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Short Communication

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Immune Response to Capsular Polysaccharide of *Streptococcus pneumoniae* in Rabbits Immunized with Pneumococcal Conjugate Vaccine

Zaenab Zaenab[1], Wisnu Tafroji[2], Korrie Salsabila[2], Yayah Winarti[2], I Gusti Ayu Inten Wulandari[2], Andi Yasmon[3], Dodi Safari[2*]

[1]*Master's Program in Biomedical Sciences, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia*
[2]*Eijkman Research Center for Molecular Biology, National Research and Innovation Agency, Cibinong, West Java, Indonesia*
[3]*Department of Microbiology, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia*

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ABSTRACT

To evaluate the immune response of a rabbit model to polysaccharide capsules of *Streptococcus pneumoniae* after primary and booster immunization with pneumococcal conjugate vaccine. Rabbits were immunized with 0.25 ml of the 13-valent pneumococcal conjugate vaccine (PCV13) on Day 0, 7, and 14. Blood of rabbits was collected to measure the specific antibodies against the native polysaccharide capsules of *S. pneumoniae* serotypes 6B (Anti-Pn6BPS antibodies) and 19F (Anti-19FPS antibodies) using enzyme-linked immunosorbent assay (ELISA). The specific anti-Pn6BPS and anti-Pn19FPS antibodies in the immunized group increased significantly compared to the control rabbit after immunization ($P<0.05$). The highest titer of anti-Pn6BPS and anti-Pn19FPS antibodies were obtained on Day 21 and Day 28, respectively. The third immunization on Day 21 is a fairly high increase in specific antibodies against polysaccharide type 6B and type 19F. The highest antibody titers against polysaccharide capsules of *S. pneumoniae* were obtained after booster immunization.

1. Introduction

Streptococcus pneumoniae is a Gram-positive commensal bacterium commonly found colonizing the upper respiratory tract of healthy individuals (Subramanian *et al*. 2019; Akpaka *et al*. 2022). Many virulence factors of *S. pneumoniae* can trigger the host's immune response, one of which is the polysaccharide capsule. The polysaccharide capsule is the dominant virulence factor and plays an important role in virulence, particularly by interfering with the elimination mechanism through the host opsonophagocytic (Brooks and Mias 2018; Paton and Trappetti 2019).

At present, there are more than 100 serotypes of *S. pneumoniae* that are immunologically distinct based on the structure of their polysaccharide capsule (Geno *et al*. 2015; Bobadilla *et al*. 2021). The distribution of capsular serotypes varies geographically, both in terms of carriage conditions, pathogenicity, and clinical presentation. However, globally, the most common infecting *S. pneumoniae* serotypes are 1, 5, 14, 6A, 6B, 19F, and 23F (Nhantumbo *et al*. 2016). In Indonesia, the most common *S. pneumoniae* serotypes are 6A/B, followed by 23F, 19F, 15B/C, 11A, 19A, 14, and 3 (Soewignjo *et al*. 2001; Farida *et al*. 2014;

* Corresponding Author
E-mail Address: dodi004@brin.go.id

Safari *et al*. 2014; Said *et al*. 2017; Kartasasmita *et al*. 2020).

The Centers for Disease Control and Prevention recommends routine administration of pneumococcal conjugate vaccine (PCV) for at-risk groups such as infants, children, and adults of 65 years and older (Pneumococcal Vaccine Recommendations | CDC 2023). Regarding the administration schedule for PCV, the World Health Organization recommends a 3-dose schedule, either as 2p+1 or 3p+0 (World Health Organization = Organisation mondiale de la Santé, 2019). In Indonesia, the PCV is administered in a 2+1 dose schedule at 2 months, 3 months, and 12 months of age ("Vaccination schedule for Pneumococcal disease," n.d.).

The distribution of PCV and its inclusion in vaccination programs have, in time, effectively reduced pneumococcal diseases, carriage, and transmission of serotypes included in the PCV (Dagan 2019). The PCV does so by eliciting an immune response in the body to create antibodies that fight against pneumococcal bacteria. Studies have shown that booster vaccines significantly increased the frequency of *S. pneumoniae* (Spn)-specific memory B cells as well as the functional antibody levels for most serotypes in the PCV13 (Chapman *et al*. 2020; Kaur and Pichichero 2020). By implementing serial primary doses of the vaccine and a booster, a functional antibody threshold can be achieved, and long-term immunity can be induced (Chapman *et al*. 2020; World Health Organization = Organisation mondiale de la Santé 2019).

Anti-capsule streptococcal antibodies can be produced by animal injection using the commercial PCV13 antigen containing purified polysaccharide capsules from 13 serotypes of *S. pneumoniae* (1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 19A, 19F, 18C, and 23F), conjugated to a nontoxic variant of diphtheria toxin (CRM197) (Galanis *et al*. 2016). In this study, rabbit immunization will be carried out to obtain polyclonal anti-PCV13 antibodies, which are heterogeneous and can bind to several different antigen epitopes. Despite its weak specificity compared to monoclonal antibodies, polyclonal antibodies have high sensitivity because of the involvement of a number of antibodies against many epitopes (Paton and Trappetti 2019). Therefore, this study aims to evaluate the immune response of a rabbit model to polysaccharide capsules of *Streptococcus pneumoniae* after primary and booster immunization with pneumococcal conjugate vaccine.

2. Materials and Methods

2.1. Rabbit Immunization

The immunization study was approved by the Animal Ethics Committee of IPB University, Bogor, Indonesia (Number: 236-2022 IPB). Eight-week-old New Zealand White female rabbits were maintained at the Animal Laboratory of PT. Biomedical Technology Indonesia, Bogor, Indonesia. Three rabbits were immunized intramuscularly with 0.25 ml of the PCV13 containing 0.11 µg/ serotype, except serotype 6B, 0.22 µg (13-valent pneumococcal conjugate vaccine, Prevnar13, Pfizer), and one rabbit was immunized with 0.25 ml of sterile distilled water on days 0, 7, and 14 (Figure 1). The serotype 6B conjugate in PCV13 has greater cross-functional reactivity with other serogroup 6, including 6A and 6C (Cooper *et al*. 2011). Blood samples were taken on days 0, 7, 14, 21, and 28 and collected in serum tubes (Fairman *et al*. 2021). Blood was stored at room temperature for one hour. Then, the serum was separated by centrifugation at 4000 rpm for 10 minutes at 4ºC. The serum was stored at -20ºC.

2.2. Enzyme-Linked Immunosorbent Assay

The enzyme-linked immunosorbent assay (ELISA) was performed to measure the antibodies to pneumococcal type 6B and 19F polysaccharide (Pn6BPS and Pn19FPS) antigens, two of the polysaccharide antigens included in the PCV13, as described previously (Galanis *et al*. 2016). Briefly, serially diluted sera were incubated for 1 h at 37 °C in flatbottom plates (Corning Inc., Corning, NY, USA), coated with 100 µL of purified polysaccharide types 6B and 19F (5 µg/ml; Staten Institute; Type 19F CPS REF:76958 and Type 6B CPS REF:76863).

A total of 5 µg/ml polysaccharide capsules of *S. pneumoniae* serotype 6B and 19F 100 µL each were coated on a 96-well plate and incubated at 37°C overnight. After washing with 1X PBS with 100 µL of 0.5% tween (PBST) three times, blocking was performed using 100 µL of 1% BSA and incubated at 37°C for 1 hour. On another 96-well plate, undiluted rabbit serum was diluted 2.5 fold using cell wall polysaccharide (CWPS) and incubated for 30 minutes at 37ºC. After that, 50 µL of incubated rabbit serum was added to each blocking well. Then, it was incubated at 37°C for 2 hours. Then, 100 µL goat anti-rabbit IgG-HRP (horseradish

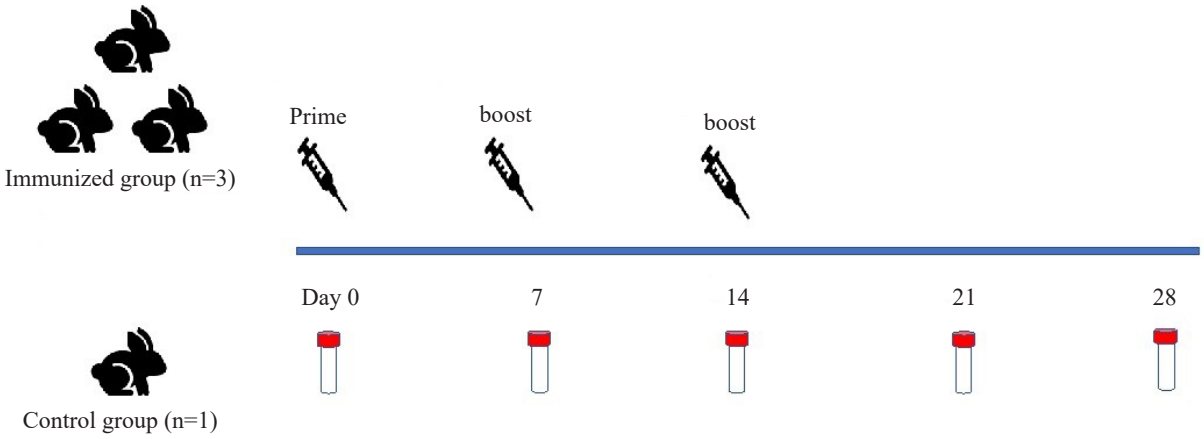

Figure 1. Rabbit immunization schedule of PCV13 vaccine. Rabbit (n=3) were immunized by In Study 1 (n = 5) mice were immunized by Intramuscular (IM) administration of 0.25 ml PCV13 containing 0.11 µg/ serotype, except serotype 6B, 0.22 µg (13-valent pneumococcal conjugate vaccine, prevnar13, pfizer) and one rabbit (the control group) received 0.25 ml of sterile distilled water on day 0 (Prime), day 7 (boost), and day 14 (boost). Blood collection was performed at 0 day, 7 days, 14 days, 21 days, and 28 days

peroxidase) substrate (Jackson ImmunoResearch Laboratories Inc.) was used as the secondary antibody and incubated at 37°C for 1 hour. After that, 100 µL of TMB substrate (3,3′,5,5′-tetramethylbenzidine) and 50 µL of stop solution, H_2SO_4 3 N were added. Each step was washed with wash buffer 100 µL three times. Optical density (OD) values were obtained with a microtiter plate spectrophotometer (BioTek Elx800) at 450 nm. Antibody titers were expressed as the OD_{450nm}.

2.3. Data Analysis

Data analysis was carried out descriptively and analytically. Measurement results statistical analysis of the titer was carried out using SPSS software to determine the cutoff value of each polysaccharide capsule of *S. pneumoniae*. Statistical analysis was used to determine the titer value of anti-capsule antibodies for pre and post-immunization.

3. Results

We compared the immunological responses for serotypes 6B and 19F of *S. pneumoniae* in the rabbit animal model. In this study, we found that the IgG antibody response analysis was carried out on control (no immunization) and experimental (immunized) groups that were monitored on a weekly basis. Antibody measurements in this study were performed using the indirect ELISA method for the IgG anti-capsule polysaccharide *S. pneumoniae* serotypes 6B (Pn6BPS) and 19F (Pn19FPS). The data in Figures 2 and 3 show that there is a statistically significant difference between the control and experimental (immunized) groups of the IgG anti-capsule polysaccharide standard for *S. pneumoniae* serotypes 6B (Pn6BPS) and 19F (Pn19FPS). When comparing the difference between the control and experimental groups between the two polysaccharide anti-capsule antibodies, the anti-capsule antibody for *S. pneumoniae* serotype 19F (Pn19FPS) shows a higher median value than *S. pneumoniae* serotype 6B (Pn6BPS).

The most prominent increase in IgG antibody titers was observed in response to serotype 6B standard polysaccharide capsules (Pn6BPS) on Day 7 after the first administration of the vaccine, with a value of p=0.017 (Figure 4A). Meanwhile, in the polysaccharide capsule of *S. pneumoniae* serotype 19F (Pn19FPS), the highest IgG antibody titer was shown on Day 28 after the first administration of the vaccine (Figure 4B). The highest antibody titers were obtained on Day 21 for *S. pneumoniae* serotype 6B (Pn6BPS) and on Day 28 for *S. pneumoniae* serotype 19F (Pn19FPS). This shows that after the 2nd booster, there is a fairly high increase in antibodies. Antibody titers on Day 28 for Pn6BPS and Pn19FPS showed similar values. However, on days 14 and 21, Pn6BPS showed higher values than the titers for Pn19FPS.

4. Discussion

This study aims to observe the immune response in rabbits to polysaccharide capsules of *S. pneumoniae* serotype 6B (Pn6BPS) and Pn19FPS conjugated with CRM197 protein in the PCV13. The 13-valent pneumococcal conjugate vaccine (PCV13) consists of a carbohydrate antigen covalently bound to a carrier

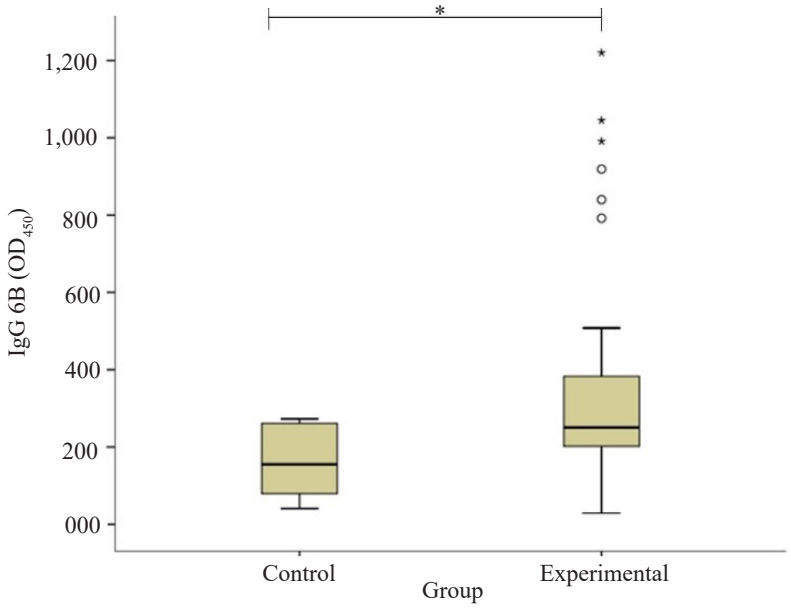

Figure 2. Comparison of IgG anti-capsule polysaccharide of the control and experimental (immunized) groups against the polysaccharide capsule of *S. pneumoniae* serotype 6B (Pn6BPS). *Results of analysis using the Mann-Whitney test ($p<0.05$)

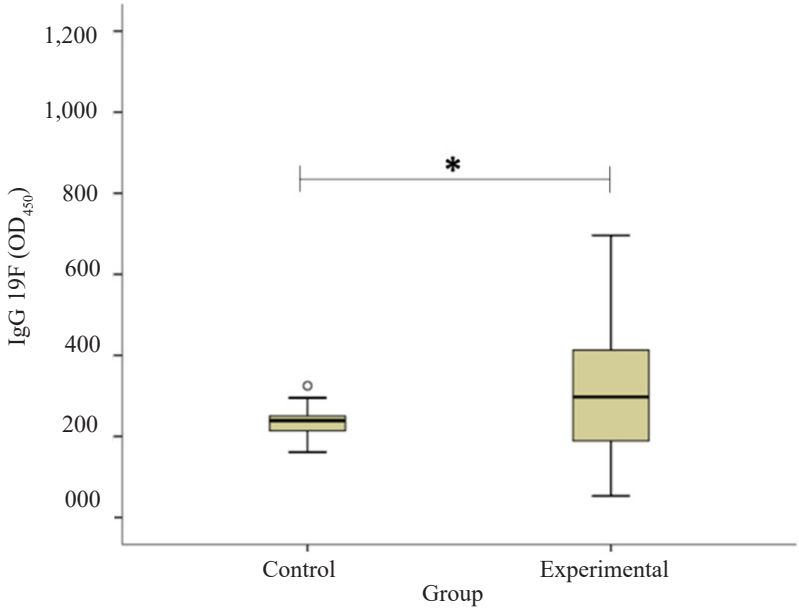

Figure 3. Comparison of IgG anti-capsule polysaccharide of the control and experimental (immunized) groups against the polysaccharide capsule of *S. pneumoniae* serotype 19F (Pn19FPS). *Results of analysis using independent t-test ($p<0.05$)

protein, diphtheria toxin CRM197. The existence of conjugation with a protein increases the immunogenic properties of this carbohydrate antigen so that it will produce a stronger primary immune response and form long-term protection. The process of evaluating the immunogenicity and safety of PCV in several pharmacological studies was carried out using New Zealand White (NZW) rabbits. This species showed a consistent immunological response to all vaccine conjugates tested, so that it can distinguish the response of conjugated polysaccharides from unconjugated polysaccharides (Fairman *et al*. 2021).

The results of the ELISA showed that the response of anti-PCV13 polyclonal antibody in the control versus experimental (immunized) rabbit to the polysaccharide capsule of *S. pneumoniae* serotypes 6B (Pn6BPS) and 19F (Pn19FPS) was significantly different. This shows that the polyclonal antibody production process

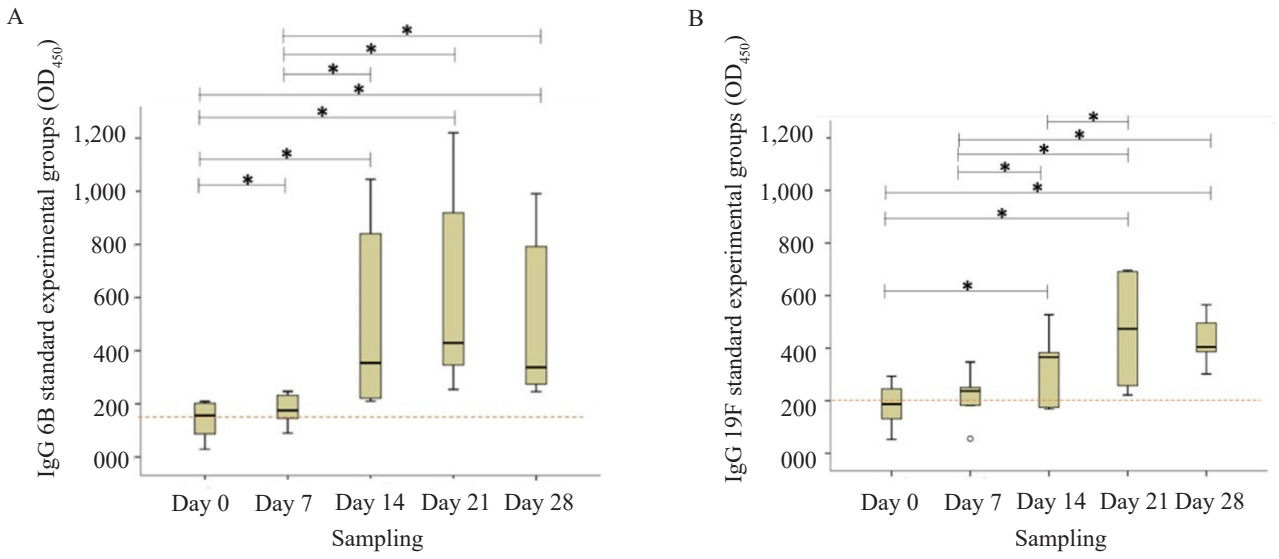

Figure 4. Comparison of IgG anti-capsule polysaccharide against (A) polysaccharide capsule of *S. pneumoniae* serotype 6B (Pn6BPS) (B) polysaccharide capsule *S. pneumoniae* serotype 19F (Pn19FPS) results between experimental (immunized) groups compared to the day of sampling. *Results of data analysis of paired sample T test ($p<0.05$)

was successful. The rabbit's immune system has recognized and reacted to the PCV13 antigen. The B lymphocytes can then develop into plasma cells and produce antibodies. The antibodies that are formed are polyclonal antibodies with varying compositions in the serum, either due to repeated immunizations or due to variations that occur during immune reactions (Tizard 2018).

The immunization process is carried out with two boosters, this will result in the activation of memory B cells, whose work is stimulated by T cells so that they can produce antibodies in large quantities. This mechanism will only occur in T-cell-dependent antigens that occur in protein antigens. Likewise, the immunization process carried out in the presence of the CRM197 carrier protein from diphtheria toxin will create a T-cell-dependent mechanism so that antibody production can increase. The B cell receptor (BCR) has two roles. The first is to start a signaling cascade due to antigen binding, in this case, PCV13. The other role is delivering antigens to intracellular sites for antigen processing so that antigenic peptides bound to MHC class II, in this case, the presence of the CRM197 carrier protein, will be returned to the surface of B cells. MHC class II complexes will be recognized by helper T cells. They will differentiate into effector T cells to express surface molecules and cytokines that, in turn, help B cells to proliferate and differentiate into antibody-secreting cells and memory B cells. The existence of help from helper T cells will increase the affinity of antibodies to antigens, and there will be class switching mechanism in the class of immunoglobulin other than IgM, one of which is the formation of IgG, which occurred in this study (Murphy and Weaver 2016).

Research data between experimental groups showed significant differences a few days after immunization. The response of anti-PCV13 polyclonal antibodies to the *S. pneumoniae* serotype 6B polysaccharide capsule (Pn6BPS) showed a significant difference between Day 0 and Day 7. Overall, the highest significant difference was obtained between Day 0 and Day 21. Meanwhile, in the response of anti-PCV13 polyclonal antibody to the *S. pneumoniae* serotype 19F polysaccharide capsule (Pn19FPS), a significant difference was observed between Day 0 and Day 14. A significant difference in value was obtained between Day 7 and Day 21. Based on the mechanism described earlier, exposure to the first antigen can activate B cells to produce antibodies. However, with the booster twice, the production of polyclonal antibodies is higher due to the presence of activated memory B cells with the help of T cells. Thus, on Day 21, a highly significant difference was observed.

This is in line with a study comparing the cellular immune response of PCV13 after primary and booster doses of the vaccine (Chapman *et al*. 2020). The study also addressed the impact of the 3rd dose and booster

vaccine on cellular immunity. Their data suggest that dose 3 did not significantly impact the number of circulating Spn-specific memory B cells compared to dose 2. However, Spn-specific memory B cells, as well as total and IgD-memory B cells, increased in frequency after booster vaccination, suggesting antigen exposure from booster vaccination increased the populations of circulating memory B cells. Another study researching serotype-specific antibody levels from infants after 2p vs. 3p doses and pre (3p+0) vs. post booster (3p+1) of PCV13 showed that after the booster, antibody levels for all the serotypes increased significantly and increased the amount of IgG antibody above the accepted protective threshold for most of the children (Kaur and Pichichero 2020). In comparison, the study found no significant difference in OPA titers compared to the 2p vs. 3p dose. Still, significant increases in functional antibodies for most serotypes were found after the booster dose.

When comparing the immune response formed against the polysaccharide capsule, the polysaccharide capsule of *S. pneumoniae* serotype 19F was higher than the polysaccharide capsule of *S. pneumoniae* serotype 6B. This is in line with research by Fairman *et al*. 2021 which compared geometric mean titer (GMT) IgG values specific to serotypes 6B and 19F on the 14th day after the primary dose and 14 days after the booster in NZW rabbits, which showed that the 19F serotype was higher than 6B (Fairman *et al*. 2021). Another study also compared the ELISA IgG concentrations between 2p vs 3p dose schedules that showed significantly lower antibody levels for some serotypes (serotype 5, 6A, 6B, 23F) for the 2p schedule but found a significantly higher concentration for serotype 19F after 2p compared to 3p (Kaur and Pichichero 2020). The highest anti-capsule PCV13 antibody titers against polysaccharide capsules of *S. pneumoniae* serotypes 6B, and 19F were obtained on days 21 and 28, respectively.

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