

## Antibody Response in Korean Raccoon Dogs Inoculated with Inactivated Rabies Vaccines

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Since sylvatic rabies was first identified in South Korea in 1993, over three million bait vaccine doses have been distributed to rabies risk regions in order to block transmission of rabies among wild animals. New progressive strategy is needed to eliminate sylvatic rabies completely in rabies risk regions. Before applying the preventive program related to eradication, immunogenicity of inactivated rabies vaccines available in Korea has to be evaluated in Korean raccoon dogs (*Nyctereutes procyonoides koreensis*). Six groups of raccoon dogs in wild rescue center of Gyeonggi-do were vaccinated intramuscularly with single dose of six inactivated commercial rabies vaccines (designated A to F). Serum samples at the time of vaccination, and two and four weeks post vaccination were obtained and analyzed by virus neutralizing assay (VNA). All raccoon dogs inoculated with vaccines C, D, E or F, showed VN antibody titers ranging from 0.5 to 13.77 IU/ml. Half of four raccoon dogs immunized with vaccine B revealed VN titer over 0.5 IU/ml, and one of four raccoon dogs inoculated with vaccine A showed protective antibody titer. This finding suggests that most of the commercially available inactivated rabies vaccines could induce protective immunity in Korean raccoon dogs and be applicable to new rabies control program.

**Key Words:** Inactivated vaccine, Immunogenicity, Rabies, Raccoon dog

### INTRODUCTION

Rabies is an important zoonosis that occurs in more than 150 nations around the world and is claiming lives of 55,000 people annually (1). Especially, mortality rate is increasing in Africa, Asia and Latin America and some countries need support of other World Animal Health

Organization (OIE) members that successfully eradicated the disease. Since the first rabies case in dogs in 1907, a number of rabies cases in Korea have been reported in several animal species such as dogs, cattle, raccoon dogs and cats at several provinces until 2011 (2, 3). As the result of the implement of Korean government's policy including intensive vaccination to dogs with live attenuated rabies vaccine (LEP Fulry and ERA strain), removal of stray dogs and carrying out rabies awareness campaigns continuously, a steady decrease in the number of animal rabies cases was observed in period between 1960 and 1984 and no rabies case was reported for 8 years from 1985 to 1992 (2~4).

Rabies is transmitted by several kinds of vectors including dogs, bats, raccoon dogs, red foxes, wolves and mongooses depending on the national situation (5~7). Dogs were

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known to be the main vector for the transmission of rabies virus (RABV) to human or other animals in Korea prior to 1984. Unfortunately, a recurrence of the rabies in dog fighting with rabid raccoon dog was identified in Gangwon-do Province in 1993 and a continuous increase of rabies cases was observed. The epidemiological study on rabies reported that wild animals such as raccoon dogs (*Nyctereutes procyonoides koreensis*) and badger (*Meles meles*) played an important role in transmitting rabies to cattle and dogs in Korea (4, 8). Since 1993, dog-to-dog transmission (urban rabies) was eliminated in Korea (2, 3), but sylvatic rabies has been identified up to 2011. In order to block rabies transmission by wild animals such as raccoon dogs, Korean government made a decision to distribute vaccinia-rabies glycoprotein (V-RG) bait vaccine in 2000 after trial application and over three million bait vaccine doses have been distributed to rabies risk provinces by a direct way (Gyeonggi-do, Gangwon-do) (3). As the result of distributing bait vaccine, rabies case has not been reported in Gyeonggi-do Province since 2007. However, it continuously has been occurring and the areas of rabies have moved eastward in Gangwon-do Province (4). Even though oral rabies vaccination (ORV) such as distribution of bait vaccine has been found to be efficacious policy in reducing rabies case, new strategy has been presented to control rabies completely. In addition, previous study reported that vaccination of raccoon (*Procyon lotor*) and skunk with inactivated rabies vaccine elicited good immunogenicity and protected them from challenge with wild RABV (9, 10). Based on the immunogenicity of commercial inactivated vaccine in Canada and USA, new strategies including trap-vaccination-release (TVR), ORV, point infection control (PIC), and population reduction (PR) programs have been applied to several provinces of the countries and made Ontario state of Canada pronounce free of rabies in 2008 (4, 11).

Therefore, the application of new rabies control program to completely eliminate rabies transmitted by raccoon dogs is needed to the rabies risk regions of Korea. Before applying the new preventive program, immunogenicity of inactivated rabies vaccine available in Korea have to be

evaluated in Korean raccoon dogs. The aim of this study was to describe immunogenicity of 6 kinds of commercial inactivated rabies vaccines after vaccination to Korean raccoon dogs and provide preliminary information associated with eradication program in Korea.

## MATERIALS AND MTEHODS

### Vaccines

The one inactivated rabies vaccine was from Korean animal vaccine company and the other five were purchased from international companies. The six vaccines used in this study were as follows: CaniShot<sup>®</sup> RV-F (ChoongAng Co., Korea), DEFENSOR<sup>®</sup> 3 (Pfizer Animal Health, USA), Nobivac<sup>®</sup> Rabies (Intervet, Netherlands), Rabdomun<sup>®</sup> (Shering-Plough, USA), Rabigen<sup>®</sup> mono (Virbac, France), Rabisin<sup>®</sup> (Merial, France), designating A, B, C, D, E, and F, respectively. They were all licensed to use in Korea for dogs, cats and cattle, but not for raccoon dogs.

### Immunization and blood sampling

All trials were conducted in wild animal rescue center located in Gyeonggi-do Province. In brief, each group consisting of four Korean raccoon dogs were inoculated with one dose of each commercial vaccine intramuscularly and control group remained without any vaccination. Bloods were taken from cephalic vein at the beginning of the experiment, and 14 and 28 days post vaccination. Clotted blood samples were centrifuged ( $1,700 \times g$ , 15 min) and sera were stored at  $-20^{\circ}\text{C}$  until use. None of them had been vaccinated before and did not have neutralizing antibody at the time of immunization. After vaccination any clinical signs appeared after immunization were observed for 28 days.

### Serological assay

Virus neutralizing assay (VNA) was determined by the fluorescent antibody virus neutralization (FAVN) test (12). In brief, a positive reference serum of WHO was adjusted to 0.5 IU/ml and used as a positive control. Each serum sample as well as the positive and negative controls were

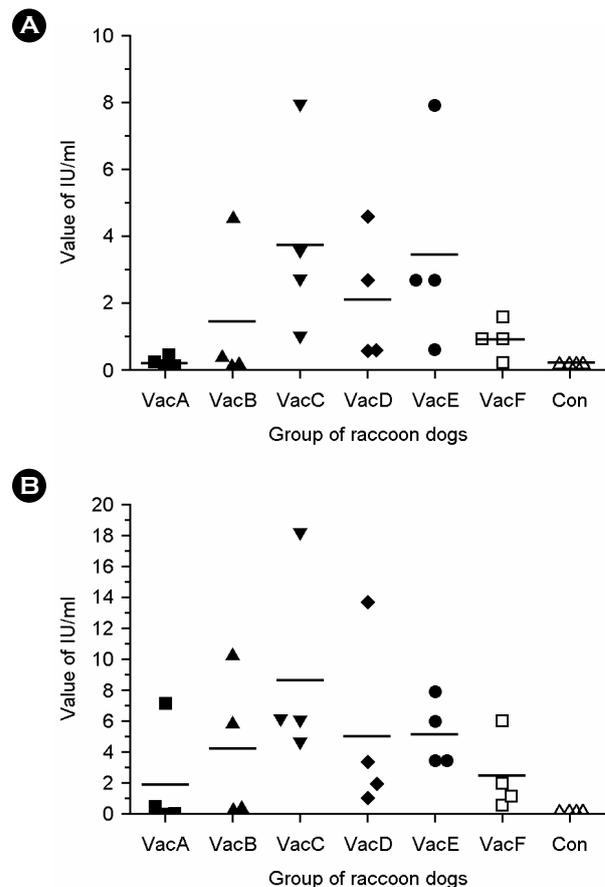
distributed in four consecutive wells, and then serially diluted. The RABV (CVS-11 strain) containing around 100 TCID<sub>50</sub>/50 µl was then added to each well. After 60 min of incubation at 37°C, 50 µl of BHK-21 cell suspension from  $4 \times 10^5$  cells/ml was added to each well and the microplate was incubated for 72 h in a humidified incubator with 5% CO<sub>2</sub> at 37°C. The microplate was fixed in cold acetone (-20°C) for 20 min. After 3 successive washings with phosphate buffer saline (PBS, pH 7.2), the microplate was reacted with specific monoclonal antibody against rabies protein (Median Diagnostics, Chuncheon, Korea) for 45 min at 37°C, and then stained with fluorescence isothiocyanate (FITC) conjugated goat-anti mouse IgG + IgM. After rinsing with PBS, the microplate was air-dried and examined at 400X 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 positivity used was 0.5 IU/ml.

### Statistical analysis

The differences in VN titers between the vaccinated or non-vaccinated groups were analyzed using nonparametric Mann-Whitney U test (SPSS, Ver. 10.1). A value of  $p < 0.05$  was considered statistically significant.

## RESULTS

None of the 24 Korean raccoon dogs inoculated intramuscularly with one dose of the commercial vaccines, 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, all raccoon dogs inoculated with vaccines C, D, E and F, respectively showed significant VN antibody titers ( $p < 0.05$ ) ranging from 0.5 to 13.77 IU/ml compared with the non-vaccinated raccoon dogs. In addition, half of 4 raccoon dogs immunized with Vaccine B revealed VN titer over 0.5 IU/ml, and one of 4 raccoon dogs inoculated with Vaccine A showed protective antibody titer. However, non-vaccinated



**Figure 1.** Antibody response in Korean raccoon dogs inoculated with inactivated rabies vaccines. Raccoon dogs were vaccinated intramuscularly with one of six commercial inactivated rabies vaccines. These vaccines were designated as VacA, VacB, VacC, VacD, VacE, and VacF. Non-vaccinated raccoon dogs were included as control. Rabies-specific antibody titers were measured by fluorescent antibody virus neutralization test at 14 days post immunization (A) and 28 days post immunization (B). Antibody titers were expressed in International Units per milliliter (IU/ml) as indicated in Materials and Methods.

raccoon dogs remained sero-negative against rabies at all times. The VN antibody titers of raccoon dogs checked at 28 days post inoculation was higher than those of raccoon dogs at 14 days post inoculation.

## DISCUSSION

All warm blooded animals can be infected by rabid animal's biting and rabid animals show high mortality with various clinical symptoms (13). It is thus essential to inoculate RABV vaccine to animals such as dogs and cattle

raised in rabies risk region. In addition, V-RG bait vaccine has been distributed to block transmission of rabies between wild animals in South Korea. Even though the preventive measure distributing bait vaccine has led to eradication in Gyeonggi-do Province since 2007, the area of rabies occurrence has moved to east northern region of Gangwon-do Province. Even though rabies cases in Korea have been decreased, animal rabies cases have been reported in Gangwon-do Province continually. Although oral vaccinations including V-RG and ONRAB (adenovirus-rabies glycoprotein recombinant oral vaccine) have been used to control rabies of raccoons and skunks in Canada and USA, the proportion of antibody positive raccoons was significantly different between vaccines (14).

Therefore, new strategies such as TVR or PIC program should be prepared to eradicate rabies in the Korean Peninsula. Earlier, it was proved that the TVR program was an effective model to eradicate sylvatic rabies in Ontario State of Canada (15). As the commercial inactivated rabies vaccines are available for dogs and cattle, they need to be demonstrated that the commercial vaccines can induce protective antibody titers in Korean raccoon dogs. To address this, we tested 6 kinds of inactivated rabies vaccines and their immunogenicity in Korean raccoon dogs.

Sobey *et al.*, reported that inoculation of inactivated rabies vaccine by intramuscular route was found to be efficacious in field evaluation of an inactivated vaccine to control raccoon rabies in Canada (16, 11). 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 also demonstrated that 87.5% (21/24) of raccoon dogs inoculated with 6 kinds of inactivated vaccines developed protective neutralizing antibodies. It is well known that animals with antibody titers  $>0.5$  IU/ml or VN titers  $>1:16$  do not develop severe clinical signs of rabies after challenge with virulent rabies virus (16). However, only 25% (1/4) raccoon dogs inoculated with Vaccine A and 50% raccoon dogs with Vaccine B induced VN titer  $>0.5$  IU/ml. Kinetics

of antibody response showed that most of raccoon dogs developed higher VN titer at 28 days post inoculation than at 14 days (Fig. 1). Peak antibody response in pet animals was generally presented in several reports between 4 and 6 weeks after inoculation (18, 19), suggesting that immunogenicity of vaccine A and B need to be evaluated at 6 weeks after application to large number of raccoon dogs. Most of raccoon dogs inoculated with Vaccine A - F showed various geometric mean VN titer ranging from 1.86 to 6.59 IU/ml at 28 days, indicating that each vaccine manufacturer used different kinds of adjuvant, rabies vaccine strain and inactivating agent for the inactivated rabies vaccine. Therefore, if the TVR program is applied to Korean raccoon dogs and is carried out multi-annually, raccoon dogs residing in rabies risk area may have protective neutralizing antibody against rabies. It also reported that raccoon dogs should be revaccinated annually as part of an effective TVR rabies control program (10).

In conclusion, most of commercial inactivated rabies vaccination could induce protective immunity in Korean raccoon dogs and be applicable to new rabies control program. The further study related to immunogenicity of inactivated rabies vaccine in wild animals such as badger will be needed to control rabies in Korea.

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