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# SURVEILLANCE FOR ANTIBODIES AGAINST SIX CANINE VIRUSES IN WILD RACCOONS (*PROCYON LOTOR*) IN JAPAN

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ABSTRACT: Raccoons (*Procyon lotor*) are found worldwide. They are frequently seen in crowded inner cities as well as in forests or wooded areas, often living in proximity to humans and their pets. We examined sera from 100 wild raccoons in Japan for antibodies to six canine viruses with veterinary significance to assess their potential as reservoirs. We also aimed to understand the distribution of potentially infected wildlife. We found that 7% of samples were seropositive for canine distemper virus (CDV), 10% for canine parvovirus type 2, 2% for canine adenovirus type 1, 6% for canine adenovirus type 2, and 7% for canine coronavirus. No samples were found to be seropositive for canine parainfluenza virus. Seropositivity rates for canine distemper virus and canine parvovirus type 2 were significantly different between areas, and younger raccoons (<1 yr old) were more frequently seropositive than older raccoons. Because raccoons belong to the suborder Caniformia, similar to dogs (*Canis lupus familiaris*), our results suggest that they can act as reservoirs for some of these important canine viruses and might be involved in viral transmission. Further study should include isolation and analysis of canine viruses in wild raccoons from a wider area.

*Key words:* Canine distemper virus, canine parvovirus, Japan, raccoons, serum.

## INTRODUCTION

Raccoons (*Procyon lotor*) belong to the order Carnivora, suborder Caniformia, together with dogs (*Canis lupus familiaris*), weasels (*Mustela* spp.), and badgers (*Meles* spp.). Raccoons are originally native to North America and were exported to European countries, mainly Germany, and Japan in the 20th century (Leśniańska et al. 2016). They escaped from their owners and settled there (Yanagihara-Agetsuma 2004; Beltrán-Beck et al. 2012). Subsequently, feral raccoons have increased in number and spread throughout these countries due to their abilities to adapt to different environments in foreign countries, even in urban environments, and their high fertility (Hohmann et al. 2001; Asano et al. 2003). They are frequently found in urban spaces near human settlements as well as in forests and wooded areas, where they can easily find alternative sources of food (Prange et al. 2003; Kresta et al. 2009). Raccoons build their nests in garrets or under the eaves of private houses (Ministry of the Environment 2011) and cause damage by attacking humans and eating field crops, such as corn, or chicken and poultry eggs on farms (Conover 1987; Dolbeer et al. 1994). Thus, in recent years, they have become more closely connected with humans and companion animals.

In Japan, raccoons escaped from a zoo located in the center of Honshu Island in Aichi Prefecture in the 1960s and were thought to have reverted to living in the wild (Agetsuma-Yanagihara 2001). Later, raccoons were imported into Japan as pets, and they settled in the wild after escaping from or being abandoned by their owners (Hagiwara et al. 2009). Presently, raccoons are widely distributed throughout Japan, mainly in the suburbs of relatively large cities (e.g., Sapporo, Tokyo, Aichi, Osaka, and Hyogo) as well as in rural areas (Ministry of the Environment 2011). The recent increase in the number of raccoons means that they are subject to control according to The Invasive Alien Species Act (Ministry of the Environment 2008).

Wild raccoons are thought to affect the lifecycles of several infectious disease agents, and they have been particularly implicated as reservoirs for pathogens of veterinary and human importance (Gordon et al. 2004; Hall et al. 2008). However, there are few reports on the involvement of raccoons in viral infections such as canine distemper virus (CDV) and coronavirus in Japan (Ishihara et al. 2009; Nakano et al. 2009; Yamaguchi et al. 2014). In this study, we focused on canine viruses with high veterinary significance: CDV, canine parvovirus type 2 (CPV), canine adenovirus types 1 and 2 (CAdV-1 and CAdV-2), canine coronavirus (CCoV), and canine parainfluenza virus (CPIV). Vaccines for these viruses are available for pet dogs and owners are advised to inoculate their animals, although the inoculation rates of these vaccines remain largely unknown. In particular, CDV, CPV, and CAdV-1 are thought to be important due to their lethal pathogenicity (da Fontoura Budaszewski and von Messling 2016; Luo et al. 2016; Walker et al. 2016). We tested for antibodies against these six canine viruses to assess whether wild raccoons in Japan are potential reservoirs to Caniformia, including pet dogs, and to understand the distribution of these viruses in wildlife.

## MATERIALS AND METHODS

### Animals

The 100 raccoons examined in this study were captured around private houses in Hyogo Prefecture, near Osaka on the western part of Honshu Island, from April 2004 to June 2006 (52 samples) and from April 2009 to May 2010 (48 samples). This prefecture is included as one of five areas in Japan (Hokkaido, Kanto, Chukyo, Kinki, and Kyushu) in which raccoons are frequently observed. Sixteen and 84 raccoons were caught in central and southern areas of Hyogo, respectively, and the distance between the two areas was >20 km. All raccoons were euthanized according to the Guideline for Raccoon Prevention of Hyogo Prefecture (National Institute for Environmental Studies 2011) by administration of pentobarbital or $CO_2$. Blood samples were collected from the hearts of each animal, and after coagulation, sera were kept at –20 C until use. The sera were inactivated by incubation at 56 C for 30 min and examined for the presence of antibodies against CDV, CPV, CAdV-1, CAdV-2, CCoV, and CPIV as described soon. The ages of the animals were estimated from the numbers of annuli formed on the teeth (Asano et al. 2003). None of the raccoons showed clinical symptoms except for a few ectoparasites found on some animals.

### Detection of virus-specific antibodies

The detection of antibodies against CDV and CCoV was performed by immunoperoxidase plaque staining test as described previously (Kai et al. 1992; Soma et al. 2001a, b). In brief, antigen plates were made by culturing Vero cells for CDV and Crandell Rees feline kidney cells for CCoV in 96-well tissue culture microplates overnight at 37 C. Cells were inoculated with 0.1 mL containing 30 plaque-forming units per well of CDV isolated from commercial canine vaccines (Solvay-Duphar, Boulevard Emile Bockstael, Brussels, Belgium) or CCoV 1-71 strain (American Type Culture Collection, Manassas, Virginia, USA) as the antigen, respectively. These antigens were confirmed to have reactivity with dog sera obtained from natural infections (Soma et al. 2001a, b). After adsorbing for 1 h, viral suspensions were removed and 0.1 mL of a minimum essential medium (Nissui Seiyaku, Tokyo, Japan) containing 2% fetal bovine serum and 1% methylcellulose was overlaid onto the cells. After 40 h of incubation at 37 C, the plates were fixed with methanol and then washed with distilled water. After drying, the plates were stored at –20 C. To determine the antibody titers against each virus, 0.05 mL of twofold serial dilutions of the sera was added to the plates after an initial 10-fold dilution for CDV or 40-fold dilution for CCoV. After 1 h of incubation at 37 C, the plates were washed with phosphate-buffered saline (pH 7.0) containing 0.1% Tween 20. Peroxidase-conjugated protein A (Sigma-Aldrich, St. Louis, Missouri, USA) was used instead of anti-dog immunoglobulin G (IgG) conjugated with peroxidase as described previously (Soma et al. 2001a, b) for detection because the reactivity of anti-dog IgG with raccoon IgG is unknown. The plates were subsequently incubated for 1 h at 37 C, and 0.1 mL of the substrate solution (0.0025% dianisidine, 0.01% $H_2O_2$, and 10 mmol/L Tris-HCl, pH 7.4) was added to the wells. The titer was determined as the inverse of the highest dilution of serum in which brown-colored plaques occurred on the wells containing the cells.

Antibodies against CPV were detected by the hemagglutination inhibition test by using CPV 916 strain (American Type Culture Collection) as CPV antigen with twofold serial dilutions of the sera after an initial 10-fold dilution (Mohri et al. 1982).

A neutralization test was used to detect antibodies against CAdV-1, CAdV-2, and CPIV

as reported previously (Emery et al. 1976; Soma et al. 2011). In brief, 50 μL of the sera at twofold serial dilutions after a fivefold dilution were incubated at 37 C and 5% $CO_2$ for 1 h with 50 μL of 50% tissue culture infectious dose of CAdV-1 D43-G strain and CAdV-2 Manhattan strain isolated from commercial canine vaccines (Kyoto Biken Laboratories, Uji, Kyoto, Japan and Pfizer, New York, New York, USA, respectively) and CPIV NL-CP1-5 strain isolated from a commercial canine vaccine (Pfizer). The treated sera were inoculated into Madin-Darby canine kidney cells (American Type Culture Collection) cultured in 96-well tissue culture–treated plates (BD Biosciences, Franklin Lakes, New Jersey, USA). After 5 d of incubation at 37 C and 5% $CO_2$, the antibody titers were estimated based on the inverse of the dilution of the sera at 50% inhibition of cytopathic effect according to the Behrens-Karber method (Karber 1931).

### Statistical analysis

Statistical comparisons between two extracted factors (e.g., year, area, sex, age, and weight) were performed by the chi-square test using StatView 5 (SAS Institute, Cary, North Carolina, USA). We considered values of $P<0.05$ to be significantly different.

## RESULTS

The results for the detection in raccoons of antibodies against the six canine viruses are summarized in Tables 1 and 2. Antibody titers of >10 for CDV and CPV; >5 for CAdV-1, CAdV-2, and CPIV; and >40 for CCoV were assessed as positive. Consequently, evidence of CDV, CPV, CAdV-1, CAdV-2, and CCoV infections was found in 6–10% of the raccoons examined, and 26 animals were found to be seropositive to one to three viruses. There was no evidence of CPIV infection. Either or both CAdV-1 and CAdV-2 antibodies were detected in seven animals (Table 2). Animal 42 and animals 4, 45, 52, 80, and 95 were thought to be infected by CAdV-1 and CAdV-2, respectively, because both viruses are known to be partially serologically cross-reactive (Soma et al. 2011). It was unclear which virus infected the remaining animal (29) because CAdV-1 and CAdV-2 antibody titers were the same (Table 2).

We compared the results between sampled years, areas, ages (younger and older than 1 yr), and weight (>6 kg and <6 kg) and sexes (Table 1). Significantly more raccoons captured from the central area were seropositive compared to those from the southern area: 31 and 2% for CDV and 38 and 5% for CPV, respectively ($P<0.001$). Raccoons estimated to be younger than 1 yr (17%) more frequently had CPV antibodies than raccoons older than 1 yr (2%, $P=0.02$).

## DISCUSSION

Raccoons are now widely distributed in Japan and have become common in areas around private houses as well as in forested areas (Yamazaki et al. 2009). Thus, raccoons may contact pet animals either directly or indirectly. The recent increase in the population of wild raccoons has led to significant agricultural damage, and eradication is being considered in some areas (Horimoto et al. 2011). Little information is available about the risk of these animals to veterinary medicine or public health in Japan. In this study, we targeted canine viruses of veterinary importance for investigation and selected two areas in Hyogo Prefecture in Japan. The surveyed areas are not considered to overlap because the activity area of raccoons is reported to be 0.1–50 $km^2$ (Ministry of the Environment 2008). We surveyed raccoons for serologic evidence of infection with canine viruses with the hypothesis that raccoons can harbor these viruses because they belong to the suborder Caniformia, as do dogs.

In the US, CDV antibodies in raccoons have been reported with a range of prevalences: 0% in the Great Smoky Mountains (Rabinowitz and Potgieter 1984) to 23% in Illinois (Mitchell et al. 1999), 54% in Missouri (Junge et al. 2007), and 84% in Maryland (Jamison et al. 1973). The seroprevalence rates of CDV antibodies in raccoons in Japan were previously reported to be 34% in Hyogo and 30% in Osaka (Nakano et al. 2009). The seroprevalence rate of 7% for CDV antibodies observed in our study is low; however, a

TABLE 1. Comparison of the prevalence of antibodies in raccoons (*Procyon lotor*) captured in Japan to six canine viruses between sampled years, areas, ages (younger and older than 1 yr old), and weight (more than and less than 6 kg).

| Parameter | No. | No. positive (%) for canine viruses[a] | | | | | |
|---|---|---|---|---|---|---|---|
| | | Canine distemper virus | Canine parvovirus type 2 | CAdV-1[b] | CAdV-2[b] | Canine coronavirus | Canine parainfluenza virus |
| Years | | | | | | | |
| 2004–06 | 52 | 5 (10) | 7 (14) | 4 (8) | 5 (10) | 5 (10) | 0 (0) |
| 2009–10 | 48 | 2 (4) | 3 (6) | 2 (4) | 2 (4) | 2 (4) | 0 (0) |
| Areas | | | | | | | |
| South | 84 | 2 (2)[c] | 4 (5)[d] | 4 (5) | 5 (6) | 5 (6) | 0 (0) |
| Center | 16 | 5 (31) | 6 (38) | 2 (13) | 2 (13) | 2 (13) | 0 (0) |
| Sex | | | | | | | |
| Male | 57 | 4 (7) | 7 (12) | 3 (5) | 4 (7) | 3 (5) | 0 (0) |
| Female | 43 | 3 (7) | 3 (7) | 3 (7) | 3 (7) | 4 (9) | 0 (0) |
| Age[e] | | | | | | | |
| <1 yr old | 54 | 5 (9) | 9 (17)[f] | 4 (7) | 4 (7) | 5 (9) | 0 (0) |
| >1 yr old | 44 | 2 (5) | 1 (2) | 2 (5) | 3 (7) | 2 (5) | 0 (0) |
| Weight | | | | | | | |
| <6 kg | 50 | 4 (8) | 4 (8) | 2 (4) | 2 (4) | 4 (8) | 0 (0) |
| >6 kg | 50 | 3 (6) | 6 (12) | 4 (8) | 5 (10) | 3 (6) | 0 (0) |
| Total | 100 | 7 (7) | 10 (10) | 6 (6) | 7 (7) | 7 (7) | 0 (0) |

[a] CAdV-1 = canine adenovirus type 1; CAdV-2 = canine adenovirus type 2.

[b] The data for CAdV-1 and CAdV-2 include one sample evaluated as seropositive for both viruses based on the same titer.

[c] Significant difference between areas ($P<0.001$, $\chi^2=17.2$).

[d] Significant difference between areas ($P<0.001$, $\chi^2=16.0$).

[e] The ages of two raccoons could not be determined, so $n=98$ for comparisons of sexes.

[f] Significant difference between ages ($P=0.02$, $\chi^2=5.5$).

significant difference in the prevalence in the south and north areas of Hyogo was observed (2.4% and 31.3%, respectively), similar to the results reported in the US. In carnivores, CDV is generally highly contagious and systemic signs include fever, diarrhea, and neurologic manifestations, often resulting in fatalities (Krakowka et al. 1985). To date, CDV has been detected in many wild animals including raccoon dogs (*Nyctereutes procyonoides viverrinus*) and foxes (*Vulpes vulpes* and *Otocyon megalotis*; Roelke-Parker et al. 1996; Kameo et al. 2012). The pathogenicity of CDV in raccoons remains unknown, although it has been reported to be lethal on occasion (Lednicky et al. 2004). Our results indicated that seropositive raccoons have been infected with CDV and supported previous reports that indicated raccoons could be a potential source of infection for other animals, including pet dogs (Lednicky et al. 2004; Nakano et al. 2009).

Antibodies against CPV were the most frequently detected antibodies in this study. Major symptoms of CPV infection are fever, vomiting, and severe or bloody diarrhea, resulting in death, especially in puppies. Interestingly, the CPV seroprevalence rate among young raccoons <1 yr old was significantly high compared with that of >1-yr-old raccoons. However, these raccoons showed no clinical symptoms, and the pathogenicity of CPV in these animals is unknown. Although we used CPV 916 strain as an antigen for the hemagglutination inhibition test, sera can also react with closely related parvoviruses, such as feline parvovirus (Osterhaus et al. 2003), previously been identified in raccoons (Nettles et al. 1980; Barker et al. 1983; Kapil et al. 2010). Therefore, isolation

TABLE 2. Antibody titers examined to six canine viruses from 26 seropositive raccoons (*Procyon lotor*) captured in Japan.

| Sample no. | Canine viruses[a] | | | | | |
|---|---|---|---|---|---|---|
| | CDV[b] | CPV[b] | CAdV-1[c] | CAdV-2[c] | CCoV[d] | CPIV[c] |
| 2 | — | — | — | — | 80 | — |
| 4 | — | — | 5 | 20 | — | — |
| 5 | — | — | — | — | 160 | — |
| 9 | — | 640 | — | — | — | — |
| 15 | — | 160 | — | — | — | — |
| 17 | 5,120 | — | — | — | — | — |
| 22 | — | — | — | — | 40 | — |
| 24 | 640 | 1,280 | — | — | — | — |
| 25 | 320 | 1,280 | — | — | — | — |
| 26 | 320 | 640 | — | — | — | — |
| 27 | — | 1,280 | — | — | — | — |
| 29 | — | — | 2,560 | 2,560 | — | — |
| 31 | 10,240 | — | — | — | — | — |
| 32 | — | — | — | — | 160 | — |
| 36 | — | 640 | — | — | — | — |
| 42 | — | — | 5,120 | 1,280 | — | — |
| 45 | — | — | — | 10 | — | — |
| 52 | — | — | 40 | 160 | 80 | — |
| 56 | — | 160 | — | — | — | — |
| 74 | 20 | — | — | — | — | — |
| 80 | — | — | 160 | 640 | — | — |
| 94 | 640 | — | — | — | — | — |
| 95 | — | — | 160 | 320 | 40 | — |
| 96 | — | — | — | — | 80 | — |
| 97 | — | 160 | — | — | — | — |
| 98 | — | 80 | — | — | — | — |

[a] CDV = canine distemper virus; CPV = canine parvovirus type 2; CAdV-1 = canine adenovirus type 1; CAdV-2 = canine adenovirus type 2; CCoV = canine coronavirus; CPIV = canine parainfluenza virus; — = negative sample.

[b] Samples were considered positive with titers of >10 for CDV and CPV.

[c] Samples were considered positive with titers of >5 for CAdV-1, CAdV-2, and CPIV and >40 for CCoV.

[d] Samples were considered positive with titers of >40 for CCoV.

and genetic characterization of the virus are needed to differentiate parvovirus infection in raccoons.

Canine adenovirus occurs as two main types, CAdV-1 and CAdV-2, that cause canine hepatitis and respiratory disease such as tracheobronchitis, respectively (Balboni et al. 2015). A CAdV-1 (previously described as canine hepatitis virus) infection can be fatal in infected dogs, and CAdV-1 infections have been reported in raccoons in the US at rates ranging from 0% to 12% based on serum neutralization tests (Jamison et al. 1973; Rabinowitz and Potgieter 1984; Junge et al. 2007). As described in a previous report (Soma et al. 2011), sera from raccoons was cross-reactive with CAdV-2. Thus, it is possible that infections reported as CAdV-1 in previous reports were actually CAdV-2.

In our study, only one animal was determined as seropositive to CAdV-1 by comparing the two antibody titers, with a seroprevalence rate of only 1%. Successful immunization of household dogs against CAdV-1 was previously reported based on high seropositive rates of 71%, and a reduced incidence of outbreaks of CAdV-1 in dogs in Japan was suggested (Taguchi et al. 2011).

The low prevalence of CAdV-1 antibodies in raccoons could reflect the success of these veterinary control measures. Commercial CAdV-2 vaccines are available in Japan, and a low percentage (2.9%) of disease incidence in household dogs was reported in a previous study (Mochizuki et al. 2008). Natural infections of wild raccoons with CAdV-2 have not been reported thus far, although raccoons have been experimentally infected with CAdV-2 (Sumner et al. 1988; Hamir et al. 1992). Our study extended the host range of CAdV-2 infection to include wild raccoons, and our results and that of previous reports (Sumner et al. 1988; Hamir et al. 1992) support the possibility of infected raccoons as reservoirs to pet dogs.

The CCoV is an *Alphacoronavirus*, one of four genera of the subfamily Coronavirinae. Infected dogs develop enteritis and gastroenteritis, and the condition is aggravated by mixed infections with other pathogens (Tennant et al. 1991). To date, serologic analysis has indicated infection with an alphacoronavirus in raccoons, although the viruses were not specifically identified (Martin and Zeidner 1992). Alphacoronaviruses include porcine epidemic diarrhea virus, transmissible gastroenteritis coronavirus, and feline coronavirus. Our results suggest that seven raccoons were seropositive for at least one alphacoronavirus because we used CCoV 1-71 strain as an antigen. Isolation of the virus from raccoons and genetic identification are required to confirm and classify the species of coronavirus.

Antibodies to CPIV were not detected in any of the examined raccoons, confirming the results of a previous report (Jamison et al. 1973). The CPIV is classified as parainfluenza virus type 5 that has a wide host range: humans, cats, hamsters, and dogs (Chatziandreou et al. 2004). Further investigation is needed to determine the potential for CPIV infection in raccoons.

In our survey, we could not compare the seroprevalence rates of these six viruses in raccoons and dogs by using sera because some dogs had been vaccinated against the viruses. Our results indicate that wild raccoons in Japan have antibodies to several canine viruses. Although our study was limited to one prefecture and the number of raccoons examined was not large, our findings suggested geographic differences in infection rates. A more thorough analysis of isolated viruses and a large-scale survey of wild raccoons and other wild animals of the order Carnivora, such as raccoon dogs, as endemic species in Japan is warranted.

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