Antibody Response in Cattle and Guinea Pigs Inoculated with Rabies Vaccines

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One hundred ninety-five rabies cases in cattle were identified in South Korea since 1993. As most of rabies cases have a relation to rabid Korean raccoon dogs (Nyctereutes procyonoides koreensis), vaccination to animals including cattle is mandatory in rabies risk region. In order to minimize fatal rabies in animals, eradication policy of the disease has been achieved by controlling reservoirs and by mass vaccination. In this study, we compared the antibody response in cattle and guinea pigs inoculated with rabies vaccines commercially available in Korea. Each group of cattle in Gangwon-do was vaccinated intramuscularly with either one of five commercial inactivated vaccines or a live attenuated rabies vaccine (designated as A to F). Serum samples at the time of vaccination and four weeks post vaccination were obtained from the cattle and guinea pigs and were analyzed with virus neutralizing assay (VNA). Each group of cattle inoculating rabies vaccines showed significant virus neutralizing antibody titers ($p < 0.05$) ranging from 1.55 to 17.8 mean IU/ml compared with the non-vaccinated cattle and guinea pigs inoculated with 1/20 dose of vaccine showed relatively low VN antibody titers ranging from 0.23 to 6.1 mean IU/ml. All cattle immunized with A, C and F showed high VN antibody titers over 0.5 IU/ml and 62.5% and 37.5% of cattle inoculated with D and E showed protective antibody titer, respectively. This finding suggests that the inactivated or live attenuated rabies vaccination commercially available in Korea could induce protective antibody response in Korean cattle, but sero-conversion rate and sero-positive rate showing VN antibody titer over 0.5 IU/ml depend on vaccines.

Key Words: Rabies vaccine, Immunogenicity, Rabies, Cattle

INTRODUCTION

Rabies is a fatal zoonosis that occurs in more than 150 nations around the world and is claiming lives of 55,000 people annually (1). Rabies is transmitted to all kinds of warm blooded animals by rabid animals in various regions of the world. In Latin America, vampire bat (Desmodus rotundus) takes responsibility for transmitting the disease to livestock; in Europe, wild animals such as foxes play a key role transmitting the disease; in several countries including India and Thailand, dogs are considered as major vector species, and in Korea, raccoon dogs (Nyctereutes procyonoides koreensis) and badger (Meles meles) are the major source of transmission (2~6).

Cattle are known to be highly susceptible to rabies.

Early clinical signs of cattle with natural infection include excessive salivation, abnormal behavior, muzzle tremors, tenemus, yawning, paraphimosis, photophobia, pica, sexual excitement, abnormal bellowing, aggression, decreased lactation, increased interest with sound or light and paralysis of the throat. In the furious form of rabies, cattle may try to attack humans or other animals. Late clinical signs can include opisthotonus, seizures, and widespread paralysis (7). In general, death occurs soon after late clinical signs.

Since the rabies case was reported again in dogs in 1993, a number of rabies cases in Korea have been identified in several animal species such as dogs, cattle, raccoon dogs and cats at only two provinces (Gyeonggi-do and Gangwon-do) until 2013 (8, 9). The number of cattle infected by rabies in Korea since 1993 has increased greater than other animals and it came to 195 cattle based on the KAHIS program (www.kahis.go.kr) in Animal and Plant Quarantine Agency (QIA). In order to control the disease, Korean government has implemented several policies including mass vaccination to dogs twice each year, distribution of bait vaccine to the rabies risk regions targeting raccoon dogs, removal of stray dogs, application of new control program such as trap-vaccination-release (TVR) and rabies awareness campaigns. It is also mandatory to inoculate rabies vaccine to cattle and dogs raised in the rabies risk area of Korea. The National Institutes of Health (NIH) rabies vaccine potency test have been wildly used to determine the efficacy of inactivated rabies vaccine in mice by challenging with virulent rabies virus and the potency of a vaccine is calculated in accordance with survival rates of mice (10, 11). Although the NIH guideline for vaccine potency test with survival rates provides accurate results, WHO has recommended to develop alternative assays to quantify the antigen concentration within rabies vaccines. The alternative assays include both serological assays for checking protective antibody titers in vaccinated animals or in vitro potency tests for determining the concentration of glycoprotein (G) within vaccines (12~14).

In the previous study, we identified the viral strain of the rabies vaccine by analyzing the nucleotide sequence of the nucleoprotein and G gene after extracting viral RNA from gel vaccines (15). Several kinds of inactivated vaccine strains such as CVS11, Pittman-Moor-NIL2, RC-HL derived from the Nishigahara strain, and Pasteur virus stain have been manufactured by companies and live attenuated rabies vaccine strains including Evelyn-Rokitnicki-Abelseth (ERA), Flury, Street-Alabama-Dufferin (SAD) strain have been produced and commercialized to immunize animals in the world (16). Currently, 14 commercial rabies vaccines have been licensed in Korea since 1980, and applied to animals for the prevention of natural infection. It has been noted that most of inactivated rabies vaccines could induce protective immunity in raccoon dogs and pet animals (17, 18). However, the immunogenicity in cattle inoculated with rabies vaccines used in Korea has not been properly investigated yet, although the aspect has been reported in cats and dogs. In this study, we investigated the comparative immune response of cattle and guinea pigs inoculated with rabies vaccines and identified G protein from commercial rabies vaccines.

MATERIALS AND METHODS

Vaccines

The five inactivated rabies vaccines and one attenuated rabies vaccine were used in this study as follows: Nobivac Rabies® (Pasteur RIV strain, MSD Animal Health, Netherland), Rabigen mono® (VP12 strain, Virbac, France), DEFFENSOR 3® (SVR-289 strain, Zoesti, USA), Rabishin® (G52 strain, Merial Animal Health, France), Canishot® RV-F (PV strain, ChoongAng Co., Korea), and Dogivac® (ERA strain, Daesung microbiology, Korea). These vaccines were designated as A, B, C, D, E, and F. The five inactivated rabies vaccines were licensed in Korea for use in animals, but the targeted animal species depend on the each vaccine brands. All vaccine except Canishot® RV-F contained adjuvant such as aluminum hydroxide to enhance immune response. One live attenuated vaccine (Dogivac®) was also licensed for dogs, cattle, horses, sheep and goats, but not cats.

Immunization and blood sampling

All trials in cattle were conducted in a Hanwoo cattle farm located in Gangwon-do Province and vaccination to guinea pigs was conducted in QIA. In brief, each group consisting of eight Hanwoo cattle were inoculated with one dose of each commercial vaccine intramuscularly (IM) and control group remained without any vaccination. Six groups comprising of four guinea pigs were divided and inoculated with 1/20 dose of each rabies vaccine IM. Blood samples of cattle and guinea pigs were taken from jugular vein and the heart at the beginning of the experiment and 28 days post vaccination. Clotted blood samples were centrifuged (1,700 x g, 15 min), heat inactivated and sera were stored at -20℃ until use. None of them had been vaccinated before. After vaccination on the cattle and guinea pigs, the clinical signs were observed for 28 days.

Serological assay

Virus neutralizing assay (VNA) was determined by the fluorescent antibody virus neutralization (FAVN) test (19). In brief, a positive reference serum of WHO adjusted to 0.5 IU/ml was used as a positive control. Each serum sample as well as the positive and negative controls were distributed in four consecutive wells, and then serially diluted. The rabies virus (RABV, CVS-11 strain) containing around 100 TCID$_{50}$/50 μl was then added to each well. After 60 min of incubation at 37℃, a volume of 50 μl of BHK-21 cells suspension containing 4×10^5 cells/ml was added to each well and the microplates were incubated for 72 hrs in a 5% CO$_2$ humidified incubator at 37℃. The microplates were fixed in 80% cold acetone (-20℃) for 20 min. After 3 successive washings with phosphate buffer saline (PBS, pH 7.2), the microplates were reacted with specific monoclonal antibody (Median Diagnostics, Chun-cheon, Korea) against rabies for 45 min at 37℃, and then stained with fluorescence isothiocyanate (FITC) conjugated goat-anti mouse IgG+IgM. After rinsing with PBS, the microplates were air-dried and were examined at 400 x using a fluorescent microscope (Nikon, Tokyo, Japan). The titers of serum samples were expressed in International Units per milliliter (IU/ml) by comparing results obtained with those of the positive standard. The threshold of protective antibody used was 0.5 IU/ml.

Identification of antigen in vaccines

One milliliter of rabies vaccine was put in tube and centrifuged at 7,000 x g for 5 min and the supernatant was collected. Another one milliliter of rabies vaccine was mixed with 0.1 g of sodium citrate on a rotator at 37℃ for 12 hrs to recover RABV antigen from the gel vaccines. The mixture was frozen and thawed three times and centrifuged at 7,000 x g for 5 min, and the supernatant was then collected. Both samples were used for the application of Western blotting. For the identification of RABV G in vaccine, the supernatant and sodium citrate treated samples were dissolved in SDS-PAGE sample buffer. Each sample was separated on a 12.5% tris-glycine gel and transferred electrophoretically onto a nitrocellulose (NC) membrane. The NC membrane was blocked with 5% skim milk solution for two hours. After washing three times with tris-buffered saline containing Tween-20 (TBST, pH 7.2), the NC membrane was incubated with a 1/1,000 dilution of anti RABV G monoclonal antibody at room temperature for 12 hrs. The NC membrane was washed three times and incubated for 1 hr with anti-mouse IgG phosphatase conjugate diluted at 1:2,000 at room temperature. After thorough washing with TBST, the membrane was developed in TMB substrate solution for membrane development. In order to compare amounts of protein between samples, sodium citrate treated samples were separated by SDS-PAGE and stained with Coomassie Brilliant Blue dye for 1 hr.

Statistical analysis

Statistical significance was assessed with one-way analysis of variance (ANOVA) for comparisons among vaccine groups. A value of $p < 0.05$ was considered statistically significant.
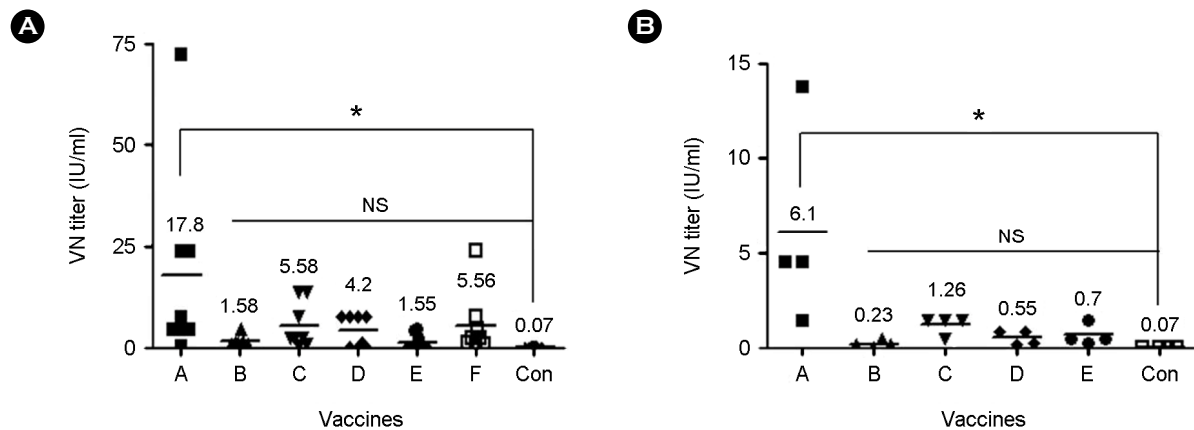
Figure 1. Antibody response in Korean Hanwoo cattle and guinea pigs inoculated with commercial rabies vaccines. Each cattle (n = 8) or guinea pig (n = 4) was vaccinated intramuscularly with one dose or 1/20 dose of commercial rabies vaccines, respectively. These vaccines were designated as A, B, C, D, E, and F. Non-vaccinated cattle and guinea pigs were included as control. Rabies-specific antibody titers were measured by FAVN test at 28 days post immunization in cattle (A) and guinea pigs (B). Antibody titers were expressed in International Units per milliliter (IU/ml) as indicated in Materials and Methods. * $p < 0.05$ (one-way analysis of variance (ANOVA)), NS = not significant.

RESULTS

Immune response of rabies vaccines in animals

None of the Hanwoo cattle inoculated intramuscularly with one dose of the commercial rabies vaccines, respectively showed the typical clinical signs of rabies, i.e., anorexia, salivation, extreme aggressive behavior, paresis, ataxia and paralysis during the 28-day post inoculation period. As the results of antibody titer are shown in Fig. 1, cattle inoculated with vaccines respectively showed significant VN antibody titers ($p < 0.05$) ranging from 1.55 to 17.8 mean IU/ml and guinea pigs inoculated with 1/20 dose of vaccine showed relatively low VN antibody titers ranging from 0.23 to 6.1 mean IU/ml ($p < 0.05$).

All cattle inoculated with Vaccine A, B, C and F revealed 100% sero-conversion rate and cattle with Vaccine D and E showed 75 and 50% sero-conversion rate at 28 days post vaccination, respectively. All cattle immunized with Vaccine A, C and F showed high VN antibody titers over 0.5 IU/ml, but only 37.5% cattle inoculated with Vaccine E showed protective antibody titer (Fig. 2A). All guinea pigs inoculated with 1/20 dose of Vaccine A and C showed high VN antibody titers over 0.5 IU/ml. All guinea pigs inoculated with Vaccine A, C, D, E and F showed 100% of sero-

conversion rate, but only 50% of guinea pigs inoculated with Vaccine D and E showed protective antibody titer (Fig. 2B). On the other hand, non-vaccinated cattle and guinea pigs remained sero-negative against rabies at all times.

### Identification of RABV antigen in vaccines

The supernatant of centrifuged rabies vaccine and the sodium citrate treated supernatant were run on poly-acrylamide SDS gels and followed by Western blotting assay with specific monoclonal antibody against G protein of RABV. Western blot assay revealed that sodium citrate treated supernatants showed better reaction than that of non-treated supernatant. Vaccine B, C, and E showed strong reaction (65 kDa) with RABV monoclonal antibody (Fig. 3).

DISCUSSIONS

All kinds of warm blooded animals can be infected with RABV and several vector hosts such as dogs, cats, foxes, vampire bats, mongooses, skunks and raccoons depending on the countries are considered the main transmitters of urban and sylvatic rabies (20, 21). Raccoon dogs are known for the major transmitters of rabies in Korea. As to the number of animal rabies cases, about 43.4% of animal rabies cases are equivalent to cattle bitten by rabid raccoon
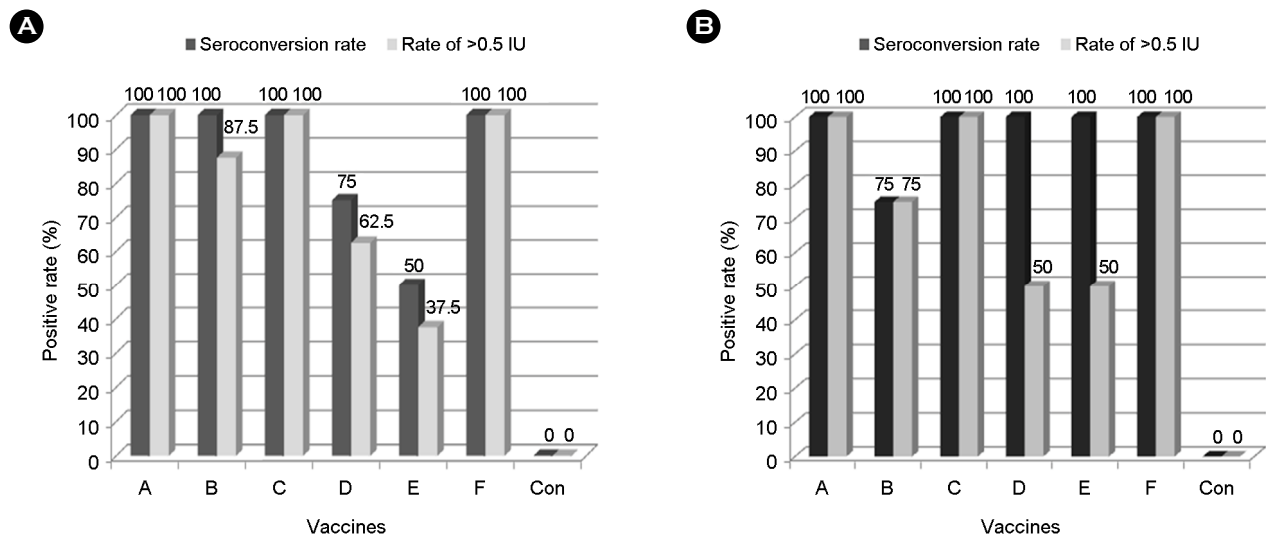
Figure 2. Sero-conversion rate against RABV in cattle (A) and guinea pigs (B) inoculated with commercial RABV vaccines. The protective antibody titer determined by FAVN test was expressed in 0.5 IU/ml. The sero-conversion rate of vaccine A, B, C and F was significantly higher than that of vaccine D and E ($p < 0.05$).
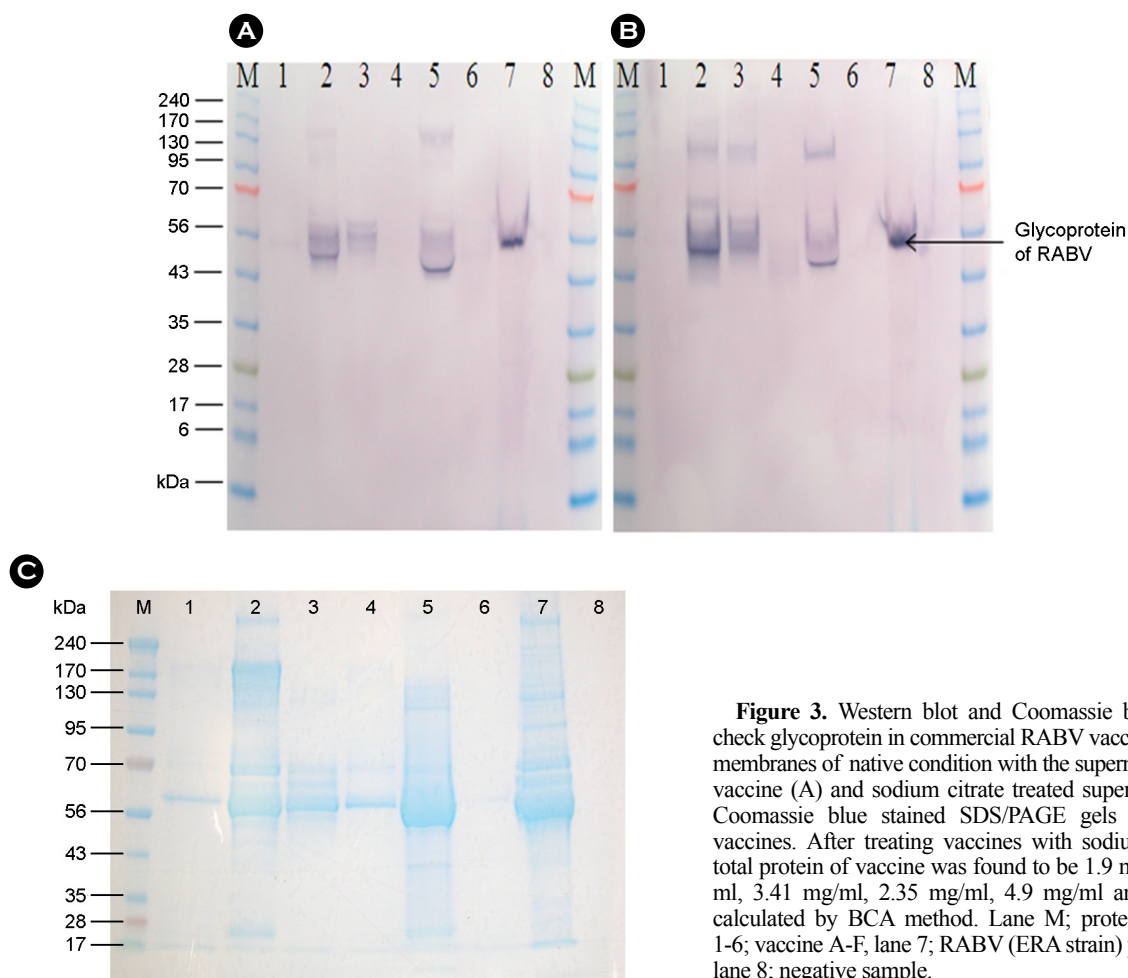
Figure 3. Western blot and Coomassie blue staining to check glycoprotein in commercial RABV vaccines. Developed membranes of native condition with the supernatant of RABV vaccine (A) and sodium citrate treated supernatant (B), and Coomassie blue stained SDS/PAGE gels (C) of RABV vaccines. After treating vaccines with sodium citrate, each total protein of vaccine was found to be 1.9 mg/ml, 5.29 mg/ml, 3.41 mg/ml, 2.35 mg/ml, 4.9 mg/ml and 1.47 mg/ml, calculated by BCA method. Lane M; protein marker, lane 1-6; vaccine A-F, lane 7; RABV (ERA strain) positive sample, lane 8; negative sample.

dogs (6, 9). The cattle infected with wild RABV show various clinical symptoms and result in death. In order to block transmission of rabies between wild and domestic animals, all animals including dogs, cats and cattle raised in rabies risk region should be inoculated with rabies vaccine. In addition, vaccinia-rabies glycoprotein (V-RG) bait vaccine has been distributed to induce immunization of wild raccoon dogs since 2000 in Korea. Even though these preventive measures have led to decrease animal rabies case, recent rabies cases have been reported in other region where rabies did not occurred during last 30 years. As the rabies occurred in the rabies free region, V-RG vaccine was distributed and all animals raised in the region have been vaccinated. Furthermore, serological survey of dogs and cattle against rabies was conducted one month after vaccination. The result of the sero-survey showed that there was significant difference of sero-positive rate ranging from 53 to 95% according to regions where several kinds of vaccines were used to immunize cattle. Therefore, we made a decision to evaluate commercial rabies vaccine in cattle and guinea pigs.

Three kinds of rabies vaccines (inactivated, live attenuated and bait) for veterinary use have been licensed by Korea Veterinary Authority. It is noted that infection of monocytes or immature dendritic cells (DCs) with an attenuated rabies vaccine results in DC maturation and a strong activation of the NFkappaB signaling pathway (22). As inactivated rabies vaccine is not affected by circulating antibody and basically associated with the quantity of antigen, to be an effective vaccine, much more inactivated viral antigen than that of live vaccine must be contained in the vaccine. Our previous study demonstrated that the nucleotide similarity of the partial G genes ranged from 91.8% to 100% among rabies vaccine strains, indicating that commercially available rabies vaccines licensed in Korean contained different RABV strains (15).

The kinetics of developing neutralizing antibodies after primary and booster inoculation of rabies vaccine have been studied in pet animals and the second inoculation of rabies vaccine induced more rapid increase and higher levels of circulating antibodies than the primary inoculation (17). Our results demonstrated that all group of cattle inoculated with one dose of each rabies vaccine developed protective VN antibody titers ranging from 1.55 to 17.8 mean IU/ml and four groups of guinea pigs inoculated with 1/20 dose of the vaccine induced VN antibody titers ranging from 0.7 to 6.1 mean IU/ml. No significant differences of immune response according to rabies vaccine were found between cattle and guinea pigs post inoculation days 28. This result is consistent with that of other studies (23). It is well known that animals with antibody titers > 0.5 IU/ml or VN antibody titers >1:16 do not develop severe clinical signs of rabies after challenge with virulent RABV (24). However, only 62.5% of cattle inoculated with Vaccine D and 37.5% of cattle with Vaccine E induced VN antibody titer > 0.5 IU/ml. Fifty percentage of guinea pigs inoculated with Vaccine D and E revealed protective antibody titer > 0.5 IU/ml. There were no significant differences of immune response between cattle and guinea pigs inoculated with rabies vaccines. Peak antibody response in pet animals was generally presented in several reports after booster inoculation (16, 25), suggesting that vaccine D and E need the second vaccination to cattle. Most cattle inoculated with Vaccine A to F showed various geometric mean VN antibody titers, indicating that each vaccine manufacturer used different vaccine formula with aluminum hydroxide, rabies vaccine strain and inactivating agent for the in-activated rabies vaccine. Therefore, if the mass vaccination program to new rabies risk region is applied to Korean cattle, the cattle should be inoculated with vaccine two times to induce complete protective neutralizing antibody against rabies.

Immunogenicity of vaccine is closely associated with concentration of RABV G within the vaccine and kinds of adjuvant. It was reported that the quantification of virus-attached rabies G has a strong correlation with VN antibody titer based on the association between the potencies of rabies vaccines tested by the NIH test and the contents of virion-attached G in the vaccine (26). In our study, G protein in three inactivated rabies vaccines was identified using Western blotting (WB) assay. However, G protein of other three vaccines was not detected when treated with sodium citrate. In this study, we were not able to verify the

reason why the G protein from vaccine A, the strongest antibody inducer, was not detected in WB assays. It could be due to different vaccine strain, adjuvant concentration, monoclonal antibody as detector, or antigen extraction method with sodium citrate. As the G protein from all kinds of inactivated RABV vaccines could not be detected in WB assay, other reagent such as isopropyl myristate may be applicable for extracting antigen from adjuvant within vaccines (15). Additional studies are needed to solve this problem. As the method of ELISA for *in vitro* potency test of rabies vaccines was evaluated (12), ELISA can be used to quantify the viral antigens in the rabies vaccines.

In conclusion, half of commercial rabies vaccines tested in this study cannot induce protective immune response in Korean Hanwoo cattle after vaccination, therefore, it is recommended that booster vaccination is needed for complete herd immunity. The guinea pigs may be useful animals in predicting immune response of rabies vaccine. In addition, development of alternative testing method *in vitro* is required for the quality control of inactivated rabies vaccine.

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