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Protective antibody response in Korean raccoon dogs (*Nyctereutes procyonoide koreensis*) administered a new rabies bait vaccine containing the ERAGS-GFP strain

Dong-Kun Yang [1,*], Choong-sik Kim [2,*], Jin Kim [2], Jongsup Yeo [2], Sungsik Yoo [2], Hokeun Won [2], Ju-Yeon Lee [1], Hye Jeong Lee [1], Yun Sang Cho [1]

[1]Viral Disease Division, Animal and Plant Quarantine Agency, Ministry of Agriculture, Food and Rural Affairs, Gimcheon, Korea
[2]ChoongAng Vaccine Laboratories Co., Ltd. (CAVAC), Daejeon, Korea

Purpose: Rabies is a deadly zoonotic disease affecting many mammals, including humans. Oral rabies bait vaccines induce an immune response without direct inoculation, and are crucial for controlling rabies in wildlife. This study evaluated the safety and immunogenicity of a new rabies bait vaccine containing a recombinant rabies virus expressing green fluorescent protein (ERAGS-GFP) in wild raccoon dogs.

Materials and Methods: To confirm the safety of the ERAGS-GFP vaccine, reversion to virulence was evaluated in 1-day-old suckling mice. The uptake, minimum effective dose, and immunogenicity of the bait vaccine were assessed in raccoon dogs, as was the persistence of post-vaccine immunity. Serum rabies virus neutralizing antibody (VNA) titers were measured using fluorescent antibody virus neutralization.

Results: No adverse effects were noted in mice, guinea pigs, dogs, or raccoon dogs administered the ERAGS-GFP vaccine orally during the test period. The glycoprotein gene of the ERAGS-GFP strain remained unchanged after five reverse passages in 1-day-old mice. Uptake of the bait vaccine was 75.8% in raccoon dogs. The minimum effective dose was at least $10^{5.0}$ $TCID_{50}$/mL. Forty-three raccoon dogs administered the ERAGS-GFP bait vaccine developed an average VNA titer of 4.23 IU/mL 28 days post-administration. Protective antibody levels were maintained for 4 months.

Conclusion: The ERAGS-GFP bait vaccine showed high uptake and strong immunogenicity in raccoon dogs, and protective antibody levels were maintained for at least 4 months. These results indicate the vaccine's potential for effective rabies control in wildlife, which can reduce the risk of transmission to humans and domestic animals.

Keywords: Rabies; Bait; ERAGS-GFP; Immunologyy; Raccoon dogs

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Corresponding authors:
Dong-Kun Yang, PhD
Viral Disease Division, Animal and Plant Quarantine Agency, Ministry of Agriculture, Food and Rural Affairs, 177 Hyeoksin 8-ro, Gimcheon 39660, Korea.
Tel: +82-54-912-0785
Fax: +82-54-912-0812
Email: yangdk@korea.kr

Hokeun Won, PhD
ChoongAng Vaccine Laboratories Co., Ltd. (CAVAC), 1476-37 Yuseong-daero, Yuseong-gu, Daejeon 34055, Korea.
Tel: +82-42-863-9322
Fax: +82-42 870-0580
Email: hokeun@cavac.co.kr

*Dong-Kun Yang and Choong-sik Kim contributed equally to this work.

KVS KOREAN VACCINE SOCIETY
KOREAN SOCIETY FOR ZOONOSES

INTRODUCTION

Rabies, one of the most important zoonotic diseases in the world, threatens a large number of people every year [1]. Most human rabies cases are caused by bites from rabid animals, and it is thus 100% preventable through animal vaccination. Animals transmitting rabies to humans are mainly dogs, but a number of other animals including, raccoons, raccoon dogs, badgers, foxes, mongooses, and bats have been linked to human transmission [2]. Vaccination of wildlife is achieved through the distribution of oral bait vaccines that are designed to induce an immune response without direct inoculation.

The first generation of commercial oral rabies vaccine (ORV) strain was based upon a highly attenuated mutant of the SAG-2 strain, which was selected from the SADBern strain using anti-rabies glycoprotein monoclonal antibodies [3]. Dogs and foxes administered the SAG-2 strain orally showed effective immunity against wild rabies virus [3-5]. The second generation of ORV to be developed was a recombinant vector vaccine, in which the glycoprotein gene of the rabies virus (RABV) was inserted into vaccinia virus or human adenovirus type 5 vectors. The recombinant vaccinia-vectored RABORAL V-RG® bait vaccine (Boehringer Ingelheim, Ridgefield, CT, USA) has been distributed globally since 1987 and prevented expansion of the raccoon rabies [6]. The recombinant adenovirus-vectored Ontario rabies bait vaccine (ONRAB®; Artemis Technologies Inc., Ontario, Canada) is also reported to have effectively prevented rabies in wild animals, including raccoons and foxes [7,8]. The third generation of ORV was developed based upon highly attenuated live rabies virus strains that also contain one or more targeted genomic modifications to prevent reversion to virulence. The genetically engineered rabies vaccine strain, SPBN GASGAS (Rabitec®; CEVA Sante Animale, Libourne, France) for foxes and raccoon dogs was found to meet the efficacy requirements set by the World Organization for Animal Health (WOAH) [9].

In the Republic of Korea (ROK), dog-mediated rabies was the main cause of human rabies before 1993; however, dog-mediated human rabies has disappeared through mass rabies vaccination of dogs. Since 1993, raccoon dogs (*Nyctereutes procynoide koreensis*) migrating south from the Korean demilitarized zone have been responsible for transmitting rabies to humans and animals in the ROK [10]. From 1993 to 2023, 7 human and 486 animal rabies cases were reported in the ROK, most of which were related to wild raccoon dogs [11]. In order to block raccoon-dog-mediated rabies, Korea's veterinary authorities have been distributing the second generation of ORV that contains an attenuated ("modified-live") recombinant vaccinia virus vector expressing the rabies virus glycoprotein gene (V-RG) to rabies risk regions since 2000. This policy of distributing rabies bait vaccine has resulted in the near elimination of animal rabies in the ROK [11]. Korean raccoon dogs, however, might have learned how to eat the bait formulation without ingesting the wax-encapsulated V-RG antigen. Only 13.7% of raccoon dogs captured in the region where the bait vaccines have been distributed had rabies antibodies [12]. In addition, there are reports that two women exposed to the V-RG antigen developed chronic skin disease [13]. Therefore, a new rabies bait vaccine that is safer and can increase the level of rabies-neutralizing antibodies in raccoon dogs is needed.

Based on recombinant technology, we previously reported the generation of the recombinant rabies virus expressing green fluorescent protein (ERAGS-GFP) strain, a recombinant rabies virus expressing green fluorescent protein in which the green fluorescence protein gene was expressed and the amino acids located at positions 194 and 333 of the rabies glycoprotein were replaced [14]. In this study, the safety of the ERAGS-GFP strain was investigated in mice, guinea pigs, dogs, and raccoon dogs. The ERAGS-GFP strain was back passaged five times in suckling mice to evaluate reversion to virulence. In addition, the uptake and immunogenicity, of the ERAGS-GFP bait vaccine and the duration of protective immunity were evaluated in raccoon dogs.

MATERIALS AND METHODS

Virus and cells

The ERAGS-GFP strain of the RABV, which was generated in 2021 using a reverse genetic system, was used for the rabies bait vaccine [14]. The ERAGS-GFP strain deposited in the Korean Veterinary Culture Collection (accession number: KVCC-VR1900060) was propagated in Vero cells (ATCC, Manassas, VA, USA). The CVS-11 strain was propagated in BHK-21 cells (ATCC) and used for rabies virus neutralizing antibody (VNA) tests. The Vero and BHK-21 cells were regularly maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum and an antibiotic and antimycotic solution.

Safety and reversion to virulence

All animal experiments involving mice and raccoon dogs were performed with the approval of the Institutional Animal Care and Use Committees at ChoongAng Vaccine Laboratories Co., Ltd (approval numbers 200511-09). To evaluate the safety of the ERAGS-GFP strain, 4-week-old mice (n=10),

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2-month-old guinea pigs (n=10), 3-month-old dogs (n=10), and 4-month-old raccoon dogs (n=10) were prepared. Dogs and raccoon dogs used for safety assessments were not vaccinated against rabies. The four species of animals were inoculated intramuscularly with the viral equivalent of 10 doses of the ERAGS-GFP bait vaccine and observed for 21 days. To assess reversion to virulence of the ERAGS-GFP strain, 0.03 mL of the virus was inoculated intracranially into 1-day-old and 4-week-old mice, and the brain tissue was collected after 5 days of observation. Back passaging was performed 5 times under the same conditions using a 10% mouse brain suspension as the inoculum. Reversion to pathogenicity of the back-passaged ERAGS-GFP strain was assessed by comparing the expression of the glycoprotein genes of ERAGS-GFP after each passage in the suckling mice (1 to 5P) was compared with that of the Evelyn-Rokitnicki-Abelseth (ERA) strain.

Determination of minimum effective dose

To determine the minimum effective dose of the ERAGS-GFP strain required to formulate the bait vaccine, three bait vaccines containing $10^{5.0}$ to $10^{7.0}$ 50% tissue culture infectious dose ($TCID_{50}$)/mL were prepared. Eleven raccoon dogs were divided into four groups. Three raccoon dogs in 3 groups were administered each bait vaccine, and two were used as controls. Blood from all raccoon dogs used in the test was collected before and 4 weeks after administration, and a rabies VNA test was performed.

Manufacturing of the rabies bait vaccine

The ERAGS-GFP strain was produced in Vero cells and prepared at a viral titer of $10^{7.5}$ $TCID_{50}$/mL or higher. The ERAGS-GFP strain, stabilizer, and additive solution were blended at a ratio of 25:25:50 and dispensed into a polyethylene film tube. The bait formulation consisted of feed, starch, gelatin, and flavor. The filled antigen bag was inserted into a molded bait formulation to manufacture the ERAGS-GFP bait vaccine, and the bait vaccine was stored at –20°C (**Fig. 1**).

Vaccine uptake

The assessment of the rabies bait vaccine uptake was conducted at three wild animal rescue centers. In total, 57 raccoon dogs were divided into 2 groups. After fasting for 1 day before the test, the ERAGS-GFP bait vaccine was supplied at 5 times the single vaccine dose. One group of 43 animals was supplied with the newly formulated ERAGS-GFP bait vaccine, and the other group of 14 animals was supplied with a commercial V-RG bait vaccine, and the uptake (%) was evaluated 1 day later.

Immunogenicity and sustainability

Assessment of the immunogenicity of the ERAGS-GFP bait vaccine was also conducted at three wild animal rescue centers. A total of 68 raccoon dogs were divided into three groups. The first group of 43 animals received the ERAGS-GFP bait vaccine, the second group of 14 received the V-RG bait vaccine, and the remaining 11 animals comprised the control group. Blood samples were collected before administration and 4 weeks later for use in rabies VNA testing. The duration of immunity after ERAGS-GFP bait vaccine administration was assessed in 13 raccoon dogs that had undergone an immunogenicity because the raccoon dogs recovered at the wild animal rescue center had to be returned into the wild: 10 animals were administered the ERAGS-GFP bait vaccine, 2 animals were administered V-RG bait vaccine, and the remaining animal was the control. The clinical signs of the raccoon dogs were observed for 4 months, and blood was collected every 4 weeks to measure rabies VNA titers.

VNA titer determination

The VNA titer was determined using a fluorescent antibody virus neutralization test with a positive reference serum at 0.5 IU/mL as the control [15]. Serum samples and controls were serially diluted in wells, followed by the addition of RABV of the CVS-11 strain and BHK-21 cells. After incubation at 37°C for 72 hours, the microplates were fixed in cold acetone, washed with phosphate buffer solution (pH 7.0), and reacted with a monoclonal antibody (Median Diagnostics, Chuncheon, Korea) against RABV. They were then stained with fluorescence-isothiocyanate-conjugated goat anti-mouse IgG+IgM (KPL Laboratories, Gaithersburg, MD, USA), air-dried, and examined under a fluorescence microscope. The VNA titers were determined by comparing the test results with the positive standard.

Fig. 1. Formulation of the ERAGS-GFP bait vaccine: approximately 20 g of the ERAGS-GFP bait vaccine is sealed within a polyethylene film sachet and is placed inside a rectangular block (27 mm × 27 mm × 12 mm).
ERAGS-GFP, recombinant rabies virus expressing green fluorescent protein.

Statistical analysis

All values were expressed as mean ± standard deviation. All statistical tests were performed using GraphPad Prism Software version 5.0 for Windows (GraphPad Software Inc., San Diego, CA, USA; www.graphpad.com). Statistical significance of the differences between the means of 2 groups was assessed with a one-tailed paired t-test. Statistical significance was defined as $p<0.05$.

RESULTS

Safety of the ERAGS-GFP strain

To determine the safety of the ERAGS-GFP strain, mice, guinea pigs, dogs, and raccoon dogs were inoculated intramuscularly with a viral titer of $10^{8.0}$ $TCID_{50}$/mL, which is equivalent to 10 doses of the ERAGS-GFP bait vaccine, and observed for 21 days. The 4 kinds of animals inoculated with the ERAGS-GFP strain did not show any clinical signs of rabies, including neurological changes, salivation, abnormal behavior, diarrhea, or anorexia (**Table 1**).

Reversion of the ERAGS-GFP strain to a pathogenic phenotype was assessed after intracerebral inoculation of 1-day-old suckling mice. The mice survived for 5 days after inoculation. The brain tissue of the surviving mice was collected and the brain tissues were re-inoculated into the brain of new 1-day-old suckling mice, which also survived for 5 days. The ERAGS-GFP strain was detected in suckling mice brains using reverse transcription-polymerase chain reaction (PCR), and the viral titer increased slightly from $10^{4.1}$ to $10^{5.5}$ $TCID_{50}$/mL after back passaging (**Table 2**). Comparison of the glycoprotein genes of the ERA, ERAGS-GFP, and ERAGS-GFP-P5 strain back-passaged in suckling mice revealed that amino acids related to pathogenicity at positions 194 and 333 were not mutated in the ERAGS-GFP-P5 (**Fig. 2**).

Determination of minimum effective dose

As a result of administering three bait vaccines formulated with different RABV strain to animals, the raccoon dogs that consumed the ERAGS-GFP bait vaccine containing a viral titer of $10^{5.0}$ $TCID_{50}$/mL developed mean VNA titers of 6.1 IU/mL, which exceeded the protective antibody titer of 0.5 IU/mL (**Fig. 3**). Therefore, the minimum effective dose of the ERAGS-GFP bait vaccine was determined to be more than $10^{5.0}$ $TCID_{50}$/mL per one dose.

Uptake

In accordance with national rabies control guidelines, and after considering the average density of raccoon dogs in

Table 1. Safety of the ERAGS-GFP vaccine in mice, guinea pigs, dogs, and raccoon dogs

Animal species	Number of animals	Route/dose	Clinical signs observed over 21 days post-administration				
			Neurologic	Salivation	Behavior	Diarrhea	Anorexia
Mice	10	IM/10 doses[a]	0/10	0/10	0/10	0/10	0/10
Guinea pigs	10		0/10	0/10	0/10	0/10	0/10
Dogs	10		0/10	0/10	0/10	0/10	0/10
Raccoon dogs	10		0/10	0/10	0/10	0/10	0/10
Mice	5	No treatment	0/5	0/5	0/5	0/5	0/5
Guinea pigs	5		0/5	0/5	0/5	0/5	0/5
Dogs	5		0/5	0/5	0/5	0/5	0/5
Raccoon dogs	5		0/5	0/5	0/5	0/5	0/5

ERAGS-GFP, recombinant rabies virus expressing green fluorescent protein; IM, intramuscular injection; $TCID_{50}$, 50% tissue culture infectious dose.
[a]$10^{8.0}$ $TCID_{50}$/mL.

Table 2. Reversion to virulence of the ERAGS-GFP strain in mice

Group	No. of mice		Route/dose ($TCID_{50}$/mL)	Clinical signs		Detection of ERAGS-GFP		Virus titer ($TCID_{50}$/mL)	
	Suckling	4W		Suckling	4W	Suckling	4W	Suckling	4W
ERAGS-GFP	10	10	IC/$10^{6.0}$	–[a]	–	+[b]	+	$10^{4.1}$	$10^{4.3}$
P1	10	10	IC/0.03 mL	–	–	+	+	$10^{4.3}$	$10^{4.3}$
P2	10	10		–	–	+	+	$10^{4.3}$	$10^{4.5}$
P3	10	10		–	–	+	+	$10^{4.9}$	$10^{4.7}$
P4	10	10		–	–	+	+	$10^{5.3}$	$10^{5.5}$
P5	10	10	IC/0.03 mL	–	–	+	+	$10^{5.5}$	$10^{5.5}$
	10	10[d]		–[c]	–	–	–	-	-

ERAGS-GFP, recombinant rabies virus expressing green fluorescent protein; $TCID_{50}$, 50% tissue culture infectious dose; P1, back passage 1; IC, intracerebral injection.
[a]Survived without any clinical signs, [b]Positive against ERAGS-GFP, [c]Negative, [d]Observation for 21 days.

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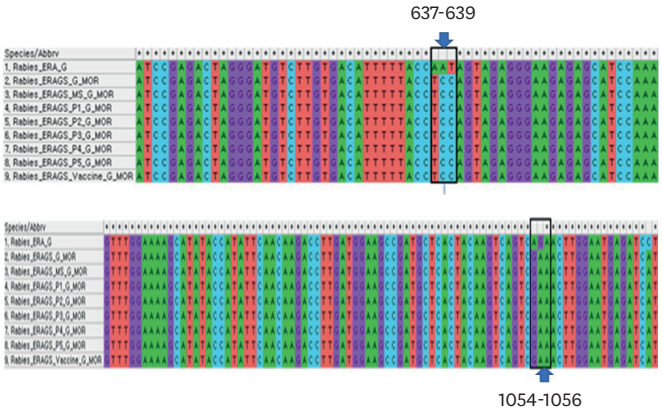

Fig. 2. Identification of the nucleotide sequence of the rabies virus G gene of the ERAGS-GFP strain that has undergone 5 back passages in mice. The nucleotide sequences (TCC, 637-639, GAA, 1054-1056) corresponding to 2 amino acids of the G gene associated with the pathogenicity of the rabies virus remained unchanged even after 5 back passages.
G, glycoprotein; ERAGS-GFP, recombinant rabies virus expressing green fluorescent protein.

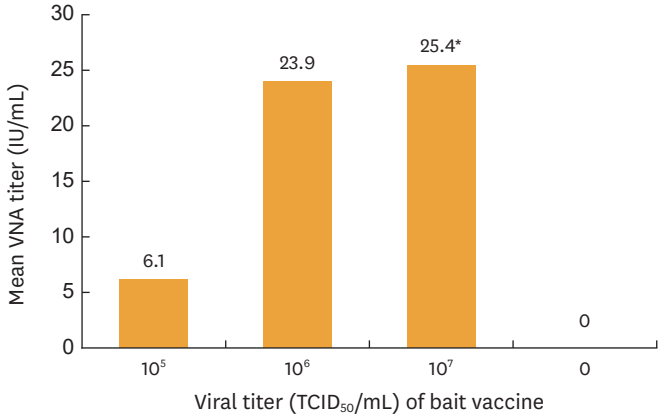

Fig. 3. Determination of the minimum effective dose of the ERAGS-GFP strain in polyethylene film bag.
ERAGS-GFP, recombinant rabies virus expressing green fluorescent protein; VNA, virus neutralizing antibody; $TCID_{50}$, 50% tissue culture infectious dose.
*$p<0.05$.

the ROK, the appropriate distribution density of rabies bait vaccine was determined to be 12 baits/km^2. Because assessment of the uptake and immunogenicity of the rabies bait vaccine for raccoon dogs was conducted at wild animal rescue centers, bait vaccines equivalent to 5 times the number of animals were supplied to raccoon dogs. Two types of rabies bait vaccines, ERAGS-GFP and V-RG bait vaccine, were supplied to 43 and 14 raccoon dogs, respectively, and the uptakes were 75.8% (163/215) and 61.4% (43/70), respectively (**Fig. 4**).

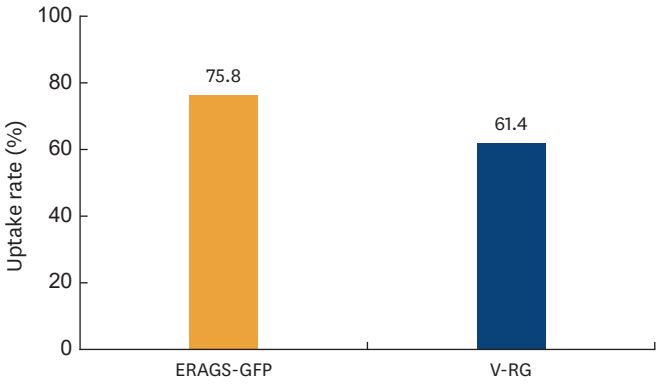

Fig. 4. Comparison of the uptake rate of the two types of rabies bait vaccines administered to raccoon dogs.
ERAGS-GFP, recombinant rabies virus expressing green fluorescent protein; V-RG, rabies virus glycoprotein gene.

Immunogenicity and sustainability

The immunogenicity of the bait vaccines was assessed 28 days after inoculation. The 43 raccoon dogs administered the ERAGS-GFP bait vaccine developed mean RABV VNA titers of 4.23 IU/mL, while the titer of the 14 raccoon dogs administered the V-RG bait vaccine was 1.96 IU/mL, and that of the 11 control raccoon was negative (0.07 IU/mL; **Fig. 5A**). None of the vaccinated or unvaccinated raccoon dogs exhibited clinical signs of rabies during the experiment. The duration of protective immunity after vaccination was assessed in 10 of the above-noted 43 raccoon dogs that were administered the ERAGS-GFP bait vaccine and 2 that were administered the V-RG bait vaccine: 4 months after vaccination, the mean VNA titers were 5.8 and 5.5 IU/mL, respectively (**Fig. 5B**).

DISCUSSION

Oral rabies vaccination programs have been highly effective in stopping or reducing the circulation of rabies in wild animals [16]. The RABV strain used in ORV must be proven safe in target and non-target animals. All warm-blooded animals are susceptible to the RABV, and humans or aircraft can deliver the ORVs directly or indirectly to target animals, such as foxes or raccoon dogs. When the ORVs as a preventive measure are distributed into rabies risk regions, only foxes or raccoon dogs do not consume the ORV. Therefore, the safety of rabies bait vaccines is essential for both target and non-target animals. The safety of the SAG-2 bait vaccine was proven in target animals such as foxes and raccoon dogs and non-target animals such as wild carnivores and rodent species [17,18]. To demonstrate the safety of the AdRG1.3 ONRAB

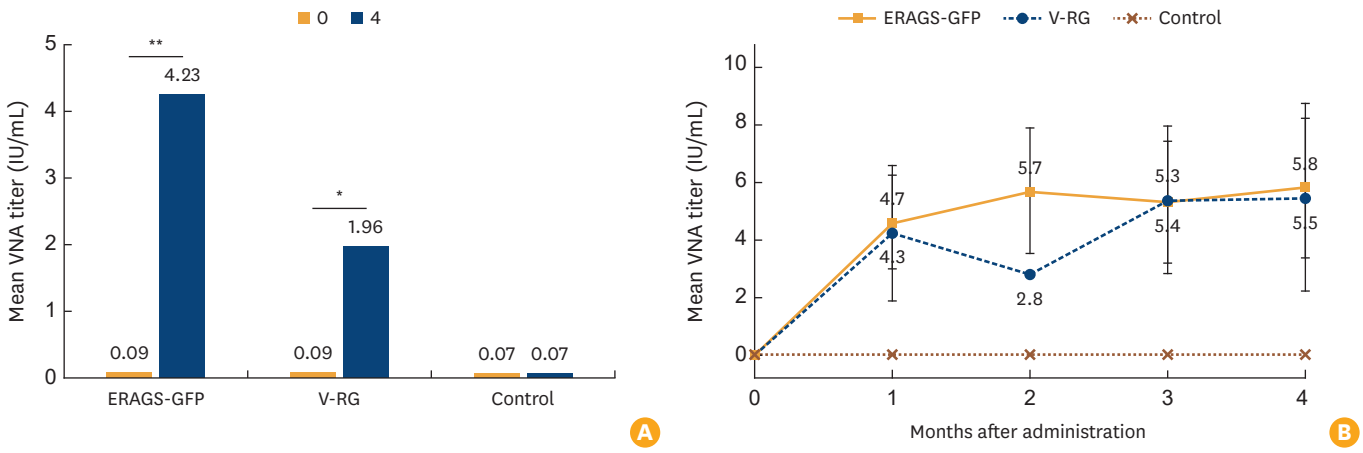

Fig. 5. Immunogenicity (A) and duration of immunity (B) after administration of two types of the rabies bait vaccine to raccoon dogs.
*p<0.02, **p<0.05.

strain, high doses of the ONRAB strain were administered orally directly to three types of rabies vector animals in Canada, such as red fox, raccoon, and striped skunk, and to laboratory animals, and no clinical signs were observed [19]. The safety of a recent oral rabies bait vaccine, SPBN GASGAS strain, was also demonstrated through excretion in feces after oral administration to 8 different target and non-target species: 7 days after administration of the vaccine, viral RNA was detected in 6.5% (50/758) of fecal samples, but no infectious virus was identified in the PCR-positive fecal samples [20]. In our study, the safety of the ERAGS-GFP strain was demonstrated by intramuscular inoculation of in mice, guinea pigs, dogs, and raccoon dogs with 10 doses ($10^{8.0}$ $TCID_{50}$/mL) of the ERAGS-GFP strain. Additionally, the ERAGS-GFP strain did not exhibit pathogenicity even after five passages in 1-day-old suckling mice, indicating that the non-pathogenicity of the ERAGS-GFP strain may be due to the substitution of arginine for glutamic acid at amino acid 333 of the glycoprotein associated with pathogenicity [21,22]. No mutations occurred in the glycoprotein gene of the ERAGS-GFP strain that was back-passaged five times in mice, suggesting that the ERAGS-GFP strain is unlikely to recover pathogenicity in a field environment. Therefore, the safety of the ERAGS-GFP strain has been established as a basis for use as a new oral rabies bait vaccine strain.

An ORV consists of a bait that attracts the target animal and a polyvinyl pack containing the rabies vaccine strain. These components of the ORV may affect the efficacy of the rabies bait vaccine. Therefore, the minimum effective dose in the vinyl pack and the percent uptake of the bait vaccine should be evaluated in target animals. The current ORVs using the SAG-2, V-RG, AdRG1.3, or SPBN GASGAS strains contain more than $10^{8.0}$ $TCID_{50}$ per sachet [10,13,17,23]. In this study, raccoon dogs administered the ERAGS-GFP bait vaccine containing $10^{5.0}$ $TCID_{50}$/mL developed mean VNA titers of 6.1 IU/mL, indicating that the vaccine can protect target animals. Nevertheless, considering losses during the production process and long-term stability of the bait vaccine, the minimum effective dose required for producing the ERAGS-GFP bait vaccine was determined to be $10^{7.0}$ $TCID_{50}$/mL or higher.

Another factor that affects the efficacy of the ORV is the uptake of the bait vaccine. The bait must be attractive enough for the target animal to consume, as the delivery of rabies immunity depends on whether the raccoon dogs puncture the polyvinyl pack inside the bait vaccine [24]. Because each species of carnivore has different food preferences, the development of new ORV requires evaluating vaccine uptake in target animals [25]. The uptake of commercial ORV varies depending on the applied animal, region, and survey period. The uptake of the V-RG and SAG-2 bait vaccines in raccoons were reported to be 79.3% and 59%–100%, respectively [10,26]. Our results demonstrated that the uptake of the ERAGS-GFP bait vaccine by raccoon dogs was 75.8% (163/215), similar to that of the SAG-2 bait vaccine.

Because the vector animal species that transmit rabies vary depending on the region, the immunogenicity of an ORV should be evaluated in the target animals of interest. Raccoon dogs administrated the SAG-2 bait vaccine developed VNA titers of 6.59–19.9 IU/mL 60 days after vaccination [7]. In areas where V-RG and ONRAB bait vaccines were distributed, 38% and 51%, respectively, of resident raccoons showed RABV-positive antibodies [27]. Our results demonstrated that raccoon dogs administered the ERAGS-GFP bait vaccine developed mean VNA titers of 4.23 IU/mL 4 weeks after administration. However, raccoon dogs administrated V-RG vaccine had a lower VNA titer of 1.96 IU/mL than those administrated the ERAGS-GFP bait vaccine. This may be

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due to differences in uptake rate between raccoon dogs, Nevertheless, raccoon dogs administrated 2 types of ORV exceeded the protective VNA titer of 0.5 IU/mL. This indicates that raccoon dogs that have taken up the ERAGS-GFP bait vaccine are protected from challenge by virulent RABV. The World Health Organization and WOAH have a goal to eliminate dog-mediated human rabies by 2030. However, we did not assess the immunogenicity of the ERAGS-GFP bait vaccine in dogs. Therefore, further study of dogs administered the ERAGS-GFP bait vaccine is needed.

In conclusion, the ERAGS-GFP bait vaccine strain was safe in suckling mice, guinea pigs, dogs, and raccoon dogs. Raccoon dogs administered the ERAGS-GFP bait vaccine had a high uptake rate of 75.8% and developed high rabies VNA titers and maintained immunity for 4 months. Therefore, the ERAGS-GFP bait vaccine is expected to contribute to eliminating animal rabies in raccoon dogs.

ORCID iDs
Dong-Kun Yang
https://orcid.org/0000-0001-5765-3043
Choong-sik Kim
https://orcid.org/0009-0000-3029-4300
Jin Kim
https://orcid.org/0009-0002-8159-3022
Jongsup Yeo
https://orcid.org/0009-0008-9649-3255
Sungsik Yoo
https://orcid.org/0009-0002-7359-6717
Hokeun Won
https://orcid.org/0000-0003-4568-4429
Ju-Yeon Lee
https://orcid.org/0000-0001-6390-8268
Hye Jeong Lee
https://orcid.org/0000-0003-2044-6176
Yun Sang Cho
https://orcid.org/0000-0003-1346-7067

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Conflict of Interest
No potential conflict of interest relevant to this article was reported.

Author Contributions
Conceptualization: Yang DK; Data curation: Yang DK; Formal analysis: Yang DK; Funding acquisition: Yang DK, Won H, Cho YS; Investigation: Yang DK; Methodology: Kim CS, Kim J, Yeo J, Yoo S, Lee JY, Lee HJ; Project administration: Yang DK, Won H; Software: Yang DK, Lee HJ; Validation: Won H, Cho YS; Writing - original draft: Yang DK; Writing - review & editing: Yang DK, Kim CS, Yoo S, Won H, Lee JY, Cho YS.

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