Jurnal Veteriner pISSN: 1411-8327; eISSN: 2477-5665
Terakreditasi Nasional Peringkat 2, Dirjen Riset dan Pengembangan,
Kemendiktisaintek RI SK No 10/C/C3/DT.05.00/2025, 21 Mar.2025

Juni 2025 Vol. 26 No.2: 221-230
DOI: 10.19087/jveteriner.2025.26.2.221
online pada http://ojs.unud.ac.id/index.php/jvet

The Effect of Inactivated Rabies Vaccine Storage Temperature on the Immune Response of BALB/c Mice Post Vaccination

(PENGARUH SUHU PENYIMPANAN VAKSIN RABIES INAKTIF TERHADAP RESPONS IMUN MENCIT BALB/C PASCAVAKSINASI)

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ABSTRACT

More than 95% of human rabies cases in the world are transmitted by dogs. Vaccination is the main program in controlling rabies in animals. During vaccine distribution, especially in remote or resource-limited areas, vaccines may be temporarily exposed to temperatures outside the recommended limits (2–8°C) due to challenges in maintaining the cold chain, such as unreliable electricity, and inadequate storage facilities. This study aims to test the thermotolerance of the inactivated rabies vaccine by evaluating its immune response through antibody titers detected in BALB/c mice after exposure to higher storage temperatures (27°C and 37°C) for two hours, simulating real-world logistical challenges. Measurement of antibody titers in serum was detected using an enzyme-linked immunosorbent assay (ELISA). Comparison of antibody titers was analyzed statistically using repeated-measures ANOVA and Post Hoc Test Pairwise Comparisons and Bonferonni. In addition, histological examination of the spleen organ was carried out by looking at the presence of germinal centers in the white pulp area which is an indication of a response to antigenic stimuli. The study demonstrates that short-term exposure (2 hours) of the inactivated rabies vaccine to higher temperatures (27°C and 37°C) does not significantly reduce its immunogenicity compared to storage at recommended temperatures (2–8°C), as evidenced by comparable antibody titers and germinal center formation in BALB/c mice.These findings suggest that the vaccine retains its antigenic potency during brief deviations outside of cold chain conditions, such as those that occur during transport or temporary storage. However, further research is needed to evaluate the impact of long-term exposure to non-recommended temperatures.

Keywords: rabies vaccine; vaccine thermotolerance; immune response; cold-chain

ABSTRAK

Lebih dari 95% kasus rabies pada manusia di dunia ditularkan oleh anjing. Vaksinasi merupakan program utama dalam pengendalian rabies pada hewan. Selama pendistribusian vaksin, terutama di daerah terpencil atau terbatas sumber daya, vaksin dapat terpapar sementara pada suhu di luar batas yang direkomendasikan (2–8°C) karena tantangan dalam menjaga rantai dingin, seperti listrik yang tidak dapat diandalkan, dan fasilitas penyimpanan yang tidak memadai. Penelitian ini bertujuan untuk menguji termotoleransi vaksin rabies inaktif dengan mengevaluasi respons imunnya melalui titer antibodi yang terdeteksi pada tikus BALB/c setelah terpapar pada suhu penyimpanan yang lebih tinggi (27°C dan 37°C) selama dua jam, dengan simulasi tantangan logistik di dunia nyata. Pengukuran titer antibodi dalam serum dideteksi menggunakan enzyme-linked immunosorbent assay (ELISA). Perbandingan titer antibodi dianalisis secara statistik menggunakan repeated-measures ANOVA dan Post Hoc Test Pairwise Comparisons dan Bonferonni. Selain itu, pemeriksaan histologis organ limpa dilakukan dengan melihat keberadaan germinal center di area pulpa putih yang merupakan indikasi respons terhadap rangsangan antigenik. Penelitian ini menunjukkan bahwa paparan jangka pendek (2 jam) vaksin rabies inaktif pada suhu yang lebih tinggi (27°C dan 37°C) tidak secara signifikan mengurangi imunogenisitasnya dibandingkan dengan penyimpanan pada suhu yang direkomendasikan (2–8°C), sebagaimana dibuktikan oleh titer antibodi yang sebanding dan pembentukan germinal center pada mencit BALB/c. Temuan ini menunjukkan bahwa vaksin mempertahankan potensi antigeniknya selama penyimpangan singkat di luar kondisi rantai dingin, seperti yang terjadi selama transportasi atau penyimpanan sementara. Namun, penelitian lebih lanjut diperlukan untuk mengevaluasi dampak paparan jangka panjang pada suhu yang tidak direkomendasikan.

Kata Kunci: vaksin rabies; *thermo-tolerance vaccine*; respon imun; *cold-chain.*

INTRODUCTION

Rabies has attacked 26 of the 38 provinces in Indonesia (Indonesian Ministry of Health, 2023). Rabies is a zoonotic disease that requires special treatment in Indonesia. This statement is contained in the Decree of the Minister of Agriculture Number 237/ KPTS/PK.400/M/3/2019 of 2019 concerning Determination of Priority Zoonoses (Decree of the Minister of Agriculture, 2019). The Rabies virus is capable of infecting all mammals and often results in death in susceptible or unvaccinated individuals (Maclachlan and Dubovi, 2010). The number of human deaths worldwide due to rabies spread by dog bites is estimated at 59,000 every year (WHO, 2018). In humans, more than 95% of rabies cases are transmitted by dogs (WHO, 2018). Eradication and control of rabies in dogs is carried out by preventing it at the source (WHO, 2018). Vaccination is the main rabies control program (WOAH, 2023).

Vaccines are sensitive biological substances that gradually lose their potency over time (WHO, 1998). The loss of this potential can be faster if exposed to high temperatures, freezing, or excessive light exposure (WHO, 2015). This loss of potential is permanent and cannot be repaired (WHO, 1998). Recommendations for vaccine storage temperatures are generally listed on the label. Most recommended vaccine storage temperatures are in the range of 2-8℃ (Milstien *et al*., 2006). However, some types of vaccines can be maintaned at freezer temperatures between -15°C and -50°C such as Mpox (Jynneos),

RSV (mResvia), and Varicella, as well as ultra cold-freezer conditions between -60°C an -90°C such as COVID-19 2024–2025: Pfizer-BioNTech (FDA, 2024; CDC, 2025). In implementing a vaccination program, several errors can occur, including: storage, transportation, prescription preparation, vaccine preparation, administration, monitoring and recording of vaccinations (Poiraud *et al.*, 2023).

Vaccine thermotolerance affects several things, including the reach of vaccination programs which are often adjusted to areas that are able to store vaccines at recommended temperatures (Lankeste *et al*., 2016). Expenditures for installation and training costs can reach 28% of the total cost of the vaccination program and this is paid for in program preparation (COVAX, 2021). This is a serious obstacle for low and middle income countries in tropical regions. These obstacles include keeping vaccines cold amidst high air temperatures and unreliable access to electricity (Karp *et al.,* 2015). Determining safe time-temperature thresholds for storing and transporting the existing inactivated rabies vaccine can help overcome logistical obstacles, especially in resource-limited settings where cold chain maintenance is challenging.

This study was aimed to observe the effect of inactivated rabies vaccine storage temperature on antibody titers in BALB/c mice which were analyzed statistically using the Repeated Measure-Analysis of Variance (ANOVA) test. Next, analyzes that show significant differences ($p<0.05$) will be tested by Post Hoc Tests-Pairwise Comparisons and Bonferroni. A descriptive analysis of histological observations of the spleen, especially the germinal center findings in the white pulp area was also included. With the data obtained, it is hoped that this research can provide data regarding the thermotolerance of inactivated rabies vaccine.

RESEARCH METHODS

Ethical Approval

This research was approved by the Animal Ethics Committee, Faculty of Veterinary Medicine, Udayana University, Bali, Indonesia (Animal Ethics Approval Number: B/85/UN14.2.9/PT.01.04/2024).

Sample Size Calculations and Classification

This research used nine 20-week old female BALB/c mice, weighing 20-25 g. The research samples used were serum and spleen tissue. Calculation of sample size used was based on the Resource Equation Approach formula (Arifin and Zahiruddin, 2017)

Preparation of Stored Vaccines and Vaccination Process

Same batch of vaccines were obtained from veterinary practicioner and stored at three different treatment groups, with a storage time of two hours. The storing conditions were: P1: vaccine stored at a temperature of 2-8ºC (refrigerator set to 2-8℃); P2: vaccine stored at 27℃ (room air-conditioned to 27ºC) and P3: vaccine stored at 37℃ (waterbath set to 37℃)

The first treatment (P1) is the recommended temperature for vaccine storage (WHO, 2006). Vaccination was carried out in all groups using the intramuscular administration route in the femoral area. The vaccine dose given was 0.2 mL (Astawa *et al*., 2018).

Sample Processing

Blood samples were collected from the orbital vein of the mice under anesthesia, a combination of ketamine (Ket-A-100®, Agrovet Market Animal Health, Lima, Peru). IM 100 mg/kg) and xylazine (Xyla-Ject®, Phoenix Pharm, Mainnheim, Germany) IM 8 mg/kg) (Virginia Tech, 2017). Blood samples (0.3–0.5 mL per collection) were taken at three times points: (i) before vaccination (pre-vaccination), (ii) the second week post-vaccination (iii) the fourth week post-vaccination (Maulina and Amalasari, 2018). The antibody titer was checked using the Rabies ELISA (Kit Elisa Rabies Pusvetma®. Balai Besar Veteriner Farma Pusvetma, Surabaya, Indonesia) according to the manufacturer's protocol. In

addition, histology sampling of the spleen was carried out on one mouse in each group after the fourth week post-vaccination. Mice were euthanized procedurally and surgery was carried out to remove the spleen (Kiernan, 2015). Histology preparations and observations were conducted at the Disease Investigation Center Denpasar (BBVet Denpasar).

Descriptive and Statistical Analysis

Antibody titers obtained from the ELISA test were analyzed using repeated-measures ANOVA. If the analysis results showed significant differences ($p<0.05$), it would be continued with the Post Hoc Test – Pairwise Comparisons and Bonferroni tests. For spleen histology preparations, a descriptive analysis was performed, especially for the white pulp germinal center area.

RESULTS AND DISCUSSIONS

Antibody Titer

The pre-vaccination antibody titer for all mice showed a value of 0.155 – 0.273 EU (Table 1). On the 2nd week of post-vaccination, the antibody titer showed a value of 0.302–0.379 EU, which is a non-protective antibody titer against RABV (<0.5 EU) (WHO, 2018). The antibody titer of mice at week four post-vaccination showed a value in the range of 0.263–0.769 EU. Interestingly, at week four post-vaccination the antibody titer of mice in group P2 showed a protective level in the range of 0.531–0.769 EU (WHO , 2018).

Histological Observations

The two main functional zones of the spleen organ, namely the red pulp and white pulp areas (Figure 2). Our histological observations showed that the marginal zone area in all treatments have a thin area. Furthermore, the marginal area of the sinus was observed to be less clear throughout the white pulp area. Observation of several areas of the white spleen indicated the presence of a germinal center.

Analyses

Based on the Repeated Measures ANOVA – Spherecity Assumed analysis, it showed that giving inactivated rabies vaccine to BALB/c mice had a significant effect on increasing antibody titers ($P< 0.05$). The formation of antibodies is shown in Figure 1.

Post Hoc Test - Pairwise Comparisons analysis was carried out based on the increase in antibody titer that occurred between time factors. Examination at pre-vaccination to the 2nd week post-vaccination showed a significant increase in antibody titer ($P < 0.05$). Furthermore, at the 2nd week and 4th week post-vaccination there was no significant change in antibody titer ($P > 0.05$).

In addition, Post Hoc Test - Pairwise Comparisons analysis was carried out to compare the antibody titers formed between treatment factors. Examination of P1 (2-8℃) with P2 (27℃) showed a significant difference in antibody titer ($P < 0.05$) with the antibody titer value of P2 (27℃) being higher than P1 (2-8℃). Furthermore, P1 (2-8℃) and P3 (37℃) did not show significant differences in antibody titers ($P > 0.05$).

Post Hoc Test – Bonferroni analysis was carried out to see the trend in antibody titers that occurred in each treatment. Based on the analysis, it showed that P1 (2-8℃) increased significantly from pre-vaccination to the 2nd week post-vaccination ($P < 0.05$) while from the 2nd to 4th week post-vaccination there was no significant change ($P> 0.05$). Furthermore, at P2 (27℃) from pre-vaccination to the 2nd week there was a non-significant increase in antibody titer values ($P>0.05$) while a significant increase occurred at 2 to 4 weeks post-vaccination ($P<0.05$). Next, at P3 (37℃) there was a significant increase from pre-vaccination to the 2nd week post-vaccination ($P < 0.05$) while from week 2 to 4 there was no significant change ($P > 0.05$).

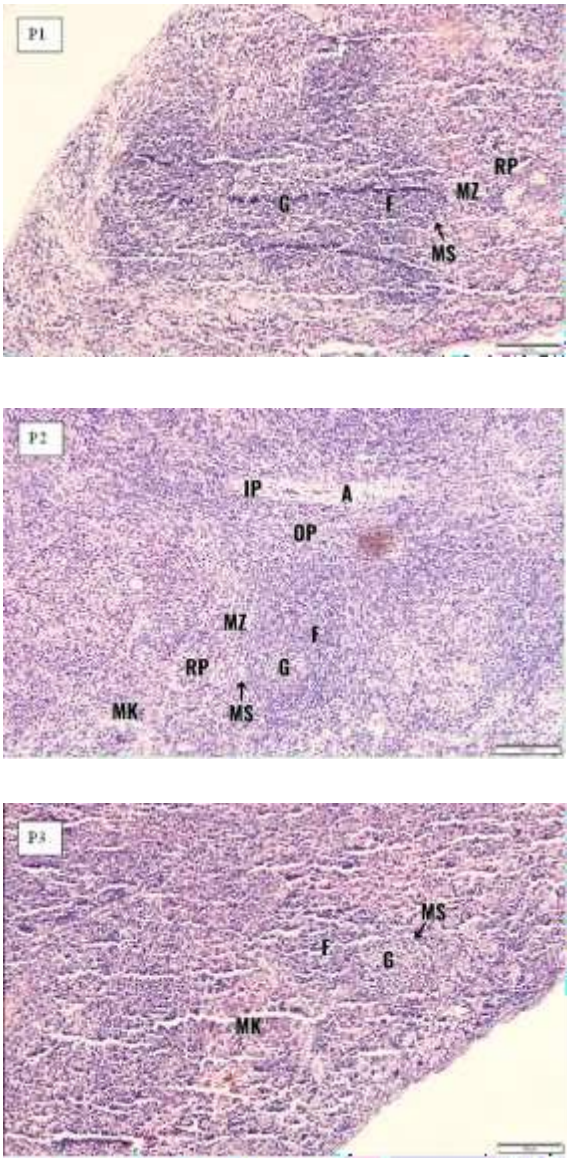

Figure 2. Histology of the spleen organ in group P1 (2-8ºC), P2 (27ºC),and P3 (37ºC) of 20-week old female BALB/c mice. G = Germinal center; F = Follicle; MS = Marginal Sine; MZ = Marginal Zone; A = Central Artery; IP = Inner PALS; OP = Outer PALS; RP = Red Pulp; MK = Megakaryocyte. HE 200X.

Discussion

According to WHO recommendations (2018), the minimum protective antibody titer value for RABV is 0.5 EU. For serum obtained during pre-vaccination, antibody titers against rabies virus (RABV) in individual mice across all groups showed values of 0.155 – 0.273 EU with an average of 0.207 EU. This titer is at a non-protective level (<0.5 EU). This antibody titer value indicates that all mice in this study had never been exposed to rabies antigen before, so any increase in antibody titer that occurred during the study can be assumed to be an induction of the inactivated rabies vaccine.

There was a significant increase in antibody titer ($p<0.05$) from pre-vaccination to week two post-vaccination in all treatment groups. This indicates that vaccines stored at temperatures of 2-8℃, 27℃ and 37℃ are able to induce antibody responses. The antibody titer found two weeks post-vaccination for all treatment groups was in the range of 0.302 - 0.379 EU. This value is a non-protective antibody titer based on WHO standards. Research by Astawa. (2018) also showed that most of the antibody titer values two weeks post-vaccination in mice after being induced by rabies vaccine and inactivated rabies virus propagated in Baby Hamster Kidney (BHK) cells were also non-protective (≤ 0.5 EU).

In the 2nd week post-vaccination, there was no significant difference ($p>0.05$) between the antibody titers against RABV induced by vaccines stored at 2-8℃, 27℃and 37℃.

This indicates that storing the vaccine above the manufacturer's recommended temperature, namely at 27℃ and 37℃ for two hours does not damage the antigenic properties of the rabies vaccine. A study by Lankester *et al*. (2016) reported similar findings, showing that the rabies vaccine (Nobivac®, Merck Animal Health, Rahway, New Jersey, USA). stored at elevated temperatures (25°C and 30°C) for 90 days still induced antibody titers not significantly lower than those stored at 2–8°C in vaccinated dogs.

On the 4th week of post-vaccination, the antibody titer of dogs induced by vaccines stored at 2-8℃ and 37℃ did not show a significant difference ($p>0.05$) with the 2nd week of post-vaccination. It can be indicated that antibody production in mice has entered the plateau phase. In this phase, antibody titers can be increased by re-vaccination or boosters. Research by

Table 1. Antibody titers against RABV in BALB/c mice expressed in equivalent units

Treatment group	Mice	Pre vaccination	2nd week after vaccination	4th week after vaccination
P1, 2-8ºC	P1.1	0.158	0.302	0.271
	P2.2	0.176	0.306	0.287
	P3.3	0.263	0.305	0.283
	Mean ± SD	0.199 ± 0.056	0.304 ± 0.002	0.280 ± 0.008
P2, 27ºC	P2.1	0.211	0.326	0.537*
	P2.2	0.196	0.312	0.531*
	P2.3	0.273	0.302	0.769*
	Mean ± SD	0.2666 ± 0.040	0.313 ± 0.012	0.612 ± 0.135
P3, 37ºC	P3.1	0.155	0.306	0.293
	P3.2	0.170	0.308	0.263
	P3.3	0.263	0.379	0.267
	Mean ± SD	0.196 ± 0.058	0.331 ± 0.041	0.274 ± 0.016

(EU). (*) indicates protective antibody titer (>0.5 E

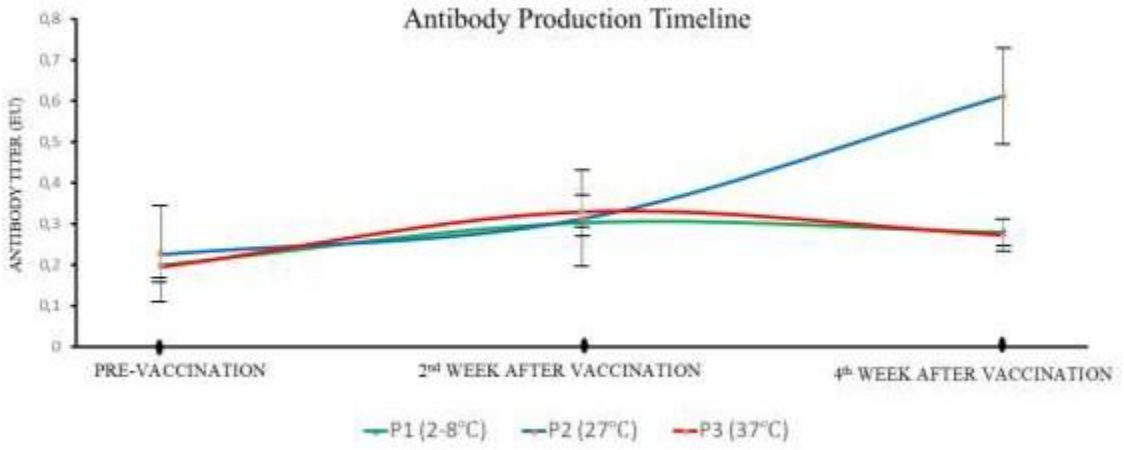

Figure 1. Curve of Antibody Responses

Aubert (1992) showed that the immune response to RABV in dogs entered a plateau after the second week post-vaccination, so a booster was needed before the antibody titer reached its lowest point. Research by Agustini *et al*. (2019) reported that rabies booster vaccination in BALB/c mice two weeks after the first vaccine could signify-cantly increase antibody titers.

The antibody titer in the 4th week post-vaccination in BALB/c induced by inactivated rabies vaccine stored at 27℃ for two hours increased compared to the 2nd week post-vaccination. In general, the anti-body titer induced by vaccines stored at 27℃ and 37℃ does not cause a decrease in antibody titer. The phenomenon of incre-asing antibody titer in the 4th week post-vaccination for P2 (27℃) group needs to be studied further. Previous study showed that storage at higher temperature renders rabies virus vaccine strain L Pasteur to lose its biological function and activity (Varianytsia *et al*., 2020). There are several factors that can cause variations in immune responses, including intrinsic factors such as genetic and physiological, as well as extrinsic factors such as vaccines, vaccine administration

techniques and the environment (Zimmermann and Curtis, 2019). For further research based on this pilot study, it is necessary to measure the uniformity of the experimental animal conditions more comprehensively. Research by Astawa *et al*. (2019) reported that there were variations in antibody titers formed in BALB/c mice after rabies vaccination. Another thing, the sample rabies vaccine uses aluminum hydroxide adjuvant which works with a depot effect. This mechanism works by slowly releasing antigens so that it can provide continuous stimulation to the immune system, which can strengthen and maintain the immune response (Kuroda *et al*., 2013; He *et al.,* 2015). This depot mechanism allows for an increase in antibody titers for a longer period after vaccine induction.

Based on the Post Hoc Test – Pairwise Comparisons analysis on the treat-ment factor, no significantly lower antibody titers were found in mice induced with vaccines stored at 27℃ and 37℃ when compared to the antibody titers of mice induced with vaccines stored at 2-8℃. Research by Lankester *et al.* (2016) reported no significant difference in antibody titers induced by rabies vaccine (Nobivac®) stored at 2-8℃ with those stored at 25℃ and 30℃ for 90 days. Sample vaccine and Nobivac® vaccines are inactivated pasteur strain vaccines with aluminum adjuvants (Daulay *et al.,* 2020; MSD Animal Health UK Limited, 2021). Several studies have shown that inactivated vaccines are stable at higher temperatures (± 40℃) for several days, but are sensitive to freezing temperatures, especially those using aluminum adjuvants (Kartoglu and Milstein, 2014; Fanelli *et al*., 2022).

In histological examination of the spleen, two main functional zones of the spleen organ were visible, namely the red pulp area and the white pulp. The red pulp functions as a blood filter that removes foreign objects and erythrocytes that have been damaged and have lost their function. This area is also a storage place for iron, erythrocytes and platelets. In rodents, this area is used for hematopoiesis, especially in animals during pregnancy and newborn animals (Cesta, 2006; Kapila *et al*., 2023; Ganz, 2016). In the white pulp follicles, antigens and pathogens transmitted through the blood are presented by antigen presenting cells. This process initiates the activation of T cells and B cells which ultimately leads to the production of antibodies (Cesta, 2006; Kapila *et al.*, 2023).

Based on our observation, the marginal zone area of the spleen of mice from all treatment groups has a thin area. The marginal zone of mice is much smaller and varies compared to the marginal zone in rats. In addition, the marginal sinus (MS) is sometimes not clearly visible in mice compared to rats which are more easily visible (Cesta, 2006). Although the marginal sinus area is more easily visible in mice, electron microscopic studies show that the marginal sinus of mice is six times larger than that of rats (Schmidt *et al.*, 1993).

Germinal center (GC) is the primary site where antigen-activated B cell clones develop and undergo hypermutation and immunoglobulin selection (Zhang *et al*., 2016). In the examination of the white pulp area, germinal centers are present in secondary follicles. This development can be an indication of an acute immune response to antigen, resulting in increased cellularity in the B cell area and increased secondary follicles containing germinal centers (Elmore, 2006; Cesta, 2006). This process begins with immature B cells, or immunoblasts, proliferating in response to antigenic stimulation (Elmore, 2006).

CONCLUSIONS

The antibody titer of mice induced by the inactivated rabies vaccine stored at a temperature of 27℃ and 37℃ for two hours was not lower than the antibody titer of mice induced by vaccine stored at a temperature of 2-8 ℃. Antibody titer increase in pre-vaccination to the 4th week post-vaccination. Observations of the spleen organ showed that there was an immune response to antigen exposure indicated by the presence of a germinal center in the secondary follicle.

SUGGESTIONS

The study demonstrates short-term thermotolerance of inactivated rabies vaccine. To strengthen the effect size and significant power, a larger sample size is needed (n=9 low). The unexpectedly higher antibody titer results in P2 (27°C) group could be explored through adjuvant kinetics or delayed antigen release. To comprehensively evaluate the antibody titers formed, future studies should prioritize vaccination trials in natural reservoir species and implement extended post-vaccination serum sampling intervals to better understand antibody trends, including fluctuations relative to the protective threshold.

ACKNOWLEDGEMENT

This research was funded by Udayana University's Institute of Research and Community Service 2022 International Research Collaboration Scheme (Contract Number : B/78.598/UN14.4.A/PT.01.03/2022). We thanked Disease Investigation Center Denpasar for histology analysis.

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