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Original Article

Serologic Survey of Rabies Virus, Canine Distemper Virus and Parvovirus in Wild Raccoon Dogs (*Nyctereutes procyonoides koreensis*) in Korea

Dong-Kun Yang[1*], Ha-Hyun Kim[1], Jin-Ju Nah[1], Sung-Suk Choi[1], Jong-Taek Kim[2], Woong-Ho Jeong[3] and Jae-Young Song[1]

[1]Animal and Plant Quarantine Agency, MAFRA, Anyang; [2]College of Veterinary Medicine, Kangwon National University, Chuncheon; [3]Gangwon Veterinary Service Laboratory Sokcho, Korea

Oral rabies vaccination (ORV) program for the wild animals in rabies risk regions of Korea has been conducted since 2000. Evaluation of ORV program under field condition and information concerning the incidence of exposure to canine distemper and canine parvovirus (CPV) are needed in wild raccoon dogs (*Nyctereutes procyonoides koreensis*). Ninety four sera of wild raccoon dogs were screened for antibodies against rabies, canine distemper virus (CDV) and CPV in Korea. The overall prevalence of antibodies against rabies virus (RABV), CDV and CPV in wild raccoon dogs was 35.1%, 89.4% and 24.5%, respectively. Comparisons of sero-prevalences of RABV, CDV and CPV were assayed in two regions (Gyeonggi-do and Gangwon-do). The Gyeonggi-do (36.4%) showed higher sero-positive rate against CPV than Gangwon-do (20.8%). In contrast, Gangwon-do (41.7% and 97.2%) showed higher sero-positive rates against RABV and CDV than Gyeonggi-do (13.6% and 63.6%). These results indicate that there was severe circulation of CDV and CPV among wild raccoon dogs in the two regions of Korea. Furthermore, raccoon dogs showing a protective antibody titer (0.5 IU/ml) were 15.9%, suggesting that new rabies control program such as trap-vaccination-release (TVR) should be launched urgently in rabies risk regions.

Key Words: Raccoon dogs, RABV, CDV, CPV, Serosurveillance

INTRODUCTION

The wild raccoon dogs (*Nyctereutes procyonoides koreensis*) have contributed to the main vector and reservoir for rabies in Europe, northern Asia and Americas and rabies cases in raccoon dogs have increased in North-eastern Europe (1). The raccoon dogs were introduced to Korea from Russia for the production of fur and pelts in the late 1920s. As fur farms for silver foxes and goats were prosperous in Asian countries, Korean raccoon dog's industry had lessened rapidly. The raccoon dogs residing in fur farms had escaped and became wild animals in Korea (2). The lack of natural predators of raccoon dogs has increased the population density of raccoon dogs and potential for transmission of disease between domestic carnivores and wild raccoon dogs if they contact infected carnivores (3, 4).

Rabies virus (RABV) belonging to the genus *Lyssavirus* of the family *Rhabdoviridae* causes a fatal disease in animals

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*Corresponding author: Dong-Kun Yang. Animal and Plant Quarantine Agency, 175 Anyang-ro, Anyang-si, Gyeonggi-do 430-757, Korea. Phone: +82-31-467-1783, Fax: +82-31-467-1797, e-mail: yangdk@korea.kr
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and humans and is classified into reportable disease to World Animal Health Organization (OIE). Since dog-to-dog transmission was eliminated in 1993, sylvatic rabies has been identified up to 2013 in Korea. The epidemiological study on rabies reported that raccoon dogs involved in transmitting rabies to cattle and dogs (5).

Canine distemper is one of the most serious diseases in Korean domestic dogs and canine distemper virus (CDV) causes a lethal disease to various animal species belonging to the *Carniovora*. The CDV is unstable outside of the body, but infected animals are known as the source of infection within a population. Based on the molecular analysis of CDV gene, CDVs were characterized as several groups such as Asia1 and 2, Europe, America and Arctic (6). Raccoon dogs are also infected with CDV spreading through aerosol or droplet exposure and resulted in lethal death (7).

Canine parvovirus (CPV) belonging to the genus parvovirus within the family *Parvoviridae* can cause fatal diarrhea in carnivores. Infected animals can excrete large amounts of virus in feces for 10 days after natural infection (8, 9). The CPV is transmitted by fecal-oral route with easy and is very stable within the environment. Since CPV was identified in the late 1970s in dogs, new CPV has been spread out worldwide and named as CPV-2 type to distinguish it from CPV prototype. Based on the amino acid residue at position 297 and 426 of VP2 gene, CPV-2a or CPV2b/c was named. Currently, CPV-2a is the main genotype circulating in the dog population in India, Germany and Korea (9, 10). The three diseases mentioned above have been associated with high morbidity and mortality rate in carnivores. Raccoon dogs have played an important role in transmitting RABV to domestic animals in Korea since 1993 (2) and are thought to be reservoirs of CDV and CPV to wild and domestic animals (6, 7).

Recently, it was reported that in comparison of ONRAB® and RABORAL Vaccinia-recombinant glycoprotein (V-RG)® bait vaccine in Canada and USA, raccoons taken up with ONRAB rather than V-RG hold higher percentage of antibody-positive against RABV (11). There was high similarity of haemagglutinin (H) gene of CDV in comparison of CDVs obtained from domestic dogs and wild raccoon dogs (7). A parvovirus genetically related to CPV type 2 was isolated from a rescued raccoon (*Procyon lotor*) (12). We have previously reported the seroprevalence of RABV, CPV in stray dogs and also the isolation of RABV, CPV-2a circulating in Korea (2, 13). The purpose of this study was to determine the seroprevalence of the RABV, CDV and CPV in wild Korean raccoon dogs.

MATERIALS AND METHODS

Collection of sera

For the seroprevalence survey, blood samples were obtained from a total of 94 wild raccoon dogs that were 0.6 month to 2 years old. Twenty two raccoon dogs were being housed in rescue centers of Gyeonggi-do in 2011 and 72 samples were obtained from wild raccoon dogs in Gangwon-do in 2012. The blood was taken from a cephalic vein. Clotted blood samples were centrifuged (3,000 × g, 10 min), and sera were stored at -20℃ until use.

Fluorescent antibody virus neutralization (FAVN) for RABV

Virus neutralizing assay (VNA) was determined by the fluorescent antibody virus neutralization (FAVN) test (14). In brief, a positive reference serum of WHO was adjusted to 0.5 IU/ml and 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 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 from 4 × 10^5 cells/ml was added to each well and the microplates were incubated for 72 h in a humidified incubator with 5% CO_2 at 37℃. The microplates were fixed in 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, Chuncheon, 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 the results obtained with those of the positive standard. The threshold of positivity used was 0.1 IU/ml and the protective antibody titer was expressed in 0.5 IU/ml.

ELISA to detect antibodies against CDV

An ELISA kit (INGEZIM MOQUILLO IgG, Madrid, Spain) was used for the detection and quantification of IgG antibody according to manufacturer's indications. The ELISA OD (optical density) results of negative (OD <0.2), low (0.2≤ OD <0.4), medium (0.4≤ OD <0.8) or high (OD ≥0.8) were equivalent to the CDV serum neutralization titers of negative, 1:20, 1:80, or >1:320, respectively. In brief, the sera samples were diluted at 1/100 dilution and added to the ELISA kit. After the incubation for 10 min at room temperature, the plates were washed 4 times with washing solution and then added 100 µl of conjugate to each well. The sealed plates were incubated for 10 min at room temperature and after discarding conjugate the plates were washed 4 times. Finally, 100 µl of substrate solution was added and the plates were kept for 5 min at room temperature. After adding 100 µl of stop solution to each well, the absorbances of each well were read with an ELISA reader (Tecan, Switzerland) at 450 nm.

Hemagglutination assay (HA) and hemagglutination inhibition (HI) test for CPV

CPV, KV0901 strain, used in this study was isolated in Korea in 2009 from feces of naturally infected puppies (13). The CPV strain belonging to CPV-2a had been passaged five times in A72 cells derived from canine fibroblast cells. The titer of CPV-2a was confirmed by an indirect fluorescent assay using an anti CPV-2 monoclonal antibody and by a hemagglutination assay (HA) using pig erythrocytes (0.6%). The CPV-2a was used as the antigen in the HI test. The HA test was carried out by preparing serial two-fold dilutions of CPV-2a in 50 µl of Sorensen buffer (pH 6.8) and with 50 µl of 0.6% pig erythrocytes. The HA titer was expressed as the reciprocal of the highest dilution of CPV-2a showing hemagglutination.

The HI test was performed in 96-well microplates as described previously (8), with slight modifications. Briefly, to remove non-specific inhibitors, 50 µl of serum was mixed with 200 µl of 25% kaolin and incubated for 30 min. After pipetting, the kaolin was removed by centrifugation (10,000 × g, 15 min). The clear supernatant was mixed with 5 µl of packed pig erythrocytes to remove any natural agglutinins. After incubation for 1 h at 37℃, the treated serum was separated from the erythrocytes by centrifugation. For the HI test, 4~8 HA units of CPV-2a (in 25 µl) were added to 25 µl of the treated serum. After incubation for 1 h at room temperature, 50 µl of 0.6% pig erythrocytes were added, and the microplates were incubated at 4℃ for 1 h. The HI titer was expressed as the reciprocal of the highest dilution of serum showing complete inhibition of hemagglutination. Serum samples showing HI titers ≥1:10 were considered positive.

Statistical analysis

The chi-square test was used to analyze differences in sero-prevalence by region. A p-value of less than 0.05 was deemed to indicate statistical significance.

RESULTS

The positive rate against RABV in 94 serum samples collected from raccoon dogs in two provinces was 35.1% in average (Fig. 1). The regional sero-prevalence was 13.6% (3/22) in Gyeonggi-do and 41.7% (30/72) in Gangwon-do and reached statistical significant (p = 0.016). Raccoon dogs showing a protective antibody titer above 0.5 IU/ml were 15.9% (15/94). The overall prevalence of antibodies against CDV and CPV was 89.4% (84/94) and 24.5% (23/94) respectively (Table 1 and 2). The regional incidence of CDV and CPV was 63.6% (14/22), 36.4% (8/22) in Gyeonggi-do and 97.2% (70/72), 20.8% (15/72) in Gangwon-do, respectively (Table 1 and 2). The raccoon dogs captured from Gangwon-do showed higher incidence rate (97.2%) of CDV than those from Gyeonggi-do and of the titers against CDV, most of raccoon dogs residing in Gangwon-do showed

medium or high titer against CDV. The statistical difference by region was only observed in CDV ($p < 0.001$), but not in CPV ($p = 0.138$). Of the raccoon dogs that had HI antibody titer of 1:10 or more, the most frequent HI titer against CPV was 1:2,560 (8.5%). In a brief conclusion, the overall prevalence of antibodies against RABV, CDV and CPV in wild raccoon dogs was 35.1%, 89.4% and 24.5%, indicating that infectious pathogens in raccoon dogs may be transferred to companion or domestic animals.

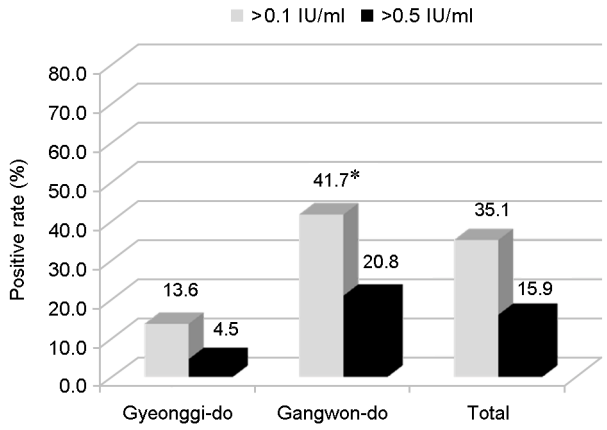

Figure 1. Sero-positive rate against RABV in Korean raccoon (n=94). Virus neutralizing antibody titer was determined by the fluorescent antibody virus neutralization (FAVN) test. The threshold of positivity used was 0.1 IU/ml and the protective antibody titer was expressed in 0.5 IU/ml. *The antibody mean titer of Gangwon-do was significantly higher than that of Gyeonggi-do ($p < 0.05$).

DISCUSSION

The wild raccoon dog is known as host and vector for rabies, sarcoptic mange, trichinellosis and echinococcosis (15, 16) and represents the majority of rescue animal among wild life in animal rescue center (17). Even though home range size in raccoon dogs was narrow ranging from 0.58 km^2 to 0.98 km^2, the population density of raccoon dogs in Korea has been consistently increased and habitat range has also expanded to urban and suburban area (3, 6). There would be high possibility in transmitting infectious pathogens with close contact between wild raccoon dog and companion animals. Thus, we conducted a survey of the

Table 1. Distribution of ELISA titer against canine distemper virus in Korean raccoon dogs

Province*	ELISA Titer against CDV				Positive rate (%)
	Neg** (OD <0.2)	Low (0.2≤ OD <0.4)	Medium (0.4≤ OD <0.8)	High (OD >0.8)	
GG	8	12	2	0	14/22 (63.6)
GW	2	9	30	31	70/72 (97.2)***
Total	10	21	32	31	84/94 (89.4)

* GG: Gyeonggi-do, GW: Gangwon-do.
** The ELISA OD results of negative (OD <0.2), low (0.2≤ OD <0.4), medium (0.4≤ OD <0.8) or high (OD ≥0.8) were equivalent to the CDV serum neutralization titer of negative, 1:20, 1:80, or >1:320, respectively, according to the manufacturer's indications.
*** The antibody mean titer of GW was significantly higher than that of GG ($p < 0.001$).

Table 2. Distribution of HI titer against canine parvovirus (CPV2a) in Korean raccoon dogs

Province*	HI titer against CPV									Positive rate (%)
	<10	20	40	80	160	320	640	1,280	2,560	
GG	14	0	2	1	1	0	1	1	2	8/22 (36.4)
GW	57	0	2	0	1	2	0	4	6	15/72 (20.8)
Total	71	0	4	1	2	2	1	5	8	23/94 (24.5)

* GG: Gyeonggi-do, GW: Gangwon-do.

presence of antibodies against RABV, CDV and CPV-2a in raccoon dogs.

The key element associated with protection in animals is the titer of VN antibody induced following vaccination. The most rabies cases do not have detectable VN antibody response until some days after demonstrating acute disease, indicating that VN antibody originated from vaccination only (18). The FAVN test has been used to monitor the success of anti-rabies oral bait policy in several countries including Korea. It has been reported that RABV sero-prevalence ranged from 38% to 55%, depending on the time and countries of survey (1, 5, 11). In this study, the overall prevalence of antibodies against RABV was 35.1% but, raccoon dogs showing a protective antibody titer above 0.5 IU/ml were only 15.9%. It assumes that even though rabies bait vaccine (V-RG) for the control of rabies in wild animals has been distributed into rabies risk regions in Gyeonggi-do and Gangwon-do of Korea, low percentage of raccoon dogs may consume the bait vaccine. This sero-positive rate may be lower than those of other countries showing over 38% of sero-positive rate (11, 19~21). At this stage, it is not clear why Korean raccoon dogs showed such a low sero-prevalence compared to those seen in other countries. However, a few suggestions could be made. First, more amount of bait vaccine should be prepared and distributed to rabies risk regions evenly. Direct distribution method by people may be replaced with aerial method. Before distributing bait vaccine, exact population density of raccoon dogs and consumption by non-target animals should be investigated. Second, new vaccine program such as trap-vaccination-release (TVR) program needs to be introduced to eliminate rabies in rabies risk regions. Despite relatively low sero-prevalences of antibody in raccoon dogs, the rabies cases have been deceased in two regions, indicating that oral rabies vaccination was helpful to decrease in number of rabies cases in Korea.

Canine distemper and canine parvovirus infections are common and serious disease in domestic dogs and wild raccoon dogs (6, 22, 23). CDV Infection has been reported in raccoon dogs and CDVs isolated from naturally infected Korean raccoon dogs belonged to the Asia-2 genotype (6, 7). Korean raccoon dogs showed high incidence rate against CDV (89.4%) and moderate incidence rate against CPV (24.5%), indicating that CDV infection is widespread and CPV infection is mild in wild raccoon dogs population. Evidence of exposure to CDV and CPV was demonstrated in raccoon dogs residing in two regions of Korea, assuming that raccoon dogs play an important role as a reservoir of CDV and CPV and these two pathogens have been spreading to other carnivores through raccoon dogs.

In conclusion, our results revealed low sero-positive rate against rabies and evidence of exposure to wild CDV and CPV in raccoon dogs. Contact between domestic dogs and raccoon dogs can play a subordinate role in the transmission of infectious pathogens. An application of TVR control program should be done urgently in rabies risk area to block circulation of wild RABV (24). The further study related to new rabies bait vaccine in Korean raccoon dogs will be needed to induce high sero-positive rate against RABV in raccoon dogs. In addition, CDV and CPV vaccines available to wild raccoon dogs should be developed in the near future.

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