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Generation of a recombinant rabies virus expressing green fluorescent protein for a virus neutralization antibody assay

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ABSTRACT

Background: Fluorescent antibody virus neutralization (FAVN) test is a standard assay for quantifying rabies virus-neutralizing antibody (VNA) in serum. However, a safer rabies virus (RABV) should be used in the FAVN assay. There is a need for a new method that is economical and time-saving by eliminating the immunostaining step.

Objectives: We aimed to improve the traditional FAVN method by rescuing and characterizing a new recombinant RABV expressing green fluorescent protein (GFP).

Methods: A new recombinant RABV expressing GFP designated as ERAGS-GFP was rescued using a reverse genetic system. Immuno-fluorescence assay, peroxidase-linked assay, electron microscopy and reverse transcription polymerase chain reaction were performed to confirm the recombinant ERAGS-GFP virus as a RABV expressing the GFP gene. The safety of ERAGS-GFP was evaluated in 4-week-old mice. The rabies VNA titers were measured and compared with conventional FAVN and FAVN-GFP tests using VERO cells.

Results: The virus propagated in VERO cells was confirmed as RABV expressing GFP. The ERAGS-GFP showed the highest titer ($10^{8.0}$ TCID₅₀/mL) in VERO cells at 5 days post-inoculation, and GFP expression persisted until passage 30. The body weight of 4-week-old mice inoculated intracranially with ERAGS-GFP continued to increase and the survival rate was 100%. In 62 dog sera, the FAVN-GFP result was significantly correlated with that of conventional FAVN ($r = 0.95$).

Conclusions: We constructed ERAGS-GFP, which could replace the challenge virus standard-11 strain used in FAVN test.

Keywords: Rabies; green fluorescent protein; virus neutralizing antibody; FAVN

INTRODUCTION

Rabies is one of the most lethal viral diseases of human and animals and is transmitted mainly via bites from infected animals. After the onset of clinical symptoms of rabies, the mortality rate approaches 100% [1]. Rabies virus-neutralizing antibodies (VNAs) block the transport of rabies virus (RABV) from the bite site to the brain. When a person is bitten by a suspected animal, the person receives hyperimmune sera treatment and vaccination for the purpose of supplying and inducing rabies VNA [2]. Vaccination is a useful and economical

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Conflict of Interest

The authors declare no conflicts of interest.

Author Contributions

Conceptualization: Yang DK; Data curation: Yang DK, Kim HH, Yoo JY; Funding acquisition: Hyun BH; Methodology: Kim HH, Park Y, Yoo JY, Park J; Project administration: Yang DK; Resources: Yang DK; Supervision: Yang DK; Validation: Kim HH; Writing - original draft: Yang DK; Writing - review & editing: Yang DK, Kim HH, Park YR, Hyun BH.

means of inducing rabies VNA in human and animals and elicits a long-term memory response. Vaccinated animals with VNA titers > 0.5 IU/mL are protected from wild RABV [3]. Monitoring of the rabies VNA titer is required to determine the rabies immune status of animals in rabies-risk regions or those being transported internationally [4].

Rabies can be controlled by vaccinating over 70% of animals living in rabies-risk areas. South Korea has maintained an animal rabies non-occurrence status by mass vaccination of domestic animals such as cattle and pet animals and distribution of a rabies bait vaccine for raccoon dogs since 2013 [5]. The importation of non-vaccinated or rabies-infected animals from other countries could remove the rabies non-occurrence status. The World Organization of Animal Health (OIE) provides strict guidelines for importing and exporting animals [6]. Quarantine authorities in many countries require dog owners to submit a rabies VNA certificate. Such certificates are issued by certified laboratories based on fluorescent antibody virus neutralization (FAVN) or rapid fluorescent focus inhibition test.

Although the FAVN test is useful for detecting RABV antibodies, it is costly and labor intensive, requiring fixed RABV, challenge virus standard (CVS)-11 strain, BHK-21 cells, cold acetone, rabies monoclonal antibody, and anti-mouse fluorescein isothiocyanate (FITC) conjugate [7]. Also, rabies CVS-11 poses a risk to the laboratory staff; for instance, if rabies-infected cells detach from a microplate during washing. Also, the anti-mouse FITC-conjugate imported from overseas can be suddenly difficult to import for several reasons. Recombinant RABV expressing green fluorescent protein (GFP) eliminates the need for an immunostaining step, simplifying FAVN test.

GFP is used to detect expression of proteins and for virus rescue [8]. Several recombinant RABVs expressing GFP have been generated by reverse genetics using the HEP-Flury, CVS-11, RC-HL, CVS-N2c, and Evelyn-Rokitnicki-Abelseth (ERA) strains [9-14]. Although these recombinant RABV had similar growth characteristics to the parent virus, the level of GFP expression varied with the genomic location of the GFP gene. Recombinant RABVs expressing GFP were detected in infected cells from 24 to 48 post-inoculation [12]. It is known that the pathogenicity of RABV is related to the 333th amino acid of the RABV glycoprotein (G). Due to the difference in the amino acid sequence of G protein of the recombinant RABV, there is a difference in pathogenicity even in the recombinant RABVs. Therefore, a safer RABV expressing GFP is required for FAVN test.

We generated and characterized a recombinant RABV expressing GFP, ERAGS-GFP, and evaluated its safety in mice. We also established a FAVN-GFP method, and compared the rabies VNA titers of serum samples determined by the FAVN and FAVN-GFP methods.

MATERIALS AND METHODS

Cell lines and viruses

BHK/T7-9 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 5% heat-inactivated fetal bovine serum (FBS), antibiotics (100 IU/mL penicillin, 10 µg/mL streptomycin), and antimycotic (0.25 µg/mL amphotericin B). The maintenance medium contains 3% FBS. BHK/T7-9 cells were used to generate recombinant RABV. BHK-21 (ATCC CCL-10) and VERO (ATCC CCL-81) cells grown in DMEM supplemented with 10% FBS were used for the propagation of RABV and for rabies VNA assay. The ERA and ERAGS, which

have mutations at positions 194 and 333 within the RABV G protein of the ERA strain, were used as comparators for safety testing of ERAGS-GFP. The CVS-11, a fixed RABV, was used as neutralizing virus in conventional FAVN test.

Sampling area and samples

Sixty-two serum samples were collected from dogs residing in Chungbuk and Gyeongbuk Provinces from 2016 to 2018. Dogs were randomly selected so as not to affect the test results. The serum samples were inactivated at 56°C for 30 min prior to use and subjected to 2 serological assays. When and where the dogs received the rabies vaccine were unknown.

Generation of ERAGS-GFP

The full ERAGS-GFP plasmid was generated by inserting the GFP gene between the G and RNA polymerase (large protein [L]) genes of ERAGS (**Fig. 1**). The open reading frame of GFP encompassed a transcription initiation start position (AACA) and polyadenylation signal (AAAAACA) based on the phosphoprotein of the ERA strain. Recovery of recombinant ERAGS-GFP virus was carried out as described previously [15]. Briefly, BHK-T7 cells constitutively expressing T7 polymerase were grown on a 24-well plate in DMEM. The medium was replaced with fresh Opti-MEM medium. The cells were transfected with pERAGS-GFP and 3 helper plasmids designated as pT7IRES-EN, pT7IRES-EP, and pT7IRES-EL using X-treme GENE HP DNA transfection reagent (Roche Diagnostics, GmbH, Germany) according to the manufacturer's instructions. After incubation for 2 days in a 5% CO₂ incubator, Opti-MEM was replaced with DMEM, followed by incubation at 37°C for 4 days. The cell culture was examined under fluorescence microscope for GFP fluorescence. Cell supernatant containing ERAGS-GFP was collected at 5 days post-transfection. ERAGS-GFP stock was prepared by infecting VERO cells with the recovered virus, aliquoted, and stored at -70°C.

Immunofluorescence assay and peroxidase-linked assay (PLA)

ERAGS-GFP was propagated in VERO cells and infected cells were fixed in 80% chilled acetone for 15 min. The cells were reacted with a monoclonal antibody (Animal and Plant Quarantine Agency, Korea) specific for the N protein of RABV for 45 min and then with a FITC-conjugated goat anti-mouse immunoglobulin G (IgG) + IgM (KPL, USA). After washing, the cells were visualized by fluorescence microscopy.

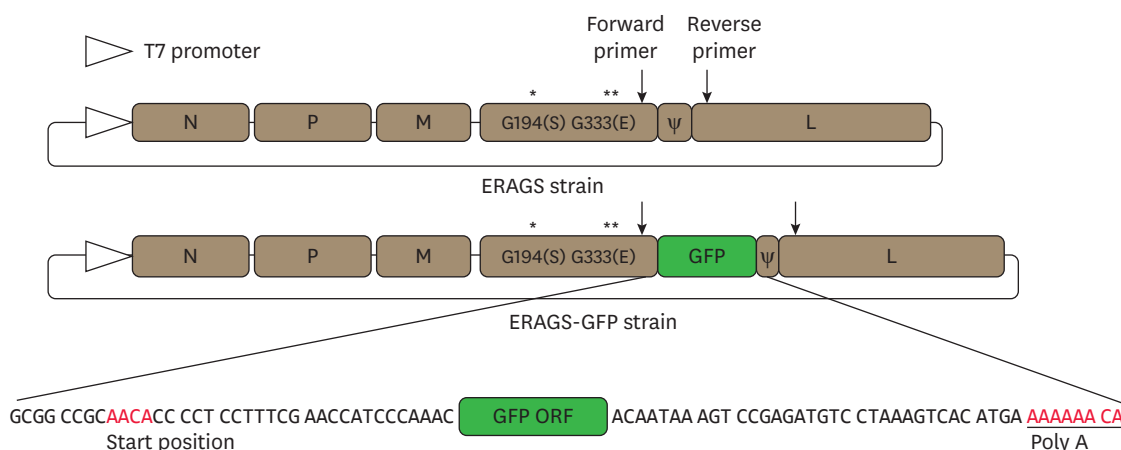


Fig. 1. Full-length plasmid map of ERAGS-GFP. The full-length complementary DNA plasmid contained the GFP gene between the G and L genes. Arrows indicate primer sites.

GFP, green fluorescent protein; G, glycoprotein; L, large protein.

*Asparagine at position 194 is replaced with serine. **Arginine at position 333 of G protein is substituted with glutamic acid.

For the PLA, VERO cells infected with ERAGS-GFP were fixed with acetone. After reaction with RABV monoclonal antibodies, the VERO cells were stained with the secondary biotinylated anti-mouse IgG in VECTASTAIN ABC kit (Vector Labs, USA) and avidin-biotin complex solution was added for 1 h. Next, 3,3'-diaminobenzidine substrate was added and the wells were washed with phosphate-buffered saline (PBS, pH 7.2). Finally, the cells were visualized by microscopy.

Reverse transcription polymerase chain reaction (RT-PCR) and sequencing

RNA from ERAGS and ERAGS-GFP was extracted using an RNA Extraction Kit (Bioneer, Korea) according to the manufacturer's instructions. A 1 step RT-PCR Premix Kit (Bioneer) was used to amplify the GFP gene with the primer listed in **Table 1**. The reaction mixture (50 μ L) comprised 5 μ L of denatured RNA, 1 μ L of forward and reverse primers (10 pmol), and 43 μ L of distilled water. The reaction profile was complementary DNA synthesis at 50°C for 30 min, followed by 35 cycles of denaturation-annealing-extension (95°C for 45 sec, 50°C for 45 sec, and 72°C for 45 sec), and a final extension at 72°C for 10 min. RT-PCR products were photo-documented in 2.0% agarose gels containing nucleic acid staining solution of RedSafe Nucleic Acid Staining Solution (iNtRON Biotechnology, Korea). The 2 purified RT-PCR products were ligated into the pGEM-T Easy Vector System (Promega, USA) and submitted to Macrogen (Korea) for sequencing. The sequencing results confirmed that the plasmid contained the GFP gene.

Electron microscope for the confirmation of RABV

VERO cells grown in a 25 cm² flask were infected with ERAGS and ERAGS-GFP and the cells were harvested 4 days after infection using a rubber policeman. After centrifuging the cells at 200 \times g for 10 min, 2.5% glutaraldehyde in PBS was used to fix the cell pellets at 4°C for 2 h. After dehydration in a graded series of ethanol and propylene oxide, the cells were embedded into spur resin. Ultrathin sections were stained with uranyl acetate and lead citrate, and virus particles in cytoplasm were visualized by electron microscopy (Hitachi 7100; Hitachi, Japan).

Growth kinetics and titration of passaged viruses

ERAGS and ERAGS-GFP (1,000 TCID₅₀/mL) were inoculated into BHK-21 and VERO cells grown in 25 cm² flasks and harvested daily for 8 days. Growth kinetics was performed as follows. After 3 consecutive freeze-thaw cycles, viral titers were determined and expressed as TCID₅₀/mL. The 2 viruses were serially passaged 30 times in VERO cells; viruses from each passage were stored at -70°C. The viral titration was carried out after every 5th serial passage, i.e, 0, 5, 10, 15, 20, 25, and 30.

Safety of ERAGS-GFP in mice

The Ethics Committee (Animal and Plant Quarantine Agency) approved the animal trials (approval No. 2019-453). Twenty-four 4-week-old BALB/c mice were divided into 4 groups. Groups 1, 2, and 3 were inoculated via intracranial route with ERA, ERAGS, and ERAGS-GFP (10^{7.0} TCID₅₀/mL). While the group 4 was kept as control. Each mouse was inoculated with 30 μ L of virus and the mice were examined for 16 days post-inoculation for the development of central nervous

Table 1. Primers for amplification and sequencing of the GFP gene and part of the G and L genes

Designation	Oligonucleotide	Expected size (bp)		Remark
		ERAGS	ERAGS-GFP	
RABVF	AGTCAATCGATCAGAACC	755	1,420	G
RABVR	AGAGTTGAGATTGTAGTC			L

GFP, green fluorescent protein; G, glycoprotein; L, large protein.

system symptoms and any abnormalities. The body weights of the mice were also measured after inoculation and their survival rates were recorded for 16 days after the inoculation.

Conventional FAVN and FAVN-GFP test

The conventional FAVN test was carried out using the standard protocol of OIE. Briefly, 50 μL of serum and reference positive (0.5 IU/mL) and negative controls (Agency for Food, Environmental and Occupational Health & Safety, France), were distributed in 4 consecutive wells of a 96-well microplate and serially diluted in 3-fold steps. Next, RABV CVS-11 (100 TCID₅₀/50 μL) was added to each well and the plate was incubated for 1 h at 37°C. Next, 50 μL of a BHK-21 cell suspension were added to each well (final density 4×10^5 cells/mL) and the plates were incubated for 48 h in a humidified incubator with 5% CO₂ at 37°C. The cells were fixed in 100 μL of 80% cold acetone for 30 min. After washing, the cells were reacted with a monoclonal antibody (Animal and Plant Quarantine Agency) against RABV N protein for 45 min at 37°C and stained with FITC-conjugated reagent. Following a wash in PBS, the microplates were examined under fluorescence microscope at magnification of 200 \times . The VNA titers of the serum samples were expressed in IU/mL by comparing the results to those of the positive standard.

FAVN-GFP test was carried out similarly to FAVN test except that ERAGS-GFP and VERO cells replaced CVS-11 and BHK-21 cells. Fixing cells in cold acetone and staining with rabies antibody and FITC-conjugate were excluded. The FAVN-GFP titers of serum samples were measured as above.

Statistical analysis

The 2-tailed paired *t*-test was used to assess the significance of differences between conventional FAVN and FAVN-GFP tests. A *p* value < 0.05 was considered indicative of significance. Simple linear regression analyses were conducted to assess the correlations of FAVN and FAVN-GFP tests. Statistical analysis and visualization were performed in GraphPad Prism software (version 8.4.2; GraphPad Software, USA).

RESULTS

Identification of ERAGS-GFP

ERAGS-GFP was generated by reverse genetics as described previously with slight modifications [15]. After transfection with the full-length genome containing the GFP gene and 3 helper plasmids (EN, EP, and EL), recombinant RABV (ERAGS-GFP) was rescued in BHK/T7-9 cells. The fluorescence of VERO cells inoculated with ERAGS-GFP were continuously observed from 24 h after inoculation (**Fig. 2A**) and were evidently expressing GFP. VERO cells inoculated with ERAGS-GFP were fixed with cold acetone at 4 days post-inoculation and stained with a specific RABV antibody. RABV-specific fluorescence was visible in the cytoplasm of VERO cells infected with ERAGS-GFP, indicating the presence of RABV (**Fig. 2C**); this was confirmed by the results of PLA (**Fig. 2E**).

The GFP gene of ERAGS-GFP was amplified by RT-PCR using primers specific for RABV. The PCR products of ERAGS and ERAGS-GFP were of 1,420 and 755 bp (**Fig. 3**), confirming the presence of the GFP gene in ERAGS-GFP.

To visualize virus particles, ERAGS and ERAGS-GFP were inoculated into VERO cells, which were sectioned and visualized by transmission electron microscopy. ERAGS and ERAGS-GFP viral particles were bullet shaped in VERO cells, a morphology typical of *Rhabdoviridae* (**Fig. 4**).

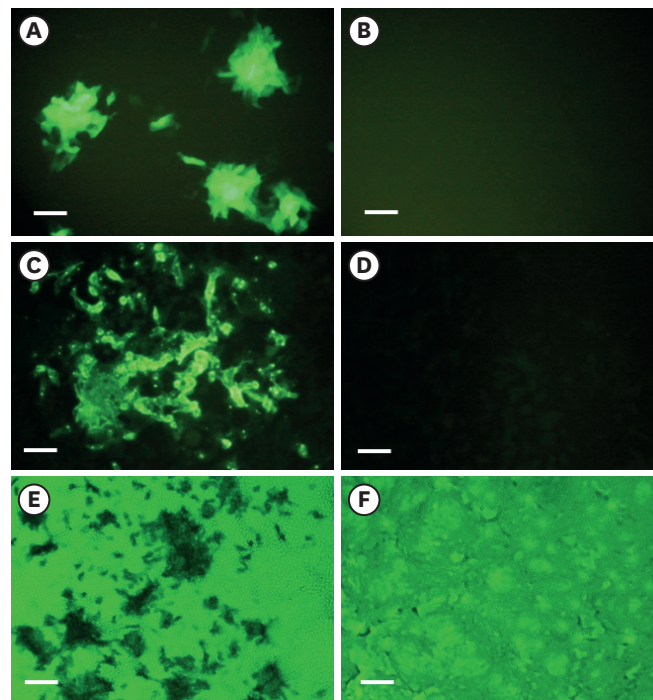


Fig. 2. Identification of ERAGS-GFP by fluorescence microscopy (A), indirect fluorescence assay, (C) and peroxidase-linked assay, (E) and normal cells (B, D, F). GFP and rabies virus-specific fluorescence was evident in the cytoplasm of VERO cells (scale bars, 100 nm; magnification, 200×). GFP, green fluorescent protein.

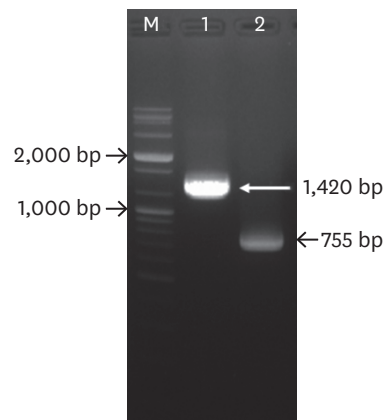


Fig. 3. Amplification of the GFP gene and parts of the G and L genes in recombinant ERAGS-GFP by polymerase chain reaction. M, 1 kb ladder; lane 1, ERAGS-GFP; lane 2, ERAGS. ERAGS-GFP showed longer amplicons than ERAGS because of the presence of the GFP gene. GFP, green fluorescent protein; G, glycoprotein; L, large protein.

Growth properties

The ERAGS and ERAGS-GFP titers were slightly higher in VERO cells than in BHK-21 cells (**Fig. 5A**). In terms of growth kinetics of ERAGS and ERAGS-GFP, the growth curve of ERAGS-GFP was similar to that of ERAGS, and they had an identical titer ($10^{8.0}$ TCID₅₀/mL) at 5 days post-inoculation (**Fig. 5B**). After 30 passages in VERO cells, the titers of both strains were $10^{7.2}$ to $10^{8.0}$ TCID₅₀/mL, indicating that the GFP gene within a pseudogene between the G and L genes did not interfere with viral growth (**Fig. 6**).

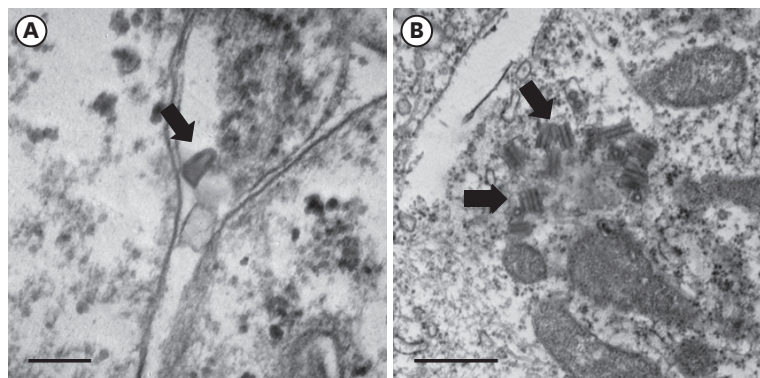


Fig. 4. Transmission electron micrographs of VERO cells infected with ERAGS (A) and ERAGS-GFP (B). Scale bars, 100 nm (A), 500 nm (B). Arrows indicate virus particles in the cytoplasm of VERO cells. GFP, green fluorescent protein.

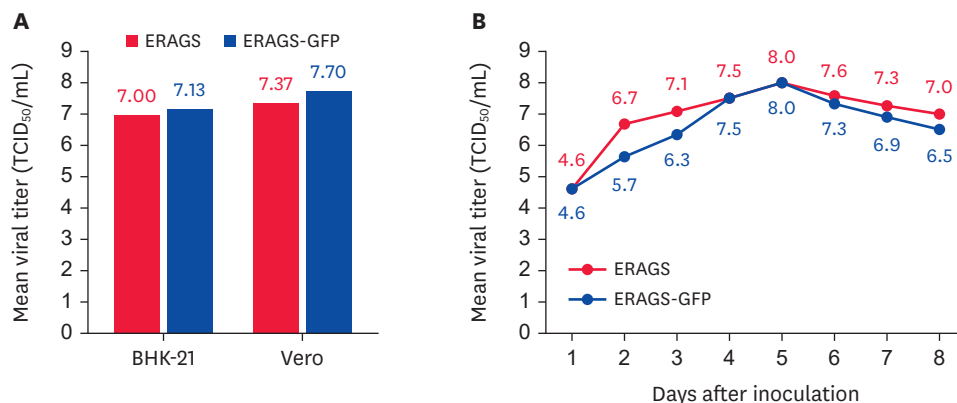


Fig. 5. Growth of ERAGS and ERAGS-GFP in VERO and BHK-21 cells (A) and growth curves of ERAGS and ERAGS-GFP in VERO cells (B). The titer of both viruses peaked at 96 h post-inoculation. GFP, green fluorescent protein.

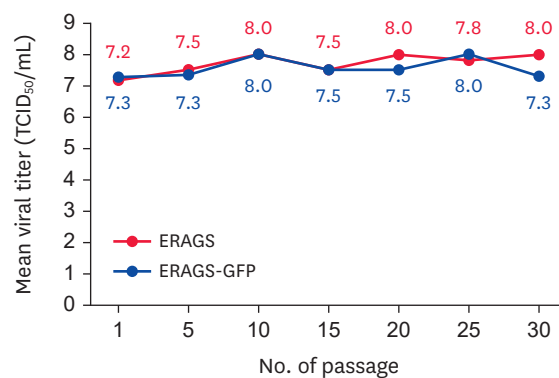


Fig. 6. Titers of ERAGS and ERAGS-GFP according to passage number in VERO cells. The titers of both strains were 10^{7.2} to 10^{8.0} TCID₅₀/mL. GFP, green fluorescent protein.

Safety of ERAGS-GFP in mice

The average body weight of mice inoculated with ERA slowly decreased until 12 days after inoculation (Fig. 6). In contrast, the mean body weights of mice inoculated with ERAGS and ERAGS-GFP increased. All mice inoculated with ERA, succumbed to rabies and died 9 to 13 days post-inoculation. In contrast, all mice inoculated with ERAGS and ERAGS-GFP and all

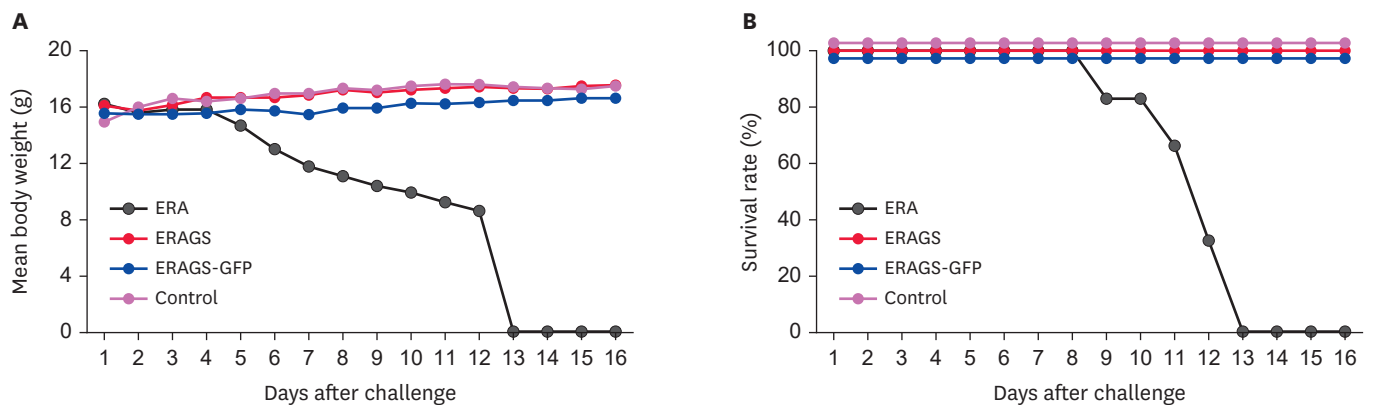


Fig. 7. Mean body weights (A) and survival rates (B) of 4-week-old mice inoculated intracranially with ERA, ERAGS, or ERAGS-GFP. The body weights of mice inoculated with ERAGS or ERAGS-GFP continued to increase whereas those of mice inoculated with ERA progressively decreased, and these mice eventually died. ERA, Evelyn-Rokitnicki-Abelseth; GFP, green fluorescent protein.

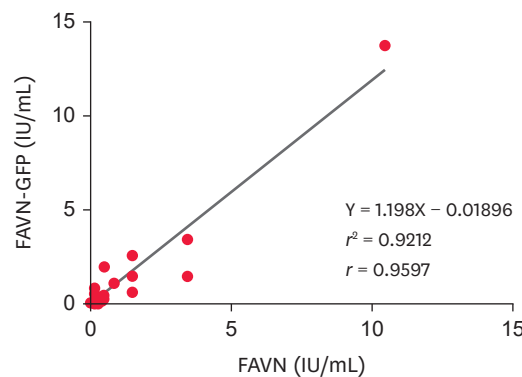


Fig. 8. Correlation of rabies virus-neutralizing antibody titers in 62 dog sera using FAVN and FAVN-GFP. The correlation is indicated by the linear regression line and *r*-value (0.95). FAVN, fluorescent antibody virus neutralization; GFP, green fluorescent protein.

control mice survived (**Fig. 7**). Therefore, the ERA was pathogenic whereas the ERAGS and ERAGS-GFP were non-pathogenic and safe for 4-week-old mice.

Comparison of FAVN with FAVN-GFP test

To evaluate accuracy, the VNA titers of 62 dog sera were measured using CVS-11 and ERAGS-GFP. **Fig. 8** shows the regression lines and correlation coefficients (*r*) between FAVN and FAVN-GFP tests. The *r*-value for FAVN-GFP test was 0.95. A 2-tailed paired *t*-test yielded a *p* = 0.2129, indicating no significant difference between the 2 methods.

DISCUSSION

Rabies can be prevented by vaccinating animals in high-risk regions, because rabies vaccine protects against all RABV variants belonging to phylogroup 1 lyssaviruses [16]. In South Korea, raccoon dogs infected with RABV are responsible for transmitting animal rabies through contact with domestic animals or pet dogs since 1993 [17,18]. In Asia, where stray dogs have transmitted rabies, an increase in the number of stray dogs is closely related to rabies outbreaks [19]. Furthermore, frequent movement of animals between countries and increasing importation of wild animals, such as raccoons and ferrets, can spread rabies to new areas

[20]. Rabies vaccination in animals can be confirmed by measuring the blood rabies VNA titer. Therefore, rabies VNA titers are important for assessing the efficacy of parenteral vaccination and monitoring overall immune status. Although the FAVN method has been approved by the OIE and World Health Organization for detecting RABV antibodies in animals and human, there is a need for a safer, more economical, and faster antibody assay method.

The FAVN method currently in use requires fixation with acetone, washing cells with PBS, and staining with a primary rabies antibody and a fluorescently labeled secondary antibody [7]. Therefore, we developed a modified FAVN method involving ERAGS-GFP to measure the serum rabies VNA titer. The rescued ERAGS-GFP virus had similar biological characteristics and growth kinetics in BHK-21 and VERO cells to the parental ERAGS. Therefore, insertion of the GFP gene between the G and L genes in the ERAGS genome did not significantly affect ERAGS-GFP replication.

The conventional FAVN method uses CVS-11 as the standard RABV [7]. CVS-11 was adapted in mice, is recommended for use in FAVN method, and is used worldwide as a challenge virus [21]. In this study, 4-week-old mice were inoculated intracranially with a high titer of ERAGS-GFP, which was safe for 16 days and did not cause loss of body weight in mice. Recombinant RABV (rRV-eGFP), which expressed enhance GFP fused with RABV phosphoprotein and was constructed from RC-HL strain, showed similar safety in 4-week-old mice [13]. This suggests that ERAGS-GFP, which derives from a genetically modified RABV (ERAGS), is safer for use in a modified FAVN assay than CVS-11, which has neurotropic and virulent characteristics.

Recombinant RABV expressing GFP constructed from CVS-11 required incubation for ≥ 48 h in cell culture [14]. Our ERAGS-GFP produces GFP fluorescence for at least 24 h (data not shown). Because ERAGS-GFP can be visualized directly under a fluorescence microscope without the need to stain, it is less time consuming, more eco-friendly (no acetone), and less costly (no conjugate) than the conventional FAVN method. The modified FAVN method uses VERO cells, which may provide a stable basis for serologic test with less frequent sub-culture. These advantages make our method suitable for testing large numbers of sera. Indeed, the rabies VNA titers in 62 dog serum samples determined by the FAVN and FAVN-GFP assays were strongly correlated ($r = 0.95$).

In conclusion, we constructed a recombinant RABV expressing GFP from ERAGS full genome plasmid using reverse genetics and investigated its growth kinetics and genetic stability. ERAGS-GFP continued to express the GFP gene until passage 30 and was safe in 4-week-old mice. ERAGS-GFP was used to develop a rapid, low-cost modified FAVN method for detecting rabies VNA.

REFERENCES

1. Dietzschold B, Schnell M, Koprowski H. Pathogenesis of rabies. *Curr Top Microbiol Immunol.* 2005;292:45-56.
[PUBMED](#) | [CROSSREF](#)
2. Li J, Ertel A, Portocarrero C, Barkhouse DA, Dietzschold B, Hooper DC, et al. Postexposure treatment with the live-attenuated rabies virus (RV) vaccine TriGAS triggers the clearance of wild-type RV from the Central Nervous System (CNS) through the rapid induction of genes relevant to adaptive immunity in CNS tissues. *J Virol.* 2012;86(6):3200-3210.
[PUBMED](#) | [CROSSREF](#)
3. Ondrejková A, Suli J, Ondrejka R, Beníšek Z, Franka R, Švrček Š, et al. Comparison of the detection and quantification of rabies antibodies in canine sera. *Vet Med (Praha).* 2012;47(8):218-221.
[CROSSREF](#)

4. Servat A, Wasniewski M, Cliquet F. Tools for rabies serology to monitor the effectiveness of rabies vaccination in domestic and wild carnivores. *Dev Biol (Basel)*. 2006;125:91-94.
[PUBMED](#)
5. Yang DK, Kim HH, Cho IS. Strategies to maintain Korea's animal rabies non-occurrence status. *Clin Exp Vaccine Res*. 2018;7(2):87-92.
[PUBMED](#) | [CROSSREF](#)
6. World Organization of Animal Health (OIE). *Terrestrial animal health code*. 29th ed. Paris (France): OIE; 2019, Chapter 8.14: Infection with rabies; 570-575.
7. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Rabies (Infection with Rabies Virus and Other Lyssaviruses) [Internet]. Paris: OIE; https://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/3.01.17_RABIES.pdf. Updated 2019. Accessed 2020 Jan 2.
8. Hu Q, Chen W, Huang K, Baron MD, Bu Z. Rescue of recombinant peste des petits ruminants virus: creation of a GFP-expressing virus and application in rapid virus neutralization test. *Vet Res (Faisalabad)*. 2012;43(1):48.
[PUBMED](#) | [CROSSREF](#)
9. Luo J, Zhao J, Tian Q, Mo W, Wang Y, Chen H, et al. A recombinant rabies virus carrying GFP between N and P affects viral transcription in vitro. *Virus Genes*. 2016;52(3):379-387.
[PUBMED](#) | [CROSSREF](#)
10. Burgado J, Greenberg L, Niezgoda M, Kumar A, Olson V, Wu X, et al. A high throughput neutralization test based on GFP expression by recombinant rabies virus. *PLoS Negl Trop Dis*. 2018;12(12):e0007011.
[PUBMED](#) | [CROSSREF](#)
11. Khawplod P, Inoue K, Shoji Y, Wilde H, Ubol S, Nishizono A, et al. A novel rapid fluorescent focus inhibition test for rabies virus using a recombinant rabies virus visualizing a green fluorescent protein. *J Virol Methods*. 2005;125(1):35-40.
[PUBMED](#) | [CROSSREF](#)
12. Qin S, Volokhov D, Rodionova E, Wirblich C, Schnell MJ, Chizhikov V, et al. A new recombinant rabies virus expressing a green fluorescent protein: a novel and fast approach to quantify virus neutralizing antibodies. *Biologicals*. 2019;59:56-61.
[PUBMED](#) | [CROSSREF](#)
13. Tang HB, Lu ZL, Wei XK, Zhong YZ, Zhong TZ, Pan Y, et al. A recombinant rabies virus expressing a phosphoprotein-eGFP fusion is rescued and applied to the rapid virus neutralization antibody assay. *J Virol Methods*. 2015;219:75-83.
[PUBMED](#) | [CROSSREF](#)
14. Xue X, Zheng X, Liang H, Feng N, Zhao Y, Gao Y, et al. Generation of recombinant rabies virus CVS-11 expressing eGFP applied to the rapid virus neutralization test. *Viruses*. 2014;6(4):1578-1589.
[PUBMED](#) | [CROSSREF](#)
15. Yang DK, Kim HH, Lee SH, Jeong WH, Tark D, Cho IS. A genetically modified rabies vaccine (ERAGS) induces protective immunity in dogs and cattle. *Clin Exp Vaccine Res*. 2017;6(2):128-134.
[PUBMED](#) | [CROSSREF](#)
16. Wera E, Mourits MC, Siko MM, Hogeveen H. Cost-effectiveness of mass dog vaccination campaigns against rabies in Flores Island, Indonesia. *Transbound Emerg Dis*. 2017;64(6):1918-1928.
[PUBMED](#) | [CROSSREF](#)
17. Kim CH, Lee CG, Yoon HC, Nam HM, Park CK, Lee JC, et al. Rabies, an emerging disease in Korea. *J Vet Med B Infect Dis Vet Public Health*. 2006;53(3):111-115.
[PUBMED](#) | [CROSSREF](#)
18. Kim JH, Hwang EK, Sohn HJ, Kim DY, So BJ, Jean YH. Epidemiological characteristics of rabies in South Korea from 1993 to 2001. *Vet Rec*. 2005;157(2):53-56.
[PUBMED](#) | [CROSSREF](#)
19. Kasempimolporn S, Sichanasai B, Saengseesom W, Puempumpanich S, Sitprijia V. Stray dogs in Bangkok, Thailand: rabies virus infection and rabies antibody prevalence. *Dev Biol (Basel)*. 2008;131:137-143.
[PUBMED](#)
20. Have P, Alban L, Berndtsson LT, Cliquet F, Hostnik P, Rodeia SC, et al. Risk of rabies introduction by non-commercial movement of pets. *Dev Biol (Basel)*. 2008;131:177-185.
[PUBMED](#)
21. Patel AC, Upmanyu V, Ramasamy S, Gupta PK, Singh R, Singh RP. Molecular and immunogenic characterization of BHK-21 cell line adapted CVS-11 strain of rabies virus and future prospect in vaccination strategy. *Virusdisease*. 2015;26(4):288-296.
[PUBMED](#) | [CROSSREF](#)