Assessing Immunogenicity of Local Dogs in Bali, Indonesia after Oral Rabies Vaccination with the SPBN GASGAS Vaccine Strain Using ELISA, RFFIT, and Serum Nuetralization Test

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Abstract

Rabies is an acute zoonotic disease that has spread throughout the world including in Bali, Indonesia. The dog population dominated by free roaming dogs is one of the main problems in achieving 70% vaccination coverage. Oral rabies vaccination (orv) is considered to be a promising alternative to increase vaccination coverage in these dogs. This field study assessed immunogenicity in local dogs in Bali after oral administration of rabies virus vaccine strain SPBN GASGAS using Enzyme Linked Immunosorbent Assay (ELISA), Rapid Fluorescent Focus Inhibition Test (RFFIT), and Serum Neutralization Test. The total of 40 dogs were bled 5 days prior to vaccination and the serum was tested using ELISA BioPro to ensure the dogs were seronegative. Then the dogs received the oral rabies vaccine either by being offered an egg-flavored bait that contained a vaccine-loaded sachet (n=10) or by direct oral administration (n=10). Another groups of dogs received a parenteral inactivated rabies vaccine (n=10) and the last group is unvaccinated control group (n=10). The dogs were bleed 30 days after vaccination and the humoral immune response was tested using ELISA BioPro, RFFIT, and Serum Neutralization Test. The seroconversion of group of dogs tested by ELISA were : bait: 90%; direct-oral: 90%; parenteral: 100%; control: 0%. The seroconversion of group of dogs tested by RFFIT were : bait: 90%; direct-oral: 100%; parenteral: 100%; control: 10%. The seroconversion of group of dogs tested by serum neutralization were : bait: 90%; direct-oral: 90%; parenteral: 100%; control: 0%. Based on statistical analysis, the parenteral vaccine had a slightly higher humoral immune response than the oral vaccine, but the level of rabies Virus Neutralizing Antibodies (rVNA) and rabies Virus Binding Antibodies (rVBA) were still detectable in most animals for all treatment groups and resulted in no significant difference in seropositivity. This study confirms that SPBN GASGAS induces a sustained detectable immune response comparable to a parenteral vaccine under field conditions in Bali, Indonesia.

Keywords: *immunogenicity; oral rabies vaccine (orv); SPBN GASGAS; ELISA; RFFIT; Serum neutralization*

1. Introduction

Rabies is one of the acute zoonotic diseases with 100% case fatality rate (CFR) and has spread throughout the world including Indonesia. Around 90% of human rabies cases are transmitted by dogs or cats, because these animals most often come into contact with humans. A mass vaccination program with a coverage of 70% has proven effective in eradicating rabies transmitted by dogs in Indonesia, but the current method is considered incapable of achieving this target due to the lack of awareness of dog owners, vaccination failure due to improper storage, the difficulty of maintaining the cold chain, especially in remote and out-of-reach areas, limited rabies vaccine stocks, and spesifically the difficulty of handling and vaccinating free-roaming dog which is the dominant dog population especially in Bali [1,2,3].

Oral rabies vaccines are considered successful in creating high vaccination coverage in free-roaming dog in several countries in Europe, Philippines, Thailand, India and Haiti in field studies. Oral rabies vaccines work by containing the rabies vaccine (usually a vaccine from a weakened or modified live virus) in a plastic sachet that is coated with an attractant. When an animal bites into the bait, it punctures the blister pack and the vaccine fluid is released and contacts tissues in the oral cavity and tonsils, and triggering an immune response [4]. The live virus in the oral vaccine must replicate first before it can induce immunity in vaccinated dogs. This replication will induce humoral immunity that lasts longer and is also an advantage of the oral vaccine compared to the parenteral vaccine [5]. Oral rabies vaccines provide more options to reach inaccessible dogs that play a key role in disease transmission. Oral rabies vaccines in several countries have made a major contribution to achieving high vaccination coverage, especially in free-roaming dog populations, as well as maintaining vaccination coverage in the overall dog population [6]. The combination of the use of parenteral vaccine and oral rabies vaccines for free roaming dogs is proposed to be tested at the field level in Indonesia, especially in Bali Island which is an endemic area for rabies. Studies related to the efficacy of oral rabies vaccine are expected to provide benefits for the development of oral rabies vaccine in Indonesia [3].

Oral rabies vaccine strain SPBN GASGAS is a highly attenuated third-generation oral vaccine which is genetically modified by site-directed mutagenesis and greatly weakened but remains a live virus that can replicate. The glycoprotein's amino acid sites 194 and 333 had all three nucleotides transformed. The glycoprotein's genetic change at amino acid position 333 renders the construct no longer harmful in adult mice following intracerebral inoculation. Mutagenesis at amino acid position 194 inhibits a potential reversal to virulence [7]. Furthermore, the construct contains a second identical glycoprotein gene that has been modified as previously disclosed. It was hypothesized that overexpressing the rabies virus glycoprotein will improve both its efficacy and safety profile by decreasing the danger of virulence reversion and increasing apoptosis [8]. Efficacy studies have shown that SPBN GASGAS meets the requirements of the European Pharmacopoeia monograph No. 0746/2014 and is able to induce a strong rabbies-specific immune response as measured by both ELISA and RFFIT at levels comparable to parenteral vaccination with Bayovac*R. 04/09/24 [9,10].

The most efficient and most frequently used method to assess the success of the oral rabies vaccine is to measure the post-vaccination antibody response in target animals. According to WHO and OIE recommendations, immunogenicity should be assessed using at least one of the serological tests. Recommended methods include the Rapid Fluorescent Focus Inhibition Test (RFFIT) method or the Fluorescent Antibody Virus Neutralization Test (FAVN test) method [11]. In addition, the Enzyme-Linked Immunosorbent Assays (ELISA) method is also often used in immunogenicity studies to detect rabies antibody binding and has been shown to provide reliable results and the obtained results from this study could be compared directly with previous studies using the same ELISA[12,13]. Based on this, the method for assessing the immunogenicity of oral rabies vaccine in this study will use the RFFIT, ELISA, and serum neutralization test.

The goals of this study was to determine if oral rabies vaccine strain SPBN GASGAS is capable to induce an appropriate immune response in Bali's local dogs as well as parenteral vaccine and also to assess the best method to measure the level of antibodies against rabies virus after vaccination.

2. Materials and Methods

Study Design

A total of 40 healthy owned male dogs (more than 3 months old) were selected with the inclusion criteria for this study were that the dogs are in good health (by visual inspection) and has never received a rabies vaccination. The dogs were fed and managed by their owners as usual. A blood sample will be collected during pre-screening (B0) to confirm that all animals will be seronegative for rabies antibodies by enzyme-linked immunosorbent assay kit (BioPro Rabies ELISA, O.K. Servis BioPro, Prague -Czech Republic).

First, 10 dogs were offered an egg-flavoured vaccine bait containing SPBN GASGAS (3.0 mL, $10^{8.4}$ FFU/mL), another 10 dogs received the same dose of SPBN GASGAS by direct oral administration (d.o.a.), and 10 dogs were targeted for vaccination by the parenteral route with a commercially inactivated rabies vaccine (Rabisin, Merial, France). Ten dogs were included as a control group and did not receive any treatment. The health of the dogs was monitored once a week by visual examination during house visits.

Blood sample (B1) was collected from the dogs 30 days post-vaccination (dpv) and tested for rabies antibodies by RFFIT, ELISA, and serum neutralization test.

Diagnostic Assays

Blood samples of at least 3 mL have been collected from the extremities' big superficial veins (e.g., V. cephalica antebrachii, V. saphena). The samples were delivered to the Disease Investigation Center (DIC) in Denpasar, Bali, at ambient temperature within 72 hours. Blood samples have been utilized to make serum, which was kept at ≤-15°C until analysis. All pre-screening (B0) sera were tested for rabies binding antibodies (rVBA) in DIC Denpasar using ELISA (BioPro Rabies ELISA, Czech the nation) essentially as described [13], using positive (PC) and negative controls (NC) offered by the manufacturer and adhering to the validity parameters and characteristics stated in the kit insert. In brief, serum samples have been placed on microtiter plates coated with rabies antigen.

After removing the sera, all wells were incubated with a fixed amount of biotin-labelled rabies-specific antibody, followed by incubation of the bound antibody with peroxidase-conjugated streptavidin and, then, chromophoric detection. A percentage of blocking (PB) lower than 40% was considered negative; a PB equal to or higher than 40% was considered positive. Sera were taken on day 30 post-vaccination (B1) would also be analyzed by ELISA BioPro.

As comparison to ELISA result, sera (B1) were also tested for the presence of rabies virus-neutralizing antibodies (rVNA) using Rapid Fluorescent Focus Inhibition Test (RFFIT) in DIC Bukittinggi. The RFFIT technique was performed by following the procedure described by the WOAH [2]. Briefy, the positive control (0.5 IU/ml) and various dilutions of serum known positive and negative were incubated for 24 h in the presence of the challenge virus standard (CVS) 11 strain suspension infecting baby hamster kidney (BHK)-21cells. Tey were grown in Dulbecco's Modifed Eagle's Medium (DMEM) (Termo Scientifc, USA) supplemented with 10% fetal bovine serum (FBS) (Termo Scientifc, USA), antibiotic and antifungal using Gibco Antibiotic-Antimycotic (Termo Scientifc, USA), and incubated in an incubator at 37°C with 50% CO_2 atmosphere. After 24 hours of incubation, the cells were washed and fxed and then incubated with fuorescein isothiocyanate (FITC)-conjugated antirabies monoclonal antibody (Fujirebio, Japan). The serum titers were expressed in IU/ml (international units per milliliter) by determining the last dilution of serum which inhibited 50% of the initial fuorescent foci. Sera were considered seropositive for rVNAs if titers were >0.5 IU/ml.

Additionally, sera (B1) were also tested for the presence of rabies virus-neutralizing antibodies (rVNAs) using Serum Neutralization Test in National Veterinary Drug Assay Laboratory (BBPMSOH), Bogor. According to the WOAH standard, 2023, serum will be tested using CVS-11 as a challenge virus with a virus titer of 200 TCID50, and neuroblastoma N2A cells. The titer value >4 is considered positive [14].

Statistical Analysis

Statistical analysis was first carried out by testing normality using the Kolmogorov-Smirnoff Test and Case Processing Summary Test, then continued with Chi-Square Tests and Crosstabs for each antibody measurement method (RFFIT, Serum Neutralization Test, and ELISA). The independent variable was the seropositivity value. While the dependent variable was the treatment given, namely oral vaccine administration through bait (ORV), direct oral vaccine administration (d.o.a), and parenteral vaccine administration. The effectiveness of each variable was then tested with MANOVA which was compared to the control to determine the difference in results between the three vaccine administrations.

Ethical Approval

This study used experimental animals. All procedures performed have met animal ethics standards that have been approved by the Airlangga University Animal Care and Use Committee (ACUC) with certificate number : 1.KEH.054.04.2024.

Study Period and Location

All the dogs were selected from local areas of the Bali Province, namely Nongan village, Karangasem District (20 dogs), representing rural areas, and Banyuning village, Buleleng District (20 dogs), representing urban areas. The study was conducted in April 2022 – August 2024.

3. Results

This immunogenicity study used 40 dogs from Karangasem and Buleleng, Bali. All animals were confirmed to be seronegative by ELISA BioPro examination on five days before the study (B0). The cut-off for the ELISA BioPro used a percent blocking of 40%. All dogs were then blooded again 30 days after treatment (B1) to examine post-vaccination antibody titers using three methods; ELISA, RFFIT, and serum neutralization test. The results of the ELISA, RFFIT, and serum neutralization tests can be seen in Table 1 where in 10 dogs that were given rabies vaccine via bait (ORV) showed that 9 (90%) were seropositive and 1 of the same dog showed seronegative results in all examination methods. Slightly different results were seen in samples of dogs that received direct oral administration (d.o.a) where ELISA and neutralization serum tests showed that out of 10 samples, 9 (90%) were seropositive and 1 (10%) of the same dog showed seronegative results, while in the RFFIT test, all dogs (100%) showed seropositive results. All dogs that received parenteral vaccines showed seropositive results (100%) in ELISA, serum neutralization, and RFFIT tests. This immunogenicity study showed similarities in the results of the three antibody titer testing methods. In control dogs, two dogs died before blood was taken after vaccination. One dog died due to suspected parvo based on clinical symptoms, while the other dog was suspected of dying from rabies based on clinical symptoms and was confirmed positive for rabies from brain sample tests. According to the owner's statement, the dog had been in contact with another dog suspected rabies. Until the end of the study, all dogs in the control group remained seronegative by ELISA and serum neutralization tests. There was one control dog that was seropositive on the RFFIT examination

Table 1. ELISA, Serum Neutralization Test, and RFFIT result.

Groups	Assay		
	ELISA	Serum Neutralization	RFFIT
A (ORV with bait)	9/10	9/10	9/10
B (ORV with d.o.a)	9/10	9/10	10/10
C (Parentral vaccine)	10/10	10/10	10/10
D (Control)	0/10	0/10	1/10

* Value of positive sample on ELISA was just above cut-off 40% inhibition; * Value of positive sample on serum neutralization was ≥ 4; * Value of positive sample on RFFIT was just above 0,5 IU/ml; d.o.a = direct oral application.

All results obtained from each method of testing the immunogenicity of oral rabies vaccine (ELISA, RFFIT, and neutralization serum) were also analyzed using Chi-Square Tests to test whether there is a statistical relationship between the variables and CrossTab to describe the distribution of these variables.

Based on testing the vaccine administration method used against the ELISA, RFFIT, and serum neutralization results, it can be seen that the Asymptotic Significance value (2-sided) in the Chi-Square column is 0.00 <0.05. Based on these data, it can be seen that there are differences in results between the methods used against the ELISA, RFFIT, and serum neutralization results.

Based on the results of antibody titer measurements using the ELISA, RFFIT, and serum neutralization method, all three vaccine administration methods had positive results, with the parenteral vaccine methods having the highest success rates, while the ORV and d.o.a method had a success rate of 90% based on the samples tested.

In addition, a MANOVA test was also conducted to determine whether one or more independent variables have a significant influence on several dependent variables. The results of the MANOVA test are shown in the tables below.

Table 2. Multivariate Tests[a]

Effect		Value	F	Hypothesis df	Error df	Sig.	Noncent. Parameter	Observed Power[d]
Intercept	Pillai's Trace	.980	843.889[b]	2.000	35.000	.000	1687.778	1.000
	Wilks' Lambda	.020	843.889[b]	2.000	35.000	.000	1687.778	1.000
	Hotelling's Trace	48.222	843.889[b]	2.000	35.000	.000	1687.778	1.000
	Roy's Largest Root	48.222	843.889[b]	2.000	35.000	.000	1687.778	1.000
METHOD	Pillai's Trace	.856	8.974	6.000	72.000	.000	53.843	1.000
	Wilks' Lambda	.174	16.337[b]	6.000	70.000	.000	98.019	1.000
	Hotelling's Trace	4.593	26.025	6.000	68.000	.000	156.148	1.000
	Roy's Largest Root	4.556	54.667[c]	3.000	36.000	.000	164.000	1.000

a. Design: Intercept + METHOD

b. Exact statistic

c. The statistic is an upper bound on F that yields a lower bound on the significance level.

d. Computed using alpha = ,05

Based on the results, it shows that the method of administering the vaccine has a significant influence on the results of the titer method as a whole. This can be seen from the test significance value <0.05.

Table 3. Levene's Test of Equality of Error Variances[a]

		Levene Statistic	df1	df2	Sig.
ELISA_POST	Based on Mean	3.375	3	36	.029
	Based on Median	.667	3	36	.578
	Based on Median and with adjusted df	.667	3	18.000	.583
	Based on trimmed mean	1.760	3	36	.172
SN	Based on Mean	3.375	3	36	.029

	Based on Median	.667	3	36	.578
	Based on Median and with adjusted df	.667	3	18.000	.583
	Based on trimmed mean	1.760	3	36	.172
RFFIT	Based on Mean	3.375	3	36	.029
	Based on Median	.667	3	36	.578
	Based on Median and with adjusted df	.667	3	18.000	.583
	Based on trimmed mean	1.760	3	36	.172

Tests the null hypothesis that the error variance of the dependent variable is equal across groups. a. Design: Intercept + METHOD

Based on the overall results, the three titer tests have a significance value of 0.029 < 0.05, this shows that the three titer tests can be effectively used to identify the results of vaccine administration.

Table 4. Multiple Comparisons

Bonferroni

Dependent Variable	(I) METHOD	(J) METHOD	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	Upper Bound
ELISA_POST	Oral Vaccine with Bait	Direct Oral Administration	.0000	.10000	1.000	-.2792	.2792
		Parenteral Vaccine	.1000	.10000	1.000	-.1792	.3792
		Control	-.9000*	.10000	.000	-1.1792	-.6208
	Direct Oral Administration	Oral Vaccine with Bait	.0000	.10000	1.000	-.2792	.2792
		Parenteral Vaccine	.1000	.10000	1.000	-.1792	.3792
		Control	-.9000*	.10000	.000	-1.1792	-.6208
	Parenteral Vaccine	Oral Vaccine with Bait	-.1000	.10000	1.000	-.3792	.1792
		Direct Oral Administration	-.1000	.10000	1.000	-.3792	.1792
		Control	-1.0000*	.10000	.000	-1.2792	-.7208
	Control	Oral Vaccine with Bait	.9000*	.10000	.000	.6208	1.1792

		Direct Oral Administration	.9000*	.10000	.000	.6208	1.1792
		Parenteral Vaccine	1.0000*	.10000	.000	.7208	1.2792
SN	Oral Vaccine with Bait	Direct Oral Administration	.0000	.10000	1.000	-.2792	.2792
		Parenteral Vaccine	.1000	.10000	1.000	-.1792	.3792
		Control	-.9000*	.10000	.000	-1.1792	-.6208
	Direct Oral Administration	Oral Vaccine with Bait	.0000	.10000	1.000	-.2792	.2792
		Parenteral Vaccine	.1000	.10000	1.000	-.1792	.3792
		Control	-.9000*	.10000	.000	-1.1792	-.6208
	Parenteral Vaccine	Oral Vaccine with Bait	-.1000	.10000	1.000	-.3792	.1792
		Direct Oral Administration	-.1000	.10000	1.000	-.3792	.1792
		Control	-1.0000*	.10000	.000	-1.2792	-.7208
	Control	Oral Vaccine with Bait	.9000*	.10000	.000	.6208	1.1792
		Direct Oral Administration	.9000*	.10000	.000	.6208	1.1792
		Parenteral Vaccine	1.0000*	.10000	.000	.7208	1.2792
RFFIT	Oral Vaccine with Bait	Direct Oral Administration	.1000	.10000	1.000	-.1792	.3792
		Parenteral Vaccine	.1000	.10000	1.000	-.1792	.3792
		Control	-.8000*	.10000	.000	-1.0792	-.5208
	Direct Oral Administration	Oral Vaccine with Bait	-.1000	.10000	1.000	-.3792	.1792
		Parenteral Vaccine	.0000	.10000	1.000	-.2792	.2792
		Control	-.9000*	.10000	.000	-1.1792	-.6208
	Parenteral Vaccine	Oral Vaccine with Bait	-.1000	.10000	1.000	-.3792	.1792
		Direct Oral Administration	.0000	.10000	1.000	-.2792	.2792
		Control	-.9000*	.10000	.000	-1.1792	-.6208
	Control	Oral Vaccine with Bait	.8000*	.10000	.000	.5208	1.0792
		Direct Oral Administration	.9000*	.10000	.000	.6208	1.1792
		Parenteral Vaccine	.9000*	.10000	.000	.6208	1.1792

Based on observed means.

The error term is Mean Square(Error) = .050.

*. The mean difference is significant at the ,05 level.

Based on the results of multiple correlation data, when compared with control samples, for all titer test methods, it is known with a significance of 1,000 > 0.05 for each variable of the vaccine administration method to the control sample, it is known that the three vaccine administration methods are effective, with no similarities to the results of the control sample. In all titer test methods, the three vaccine administration methods are known to have good effectiveness in identifying antibodies in the sample.

Based on significance, for the control sample it has a significance of 0.00 <0.05 where it is known that the three methods of vaccine administration have differences with the control, while for the three methods of administration the most significant is the parenteral vaccine method with a significance of 0.750> 0.05. For the d.o.a and ORV methods with bait, they have similar results with a significance of 1,000> 0.05 which shows great significance for both methods. In addition, a Multinominal logistic statistical analysis was also carried out to determine the most effective method of vaccine administration with the results shown in the table below.

Table 5. Multinominal logistic Test

Classification

	Predicted				
Observed	Oral Vaccine with Bait	Direct Oral Administration	Parenteral Vaccine	Control	Percent Correct
Oral Vaccine with Bait	0	0	9	1	0.0%
Direct Oral Administration	0	0	9	1	0.0%
Parenteral Vaccine	0	0	10	0	100.0%
Control	0	0	0	10	100.0%
Overall Percentage	0.0%	0.0%	70.0%	30.0%	50.0%

Based on the multinominal logistic test, the largest percentage of vaccine administration methods was obtained using parenteral vaccines. However, the other two methods also showed a high success rate although not as large as parenteral vaccines. Based on all statistical analyses carried out, it can be seen that the three antibody titer measurement methods can be used to see the results of the vaccine reactions that have been given, this is known by the difference in results between samples given vaccine treatment and control samples. To determine the most optimal vaccine administration method, testing was carried out by comparing the results of the post-vaccination ELISA test, Neutralization Serum, and RFFIT with the vaccine administration method. From the results of the test, similar results were obtained where the three methods had significantly different results with the control sample, where the most effective vaccine administration method used was parenteral vaccination. The ORV and d.o.a methods can also be used in administering rabies vaccines with an error rate of 10% for each sample used.

4. Discussion

Several experimental studies in the field have been conducted on the immunogenicity of various types of oral rabies vaccines in dogs [15, 16, 17, 18, 19, 20, 21]. An experimental study in Bali using the second generation oral rabies vaccine SAG2 showed that local dogs became seropositive after consuming the bait vaccine [5]. This study is the first study to test immunogenicity in dogs in Bali. Immunological studies in local dogs (which roam freely) are considered important because the quality and/or quantity of their food is low, which may have a negative impact on the immune response [22, 23]. In addition, the presence of endoparasites and ectoparasites and other conditions that weaken immunity can cause immunosuppression [24]. Stress factors can also affect seroconversion, including the duration and level of antibodies detected after rabies vaccination [25].

As in previous studies [10], dogs vaccinated orally with the SPBN GASGAS strain in this study were shown to be able to induce a protective immune response as seen from the high number of seropositive dogs after oral vaccination both through bait and d.o.a. Efficacy studies showed that SPBN GASGAS is immunogenic in foxes and raccoons and induces humoral responses and long-term protection after being challenged with a highly virulent rabies virus, thus meeting the requirements of the European Pharmacopoeia monograph No. 0746/2014 [9].

Although the seropositive results in parenteral vaccination showed the best results (100%), the oral vaccination method (either through bait/ORV or direct oral administration) also showed very good results in this study, reaching 90%. Therefore, the use of oral rabies vaccine as a complement to parenteral vaccination in mass vaccination campaigns can be implemented in Bali in order to achieve herd immunity, especially for stray dogs or dogs that are difficult to control so that animal welfare issues can also be minimized. Although there were variations in the number of antibody titers measured in this study, how high the antibody level in each dog was not too crucial, what was more important was the status of the dog being protected from rabies infection (protective) [26].

Based on the results of antibody titer examinations using three methods, there were several animals that were seronegative after vaccination, although the number was not more than 10%. The lack of seroconversion in several animals vaccinated orally does not always indicate vaccine failure, because many factors can cause the absence of protective antibodies in animals that do not respond to vaccination [27]. It has been shown that oral vaccination induces slower antibody development compared to parenteral vaccination, but lasts longer. In a study of dogs in Thailand, not all dogs vaccinated orally developed antibodies detectable using ELISA until 4 weeks after vaccination, while all dogs vaccinated parenterally had detectable antibodies since 7 days after vaccination [5, 10]. The short time between vaccination and sampling (17-20 days post-vaccination) might be responsible for the low seroconversion rates obtained in the Haitian study [28]. The seroconversion rates observed in this investigation were higher than those observed in dogs in Haiti and Namibia who were similarly immunized with SPBN GASGAS and examined using the same ELISA BioPro assay [28, 29].

A recent immunogenicity investigation of local Thai dogs sheltered in a dog shelter and vaccinated with the same vaccine and ELISA test found that all dogs infected orally showed protective antibody levels 28 days after vaccination. The Thai dogs had been vaccinated against numerous common infectious diseases, which differed from the findings of this research. They were also dewormed at 3 months of age. These animals were fed daily with commercially available high-quality animal food and thus the dogs were in excellent condition [10]. The differences in seroconversion in several studies after oral vaccination with the same SPBN GASGAS strain vaccine were not solely influenced by the physical condition of the dogs. There are many other factors that can influence the differences in study results, such as study design, quality of blood samples collected, vaccinator skills, dog characteristics, and environmental conditions. Dogs that usually roam freely and are surrounded by a vaccination team will make the dogs anxious, which may have a negative impact on the handling and consumption of bait by the dogs, thus affecting the shedding of the virus into the oral cavity, which as previously mentioned is a prerequisite for successful vaccination [28].

The effectiveness of oral vaccination attempts is influenced not only by the quality of the vaccine and the appealing factor of the bait, but also by external factors such as how the bait is delivered to the dog. A different situation occurred in a study in Namibia, where blood samples were collected several weeks before vaccination, and most animals were not offered the bait on their own premises due to logistical reasons, but generally free-ranging dogs were brought to a collection point for vaccine administration. The Namibian study's bait acceptance level (61%) was significantly lower than in previous experiments using the same bait [29]. It is probable that differing environmental conditions and stress levels influenced individual dogs' bait acceptance. Many dogs were stressed because they were on a leash, which they were not accustomed to. They were in an unusual environment, surrounded by other dogs and humans [30]. Suboptimal circumstances are thought to impede with bait absorption. As a result, the vaccine was not fully released into the oral cavity, and no immune response occurred following bait eating. In contrast, a recent field research in Thailand found that when free-ranging dogs were presented bait directly, the majority of them happily took it and chewed it until the packaging was ruptured [31].It can be assumed that with careful management under field scenario conditions, effective bait uptake can be optimized, resulting in high post-vaccination seroconversion rates, as observed in this study in Bali.

Assessment of the presence of antibodies to the rabies virus is necessary in determining the immunity status achieved after rabies vaccination. Several serological tests have been developed to detect rabies virus neutralizing antibody (rVNA). Virus detection by RFFIT or FAVN is the gold standard [32]. Although RFFIT is known as the most reliable test to evaluate vaccination success, unfortunately both the RFFIT and serum neutralization methods are time-consuming, expensive, and require live rabies virus, sometimes, the results cannot be read due to cytotoxic effects on cells. Virus neutralization technically requires highly skilled laboratory personnel, is difficult to standardize, there are some variations between laboratories, is difficult to perform at weekly intervals, and requires special facilities that can only be performed in reference laboratories that meet high safety standards [33].

To overcome this, ELISA is worthy of being developed as an alternative to RFFIT because it is technically considered simpler, more affordable, safer, and faster than RFFIT. ELISA does not require live viruses and high containment facilities, is easy to validate, and has a guarantee of more consistent results [34]. RFFIT and ELISA have also been found to have good compatibility with each other. ELISA is very suitable for routine serological testing with a large number of samples, making it very suitable for monitoring antibody titers after rabies vaccination [33]. Oral vaccination with SPBN GASGAS either by direct administration or through bait is able to induce a strong rabies-specific immune response as measured by both ELISA and RFFIT [10]. This is in accordance with this study where based on the results of the statistical analysis carried out, it appears that the three methods of measuring antibody titers (RFFIT, serum neutralization test, and ELISA) can be used to see the results of the vaccine reaction that has been given with almost similar results in each method (90%), so that its use can be adjusted to the capabilities and conditions of each in the field.

Conclusion

This study confirms that local dogs in Bali develop an adequate immune response after a single oral vaccination with the third-generation oral rabies vaccine strain SPBN GASGAS. The oral rabies vaccine strain SPBN GASGAS in this study was shown to induce antibodies that were as protective as the parenteral vaccine. The immunogenicity of the oral rabies vaccine can be measured well using the RFFIT, ELISA, and Serum Neutralization Test methods, where the results of antibody titer measurements with the three methods in the study showed very similar results.

Rabies eradication in Bali have received international attention, but the parenteral vaccination method has so far failed to achieve adequate vaccination coverage. The use of this oral rabies vaccine is suitable for increasing vaccination coverage in Bali considering the high population of stray and free released dogs, allowing field officers to reach inaccessible dog populations while still paying attention to animal welfare.

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