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SECONDARY ANTIBODY TITER RESPONSE IN PIGS FOLLOWING *STREPTOCOCCUS SUIS* VACCINATION WITH VARIOUS ADJUVANTS: DETECTION USING SUPERNATANT ANTIGEN-BASED ELISA

Respons Titer Antibodi Sekunder Pada Babi Setelah Vaksinasi *Streptococcus suis* dengan Berbagai Adjuvan: Deteksi Melalui Uji Elisa Berbasis Antigen Supernatan

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Abstract

Streptococcus suis is a pathogenic bacterium that causes severe disease in pigs and poses a zoonotic risk to humans. Vaccination is one of the primary preventive strategies, and its effectiveness can be enhanced through the use of appropriate adjuvants. This study aimed to evaluate the secondary humoral immune response in pigs vaccinated with a locally derived *S. suis* vaccine formulated with two different adjuvants: Montanide ISA 201 VG and Montanide Gel-01. In addition, the study observed the dynamics of antibody titters over time post-vaccination. Twelve pigs were divided into three treatment groups: a control group (unvaccinated), a group vaccinated with Montanide ISA 201 VG, and a group vaccinated with Montanide Gel-01. Blood samples were collected weekly for five weeks following the booster vaccination, and antibody titers were measured using ELISA based on culture supernatant antigens. Data were analyzed using analysis of variance and linear regression. The results showed that both adjuvants significantly increased antibody titers compared to the control ($p < 0.05$), with Montanide Gel-01 producing the highest titer (0.718 ± 0.0397), followed by ISA 201 VG (0.703 ± 0.0320), and the control group (0.459 ± 0.0419). Furthermore, the timing of serum collection significantly influenced antibody titer levels ($p < 0.005$). This study concludes that the use of Montanide adjuvants, particularly Gel-01, is effective in enhancing the humoral

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immune response to *S. suis* vaccination. Further research is recommended to evaluate the effects of increasing antigen concentration for achieving a more robust immune response.

Keywords: *Streptococcus suis*; vaccine; pigs; antibody titer; ELISA

Abstrak

Streptococcus suis merupakan bakteri patogen yang menimbulkan penyakit serius pada babi dan bersifat zoonosis. Vaksinasi menjadi salah satu strategi pencegahan utama, di mana efektivitasnya dapat ditingkatkan melalui penggunaan adjuvan yang tepat. Penelitian ini bertujuan untuk mengevaluasi respons imun humoral sekunder pada babi yang divaksinasi dengan vaksin *S. suis* berbasis strain lokal menggunakan dua jenis adjuvan berbeda, yaitu Montanide ISA 201 VG dan Montanide Gel-01, serta untuk mengamati dinamika titer antibodi pascavaksinasi. Sebanyak 12 ekor babi dibagi ke dalam tiga kelompok perlakuan: kelompok kontrol (tanpa vaksin), kelompok vaksin dengan adjuvan Montanide ISA 201 VG, dan kelompok vaksin dengan Montanide Gel-01. Sampel darah dikoleksi setiap minggu selama lima minggu setelah vaksinasi booster, dan titer antibodi diukur menggunakan metode ELISA berbasis antigen supernatan. Data dianalisis menggunakan analisis ragam dan regresi linier. Hasil menunjukkan bahwa kedua jenis adjuvan meningkatkan titer antibodi secara signifikan dibandingkan kontrol ($p<0,05$), dengan Montanide Gel-01 menghasilkan titer tertinggi (0,718± 0.0397), diikuti oleh ISA 201 VG (0,703±0.0320), dan kontrol (0,459± 0.0419). Selain itu, waktu pengambilan serum berpengaruh nyata terhadap peningkatan titer antibodi ($p < 0,005$). Penelitian ini menyimpulkan bahwa penggunaan adjuvan Montanide, khususnya Gel-01, efektif dalam meningkatkan respons imun humoral terhadap vaksin *S. suis*. Studi lanjutan disarankan untuk mengevaluasi peningkatan konsentrasi antigen guna memperoleh respons imun yang lebih optimal.

Kata kunci: *Streptococcus suis*; vaksin; babi; titer antibodi; ELISA

INTRODUCTION

Streptococcus suis (*S. suis*) is a pathogenic bacterium that affects both pigs and humans, exhibiting distinctive morphological, physiological, and pathogenic characteristics (Huong *et al*., 2014). Morphologically, *S. suis* is a Gram-positive coccus that forms chains. The bacterium grows well on blood agar media incubated at 37°C. In pigs, *S. suis* infection can lead to various severe diseases such as meningitis, pneumonia, sepsis, and arthritis (Kasianenko dan Liu, 2023). Infected pigs often exhibit symptoms such as loss of appetite, lethargy, and in many cases, sudden death (Setiarto dan Karo, 2021). Transmission of *S. suis* infection may occur through direct contact with infected pigs or indirectly via contaminated equipment, farm personnel, or veterinary workers (Handayani, 2024).

S. suis infection has significant public health and economic impacts, particularly within the food production system. Pork derived from infected animals is unsafe for consumption, and numerous human infection cases have been linked to the consumption of undercooked pork (Besung *et al.*, 2022). In humans, the infection often results in permanent hearing loss, meningitis, or other serious complications (Hlebowicz *et al*., 2019). Globally, *S. suis* has become a growing concern in food safety systems, especially in Southeast Asia and several European countries (Salasia dan Mangkoewidjojo, 2021). In Indonesia, preventive measures against *S. suis* remain suboptimal due to the absence of a commercially available vaccine to control the spread of the disease (Hardi *et al*., 2020). Vaccination is considered a key preventive strategy, as it stimulates the immune system without causing disease (Segura, 2020). However, there is still a pressing need for an effective *S. suis* vaccine (Maes *et al*., 2021).

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This study aims to evaluate the effectiveness of an *S. suis* vaccine in eliciting immune responses, particularly by employing different adjuvants. Montanide ISA™ 201 VG and Montanide™ Gel-01 are two adjuvants with distinct properties in stimulating both humoral (antibody-mediated) and cellular (T-cell-mediated) immune responses (Susmiarsih, 2018). The evaluation of vaccine effectiveness was conducted by measuring antibody titers using an ELISA method. The assay utilized supernatant antigens, which are superior to pellet-derived antigens due to the absence of cellular debris that may interfere with ELISA reactions (Li *et al*., 2021). Accordingly, this study is expected to provide new insights into the efficacy of *S. suis* vaccination and contribute to improved preventive measures against infection in both pigs and humans.

RESEARCH METHODS

Ethical Approval

This study was conducted in accordance with the ethical guidelines established by the Animal Ethics Committee of the Faculty of Veterinary Medicine, Udayana University, Denpasar, Bali, Indonesia (Approval No. B/181/UN14.2.9/PT.01.04/2024, dated October 25, 2024).

Experimental Animals

A total of 12 pigs aged two months were used in this study. The animals were acclimatized for one week in pens before the start of the experiment. Each pig received two doses of vaccine: the primary vaccination, followed by a booster four weeks after the first dose.

Experimental Design

This study utilized a completely randomized factorial design with two factors. The first factor was vaccination treatment: no vaccination (P1), vaccination with Montanide™ Gel-01 adjuvant (P2), and vaccination with Montanide™ ISA 201 VG adjuvant (P3). The second factor was the serum collection period, consisting of five time points: prior to vaccination (Week 1), and at weekly intervals post-vaccination Week 2, Week 3, Week 4, and Week 5. Each treatment combination was replicated four times, involving a total of 12 pigs.

Bacterial Culture

The *Streptococcus suis* isolate used in this study was obtained from a field case and preserved in the Biomedical Laboratory of the Faculty of Veterinary Medicine, Udayana University. The isolate had been previously identified through PCR testing. Five bacterial colonies were cultured in 500 mL of Tryptic Soy Broth (TSB) and incubated for 48 hours in a shaker incubator. The bacterial concentration was then adjusted using a 0.5 McFarland standard. Subsequently, the culture was plated on Sheep Blood Agar and subjected to Gram staining to confirm bacterial purity (Kasianenko and Liu, 2023).

Bacterial Inactivation

The *S. suis* culture was transferred into two 50 mL Eppendorf tubes and centrifuged for 10 minutes at 5,000 rpm. The supernatant was discarded, and the bacterial pellet was retained. This step was repeated until the entire TSB culture was processed. The pellet was then resuspended in NaCl solution to a final volume of 80 mL. Bacterial inactivation was performed using sonication (70% amplitude for 20 minutes) followed by heat treatment (80°C for 2 hours). To confirm complete inactivation, the treated suspension was cultured on Mueller Hinton Agar and incubated at 37°C for 24 hours (Besung *et al*., 2019).

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Adjuvant Addition and Vaccination

The *S. Suis* vaccine was formulated with two types of adjuvants. For Montanide™ Gel-01, the formulation consisted of 2.5% adjuvant, 47.5% NaCl, and 50% antigen. For Montanide™ ISA 201 VG, the formulation consisted of 50% adjuvant and 50% antigen. Each mixture was stirred using a magnetic stirrer at 1,500 rpm for 25 minutes until a stable emulsion was formed (Pramesti *et al*., 2022). The vaccine was administered intramuscularly at a dose of 2 mL per pig, given on the fourth week following the initial vaccination.

Serum Collection

A total of 3 mL of blood was collected weekly from the jugular vein of each pig using vacuum tubes. Serum was obtained by centrifugation at 5,000 rpm for 10 minutes and subsequently stored under refrigeration for ELISA analysis (Muhandis *et al*., 2022)

ELISA Assay

Antibody titers were determined using an indirect ELISA with a supernatant-derived antigen at a concentration of 22.3 µg/µL, diluted 1:10. Microplate wells were coated with 50 µL of the antigen solution and incubated overnight at 4°C. Plates were then washed with PBS containing 0.05% Tween-20 (PBS-T). Blocking was performed using 10% skim milk (100 µL/well) for 1 hour at room temperature, followed by washing with PBS-T. Serum samples were diluted 1:100, and 1 µL of the diluted serum was added to each well, incubated for 1 hour at room temperature, and then washed with PBS-T. A conjugated secondary antibody (mouse anti-pig IgG H+L, alkaline phosphatase; Sigma-Aldrich) was added at a volume of 50 µL per well with a dilution of 1:1,000, followed by another PBS-T washing step. Subsequently, 50 µL of p-nitrophenyl phosphate (p-NPP) substrate was added to each well, and the plates were incubated at room temperature for 15 minutes until a color change was observed. Optical density (OD) values were measured at the appropriate wavelength using an ELISA microplate reader (Jeffery *et al*., 2024).

Data Analysis

Differences in secondary antibody titers between adjuvant treatment groups and temporal changes over the sampling periods were analyzed using a factorial analysis of variance (ANOVA). Post hoc comparisons were performed using the Least Significant Difference (LSD) test. In addition, linear regression analysis was conducted to assess the trend of antibody titer increases over time. A significance threshold of $P < 0.05$ was applied. All statistical analyses were performed using IBM SPSS Statistics software, version 29.

RESULTS AND DISCUSSION

Result

The antibody titer data from the *S. Suis* vaccine trial using different adjuvants are presented in Figure 1. The groups included: P1 (control), P2 (Montanide ISA 201 VG), and P3 (Montanide Gel-01). The results showed a progressive weekly increase in antibody titers in group P3. In contrast, group P1 exhibited a decline in week 4, while group P2 showed a decrease in week 5.

Two-way ANOVA analysis (Table 1) indicated that the vaccine treatment had a statistically significant effect on antibody titers ($P < 0.05$). The time of serum collection also showed a significant influence ($P < 0.05$). However, there was no significant interaction between vaccine treatment and time ($P > 0.05$). Post hoc Least Significant Difference (LSD) test results (Table 2) revealed that the antibody titers in the control group (P1) were significantly different from those in the adjuvant-treated groups P2 and P3 ($P < 0.05$). However, no significant difference was found between P2 and P3 ($P > 0.05$). Table 3 shows that antibody titers in weeks 3, 4, and

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5 were significantly higher compared to week 1 ($P < 0.05$). Additionally, titers in week 5 were significantly higher than those in week 2 ($P < 0.05$).

Regression analysis demonstrated that in the control group, the relationship between antibody titer and time was not significant ($P > 0.05$), with a regression equation of $Y = -1.409 + 9.589X$ and a coefficient of determination (R^2) = 0.077. In contrast, a significant correlation was observed in the Montanide ISA 201 VG group ($P < 0.05$), with the regression equation $Y = -11.433 + 20.503X$ and $R^2 = 0.205$. The Montanide Gel-01 group also showed a significant relationship ($P < 0.05$), with the equation $Y = -18.063 + 29.335X$ and a higher $R^2 = 0.645$, indicating a strong linear trend in titer increase over time.

Discussion

Vaccination is a preventive strategy that effectively enhances individual immunity against infectious diseases. It involves the administration of antigens either attenuated, inactivated, or subunit proteins to stimulate the immune system and induce a specific immune response (Lestari dan Raveinal, 2020). The success of vaccination is influenced by various factors, including antigen type, vaccine formulation, and the use of adjuvants. Adjuvants play a critical role in improving vaccine efficacy by prolonging antigen release, enhancing immune cell activation, and modulating the type of immune response elicited.

Figure 1 demonstrates that vaccination with *Streptococcus suis* combined with either Montanide ISA 201 VG or Montanide Gel-01 adjuvants induced antibody production. During the early weeks, antibody titers in both adjuvant groups followed a similar trend; however, by the final week, antibody titers in the Montanide Gel-01 group were significantly higher. This difference may be attributed to the distinct mechanisms of action of each adjuvant. Montanide ISA 201 VG, a water-in-oil-in-water (W/O/W) emulsion, facilitates local antigen retention and gradual release, thereby prolonging interactions between T and B cells, which ultimately enhances both humoral and cellular immune responses over time (Obradovic *et al*., 2021). In contrast, Montanide Gel-01 creates a depot effect at the injection site, enabling slow and sustained antigen release. This prolonged exposure allows continuous stimulation of the immune system, supporting robust activation of humoral and cellular immunity and resulting in higher and more sustained antibody production (Pedro *et al*., 2021).

Two-way ANOVA analysis (Table 1) confirmed that both vaccine treatment and sampling time significantly influenced antibody titers ($P < 0.05$). However, no significant interaction was observed between the treatment and time factors ($P > 0.05$), indicating that the effects of vaccination and sampling time on antibody titers were independent; in other words, one factor did not modify the effect of the other (Bogovič *et al*., 2019).

Post hoc LSD test results (Table 2) revealed significant differences between the control group and both adjuvant-treated groups ($P < 0.001$). However, no statistically significant difference was observed between the Montanide ISA 201 VG and Montanide Gel-01 groups ($P > 0.05$), suggesting comparable effectiveness of the two adjuvants in enhancing immune responses. The observed outcomes also indicate that the performance of different adjuvants may vary depending on the timing and methodology of antibody titer measurement (Remic *et al*., 2020).

As shown in Table 3, antibody titers at weeks 3, 4, and 5 were significantly higher ($P < 0.05$) than at week 1. Additionally, titers at week 5 were significantly higher than at week 2. These results indicate a clear increase in antibody production beginning in week 3, underlining the importance of time post-vaccination in evaluating vaccine efficacy (Jungbauer *et al*., 2021).

Regression analysis revealed that in the control group, antibody titers did not significantly increase over time ($P > 0.05$), with a regression equation of $Y = -1.409 + 9.589X$ and a low

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coefficient of determination ($R^2 = 0.077$), suggesting that only 7.7% of the variation in antibody titers could be explained by time. In contrast, the Montanide ISA 201 VG group showed a significant association between titer and time ($P < 0.05$), with a regression equation of $Y = -11.433 + 20.503X$ and $R^2 = 0.205$. The Montanide Gel-01 group showed the strongest correlation ($P < 0.05$), with a regression equation of $Y = -18.063 + 29.335X$ and a high R^2 value of 0.645, indicating that 64.5% of the variation in antibody titers was explained by time.

These findings suggest that in the absence of an adjuvant, antibody titers do not increase significantly over time, indicating insufficient immune stimulation. In the Montanide ISA 201 VG group, the moderate R^2 value implies that other factors beyond time may contribute to the immune response. Nonetheless, the regression coefficient of 20.5% reflects a clear enhancement in antibody titers relative to the control group. The Montanide Gel-01 group, with a higher regression coefficient (29.3%) and R^2 value, exhibited superior effectiveness in boosting immune responses over time, likely due to its slow-release mechanism (Dessalegn *et al*., 2021).Montanide Gel-01 was the most effective adjuvant in enhancing immune responses to the *S. Suis* vaccine when compared to Montanide ISA 201 VG. This conclusion is supported by its higher regression coefficient and greater explanatory power (R^2), indicating a faster and more sustained increase in antibody titers (Ou *et al*., 2022).

CONCLUSION AND RECOMMENDATIONS

Conclusion

This study demonstrated that the addition of Montanide ISA 201 VG and Montanide Gel-01 adjuvants to the *Streptococcus suis* vaccine significantly enhanced antibody titers compared to the control group ($P < 0.05$). Moreover, the timing of serum collection post-vaccination also had a significant effect on antibody titers ($P < 0.005$). These findings indicate that both adjuvant formulation and sampling time are critical factors in determining the effectiveness of immunization.

Recommendations

Further research is recommended to explore the effects of increasing antigen concentration in the vaccine formulation in order to achieve higher antibody titers. Additionally, extending the observation period is essential to monitor the dynamics of antibody decline, providing a more comprehensive understanding of post-vaccination immune durability.

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Figure

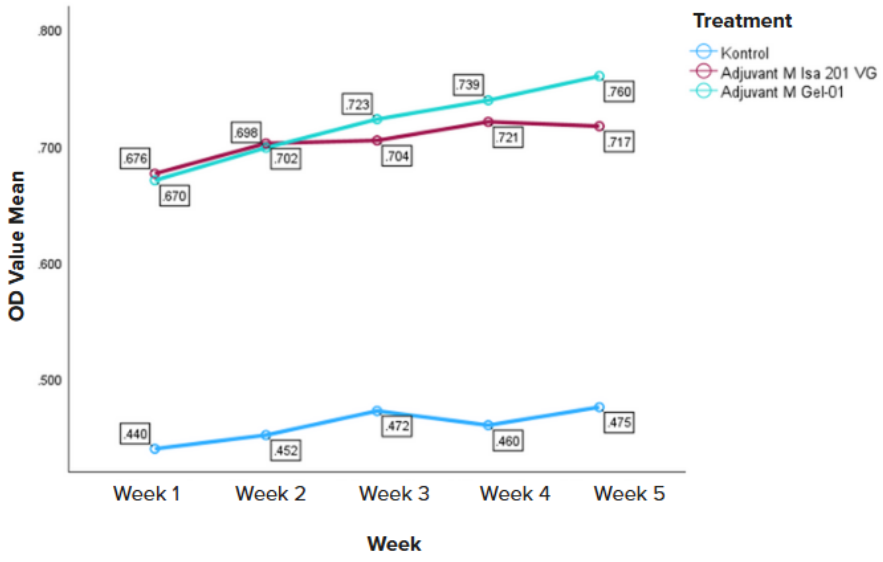

Figure 1. Antibody titers in pig serum following vaccination with *S. suis* using various adjuvants across different observation periods.

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Tables

Table 1. Two-way ANOVA analysis

Source	Type III Sum of Squares	df	Mean Square	F	Significant
Corrected Model	0.871[a]	14	0.062	50.947	<0.001
Intercept	23.607	1	23.607	19330.181	<0.001
Treatment	0.843	2	0.422	345.210	<0.001
Week	0.022	4	0.006	4.570	0.003
Treatment *Week	0.006	8	0.001	0.570	0.797
Error	0.055	45	0.001		
Total	24.533	60			
Corrected Total	0.926	59			

Table 2. LSD test results of antibody titers in pig serum following *S. suis* vaccination with various adjuvants.

(I) Treatment	(J) Treatment	Mean Difference	Significant
Kontrol	Adjuvan M 201 VG	-0.24415*	<0.001
	Adjuvan M Gel-01	-0.25820*	<0.001
Adjuvan M ISA 201 VG	Adjuvan M Gel-01	-0.01405	0.210

Table 3. Antibody titers in pig serum following *S. suis* vaccination across different observation periods.

(I) Time Treatment	(J) Time Treatment	Mean Difference	Significant
Week 1	Week 2	-.02192	.131
	Week 3	-.03783*	.011
	Week 4	-.04450*	.003
	Week 5	-.05533*	<.001
Week 2	Week 3	-.01592	.270
	Week 4	-.02258	.120
	Week 5	-.03342*	.024
Week 3	Week 4	-.00667	.643
	Week 5	-.01750*	.226
Week 4	Week 5	-.01083	.452

Terakreditasi Nasional Sinta 4, berdasarkan Keputusan Direktur Jenderal
Pendidikan Tinggi, Riset, dan Teknologi No. 158/E/KPT/2021