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The histopathological features and bacterial counts after exposure to *Streptococcus pneumoniae* serotypes 2,3,4 and 19 F in the lung of Balb/c mice



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ABSTRACT

Background: Pneumococci cause mild or severe infections that begin with colonization in the nasopharyngeal area. Intranasal transmission is a natural route of bacterial infection in the host. This study aims to determine the type of serotype that can infect and provide an overview of inflammation in the lungs of mice after exposure to 107 bacteria *S. pneumoniae* serotypes 2, 3, 4, 19F and ATCC 6030 intranasally in animals try Balb / c mice.

Methods: True experimental study was conducted using Randomized Posttest Only Control Group Design among 30 Balb/c mice divided into 3 groups. The intervention used in this study was carried out twice, namely at 24 hours and 48 hours with 50 µl suspension of *Streptococcus pneumoniae* bacterial inoculum via

intranasal drop by drop. Lung histopathology and CFU analysis of infected mice were evaluated. TNF-α was examined using ELISA. Data were analyzed using SPSS software version 17 for windows.

Results: The results showed that *S. pneumoniae* serotype 3 could infect Balb / c mice and found about 5x10⁴ CFU (SD ± 7x10⁴ CFU) at 101 dilutions and was still detected at 104 dilutions i.e. 0.5 CFU (SD ± 0.7 CFU) at 24 hours post-infection as well at 48 hours post-infection, accompanied by infiltration of neutrophil cells in the lung tissue at the same time. The TNF-α levels did not significantly differ between the treatment group (P>0.05)

Conclusion: The results of this study indicate that not all *S. pneumoniae* serotypes can infect experimental animals.

Keywords: Animal models, Intranasal challenge, *Streptococcus pneumoniae*, Colony Forming Unit/CFU

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INTRODUCTION

Streptococcus pneumoniae is a significant cause of bacterial pneumonia, meningitis, bacteraemia, and otitis media. Infectious disease by *Streptococcus pneumoniae* is a disease that can cause high mortality rates among vulnerable groups, such as infants, children, and older people.¹ The infection caused by the *Streptococcus pneumoniae* begins with the colonization of these bacteria in the nasopharynx which is the initial contact between the bacteria and the host where the transmission of these bacteria through the respiratory droplet.² If the host's immune system is susceptible to infection, then this bacteria will be able to attack the mucosal surface around the nasopharynx and then invade the lungs, pass through the central nervous system and also as a reservoir to transmit these bacteria horizontally to the population.^{3,4}

The respiratory tract is continuously exposed to the environmental antigens, which can be dangerous for the host. Bronchial and tracheal

epithelium are major mechanical barriers to incoming pathogens and, together with alveolar macrophages and dendritic cells, work as the first line of defences to prevent infection.⁵ The *S. pneumoniae* expresses several virulence factors which are used to avoid the immune response from the host.⁶ The immunological response to *S. pneumoniae* begins with the introduction of bacteria by alveolar macrophages and epithelial cells, which actively secrete chemokines and cytokines to stimulate infiltration of monocytes, neutrophils, and lymphocytes into the lungs.⁶ Neutrophils are the primary defence of host against *S. pneumoniae*, as effector cells that clear pathogens and contribute to the pro-inflammatory and immunopathological conditions of increased lung damage caused by the recruitment of high neutrophils into the lungs.⁷ The initial immune response to *S. pneumoniae* infection is dominated by phagocytes, although the relative contribution of neutrophils and macrophages is not determined. However the role of Tumor Necrosis Factor (TNF) is widely reported

as an important component in host defence against intranasal pneumococcal infection.⁸ TNF- α is a proinflammatory cytokine that activates the immune and inflammatory response, which has a beneficial and harmful response to the host when an infection occurs.⁹

The model of infection in mice closely resembles the natural route of *S. pneumoniae* infection. Animal models such as mice can be used to test alternative vaccination strategies, test new adjuvants used for vaccines, antibiotic efficacy, natural and adaptive immune mechanisms, used for screening and testing virulence factors.¹⁰ There are various ways to inoculate *S. pneumoniae* bacteria to the experimental animals such as mice to make these experimental animals infected. The target of infection in the organ will also determine the exposure technique in experimental animals.¹¹ Intranasal exposure is the most commonly used for exposure, which has the advantage that this route closely resembles to the route of pneumonia infection in humans. About more than 80% of mice will produce infection at one time of exposure, slightly less virulent compared with peritoneal exposure making it easier to detect effects protection from immune cells.¹⁰

The biggest obstacle in developing a model of infection with experimental animals is that not all virulent serotypes in humans are also virulent in animals.¹² In this study, intranasal exposure to *S. pneumoniae* serotypes 2,3,4 and 19F is carried out to determine the presence of pulmonary infection and to describe the immunological response in animals trying to Balb/c mice. The initial data obtained in this study is expected to encourage further research shortly.

METHODS

Research Design

A true experimental research study using the Randomized Posttest Only Control Group Design was conducted. The minimum sample size in this study was divided into 3 sample groups. Observation after treatment in this study was carried out twice, namely at 24 hours and 48 hours. The number of samples was 15 per observation time interval so that the total sample of Balb / c mice in this study was 30

Preparation of Mice

The samples used in this study were 6-week-olds Balb / c mice weighing 25-30 grams originating from the Animal Unit Laboratory, Department of Pharmacology, Faculty of Medicine, Universitas Udayana, Bali, Indonesia. Animals were kept in individual cages measuring 15x25x35 cm, conditioned by a light-dark cycle every 12/12 hours (light from 8 AM to 8 PM and dark from 8 PM to 8

AM) and the room temperature was allowed at $22 \pm 2^\circ\text{C}$ and humidity around $50 \pm 10\%$. All of animals are given free access to food and water and left in this situation for 2 weeks before intervention.

BACTERIAL PREPARATION

S. pneumoniae serotype 2,3,4 originating from the Jakarta Eijkman Institute and serotype 19F isolated from clinical specimens at Sanglah Hospital Denpasar Clinical Microbiology Laboratory stored in the -80°C freezer. The bacteria were planted on 5% goat blood agar and incubated at 37°C at 5% CO_2 incubator for 18 - 24 hours. Bacterial colonies were taken and put into 200 PBL PBS to make bacterial suspensions, then put into TSB (Tryptisoy Broth) medium and incubated at 37°C in an incubator of 5% CO_2 in 6 hours. Then a 0.5 MacFarland bacterial suspension (1.8×10^7) was made.

Infection

The mice were given anaesthesia Ketamine 40 mg/kg and Xylazine 5 mg/kg through intramuscular injection; then the mice were exposed with 50 μl suspension of bacterial inoculum through intranasal drop by drop. These mice were bound by straps from their front teeth and allowed the suspension of bacterial inoculums to be aspirated by the mice for 10 minutes before the mice were returned to the cage to be restored. Mice were randomly selected as controls and treated. For survival rate, the mice were evaluated every 24 hours during 14 days.

Histopathology of Lung dan CFU Analysis

The lung histopathology and CFU analysis of infected mice were evaluated at 24- and 48-hour intervals. Mice were given anaesthesia Ketamine 40 mg/kg and Xylazine 5 mg / kgBW through intramuscular injection; then lung samples were taken for Hematoxylin and Eosin (H&E) staining and culture. Lung tissue for HE staining is cut 15 μm with a microtome at -18 to -25 degrees Celsius. These tissues were allowed to dry at room temperature for 20 minutes then the tissue was stained with haematoxylin and eosin (H&E) and fixed with DPX mountant (BDH) for long time storage. Pieces of tissue that have been stained are seen under a 400x magnification light microscope. The pulmonary lung of the mice was crushed and homogenized with sterile PBS, and then a serial dilution was made for CFU analysis of bacteria. Serial dilution of lung tissue is planted in 5% agar blood media, incubated at 37°C , 5% CO_2 for 24 hours and then bacteria are counted.

TNF Alpha Analysis

Each blood mice were taken in the retro-orbital

region after being infected with *S. pneumoniae* bacteria at intervals of 24 and 48 hours. Before drawing blood of mice, the mice were anaesthesia first. The blood obtained was centrifuged at 1500 rpm for 30 minutes, took the supernatant into a 1.5 ml microcentrifuge tube and stored at -80oC as long as the examination had not been carried out. TNF alpha examination was carried out by the ELISA method.

Statistical analysis

This study was analyzed with SPSS version 25 for Windows using mean, standard deviation, and international units of parameters assessed quantitatively.

RESULTS

Thirty Balb/C mice used in this study were healthy for 2 weeks before 107 CFU were exposed to five *S.*

pneumoniae bacterial serotypes namely Serotypes 2, 3, 4, 19F and *S. pneumoniae* ATCC 6030. After intranasal bacteria exposure, Balb / C mice were harvested in 24 hours, 48 hours and 14 days after infection. After 48 hours after infection, some mice showed signs of infection wherein the mouse was weak and bent over (Table 1).

On examination of the number of bacteria (bacterial load) of the lung tissue after 24 hours of treatment, the highest number of bacteria in the lung tissue after exposure to *S. pneumoniae* serotype 3 bacteria was 5×10^4 CFU (SD $\pm 7 \times 10^4$ CFU) at 10^1 dilutions and still detected at 10^4 dilution i.e. 0.5 CFU (SD ± 0.7 CFU). The number of bacteria in the lungs was also shown in exposure to *S. pneumoniae* ATCC 6030 bacteria. After determining the serotype in *S. pneumoniae* ATCC bacteria using Polymerase Chain Reaction (PCR) technique, *S. pneumoniae* ATCC bacteria were serotype 3. Data

Table 1. The number of bacteria in the lung tissue (CFU), the number of PMN in the lung tissue (HE) and the TNF alpha level (ng/ μ l) after 24 hours, 48 hours, and 14 days after exposure to *S. pneumoniae* serotype 2 (1), 3 (2), 4 (3), 19F (4), ATCC 6030 (5), and control.

Sample	Bacterial load (dilution) (CFU)				Histopathology	TNF- α (ng/ μ l)	P-value
	10^1	10^2	10^3	10^4			
After 24 hours							
1	$5 \times 10^3 \pm 7 \times 10^3$	$1.8 \times 10^1 \pm 2.5 \times 10^1$	1.5 \pm 2	0.5 \pm 0.7	+	8.7 \pm 0.14	>0.05
2	$5 \times 10^4 \pm 7 \times 10^4$	$1.2 \times 10^1 \pm 1.5 \times 10^1$	2.5 \pm 3.5	0.5 \pm 0.7	++	9.0 \pm 0.0	
3	8 \pm 11	0	0	0	+	11.03 \pm 0.15	
4	0	0	0	0	++	9.54 \pm 0.8	
5	$5 \times 10^4 \pm 7 \times 10^4$	$5 \times 10^2 \pm 7 \times 10^2$	$5 \times 10^1 \pm 7 \times 10^1$	$1.2 \times 10^1 \pm 1.6 \times 10^1$	+	10.4 \pm 0.4	
After 48 hours							
1	$1 \times 10^5 \pm 0.0$	$1 \times 10^4 \pm 0.0$	$1 \times 10^3 \pm 0.0$	$5.8 \times 10^1 \pm 6 \times 10^1$	+	8.04 \pm 1.3	>0.05
2	$5 \times 10^1 \pm 7 \times 10^1$	9 \pm 12	1 \pm 1.4	0	++	8.04 \pm 1.3	
3	0	0	0	0	+	8.04 \pm 1.3	
4	0	0	0	0	+	8.04 \pm 1.3	
5	$5 \times 10^4 \pm 7 \times 10^4$	$5 \times 10^2 \pm 7 \times 10^2$	$5 \times 10^1 \pm 7 \times 10^1$	$1.2 \times 10^1 \pm 1.6 \times 10^1$	++	8.04 \pm 1.3	
After 14 days							
1	0	0	0	0	+++	7.52	>0.05
2	0	0	0	0	+	8.04 \pm 1.3	
3	0	0	0	0	+++	8.04 \pm 1.3	
4	0	0	0	0	+++	8.76	
5	0	0	0	0	+++	6.49	
Control	0	0	0	0	+	9.8	

CFU: Colony forming units; +: 1-5 cells/field; ++: 6 – 10 cells/field; +++: > 10 cells/field, micro-abscess

on the number of bacteria in the lungs of mice after 24 hours of exposure can be seen in [Table 1](#).

The bacterial growth in lung tissue samples after 48 hours of exposure to *S. pneumoniae* was demonstrated in mice exposed to *S. pneumoniae* serotypes 2, 3 and ATCC 6030, respectively 1×10^5 CFU (SD ± 0.0), 5×10^1 (SD $\pm 7 \times 10^1$) and 5×10^4 (SD $\pm 7 \times 10^4$) at dilution 10^1 . The number of bacteria in the lungs can still be detected until dilution 10^4 . Data on the number of bacteria in the lungs of mice after 48 hours of exposure can be seen in [Table 1](#).

The exposure of *S. pneumoniae* bacteria in mice also shows the infiltration of neutrophil cells (PMN) in the lung tissue of mice which can be seen by H&E staining. The amount of neutrophil infiltration in lung tissue varies with the *S. pneumoniae* serotype. At 24 hours after exposure to *S. pneumoniae*, the bacteria showed neutrophil infiltration in lung tissue and the number of neutrophil cells was most indicated on serotype 3 exposure ([Table 1](#)). On the 14th day of infection, *S. pneumoniae* ATCC 6030 which is serotype 3 showed micro-abscess ([Table 1](#)). In the staining of HE can be seen neutrophil infiltration in the area of bronchioles and perivascular after 24 hours after infection with *S. pneumoniae*, especially serotype 3.

The results of the TNF alpha in this study did not differ significantly from each treatment group ($P > 0.05$), but there was a fact that there was a decrease in the amount of TNF-alpha in the blood in mice with micro pulmonary abscesses 14 days after being infected by *S. pneumoniae* serotypes 2 and 3, 7.52 ng/ μ l and 6.49 ng/ μ l ([Table 1](#)).

DISCUSSION

The intranasal technique (IN) used in this study in infecting mice is because the procedure is straightforward. Based on the previous studies, intranasal infection (IN) is straightforward and fast to do without sophisticated and invasive surgical techniques as well as mimic the route of infection naturally. For this reason, IN infection is the most commonly used method and applicable in this study.⁹

The results in this study are almost the same with the previous studies, where serotype 3 can infect the lungs of mice 24 hours after infection with bacterial concentrations of 107 CFU. Research by Seland et al. showed that the occurrence of pulmonary infections and bacteremia in intranasal exposure was from the serotypes 1, 3, 6A, and 8 with high bacterial concentrations (107-108 CFU).³ Likewise, as shown in the study of Calboa et al., all mice had a lung infection and bacterium with 100% mortality after being infected with 107 CFU *S. pneumoniae* serotype 3.¹³ In experimental animals

such as mice, host resistance to *S. pneumoniae* is most often studied after being infected with highly encapsulated serotypes, such as 2 and 3 and causing high mortality.⁸

After being infected with *S. pneumoniae*, in the early stages these bacteria will be recognized by alveolar macrophages, epithelial cells, dendritic cells and B cells in the alveoli, which emit cytokines and pro-inflammatory chemokines that produce neutrophil and monocyte explosions.⁷ Neutrophils begin to accumulate in the lungs around 12 to 16 hours after infection with *S. pneumoniae*.¹⁴ Following the previous results, the neutrophil counts increase 12 hours after pneumococcal infection and gradually decrease up to 14 days after infection.¹⁵ A similar result was also found in our study whereas the neutrophil infiltration of the lungs appeared 24 h post-infection. The role of neutrophils is indispensable for the process of cleaning *S. pneumoniae* in lung tissue, with several specific procedures, including adhesion to blood vessel walls, chemotaxis, phagocytosis, and killing of microbes.¹³ Histological analysis of lung tissue sections from mice infected with *S. pneumoniae* showed inflammation and infiltration of inflammatory cells centered around the bronchioles and perivascular areas. The focus of inflammation is limited to certain perivascular bronchioles and regions close to these bronchioles 24 hours after infection. Inflammation presents itself as bronchial wall hypertrophy, severe inflammatory cell infiltration around the bronchioles and some pulmonary oedema.¹⁴

In this study shows that TNF-alpha results between the treatment groups were not significantly different. Tumor necrosis factor-alpha (TNF alpha) is a proinflammatory cytokine that activates an immune response to infection, invasion, injury, or inflammation. Previous studies have also shown that TNF-alpha has an important role in protecting hosts from systemic infection with *S. pneumoniae*.¹⁶ In our study, experimental animals did not show systemic infections but only showed localized infections in the lungs where serum was taken 24- and 48-hours post-infection. This result indicated that the value of serum TNF alpha does not increase significantly. This is supported by previous studies, serum TNF-alpha levels began at 3 days after infection, respectively, together with an increase in the number of bacteria in the lungs.¹⁷

CONCLUSION

Experimental animals such as mice are the most commonly used experimental animals in studying the pathogenesis of *S. pneumoniae* infection. Each strain of mice gives different immunological and

pathological responses when exposed by various serotypes or strains of *S. pneumoniae* bacteria. In this study, it was shown that *S. pneumoniae* serotype 3 could infect Balb/c mice compared to other serotypes.

CONFLICT OF INTEREST

There is no competing interest regarding the manuscript.

ETHICAL CLEARANCE

Ethics approvals have been obtained prior to the study being conducted from Ethics Committee, Faculty of Medicine, Universitas Udayana, Bali, Indonesia.

FUNDING

None.

AUTHOR CONTRIBUTION

All of the authors are equally contributed to the study from the conceptual framework, data gathering, data analysis, until reporting the results of study.

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