L.^[19,20] TGF- β , together with IL-5, IL-6, and IL-21 stimulate IgA

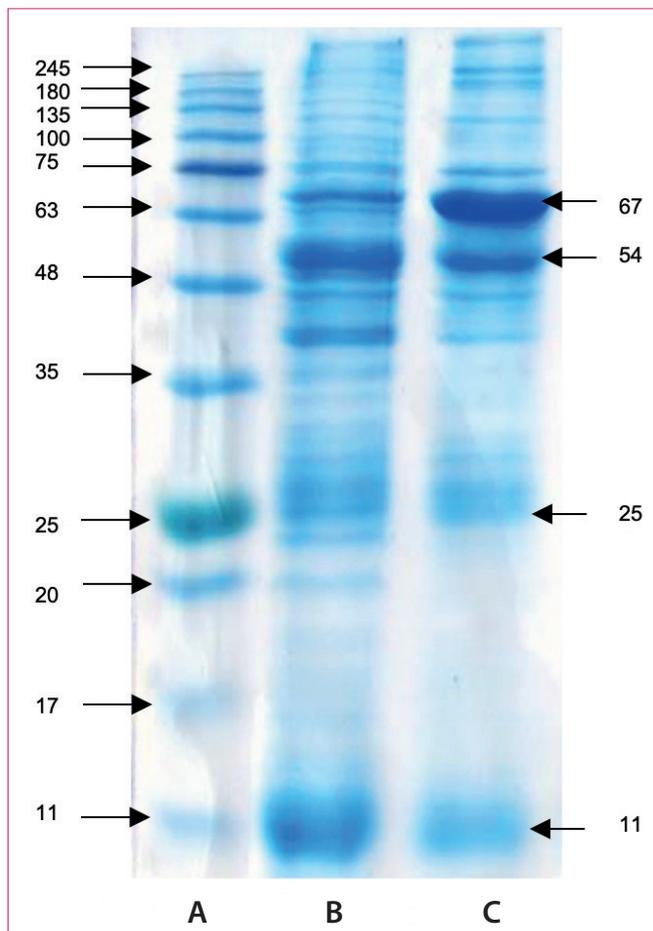


Figure 1. Profile MW pilus proteins of *S. pneumoniae* in the SDS-PAGE. Protein marker (kDa) (a), whole bacteria (b), and pilus (c).

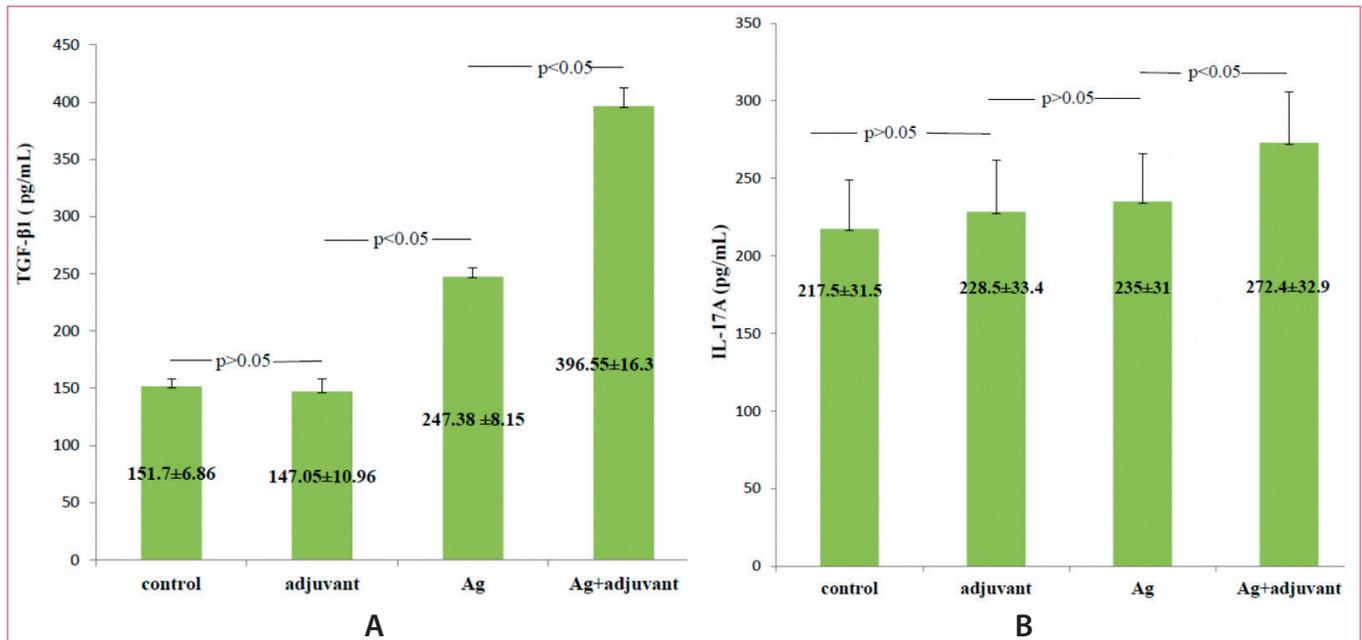


Figure 2. ELISA results of immunized Wistar. The concentration of TGF-β1 nasal washing of control and treatment rats (a). The concentration of IL-17A nasal washing of control and treatment rats (b). Data are mean ± standard deviation values of 7 rats in each group. Significant difference based on LSD's high significant differences test at a 95%.

and B cells to differentiate into plasma cells that produce dimeric-IgA. [21,22] TGF-β1 and IL-1β play a role in T-cell differentiation into Th17. [17]

Differentiation of naïve T cells into Th17 can be induced by antigen immunization. The differentiation can be identified by the increase of cytokines expressed by Th17 cells such as IL-17A, IL-17F, IL-22, IL-26, and CCL-20.

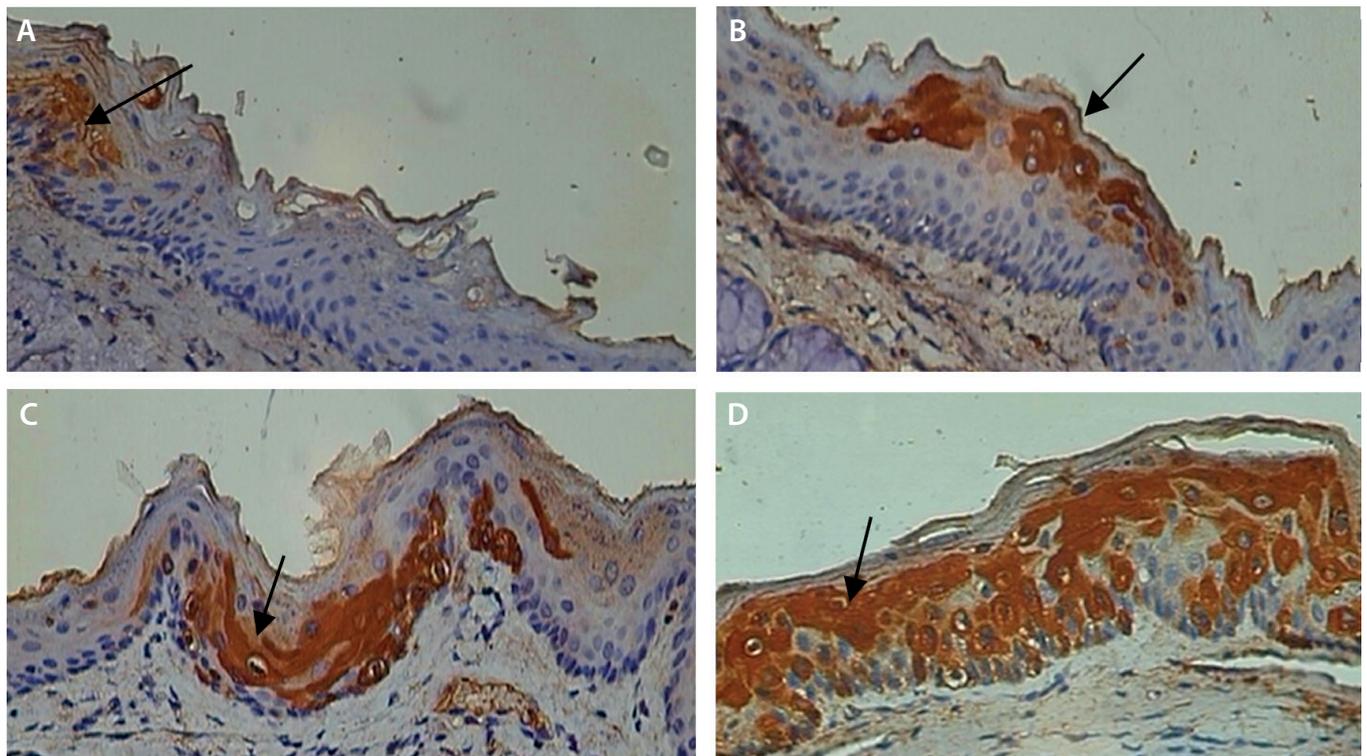


Figure 3. The expression pIgR nasopharynx after immunized with immunohistochemistry staining. pIgR expression: Control rats (a). Rats immunized with the adjuvant (b). Rats immunized with Ag (c). Rats immunized with Ag + adjuvant (d). The nasopharyngeal tissue saw with a CXC-3 Olympus microscope (M=400×). The arrows indicate epithelial cells expressing pIgR.

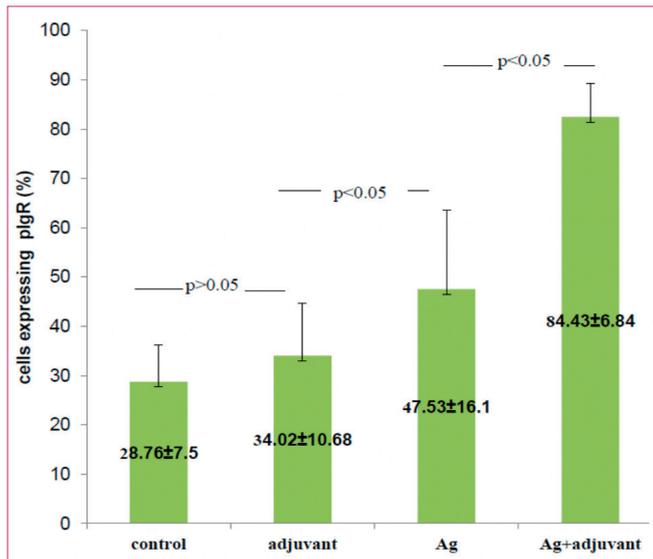


Figure 4. IHC results of pIgR nasopharynx Wistar rats that had immunized. Data are mean \pm standard deviation values of 7 rats in each group. Significant difference based on LSD's high significant differences test at a 95%.

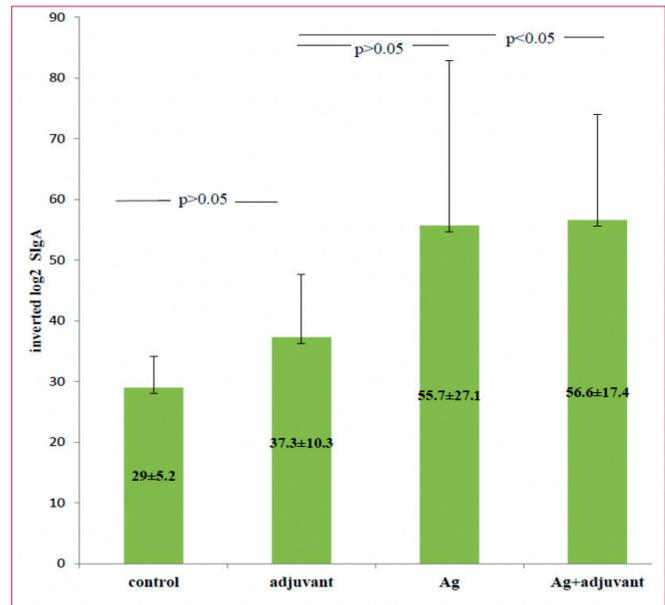


Figure 5. ELISA results of sIgA nasal lavage fluid washing Wistar Rats after immunization. Data are mean \pm standard deviation values of 7 rats in each group. Significant difference based on LSD's high significant differences test at a 95%.

In this study, there was an increase in the expression of IL-17A (Fig. 2B) in rats intranasal immunized with Ag plus adjuvant, so it can be concluded that intranasal immunization using 54 kDa *S. pneumoniae* pili protein with adjuvant CTB was able to induce differentiation of naïve T cells into Th17. Th17's ability to express cytokines varies depending on the Th17 subset and the vaccination route. Th17 mature cells express IL-17A and IL-17F but do not express IL-22.^[23] The intranasal immunization route will activate Th17 cells in the lung and nasal cavity by more than 10%.^[8]

IL-17A can increase pIgR expression. In rats immunized with pilus protein plus adjuvant, there was a significant increase in pIgR expression (Fig. 4). These results are similar to those found by Jaffar that rats given intranasal IL-17A have significantly elevated pIgR levels compared with controls.^[9] Another study also showed that human colon epithelial cells treated with IL-17 increased pIgR expression. Increased pIgR expression by IL-17A occurs through activation of the NF- κ B and P13K pathways.^[10] Based on these results, it can be concluded that IL-17A is an important cytokine for increasing pIgR expression.

Polymeric receptor immunoglobulin (pIgR) is a type 1 transmembrane protein with a molecular weight of 120 kDa. pIgR can be divided into three parts: extracellular, transmembrane and cytoplasmic. These parts comprise

623, 23 and 103 amino acids, respectively. Five domains of homologous immunoglobulin (i.e., domains 1–5) and one homolog domain (i.e., domain 6) make up the extracellular part. One is a domain that binds to dAgA so that the amino acid sequence (AA) of its constituents is maintained. Six are located near the transmembrane protein; its AA sequences vary the most among species and this domain is a site that is broken down by proteolytic enzymes.^[24]

Structurally, the s-IgA consists of the IgA dimeric, and the extracellular domain pIgR which is known as the secretory component (SC).^[25-27]

The increase of pIgR is expected to increase transcytosis of pIgA, thus raising s-IgA levels. In this study, increased pIgR was followed by raised s-IgA in nasal wash, which suggested that rats immunized with Ag and adjuvant had higher sIgA than control and other groups (Fig. 5). These results are similar to those of Malley, et al.^[13], in which mice immunized nasally with the 40 kDa outer membrane protein of *Porphyromonas gingivalis* combined with cholera toxin increased s-IgA in saliva and nasal wash. The results of this study are also similar to those of Christensen, et al.^[28], who found that intranasal immunization using Group A streptococcal C5a peptidase (SCPA) + cationic adjuvant formulation No. 1 (CAF01) increased s-IgA in lungs, although the highest yield of sIgA was found when

administering vaccines subcutaneously, followed by a booster administration intranasally. The study of Cai, et al.^[14] shows that by increasing the expression of IL-17A there is an increase in s-IgA levels and vice versa (i.e. with the neutralization of IL-17A there is a significant decrease in s-IgA levels). The results of this study, supported by various other studies show that intranasal immunization increases levels of s-IgA in the respiratory tract.

Secretory Immunoglobulin A prevents infection by blocking bacteria and its toxin, which adheres to the epithelial mucosa of target cells, hence preventing epithelial disruption, bacterial colonization, and invasion. Early-stage adherence of bacteria to the epithelial mucosa is consecutively mediated by pili and outer membrane proteins (OMP). Hemagglutinin adhesin protein pili and OMP of *S. flexneri* can cross-react with OMP of *S. dysenteriae*.^[29] *S. dysenteriae* pili proteins acting as adhesive molecules may protect fluid movement by using the rat Ties Ilea Loop Model.^[30] Market able Pertussis a cellular vaccine, Diphtheri, Pertussis, and Tetanus (DPT) contains molecule adhesion *Bordetella pertussis*.^[31] Therefore, immunization using vaccines containing adhesive pili proteins is likely to prevent infection, making such proteins suitable as a vaccine against *S. pneumoniae* infection. This research suggests that the 54 kDa *S. pneumoniae* pilus protein is a suitable candidate for a vaccine to prevent the many important diseases caused by this bacterium.

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