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Effectiveness of Field Vaccination Against Jembrana Disease Virus in Bali Cattle in West Sulawesi, Indonesia

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ABSTRACT

Jembrana disease is an infectious disease caused by the Jembrana disease virus (JDV). This disease predominantly infects Bali cattle, thus threatening the Bali cattle population in Indonesia, especially in West Sulawesi. This study aims to assess the effectiveness of JDV vaccination by measuring the antibody response via enzyme-linked immunosorbent assay (ELISA), then carrying out a serum neutralization test (SNT) and molecular confirmation. the data was collected from 120 Bali cattle sera which had been vaccinated one, three, and six months prior to sampling. The amount of antibodies produced was analyzed by the ELISA test, while the neutralization assay of antibodies was analyzed by the SNT test in Vero cell cultures and verified by reverse transcriptase-PCR. the results indicated that at one month post-vaccination, the amount of antibodies was highest (mean optical density of 1.704 units), and then reduced at three months (1.223) and six months (0.672) post-vaccination. Seroprotective antibodies against Bali cattle at one month post-vaccination indicated 75% but reduced to 2.5% at three months post-vaccination and no longer existed at six months post-vaccination. SNT results from samples with seroprotective titters exhibited positive neutralization activity. Confirmation results utilizing the reverse transcriptase-PCR method indicated the absence of viral nucleic acid in seroprotective samples. The research findings indicate that JDV vaccination successfully elicits a humoral immune response; however, its duration of protection is constrained. To help get rid of Jembrana disease in the Bali cattle population, it is important to give booster vaccinations every 3 to 4 months and put in place integrated biosecurity measures.

Keywords: Jembrana disease virus, Bali cattle, Indonesia, vaccination, antibody

INTRODUCTION

Bali cattle (*Bos javanicus*) are one of Indonesia's native breeds of cattle that are very valuable. Its population is spread out across Indonesia, and it plays a big part in meeting the country's need for beef. According to the Directorate General of Livestock and Animal Health, there were 17,118,650 Bali cattle in Indonesia in 2021, spread out over 38 provinces. In 2022, the population of West Sulawesi Province was said to be 115,090 head, but by 2023 it had dropped to 113,251 head. This downward trend happened at the same time as an increase in sudden deaths of cattle, which many people thought were caused by infectious diseases that were affecting Bali cattle.

Jembrana disease (JD) is one of the most dangerous infectious diseases that can affect Bali cattle. It is a big problem for cattle farming in Indonesia, especially in West Sulawesi. Since the outbreak in early 2022. JD has spread quickly, especially on small farms that keep animals. The disease is caused by the Jembrana disease virus (JDV) and has a death rate of up to 20%, a recovery rate of about 26%, and a reported treatment success rate of 68% (**1**). In the

acute phase, there is a potential rise in mortality up to 71% within 1-2 weeks post-infection (**2**). The number of Bali cattle that died due to JD, based on official reports from the West Sulawesi Province Livestock Service in 2023, is supportive of this finding. There was a total mortality of 954 cattle.

Jembrana disease is an acute immunosuppressive condition that is characterized by a wide range of symptoms, including: severe fever, lethargy, anorexia, oral mucosal erosions, enlargement superficial lymph nodes, hypersalivation, nasal discharge, hemorrhagic diarrhea, pale mucous membranes, discharge from the lacrimal sac, and blood sweating, in addition to others (**3**). The clinical symptoms appear between 4-12 days after infection and remain evident for 5 to 10 days post-infection (**4**). Moreover, the JDV may remain in the blood for a period of up to two years, thus it will be easier for the virus to transmit from one cattle to another for a prolonged period of time (**5**).

The Indonesian government has stated that vaccination is the best method to halt Jembranna disease since 2023. Such a method has been shown to reduce the severity of clinical signs, and control the spread of the disease among Bali cattle. ELISA remains the most common method for detecting antibodies and monitoring immune responses following vaccination. However, there is a general deficiency in scientific reports evaluating vaccine effectiveness under field conditions. A serological survey conducted in Sumatra showed that less than 1% of Bali cattle attained post vaccination seroprotective status against JDV. In another similar survey, it was observed that 40.63% cattle were protected by seroprotecting 14 days after vaccination. However, this declined to 15.63% on day 60 (**2**).

The lack of field data on the effectiveness of JDV vaccination serves to highlight the importance of the current study, which is particularly true for regions such as West Sulawesi, which is an endemic region for the disease. The current study was conducted to determine the effectiveness of field vaccination of cattle against JDV by measuring the antibody levels in Bali cattle for a period of six months post-vaccination. the results of this study are anticipated to confer scientific validity to governmental initiatives designed to control and finally eliminate Jembrana disease in Indonesia.

MATERIALS AND METHODS

Ethical Approval

All experimental procedures were reviewed and approved by the Animal Care and Use Committee (ACUC) of the Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia (ethical approval no. 1.KEH.096.07.2024).

Population and Sample Collection

The study population consisted of all Bali cattle that had received a complete JDV vaccination in West Sulawesi Province. Samples were collected using purposive sampling based on the criterion that the sera originated from Bali cattle with a complete JDV vaccination status. The number of samples was determined using Slovin's formula with a 10% margin of error. Blood samples were obtained via the jugular vein, and sera were separated from clots by centrifugation and stored in a freezer at -80°C (Esco Lifesciences, Singapore) until ELISA testing was performed. Positive and negative control sera prepared by Maros Veterinary Laboratory (BBVet Maros) were used to act as reference standards in an ELISA test

Serum Collection and ELISA Assay

A total of 120 sera were collected from Bali cattle and were pooled into three different groups based on the post-vaccination period: 1 month (A), 3 months (B), and 6 months (C). The sera were heat-inactivated at 56°C for 30 minutes. Test and negative control sera were diluted 1:100, whereas positive control sera were diluted at 1:100, 1:200, and 1:400 in 5% skim milk. Antibody titers were determined by ELISA according to Bagenda (**8**).

The recombinant Jgag-6-histidine protein coated the plates at 50ng/50µL in carbonate-bicarbonate buffer incubated overnight at 4°C. the plates were washed three times with phosphate buffered saline containing Tween 20 (PBST). Five percent skim milk in PBST was used to block the plates for one hour at room temperature and then cleansed. Fifty microliters of serum samples and controls were added into the wells incubated at 30°C for an hour and then rinsed. Added 50µL of the anti-bovine IgG HRP conjugate Sigma, diluted 1:1000 to each well, let it sit at 37°C for one h, after which washed it. HRP substrate Biorad was added and the reaction stopped with 2% oxalic acid after 2 min. An ELISA reader (Biosan®) was used to detect optical density (OD) at 405 nm. The cut-off value for each ELISA assay was established by calculating the mean OD plus two standard deviations (2 SD) of normal/negative control samples; those exhibiting an OD exceeding the cut-off were deemed positive (**9**). Seropositivity cut-offs were determined following Ardiawan (**4**). Seropositivity is divided into weak and strong seropositivity. The OD of the negative control serum and the OD of the maximum dilution (1:400) of the positive control are used to set the seropositivity cut-off. Based on the OD values, antibody responses were classified into three categories: Seronegative: OD ≤ 0.521; Weak seropositive: OD 0.522–1.161; Strong seropositive (seroprotective): OD ≥ 1.162. Vaccination is considered successful when strong seropositive (seroprotective) titers were induced (**10**).

Serum Neutralization Test (SNT)

Vero cells were grown in 24-well microplates in Dulbecco's Modified Eagle Medium and incubated at 37°C in the presence of 5% CO_2 until the cells reached 80% confluency. Serum samples were inactivated by heat at 56°C for 30 min and then diluted in a series of 1:32, 1:64, 1:128, and 1:256 in media with 1% bovine albumin and penicillin-streptomycin. The diluted serum and antigen were mixed in equal proportions (1:1) to ensure uniform reaction condition, let them sit for an hour at 37°C with 5%

CO_2, and then added them to Vero cells that were already growing. Plates were incubated for 5 days at 37°C with 5% CO_2, and cytopathic effects (CPE) were monitored every 12 h (**11**).

RNA Extraction and Reverse Transcription-PCR

Viral RNA was extracted from 140 µL of clarified cell culture supernatant obtained from JDV-infected Vero cells using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and eluted in 60 µL of nuclease-free water. The extracted viral RNA was reverse transcribed into complementary DNA (cDNA) using the GoScript™ Reverse Transcription System (Promega, USA) in a separate reaction following the manufacturer's protocol. The synthesized cDNA served as the template for conventional Reverse Transcription Polymerase Chain Reaction (RT-PCR) to amplify the JDV-specific gene fragment (360 bp) using a thermal cycler (Bio-Rad Laboratories, USA) and the primers JDV1 (5′-GCAGCGGAGGTGGCAATTTTAATAGGA-3′) and JDV3 (5′-CGGCGTGGTGGTCCAAAAACTG-3′) (**12**). To validate the accuracy of the RT-PCR process, a negative control and a positive control were also added to each reaction. To create the negative control (K-), nuclease-free water was used instead of the viral genome, for the positive control (K+), a sample of JDV was added. The PCR amplification was conducted in a total reaction volume of 20 µL, comprising 10 µL of 2× PCR master mix, 0.8 µL of forward primer, 3 µL of cDNA template, and nuclease-free water. The conditions for cycling included an initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 20 sec, annealing at 55°C for 45 sec, and extension at 72°C for 5 min. Agarose gel from Bio-Rad Laboratories, USA, was used in electrophoresis at 1.5%, 100 V, for 30 min, and then visualized using the Gel Doc™ EZ Imager from Bio-Rad Laboratories, USA. A 360 bp band showed that JDV was present (**13**).

Statistical Analysis

The data were statistically evaluated and the results were compared based on the antibody titer data, which were evaluated employing the Welch ANOVA test with Games-Howell test statistics when there were significant differences. All the evaluations were carried out with the help of SPSS software (version 26.0; IBM Corporation, Armonk, NY, USA). The serum neutralization test (SNT) that showed negative cytopathic effects (CPE) in cell culture also needed to be confirmed by RT-PCR results imply that the virus had already been neutralized by the JDV vaccine. In addition, categorical data related to seropositive and seronegative propositions were analyzed using the Chi-square (χ^2) test.

RESULTS

The results of the analysis using the Welch ANOVA test showed a significant difference in antibody titers between observation periods ($P < 0.05$). Further Games–Howell tests showed that the mean antibody titer at 1 month post-vaccination (1.347 ± 0.240) was significantly different compared to 3 months (0.758 ± 0.227) and 6 months (0.386 ± 0.146) (**Table 1**). Antibody titer values decreased significantly with increasing time post-vaccination.

Table 1. Antibody titer of Bali cattle against JDV vaccine at different post-vaccination time points

Post Vaccination JD	N	Mean±SD	Max	Min	SE
A (1 Month)	40	1.347 ± 0.240[a]	1,704	0,845	0.038
B (3 Months)	40	0.758 ± 0.227[b]	1,223	0,387	0.036
C (6 Months)	40	0.386 ± 0.146[c]	0,672	0,109	0.041

Mean (± SD) antibody titers of Bali cattle at different post-JDV vaccination periods. Different superscript letters in the same column indicate significant differences between groups according to the Games–Howell post-hoc test ($p < 0.05$). Mean values ± SD indicate the mean and standard deviation of OD of antibodies measured by ELISA at 1, 3, and 6 months post-vaccination

Antibody titers were classified according to OD values: seronegative (≤ 0.521), weakly seropositive (0.522–1.161), and strongly seropositive (≥ 1.162). The distribution of antibody titer categories (**Table 2**) showed that at 1month post-vaccination, 75% of the cattle had strong seropositive titers (≥ 1.162), and 25% had weak seropositive titers (0.522–1.161). At this time, none of the animals were seronegative.

Table 2. Distribution of Antibody Titers in Vaccinated Bali Cattle Based on Antibody Titer Categories

		Antibody Titer (OD, Category)						
		Seronegative (≤0,521)		Seropositive Weak (0,522-1,161)		Seropositive Strong (≥1.162)		seroprotective
Post-Vaccination	Samples	n	%	n	%	n	%	%
A (1 month) [a]	40	0	0	10	25	30	75	75
B (3 months) [b]	40	8	20	31	77,5	1	2,5	2,5
C (6 months) [c]	40	30	75	10	25	0	0	0

At 3 months, the proportion of strong seropositive animals decreased sharply to 2.5%, with the majority (77.5%) classified as weak seropositive and 20% becoming seronegative. At 6 months post-vaccination, none of the animals retained seroprotective titers; 75% were seronegative and 25% weak seropositive. This pattern shows that immunity drops a lot between 3 and 6 months after vaccination (**Figure 1)**

Table 2 Shows how the antibody titer groups are spread out among Bali cattle that have been vaccinated at different times following immunization. The χ^2 test shows that there is a significant difference between those values with different superscript letters in each column. This indicates values that are different from each other. The data showed a highly significant difference in the distribution of antibody response between the groups ($\chi^2 = 111.59$, df = 4,

$P < 0.001$). A month post-vaccination, most of the cattle had strong seropositive antibody titers, meaning 75% had seroprotective response. But as time went on, the levels of antibodies declined a lot. This is seen from the substantial increase in the frequency of seronegative and weak seropositive categories at subsequent sample site.

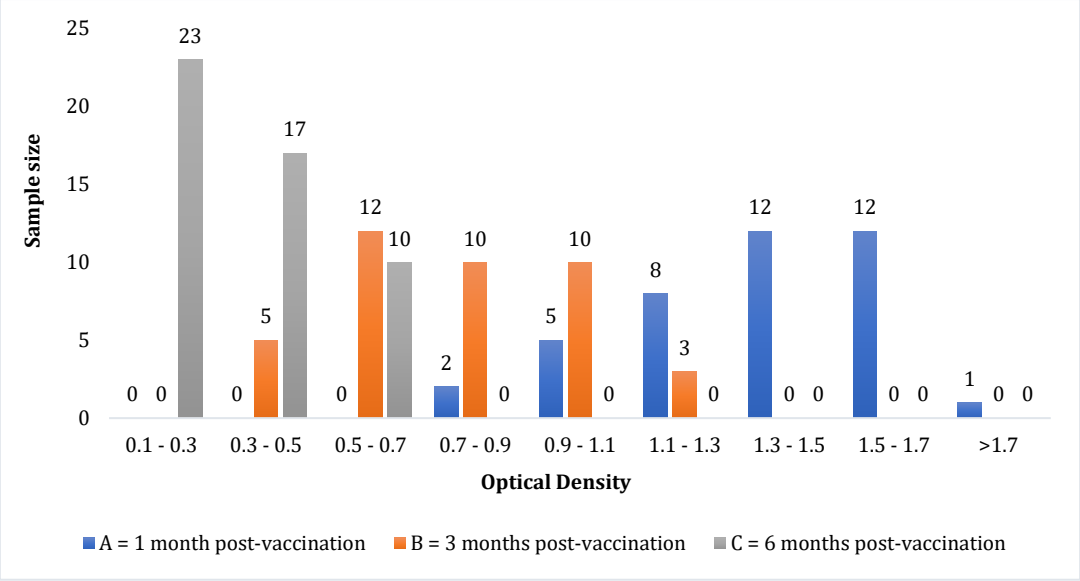
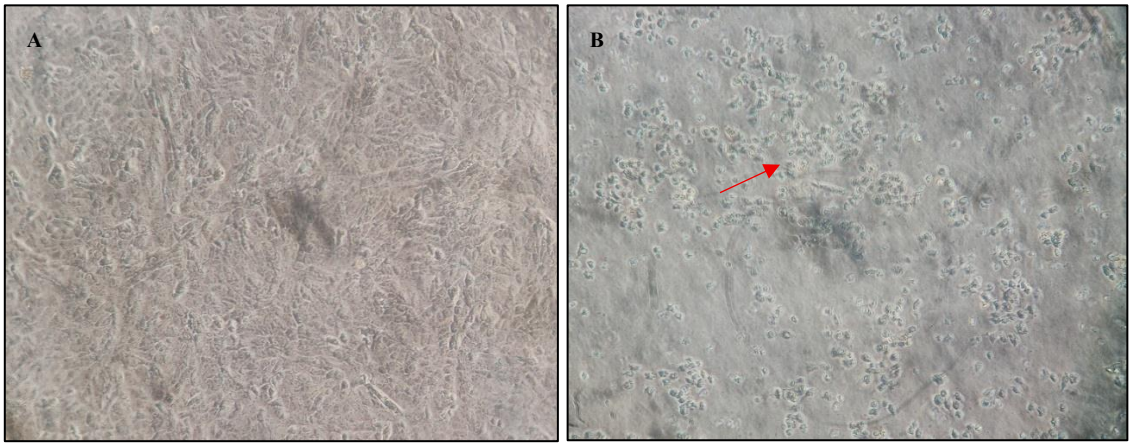


Figure 1. The dynamics of antibody titers in Bali cattle following veccination with Jembrana Disease Vaccine. The dynamics were monitored by performing an ELISA 1 month (A), 3 months (B), and 6 months (C) following vaccination. The data indicate a temporal shift in response distribution. A strong peak in seropositivity was detected 1 month following vaccination, manifested by hidh antibody titers in blue-colored bars. A clear decline was recorded 3 months following vaccination, as indicated by orange-colored bars. The lowest values were recorded 6 months following vaccination, suggesting a gradual reduction in humoral response.



Figur 2. Serum neutralization test results on Vero cell culture. A. Vero cell culture showing no cytopathogenic effect (CPE) indicating a positive SNT result (neutralization). B. Positive control demonstrating CPE on Vero cells subsequent to JDV infection. In Vero cell, the cytopathic effects (CPE) were cell rounding, shrinking, deteching from the culture surface, losing the integrity of the monolayer, and finally cell lysis. This proved that the virus could get inside the cells

The serum neutralization test (SNT) on Vero cells yielded two primary conditions (**Figure 2**). Cell cultures that exhibited no cytopathogenic effect (CPE) demonstrated positive SNT results for Jembrana virus protective antibodies (**Figure 2A**). Conversely, cell cultures in the positive control showed morphological changes in the form of cell clustering, shrinkage, and lysis, indicating a positive CPE due to Jembrana virus infection (**Figure 2B**).

The SNT results at three sampling times (1, 3, and 6 months post-vaccination) showed that the percentage of neutralizing antibodies went down as the serum dilution went up (**Figure 3**). At a serum dilution of 1:32, the antibody neutralization rate was 95% one month after vaccination, 80% three months later, and 50% six months later. In this serum dilution at 1:256, the percentage of neutralizing antibodies fell to 80%, 50%, and 20% at the time of sampling. This research indicates that the resistance against viruses deteriorates with time, although there is still considerable amount of antibodies up to six months after immunization.

On one, three, and six months post-immunization, we measured the concentration of neutralizing antibodies to JDV. These concentrations are defined as the highest serum dilutions that can neutralize the viruses. Indeed, as it can be observed from the experiment, the neutralizing effect naturally dwindled gradually as the dilutions become higher and as it progresses in time, indicating that the amount of the antibodies naturally dwindled gradually. This indicates that it is possible to detect some amount of antibodies a few months after immunization, but they are less efficient in the long run.

The results showed that the majority of the serum samples obtained one month after vaccination had high titers of neutralizing antibodies. This was considerably reduced three months later, and after six months, the reduction was more pronounced. This therefore tends to explain the remarkable reduction in the titers of neutralizing antibodies with time. The antibody titer obtained through the ELISA method supported this result, describing a correlation between the humoral immune response and the neutralizing efficiency of antibodies present in serum.

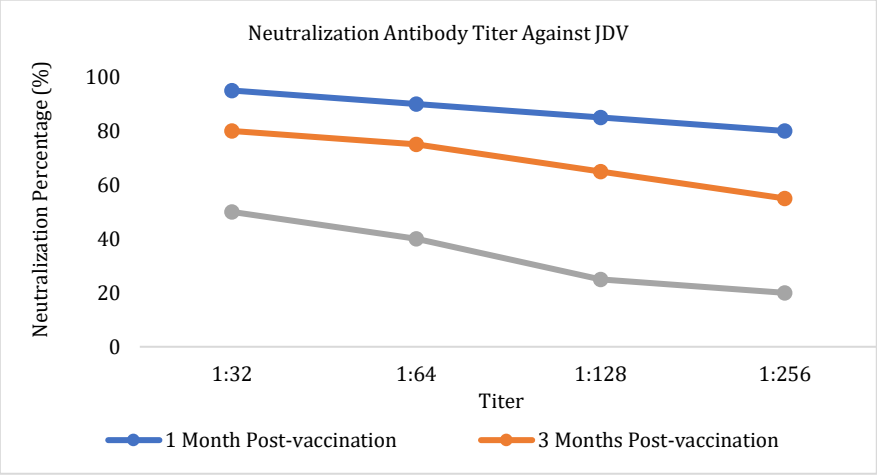


Figure 3. Percentage of neutralizing antibodies against JDV at various serum dilutions with different sampling times (1, 3, and 6 months post-vaccination). Neutralizing antibody activity decreased with increasing serum dilution, indicating a decrease in antibody activity over time

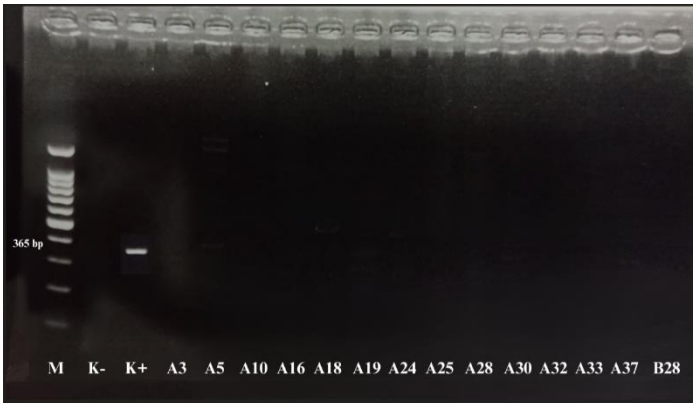


Figure 4. Amplification of the JDV gene (365 bp) by reverse transcription-PCR visualized on a 1.5% agarose gel. Lane M: 100bp DNA marker; K-: no-template negative control; K+: JDV-positive control exhibiting the expected 365 bp amplicon; lanes A3-B28: samples that protect against disease. The positive control amplified effectively, but the negative control did not. This means that the PCR and sample loading were done correctly. The lack of JDV-specific bands in seroprotective samples indicates authentic PCR-negative results rather than loading failure

Confirmation of seroprotective serum samples previously evaluated by SNT using RT-PCR showed a DNA band at 365bp, which is the same as the JDV target. On the electrophoresis gel (**Figure 4**), the positive control (K+) showed a clear band at 365 bp, while the negative control (K-) showed no amplification. A few samples (A3, A10, A16, A18, A24, A25, A28, A30, A32, A33, and A37) had smeared bands, but they weren't at the right place for JDV. Because of this, the results were thought to be JDV negative. Conversely, samples A5, A19, and B28 did not show

amplification at the JDV target position and were therefore also considered JDV negative.

DISCUSSION

A significant decrease in JDV antibody titers 1 to 6 months after vaccination of Bali cattle indicates that the humoral immune response induced by JDV vaccination is not long-lasting. One month following immunization, the humoral immune response was the strongest, with a seroprotective titer percentage of 75%. The humoral immune response, on the other hand, went down a lot after three months and was gone totally after six months. This means that boosters are necessary to keep the immune response running for a long time.

This decrease may be due to the natural decrease in antibodies when there is no more antigenic stimulation. All the above results are in line with the findings of Unsunnidhal (**14**) that the titers of the antibodies to the vaccine begin to decrease around three months after vaccination and may become completely depleted without the need for a booster dose. Similarly, other studies have proved that the immunity brought about by the JDV vaccine has been found to be temporary and therefore the need for booster doses to ensure continued seroprotecting (**15**).

The transition from robust to diminished seropositivity corresponds to the immune response to inactivated vaccines characterized by an initial peak a short period following vaccination course will, therefore, be necessary, especially in endemic areas, to prevent deterioration of herd immunity. Regular serological testing can also be used as one of the criteria for determining the optimal booster interval (**16,17**). These results emphasize the need to integrate vaccination campaigns with disease surveillance through serological and molecular testing to enhance efforts toward the control and elimination of JD in Indonesia.

Serum neutralization testing is considered the gold standard for determining the presence of an immune response to viral infection (**18**). The findings of this present study reveal that antibodies generated following vaccination indeed provide a high degree of protection within the first month, as indicated by a total absence of CPE in the Vero cell cultures. However, by the third month, the levels of neutralizing antibodies had waned and become undetectable at six months. This indicates that the protective immunity was gradually diminishing over time course (**12**).

This reduction in the ability to be neutralized corresponds to the graphical representation of the antibody titer, as can be seen in the ELISA results. Several similar studies have shown that the protective immune response drops three to six months after vaccination for both viral diseases in ruminants and other infectious diseases (19). The length of immunity can be affected by a number of things, such as the half-life of antibodies, how well booster shots work, and how the host's immune system responds (**20**).

The implications of this study underscore the need to regularly assess protective antibody levels to schedule a booster vaccination schedule. Further, as the SNT process detects a verification of action and the ELISA process determines total antibodies, the implication of this study on the validation of antibody titer detected by both processes receive increased credibility (**21**).

The non-detection of 365bp in the seroprotective samples during RT-PCR also supports the result of the SNT showing the presence of antibody titers indicating protection against JDV. This proves that there was no genetic material of JDV present in this sample because in early or subclinical infections, RT-PCR is a highly sensitive and specific method to detect viruses (**22**). Since SNT measures neutralizing antibodies, which may not always indicate the presence of an active virus, using RT-PCR as a confirmatory test is crucial (**23**).

The above pattern of observed bands also indicates that some of the animals that were found to possess protectively immune antibody levels, as indicated by SNT, were found to lack evidence of infection as determined by RT-PCR. This could be due to various factors related to immune responses, timing, and possibly a small amount of viral genomic material that might exist in a specimen that is below the detection limit of RT_PCR (**24**). Moreover, serological and genomic diagnostic methods for JDV would increase its accuracy in a field situation, especially in a vaccination monitoring evaluation scenario.

Jembrana disease virus vaccination successfully induces a humoral immune response in Bali cattle, with peak titers at 1 month post-vaccination and lower titers at both 3 and 6 months post-vaccination. The decrease in Jembrana virus-specific antibodies over time was also demonstrated through SNT assay. RT-PCR assay showed that seroprotective vaccination is possible. The results suggest that vaccine effectiveness also depends on time. A booster vaccination done at around 3-4 months post-vaccination is advised for maintenance of vaccine-induced high titers of neutralizing antibodies and/or serum antibodies. This is in line with Jembrana disease control schemes.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Investigation: D.U.F., M.M., F.A.R., N.S.S.; Formal Analysis: D.U.F.; Writing – Original Draft: D.U.F.; Writing – Review & Editing: N.S.S. All authors have read and approved the final version of the manuscript.

Artificial Intelligent Declaration

The authors declare that they are responsible for the accuracy and integrity of all content of the manuscript, including part generated by AI, and it is not used as a co-author.

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