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Detection and genotyping of canine coronavirus RNA in diarrheic dogs in Japan

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ABSTRACT

To clarify the prevalence of canine coronavirus (CCoV) infection in Japan, faecal samples from 109 dogs with diarrhoea were examined for CCoV RNA together with canine parvovirus type 2 (CPV-2) DNA. The detection rates of CCoV and CPV-2 for dogs aged less than 1 year were 66.3% and 43.8%, while those for dogs aged 1 year or older were 6.9% and 10.3%, respectively, which were significantly different ($p < 0.0001$ and $p = 0.0003$, respectively), indicating not CPV-2 but CCoV is an important diarrhoea-causing organism in juvenile dogs. Among the CCoV-positive dogs, 65.5% and 72.7% showed to be positive for CCoV types I and II, respectively, and simultaneous detection rate of both types was high at 40.0%. Furthermore, transmissible gastroenteritis virus (TGEV)-like CCoV RNA was detected from 8 dogs. These findings indicate that CCoV type I and TGEV-like CCoV are already circulating in Japan, though no reports have been presented to date.

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Canine coronavirus (CCoV) is a positive stranded RNA virus, and has been classified as a Group 1 coronavirus along with feline coronavirus (FCoV), transmissible gastroenteritis virus (TGEV), and human coronavirus 229E (Pratelli, 2006). CCoV infection occurs mainly by oral ingestion of the virus in excreted faeces, and the major symptoms are digestive conditions such as diarrhoea and vomiting. Juvenile dogs and dogs under stress tend to be affected more frequently. Furthermore, the symptoms become more severe in dogs co-infected with canine parvovirus type 2 (CPV-2) (Appel, 1988).

CCoV was initially isolated from military dogs with diarrhoea in 1971 (Pratelli, 2006). The virus has spread extensively throughout the world and in Japan it was first isolated in 1982 (Yasoshima et al., 1983). Thereafter, evidence of CCoV infections in Japan has been confirmed by serological and genetic studies (Bandai et al., 1999; Mochizuki et al., 1987, 2001; Soma et al., 2001). CCoV has long been recognized as a single genotype. However, Pratelli et al. (2002b, 2003) reported two genotypes of CCoV; atypical CCoV, designated as CCoV type I with a spike-protein gene similar to that of FCoV, and the well-known typical CCoV, designated as CCoV type II. It has been confirmed that CCoV type I is in circulation in Europe and China (Benetka et al., 2006; Decaro et al., 2010; Pratelli et al., 2003; Stavisky et al., 2010; Yesilbag et al.,

2004; Wang et al., 2006). Furthermore, Wesley (1999) reported a novel CCoV type II named TGEV-like CCoV, which possesses an S gene similar to that of TGEV, and this virus also appears to be circulating in dogs in Europe and the USA (Decaro et al., 2009, 2010; Elia et al., 2010; Erles and Brownlie, 2009). It is highly likely that CCoV type I and TGEV-like CCoV are already in circulation in Japan, though no reports have been presented to date. In the present study, for the purpose of clarifying the present state of CCoV infection in Japan, faecal samples of dogs with diarrhoea were examined for CCoV RNA and detected CCoV strains were examined for genotype. In addition, CPV-2 DNA was investigated and its detection rate was compared with that of CCoV RNA.

Rectal swab samples were collected from a total of 109 privately owned (not kennelled) family pet dogs with diarrhoea which visited veterinary clinics in Japan in the period from September 2007 to December 2009. None had received CCoV live vaccine and CPV-2 vaccine derived from CPV-2 new type, the antigenic variants referred to as types 2a, 2b and 2c, though some dogs had received the vaccine from the original CPV-2 (old type). The swab samples were soaked in a small amount of sterile saline and immediately preserved in a frozen state. Viral RNA was extracted from the swab samples using QIAamp Viral RNA Mini Kit (Qiagen, Tokyo, Japan) according to the manufacturer's instruction. RT-PCR for detecting CCoV RNA was performed using QIAGEN OneStep RT-PCR Kit (Qiagen, Tokyo, Japan). About 2.5 μ L of the extracted RNA was added to the 25 μ L reaction mixtures containing 5 μ L of 5 \times

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buffer (finally 1.5 mM MgCl₂), 0.4 mM of each deoxynucleoside triphosphate (dNTP), 10 U RNase inhibitor (Qiagen, Tokyo, Japan), 0.8 μM of the CCV1–CCV2 primer pair providing a 409 bp amplicon of CCoV-M region (Pratelli et al., 1999) and 1.0 μL enzyme mix. The RNA was reverse transcribed at 50 °C for 30 min, followed by inactivation of reverse transcriptase and denaturation of cDNA template at 95 °C for 15 min. The cDNA was amplified in 35 sequential cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min, followed by a final extension of 72 °C for 10 min. The PCR products were electrophoresed at 100 V for 40 min in a 2% agarose gel. Then, they were stained with ethidium bromide, and were visualized using a UV transilluminator at 300 nm. The RT-PCRs for CCoV genotyping were performed using EL1F–EL1R, S5–S6 and CEPol-1–TGSp-1 primer pairs, which amplify CCoV type I, CCoV type II and TGEV-like CCoV genes, respectively (Erles and Brownlie, 2009), in a manner similar to the CCV1–CCV2 primer pair except for annealing temperature of CEPol-1–TGSp-1 primer pair (50 °C). The EL1F–EL1R, S5–S6 and CEPol-1–TGSp-1 primer pairs provided 346, 694 and 370 bp amplicons, respectively. In addition, CPV-2 PCR was performed with FY3–RY1 primer pair, which can detect CPV-2 new type (CPVs-2a, 2b and 2c) specifically (Senda et al., 1995). In brief, viral DNA extracted by QIAamp DNA Mini Kit (Qiagen, Tokyo, Japan) was amplified in 30 cycles at 94 °C for 30 s, at 55 °C for 2 min and at 72 °C for 2 min, followed by the agarose gel-electrophoresis and the ethidium bromide-staining.

CCoV RNA (M gene) and CPV-2 DNA were detected in 55 (50.5%) and 38 (34.9%), respectively, of 109 dogs, while both were detected in 22 dogs (20.2%). The detection rates of CCoV-M and CPV-2 in dogs aged less than 1 year were 66.3% (53/80) and 43.8% (35/80), respectively, while those in dogs aged 1 year or older were 6.9% (2/29) and 10.3% (3/29), respectively, which were significantly different ($p < 0.0001$, $\chi^2 = 30.0$ and $p = 0.0003$, $\chi^2 = 12.9$, respectively) (Table 1). No significant differences were observed for CCoV-M detection rates in regard to gender, breed, or season ($p > 0.05$) (data not shown). The genotypes of CCoV-M positive samples were also examined, as shown in Table 1. CCoV type I was detected in samples from 36 dogs (65.5%), CCoV type II in 40 (72.7%), and both in 22 (40.0%). Of the 36 dogs positive for CCoV type I, 7 (19.4%) were negative for CCoV type II and CPV-2. TGEV-like CCoV was detected in 8 dogs (14.5%), 2 and 6 dogs of which were positive for CCoV type II alone and both types I and II, respectively.

CCoV RNA detection rates determined by RT-PCR in previous reports ranged from 15.6% to 57.3% (Bandai et al., 1999; Decaro et al., 2005, 2010; Mochizuki et al., 2001; Wang et al., 2006; Yesilbag et al., 2004) and the present results (50.5%) were within that range. In previous serological examinations, the seropositivity of juvenile dogs was found to be higher than that of adult dogs, however the difference between those age groups was not as remarkable as that observed in the present study (Priestnall et al., 2007; Soma et al., 2001). As for the causal factors, it is considered that the antibody

is not detected immediately after infection, whereas it is sometimes detected for a prolonged period even after the infection has been terminated. On the other hand, it is considered that detection of virus genes is a more accurate method of determining infection because it reflects viral replication, though CCoV infection have persisted for weeks to months after disappearance of clinical signs in some cases (Pratelli et al., 2002a; Tennant et al., 1993). In the present study, CCoV infection was quite frequent in dogs aged less than 1 year, while it was extremely rare in dogs aged 1 year or older, indicating that CCoV is an important causative organism of diarrhoea in juvenile dogs. Furthermore, simultaneous infections with CCoV and CPV-2 were observed in quite a few dogs (20.2%). On the basis of these findings, it is necessary to use vaccines to prevent not only CPV-2, but also CCoV, especially in juvenile dogs.

Currently, CCoV type I has been reported to be circulating in Europe and China (Benetka et al., 2006; Decaro et al., 2010; Pratelli et al., 2003; Stavisky et al., 2010; Wang et al., 2006; Yesilbag et al., 2004), while the present results indicate that it is also effectively circulating in Japan. The pathogenicity of CCoV type I in dogs has not been clarified, because of failure to isolate the organism (Pratelli, 2006). However, it has been observed that diarrhoea tends to be more serious in dogs with simultaneous infection with CCoV types I and II as compared to in those infected with only CCoV type II (Decaro et al., 2005). The simultaneous infections have been observed in 34.2–76.8% of CCoV RNA-positive dogs (Pratelli et al., 2004; Decaro et al., 2005, 2010). The present result (40.0%) was in the range of those previous reports. On the other hand, 7 of positives for CCoV type I (19.4%) were negative for CCoV type II and CPV-2, indicating that CCoV type I infection may be a potential cause of diarrhoea, though some other factors play roles in diarrhoea.

Thus far, TGEV-like CCoV has been detected in dogs from Europe and the USA (Decaro et al., 2009, 2010; Elia et al., 2010; Erles and Brownlie, 2009). In the present study, this viral gene was detected in 7.3% (8/109) of the dogs with diarrhoea, indicating that this virus is also circulating in Japan, and the prevalence was similar to that reported previously (6.7%) (Decaro et al., 2010). TGEV-like CCoV has poorly serological reactivity with classical CCoV type II, from which CCoV vaccines available currently are derived (Decaro et al., 2009), therefore, the effect of the vaccination may be re-evaluated *in vivo*. However, Erles and Brownlie (2009) detected the viral gene in 12.5% (5/40) of asymptomatic dogs by RT-PCR using the same primer pair as in the present study, indicating that the pathogenicity of TGEV-like CCoV may be low, though it has been observed that this virus induced mild diarrhoea in an experimental infection (Decaro et al., 2010).

Few reports have been published regarding infections with CCoV type I and TGEV-like CCoV. Thus, it is necessary to investigate more cases in the future to clarify the prevalence and pathogenic roles of those viruses.

Table 1
Prevalence of CCoV RNA and CPV-2 DNA in diarrheic dogs.

| Age | CCoV | | | | | CPV-2 | CCoV + CPV-2 |
|--------------------------|------------|-------------------------|-------------------------|-------------------------|------------------------|------------|--------------|
| | M | Type I | Type II | Type I + II | TGEV-like | | |
| Less than 1 year | | | | | | | |
| 1–2 months (n = 60) | 40 | 27 | 31 | 19 | 7 | 32 | 20 |
| 3–4 months (n = 16) | 10 | 7 | 6 | 3 | 1 | 3 | 2 |
| 5–11 months (n = 4) | 3 | 2 | 1 | 0 | 0 | 0 | 0 |
| Subtotal (n = 80) | 53 (66.3%) | 36 (67.9%) ^a | 38 (71.7%) ^a | 22 (41.5%) ^a | 8 (15.1%) ^a | 35 (43.8%) | 22 (27.5%) |
| 1 year or older (n = 29) | 2 (6.9%) | 0 | 2 (100%) ^a | 0 | 0 | 3 (10.3%) | 0 |
| Total (n = 109) | 55 (50.5%) | 36 (65.5%) ^a | 40 (72.7%) ^a | 22 (40.0%) ^a | 8 (14.5%) ^a | 38 (34.9%) | 22 (20.2%) |

^a The percentages of type I, type II, type I + II and TGEV-like indicate the prevalence among CCoV-M gene positives.

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