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COMPARISON OF BALB/C MICE IMMUNE RESPONSE WHICH HAS BEEN INDUCED WITH DIFFERENT RABIES VACCINE

Perbandingan Respons Imun Mencit BALB/c yang Diinduksi dengan Vaksin Rabies Berbeda

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Abstract

Rabies is a fatal zoonotic disease that remains incurable, making vaccination the primary strategy for prevention in veterinary and human public health. Although multiple commercial rabies vaccines are widely used, comparative data on their immunogenicity are limited and often inconsistent. This study evaluated and compared the humoral and cellular immune responses induced by three commercially available rabies vaccine brands using a BALB/c mouse model. Ten 20-week-old female BALB/c mice were allocated into treatment groups and immunized accordingly. Blood samples were collected at week 0 (pre-vaccination), week 2, and week 4 post-vaccination to determine specific anti-rabies antibody titers using enzyme-linked immunosorbent assay (ELISA). At the end of the study, spleens were harvested for histopathological examination with hematoxylin-eosin staining to assess cellular immune responses. Antibody titers were analyzed using analysis of variance (ANOVA). All three vaccines induced a significant and comparable increase in antibody titers up to two weeks post-vaccination, with no statistically significant differences among brands. By week 4, a decline in antibody levels was observed across all groups. Histopathological analysis revealed similar activation of cellular immunity among vaccines, characterized by lymphoid proliferation and plasma cell activity, without notable inter-group differences. In conclusion, the three commercial rabies vaccines demonstrated equivalent immunogenicity in inducing both humoral and cellular immune responses in BALB/c mice. These findings suggest that no single vaccine brand was superior, and that rabies vaccination strategies should prioritize operational factors such as schedule adherence, booster administration, and proper vaccination techniques rather than brand selection.

Keywords: BALB/c mice, immune response, immunology, rabies vaccines, vaccination

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Abstrak

Rabies merupakan penyakit zoonosis fatal yang hingga kini belum dapat diobati, sehingga vaksinasi menjadi strategi utama pencegahan dalam kesehatan masyarakat veteriner dan manusia. Meskipun berbagai vaksin rabies komersial telah digunakan secara luas, data komparatif mengenai imunogenisitasnya masih terbatas dan sering tidak konsisten. Penelitian ini bertujuan mengevaluasi dan membandingkan respons imun humoral dan seluler yang diinduksi oleh tiga merek vaksin rabies komersial menggunakan model tikus BALB/c. Sepuluh ekor tikus BALB/c betina berusia 20 minggu dibagi ke dalam kelompok perlakuan. Sampel darah dikoleksi pada minggu ke-0, ke-2, dan ke-4 pascavaksinasi untuk pengukuran titer antibodi rabies menggunakan ELISA. Pada akhir periode pengamatan, limpa diambil untuk analisis histopatologi dengan pewarnaan Hematoksilin–Eosin guna menilai respons imun seluler. Data titer antibodi dianalisis menggunakan uji ANOVA. Hasil menunjukkan bahwa ketiga vaksin mampu meningkatkan titer antibodi secara signifikan dan setara hingga dua minggu pascavaksinasi tanpa perbedaan statistik antar kelompok. Pada minggu ke-4, seluruh kelompok menunjukkan kecenderungan penurunan kadar antibodi. Analisis histopatologi limpa memperlihatkan aktivasi respons imun seluler yang sebanding, ditandai oleh proliferasi sel limfoid dan aktivitas sel plasma, tanpa perbedaan bermakna antar vaksin. Disimpulkan bahwa ketiga vaksin rabies komersial yang diuji memiliki imunogenisitas yang setara dalam menginduksi respons imun humoral dan seluler pada tikus BALB/c. Oleh karena itu, strategi vaksinasi rabies sebaiknya lebih menekankan aspek operasional seperti kepatuhan jadwal, pemberian booster, dan teknik administrasi yang tepat dibandingkan pemilihan merek vaksin.

Kata Kunci: imunologi, mencit BALB/c, respon imun, vaksinasi, vaksin rabies

INTRODUCTION

Rabies is a viral infection that causes encephalitis in mammals, including humans (Dietzschold, 2005). This disease is highly lethal and zoonotic, meaning it can be transmitted from animals to humans. Transmission occurs when viral particles present in the saliva of infected animals such as dogs, cats, monkeys, bats, and raccoons - enter the human body or other susceptible animals, typically through bites or scratches, or when the saliva contacts the eyes, mouth, nose, or broken skin (CDC, 2019). The incubation period between viral exposure and symptom onset typically ranges from one to three months, but it can vary from less than one week to over a year, depending on the distance the virus must travel from the peripheral nerves to the central nervous system (WHO, 2013).

Vaccination remains the primary preventive measure against this fatal infection. Various commercial rabies vaccines are available, differing in formulation, immunogenicity, and cost (Natesan *et al.*, 2023). Understanding both the pathogenesis of rabies and the characteristics of available vaccines is essential for optimizing vaccination strategies and ensuring effective disease control.

Previous research of similar nature has been done before (Astawa et al, 2018), which also uses BALB/c mice, although that particular research only studied the effect of one vaccine on the test subject. Other researches had been done that aims to see the effectiveness of certain vaccines, but does not directly compare one vaccine against another. Other studies use suboptimal animals with variables that might affect the result. While some other does not directly compare one vaccine with another using the same test animal. There remains a need for studies that directly compare multiple vaccines under standardized and controlled conditions.

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By conducting immune response tests in BALB/c mice induced with various vaccine formulations under laboratory conditions, and by utilizing BALB/c mice, which are assumed to be uniform in their biological state, we can eliminate the variables typically encountered in the field or in test animals (Potter *et al.*, 1985). This allows for a better understanding of the vaccines and enables the selection of the most suitable and efficient vaccine to aid in eradicating the disease. This research aims to directly compares 3 different vaccines to see which, if any is more effective in inducing antibody response in the test animal, BALB/c mice.

RESEARCH METHODS

Animal Ethics Approval

This research was approved by the Animal Ethics Committee, Faculty of Veterinary Medicine, Udayana University, Bali, Indonesia (B/109/UN14.2.9/PT.01.04/2024). This study was conducted from January to April 2024. Animal maintenance, treatment administration, sample collection, and serological examinations were carried out in the Immunology Laboratory of the Faculty of Veterinary Medicine, Udayana University. Histological examinations were performed at the Denpasar Disease Investigation Center (DIC).

Research Object

This study utilized nine female BALB/c mice aged 20 weeks (5 months) with an average weight of 20-25 grams (Charles River, 2015). The mice were housed in three cages (4 mice per enclosure), each measuring 47 x 33 x 15 cm. Food and water were provided ad libitum. Female mice were selected for this study as they are known to be more sensitive to infection compared to male mice (Klein & Flanagan, 2016). The sample was divided into three experimental treatment groups, with three mice in each group. Calculation of sample size used was based on the Resource Equation Approach formula, and taking into consideration ethics, and ELISA test kit availability (Arifin and Zahiruddin, 2017)

The vaccines used in this study are vaccine A, Vaccine B, and vaccine C, all three have different composition, manufactured by different company, and using different adjuvant. All three vaccines are the same type of rabies vaccine, using inactivated rabies virus (VMD UK, 2021). Prior to vaccination, serum samples were collected from each group to serve as sero-negative controls for the rabies virus (Makoschey, 2015). The BALB/c mice were vaccinated following the collection of pre-vaccination samples. Vaccination was administered intramuscularly at a dose of 0.1 mg per 10 grams of body weight, targeting the femur muscle of the mice (Thomas, 2021).

Study Design

This study was carried out using a Completely Randomized Design (CRD) with a tiered treatment structure, involving ten BALB/c mice divided into three groups. Each group received a different rabies vaccine formulation. Blood samples were collected prior to vaccination, as well as two weeks and then four weeks post-vaccination, and subsequently analyzed using ELISA. Following the sampling period, the animals were humanely euthanized and necropsied, and the spleen, heart, and kidneys were collected for further examination.

Research Variables

The variables used in this study were as follows:

a) Independent variable: The brand/type of rabies vaccine administered.

b) Dependent variable: The immune response of BALB/c mice, measured through antibody titers and histological features of the spleen.

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c) Controlled variables: Mouse body weight, age, feed and water intake, husbandry management, and environmental conditions.

Data Collection Method

Blood samples were collected serially from the orbital sinus in volumes of 0.5–1 ml at three time points: pre-vaccination (week 0), two weeks post-vaccination (week 2), and two weeks after that (week 4) (Kimball, n.d.). Blood sampling from the orbital sinus was performed under sterile conditions, with the experimental animals anesthetized using a mixture of ketamine-xylazine-acepromazine at a dose of 0.1 ml per 10 grams of body weight (Ogston, 2014).

Blood samples were centrifuged until the serum was completely separated. The serum was then tested using an Enzyme-Linked Immunosorbent Assay (ELISA), using the Pusvetma™ rabies elisa kit (catalogue No. D101CE04). The ELISA procedure began by adding 100 µl (in duplicate) of positive control serum, ST 1 EU control serum, negative control serum, and diluted sample serum into the wells of a microtiter plate, following the specified sequence. In wells H11 and H12, 100 µl of PBST was added as a blank. The microtiter plate was then covered with an adhesive plastic sheet and incubated at 37℃ for 1 hour.

After incubation, the adhesive cover was removed, the liquid in the microtiter plate was discarded, and the plate was washed with a minimum of 200 µl of PBST per well 4–5 times. The plate was tapped to remove any remaining water bubbles. Next, 100 µl of Protein A Conjugate, diluted 1:16,000, was added to all wells. The plate was again covered with an adhesive plastic sheet and incubated at 37℃ for 1 hour. After incubation, the adhesive cover was removed, the liquid in the microtiter plate was discarded, and the washing process was repeated. Subsequently, 100 µl of Substrate solution was added to each well, and the plate was placed in a dark environment. Upon the appearance of a specific color change (± 10 minutes), 100 µl of Stop solution was immediately added to each well. The microtiter plate was then read using an ELISA reader at a wavelength of 402 nm.

Histologic Examination

Organ samples were collected from one mouse in each treatment group during the fourth week post-vaccination. The mice were physically euthanized via cervical dislocation while still under anesthesia following blood collection. Subsequently, necropsy was performed on the euthanized mice to obtain the spleen as an organ sample for histological preparation. The samples were immersed in 10% Neutral Buffered Formalin (NBF) to halt biological processes (enzymatic activity, cell replication, and tissue degradation) and to preserve the tissue structure. The organ samples were sliced into thin sections, placed in tissue cassettes, and fixed in 10% NBF for 24 hours to ensure optimal preservation.

Following fixation, the organ samples underwent dehydration through a graded series of alcohol concentrations (70%, 80%, 90%, 96%, and absolute alcohol (100%)) over several hours to remove water from the tissues. The clearing process was then carried out by immersing the spleen sections in xylol to eliminate alcohol from the tissues, followed by impregnation with liquid paraffin. The organ samples were embedded in paraffin blocks to facilitate sectioning. These paraffin blocks were sectioned using a microtome at a thickness of 3.5 microns. The tissue sections were mounted onto glass slides and incubated for one day at 50℃ to dry the tissues. Finally, Hematoxylin and Eosin (HE) staining was performed to visualize tissue structures. The slides were mounted with coverslips using Permount liquid medium to protect the samples and enhance image clarity. The stained organ samples were then observed and compared under a microscope.

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Data Analysis

The numerical values of induced rabies antibody *optical density* (OD) produced by ELISA is then processed statistically through ANOVA on the SPSS application to get a direct statistical comparison of all three vaccine. Meanwhile the result of the histological examination is displayed as a picture.

RESULT AND DISCUSSION

Result

Antibody titers against the rabies virus induced by the rabies vaccine were measured through an ELISA test. The vaccine formulations injected into the mice were randomized and referred to only as vaccines A, B, and C. The optical density (OD) values obtained from the ELISA were converted into ELISA Unit (EU) using the formula provided in the ELISA Kit Protocol from Pusvetma™.

Pre-vaccination antibody titers for all mice ranged from 0.2648 to 0.263 EU. These titers were non-protective, indicating no prior exposure to the rabies virus before treatment. It can be assumed that any increase in antibody titers was a direct result of the vaccine administration.

A One-Way ANOVA statistical analysis was conducted to examine the differences in antibody titers from pre-vaccination to two weeks post-vaccination. Antibody titers at two weeks post-vaccination showed a significant increase compared to pre-vaccination levels ($p < 0.05$; One-Way ANOVA). However, antibody titers at four weeks post-vaccination decreased compared to the second week, indicating a reduction in the vaccine's effect, suggesting the need for a booster dose.

Another One-Way ANOVA was performed to compare antibody titers at two- and four-weeks post-vaccination. The p-value obtained for week 2 was 0.2009 EU, while for week 4, it was 0.2084 EU. Since both p-values were greater than 0.05, this indicates that there was no statistically significant difference between the effectiveness of the three vaccines at both time points. This suggests that the three vaccines had similar efficacy over the time frame tested. Seeing the highest increase in week 2, another One-Way ANOVA then was conducted on the sample data to compare the rise in antibody titers among the three vaccines. At this time point, the differences in antibody titers between the three vaccines were not statistically significant ($p>0.05$).

No major changes be it discoloration, congestion, or other pathological changes was observed in the spleen. Histological test results showed that the white pulp (WP) appeared darker, indicating the dominance of nucleated lymphoid cells. Lymphoid cell proliferation was evident from the increased purple coloration in the white pulp, suggesting heightened cellular activity triggered by an active immune response in that area.

As lymphoid cells proliferated, the number and size of lymphoid follicles increased, leading to a visible rise in the amount of white pulp. Images from all three spleens demonstrated lymphoid follicle proliferation, as seen by the abundance of white pulp. This indicates that all three vaccines were able to induce a cellular immune response in the mice. However, the degree of proliferation, as well as the number and size of lymphoid follicles, was difficult to quantify. Therefore, it was challenging to distinguish between the mice's immune reactions to different rabies vaccines based on spleen histology without using a microscope capable of automatic counting.

The results of this study demonstrated an increase in antibody titers at two weeks post-induction in all three rabies vaccine groups, indicating that the vaccines were able to stimulate

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an immune response in the mice. However, the titers obtained (0.4019 EU; 0.3073 EU; 0.338 EU) did not reach the protective threshold. These findings are consistent with Astawa *et al.* (2018), who reported that most antibody titers measured two weeks after vaccination in mice remained below the protective level (≤0.5 EU).

Statistical analysis showed that the p-values between the three vaccine groups were greater than 0.05, indicating no significant differences among the groups at either two- or four-weeks post-vaccination. All three vaccines, however, produced a significant increase in antibody titers from pre-vaccination to two weeks post-vaccination ($p < 0.05$).

Discussion

Vaccination remains a key strategy for rabies control. The WHO (2008) recommends a minimum protective antibody titer of 0.5 EU. In this study, antibody titers measured using the Pusvetma Rabies ELISA Kit increased at two weeks post-immunization for all three vaccines, confirming their ability to induce a primary immune response in mice. However, the titers (0.4019; 0.3073; 0.338 EU) remained below the protective threshold, consistent with Astawa *et al.* (2018), who reported subprotective titers at two weeks post-vaccination in mice.

Statistical analysis showed no significant differences among the three vaccine groups at either two or four weeks ($p>0.05$), as confirmed by ANOVA. All vaccines elicited significant increases from baseline to week two ($p<0.05$). Visual differences in the plotted curves were attributable to axis scaling rather than true biological variation.

The rise in titers at week two reflects activation of the primary immune response, in which dendritic cells present vaccine antigens to T cells in lymph nodes, subsequently activating B cells to differentiate into plasma cells that secrete antigen-specific antibodies (Janeway, 2001; Plotkin *et al.*, 2023). By week four, titers declined, corresponding to the normal transition from IgM-dominated early responses to IgG production and the contraction phase of the primary response. Only vaccine B showed a significant decrease ($p<0.05$). This plateau phase aligns with findings by Aubert (1992) and indicates the need for a booster dose to achieve protective immunity. Research by Agustini *et al.*, (2019), reported that rabies booster vaccination in BALB/c mice two weeks after the first vaccine could significantly increase antibody titers.

Overall, the three vaccines demonstrated comparable immunogenicity, with observed variation likely attributable to random differences among individual mice. The generated antibodies follow the expected pathway of antigen presentation, B-cell activation, and plasma-cell–mediated antibody secretion, establishing the basis for immunological memory (Roy *et al.*, 2021; NCBI, 2023).

CONCLUSION AND RECOMMENDATIONS

Conclusion

All three rabies vaccines induced a significant increase in antibody titers and a cellular immune response, with no statistically significant differences in effectiveness, though the significant decline($p<0.05$) in antibody levels by week four suggests the potential need for a booster dose to reach the protective threshold of 0.5 IU/mL (WHO 2013).

The results of this study indicate that there are no statistically significant differences among the three vaccines in their ability to induce an antibody response, as evidenced by both antibody titer measurements and histological analysis of the spleen. Therefore, based on these findings, vaccination efforts should prioritize administration techniques rather than vaccine brand selection, as no rabies vaccine demonstrated superior efficacy over the others.

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Recommendations

Further research needs to be carried out with revaccination or boosters for experimental animals that have antibody titers below the protective titer threshold of 0.5 EU according to WHO.

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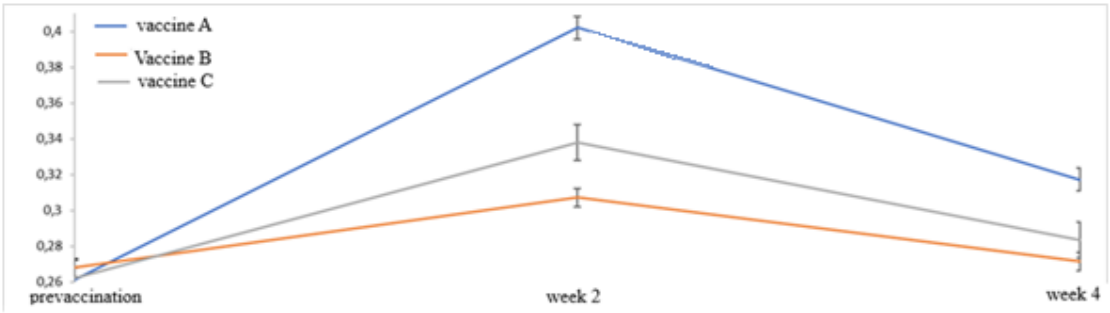
Table

Table 1. Antibody Titers in Mice Following Induction with Vaccines A, B, and C

Vaccine	Mice	Prevaccination(EU)	Week 2 (EU)	Week 4 (EU)
A	1.1	0.2688	0.4475	0.3334
	1.2	0.2587	0.3123	0.2749
	1.3	0.2572	0.4461	0.3433
	Mean±SD	0.2615±0.0063	0.4019±0.0776	0.3172±0.0369
B	2.1	0.2648	0.3152	0.27088
	2.2	0.2668	0.3078	0.26886
	2.3	0.2729	0.2989	0.27491
	Mean±SD	0.2681±0.0042	0.3073±0.0081	0.27155±0.0030
C	3.1	0.2648	0.4089	0.3212
	3.2	0.2708	0.2974	0.2648
	3.4	0.2509	0.3078	0.26482
	Mean±SD	0.2621±0.0102	0.338±0.06159	0.2836±0.0325

Graph



Graph 1. Graph of Mean Antibody Titers Against the Rabies Virus in Mice

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Figure

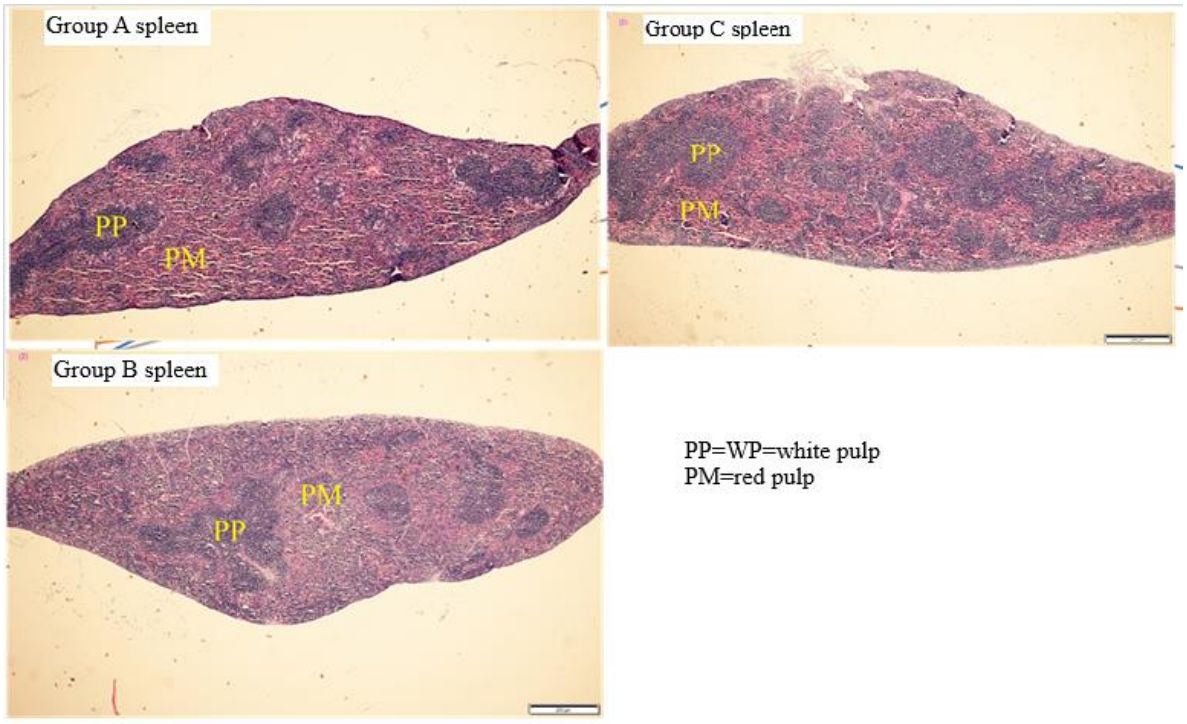

Figure 1. Histological Analysis of Mouse Spleen After Rabies Vaccine Induction (40x objective magnification). Spleen samples were collected from one mouse per treatment group four weeks post-vaccination. Samples were stained with HE staining. The spleen showed no major pathological changes. Histologic examination revealed darker white pulp, indicating lymphoid cell proliferation and heightened immune activity.