Canine Coronavirus Infections in Japan: Virological and Epidemiological Aspects

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ABSTRACT. Ten strains, eight field and two reference laboratory strains, of canine coronavirus (CCV) were comparatively examined with respect to antigenic relationships and pathogenic potential in dogs. With monoclonal antibodies and hyperimmune antisera to feline coronavirus and CCV, respectively, varying degrees of antigenic diversities were found among the strains by neutralization and immunofluorescence assays, but it was felt that they belong to one serotype. Specific-pathogen-free puppies experimentally inoculated with some CCV strains manifested clinical symptoms, but there was a difference in their virulence. In order to elucidate the prevalence of CCV infections in dogs in Japan, we tested for neutralizing antibodies to CCV in 467 field dogs, and found a prevalence of 44.1%. Moreover, by using nested reverse transcriptase—polymerase chain reaction on rectal swabs of 100 diarrheic dogs recently presented in veterinary clinics, evidence of CCV in 16% of these specimens was found. The results suggested that CCV infection is more widespread than expected in dogs, and that CCV is a significant etiologic factor in canine diarrhea also in Japan.—KEY WORDS: canine, canine coronavirus, diarrhea, enteritis.

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Canine coronavirus (CCV) was first isolated in 1971 by Binn and coworkers from dogs with diarrhea [6]. Young pups, experimentally challenged with this coronavirus (designated as strain 1–71), developed acute diarrhea. The virus could be demonstrated in the small and large intestines and was excreted in the feces [6, 17, 25]. Since then several CCV strains have been isolated and studied, and as a result it is now generally recognized that CCV is an important etiologic agent which causes diarrhea, vomiting and dehydration, loss of appetite, lethargy, and occasionally sudden death of younger dogs. The persistence and severity of signs can often be related to stress and the presence of parasites, bacteria or infection with other enteric viruses such as canine parvovirus (CPV) [1, 3, 4, 8, 9].

Porcine transmissible gastroenteritis virus and feline coronavirus (FCoV) are serologically cross-reactive with CCV [16, 22]. Genomic analysis also shows a close relationships between these viruses [27], but the number of previously examined CCV isolates is still small because of their presumably fastidious nature and technical difficulties in isolating the viruses in tissue cultures from contaminated feces. Consequently, the antigenic properties of CCV strains have not been examined sufficiently, leaving the possibility that serotypes exist among CCV, as is the case with FCoV, for example.

Meanwhile, CCV was first isolated in Japan in the early 1980s [28], but the antigenic and pathogenic properties of these strains were not studied in detail. Furthermore it is not clear how widespread CCV infection is in dogs in Japan nowadays. In an attempt to understand about CCV infection, we examined eight strains newly isolated from diarrheic dogs, and two American strains as reference with respect to their antigenic and pathogenic characteristics. For serological epidemiology of CCV infection, neutralizing antibodies to CCV in dog serum samples recently collected were determined. In addition, we applied nested reverse transcriptase—polymerase chain reaction (RT-nPCR) for detection of CCV in the recent field diarrheic dog feces.

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MATERIALS AND METHODS

Virus isolation and reference CCVs: Isolation of CCV was tried for feces of diarrheic dogs collected from various parts of Japan during the years 1990 to 1997. A rectal swab was placed in 1 m*l* of Eagle's minimal essential medium (Eagle's MEM), the extract was clarified by centrifugation at 8,500 g for 10 min, and supernatant was inoculated onto the *felis cutus* whole fetus-4 (fcwf-4) cell monolayer in a culture dish. After 1hr of adsorption, the inoculum was removed and the conditioned Eagle's MEM containing 5% fetal calf serum, 10% tryptose phosphate broth and antibiotics (penicillin 200 U, streptomycin 200 μg and kanamycin 20 μg/m*l*) was added. Then they were incubated at 37°C in a humidified chamber containing 5% CO_2. When a cytopathic effect (CPE) was observed, an isolate was identified by an indirect immunofluorescence assay (IFA) with specific monoclonal antibodies (MAb) to FCoV, as described previously [13].

Reference CCV strains 1–71 and TN449 were obtained from Tokyo University, Tokyo and Fort Dodge Laboratories, Iowa, respectively.

MAbs and hyperimmune antisera: A MAb reagent, which is a mixture of MAbs against each nucleocapsid, integral membrane and spike protein of FCoV, was used for

identification of the isolate as a coronavirus. Each was selected from a panel of MAbs previously described [13]. MAbs 5–6–2, 6–4–2, 7–4–1 to FCoV spike protein have been previously described by Hohdatsu *et al.* [14].

Hyperimmune antisera to fc4, fc9, fc76, fc100, 1–71 and TN449 strains were prepared as follows. The virus was grown in the fcwf-4 cell culture and the culture fluid was concentrated by ammonium sulfate precipitation, layered onto a discontinuous sucrose density gradient, and centrifuged at 30,000 rpm for 90 min. The virus band was collected and suspended at 1:1 (vol/vol) in Freund's incomplete adjuvant. This virus antigen was used to immunize guinea pigs twice with a 2-week interval. Blood samples were collected weekly and tested for serum-neutralizing antibodies by a micro-neutralization test (MNT). When the antibody titer reached a plateau, the serum was collected. Prior to use, sera were heat-inactivated at 56°C for 30 min.

IFA: The virus was inoculated onto nearly confluent fcwf-4 cell monolayer cultivated on microslide glasses. When CPE became noticeable, the cells were treated with absolute acetone at room temperature for 10 min, and then they were overlaid with the MAb. After incubation at 37°C for 1 hr in the chamber, they were washed with phosphate-buffered-saline (PBS), and overlaid with fluoresceine isothiocianate conjugated anti-mouse IgG antiserum at 37°C for 1 hr. They were washed again with PBS, mounted in glycerol buffer, and observed under a fluorescence microscope.

Neutralization test: The plaque reduction neutralization test (PRNT) and MNT were described previously [21] and the conditioned Eagle's MEM was used for both tests. Briefly, PRNT was performed by mixing equal volumes of two-fold dilutions of serum and virus suspension containing 100 plaque forming units. After incubation at 37°C for 1 hr, the mixtures were inoculated onto the fcwf-4 cell monolayer. After 1hr adsorption at 37°C, an overlay medium containing 1% noble agar was added. The plate was incubated at 37°C for 2 days in the chamber and stained with 0.1% crystal violet solution. The titer was expressed as the highest serum dilution which showed 75% or more plaque count reduction.

For MNT, 50 μl of serum was serially diluted two-fold in microwells. To each of the wells 50 μl of a virus suspension containing 200 $TCID_{50}$/50 μl was added. After the plate was incubated at 37°C for 1 hr in the chamber, 100 μl of a fcwf-4 cell suspension (150,000 cells/m*l*) was added to each well and the plate was incubated at 37°C for 5 days. The titer was defined as the highest serum dilution at which CPE had been suppressed completely.

Dog serum samples for seroepidemiology: For a seroepidemiological survey, 467 serum samples were collected from dogs which were in the animal shelters in Ibaraki, and were presented in veterinary clinics of various parts of Japan during the years 1993 to 1996. Anti-1–71 neutralizing antibodies were determined by the MNT.

Experimental infection of dogs: A total of eight specific-pathogen-free (SPF) dogs approximately 5-month-old were divided into four groups. They were examined and found to be free of anti-CCV neutralizing antibody before challenge. Two dogs in each group were challenged by either oral or oral and intranasal routes with the strains fc100, fc97–022, 1–71 and TN449, respectively. Doses and routes, as well as virus passage history are summarized in Table 4. On 14 consecutive days after inoculation, clinical conditions including body temperature were monitored. Blood samples were collected before, and on days 7 and 14 after inoculation, and neutralizing antibody titer for each inoculum was determined by MNT. Rectal swabs were also collected and examined for virus excretion by the cell-culturing method daily for 14 days after inoculation.

RT-nPCR assay: The RT-nPCR assay for detection of FCoV previously described by Herrewegh *et al.* [12] was modified for CCV. The oligonucleotide primer sequences were chosen from the highly conserved 3'-untranslated region of coronaviruses [15] (first amplification outer primers 5'-CACTAGATCCAGACGTTAGCTC-3' and 5'-GGCAACCCGATGTTTAAAACTGG-3', and nested internal primers 5'-GCTCTTCCATTGTTGGCTCGTC-3' and 5'-CCGAGGAATTACTGGTCATCGCG-3').

The assay was carried out as follows. A rectal swab was placed in 2 m*l* of PBS and extracted vigorously by vortexing. Insoluble components were removed by centrifugation as mentioned before, then 8.5 μl of the supernatant fraction was added to 3.5 μl of the first premix, consisting of 3 μl of 5x reverse transcriptase (RT) buffer (25 mM Tris-HCl :pH 8.3, 50 mM KCl, 2 mM DTT, 5 mM $MgCl_2$) and 0.5 μl of a diethyl pyrocarbonate-ethanol mixture (0.8% [vol/vol] diethyl pyrocarbonate in 96% ethanol). After leaving at room temperature for more than 5 min, it was incubated at 100°C for 10 min, and then placed on ice. 6.5 μl of RT-mixture, consisting of 5x RT buffer, 10 mM of each dNTP, 30 U of ribonuclease inhibitor (TaKaRa, Tokyo, Japan), 5 μM of primer and 200 U of Moloney murine leukemia virus RT (Wako, Osaka, Japan) was added to the reaction mixture. This reaction was carried out by incubating at 37°C for 60 min. One μl of the product was added to 49 μl of PCR reaction mixture for amplification. This mixture consisted of PCR buffer (100 mM Tris-HCl: pH 8.3, 500 mM KCl, 15 mM $MgCl_2$), 15 mM of each dNTP, 150 mM of each primer and 2 U of Taq DNA polymerase (TaKaRa, Tokyo, Japan). Amplification was performed as follows; preheating at 90°C for 5 min followed by 40 cycles of denaturation at 94°C for 50 sec, primer annealing at 55°C for 1 min, and primer extension at 72°C for 1 min. One μl of the first PCR product was used for the second round amplification with the nested pair of primers in a 50 μl reaction. For the nested PCR, 35 cycles of denaturation at 94°C for 50 sec, primer annealing at 55°C for 1 min, and primer extraction at 72°C for 1 min were employed. The PCR product was analyzed on a 2% agarose gel electrophoresis. Since the final amplified product was predicted to be approximately 180 base pairs in length containing a *Dra* I restriction site the specificity of the product was confirmed by *Dra* I digestion test.

Table 1. CCV strains examined in this study

Strain	Date & place of sample collected		Age of dog	Other viral agents in feces
Field isolates				
fc1	1990	Hyogo	50 days	–
fc4	1990	Hyogo	45 days	–
fc7	1990	Hyogo	46 days	–
fc9	1990	Hyogo	46 days	CPV[a)]
fc76	1990	Aichi	puppy	–
fc100	1991	Chiba	2 months	CPV
fc94–039	1994	Aichi	puppy	–
fc97–022	1997	Tochigi	2 months	–
Reference				
1–71	1972	U.S.A.		
TN449	1980	U.S.A.		

a) CPV: canine parvovirus.

Sensitivity and specificity of the RT-nPCR assay was determined by comparing to the cell culturing method by using CCV 1–71, and the assay was also utilized for 100 fecal swab samples from dogs with diarrhea collected in 1997. These samples were submitted to our laboratory from veterinary clinics in various parts of Japan.

RESULTS

Isolation of virus: Eight CCV strains were isolated from the feces of dogs with diarrhea (Table 1). All reacted positively with the coronavirus-specific MAb reagent in IFA and were identified as coronavirus. They grew in fcwf-4 cells efficiently and showed lytic CPE characterized by syncytium formation but varied in both their infectivity to the cells and their plaque-characteristics (data not shown). As shown in Table 1, CPV was detected in the samples which yielded CCV strains fc9 and fc100. No virus other than CPV was detected in them.

Reactivity with CCV-specific antibodies: The reactivity of three MAbs with ten CCV strains is shown in Table 2. Strain fc76 did not react with MAb 7–4–1, whereas strains fc9 and fc100 did not react with MAb 5–6–2. Strain TN449 showed an ambiguous reaction with MAb 5–6–2.

Reactivity of the six hyperimmune antisera with ten CCV strains by PRNT is shown in Table 3. Each strain was antigenically related to all other strains, but showing various degrees of neutralization. Remarkable reactive titers are indicated by asterisks in the table, and these came out mainly when fc9 and fc100 strains were examined.

Clinicopathological features of experimentally inoculated dogs (Table 4): All dogs inoculated with either TN449 or fc97–022 strain showed signs of diarrhea, vomiting and dehydration 2 days after inoculation, and recovered within 2 to 4 days. On the other hand, the dogs inoculated with strains fc100 and 1–71 did not show any clinical signs of disease. On day 14 post inoculation, 1:8 to 1:32 neutralizing antibody titers were detected in the sera of seven dogs, but not detected in No. 4 dog orally inoculated with the strain 1–71. CCV was recovered from the rectal swabs of only Nos. 5 and 6 dogs infected with fc97–022 on days 2 to 5 post inoculation.

Prevalence of CCV infections: In a seroepidemiological

Table 2. Monoclonal antibody typing of CCV strains by immunofluorescence assay

Strain	Monoclonal antibodies		
	5–6–2	6–4–2	7–4–1
fc1	+[a)]	+	+
fc4	+	+	+
fc7	+	+	+
fc9	–	+	+
fc76	+	+	–
fc100	–	+	+
fc94–039	+	+	+
fc97–022	+	+	+
1–71	+	+	+
TN449	±[b)]	+	+

a) Specific fluorescence was present.
b) Fluorescence was ambiguous.

Table 3. Antigenic relationships among CCV strains determined by plaque-reduction neutralization test

Strain	Immune sera against					
	fc4	fc9	fc76	fc100	1–71	TN449
fc1	320	20	1280	1280	320	1280
fc4	1280	640	1280	1280	1280	640
fc7	640	40	2560	640	160	2560
fc9	160*[a)]	80	320	80*	80*	160*
fc76	1280	640	1280	1280	320	1280
fc100	40**[b)]	20	160*	640	20**	80**
fc94–039	320	40	1280	320	160*	640
fc97–022	320	40	1280	640	320	1280
1–71	640	320	1280	1280	1280	1280
TN449	1280	160	1280	2560	640	2560

a) *: 4–20 × reduction of titers compared to homologous strain.
b) **: ≥ 20 × reduction of titers compared to homologous strain.

Table 4. Experimental infection of SPF dogs with CCVs

Dog No.	Strain	Passage history	Dose & route[a)]	Clinical signs	Antibody titer[b)]	Virus shedding[c)]
1	fc100	5	6.11 P.O.	–	16	–
2				–	8	–
3	1–71	unknown[d)]	7.51 P.O.	–	8	–
4				–	<2	–
5	fc97–022	4	6.30 P.O.+I.N.	+	16	4
6				+	8	2–5
7	TN449	13	6.51 P.O.+I.N.	+	32	–
8				+	16	–

a) P.O.; per os, I.N.; intranasal.
b) Neutralizing antibody titer in the serum collected 14 days after inoculation.
c) Viral recovery from rectal swabs was shown by the day after inoculation.
d) The strain was passaged in the fcwf-4 cell culture six times in our laboratories, but previous passage history is unknown.

survey, anti-1–71 neutralizing antibodies were determined in 467 dogs by the MNT, and a titer less than 1:4 was temporarily regarded as negative. The results of this study found a positive rate of 44.1% (206/467), and neutralizing antibody titers between 1:4 and 1:512 were detected with a 1:19.3 geometric mean titer.

To determine whether the RT-nPCR assay applied in the present study detects CCV correctly, *in vitro* passaged CCV strains were subjected to RT-nPCR, and PCR products of expected size were obtained in all strains. Digestion of the products by *Dra* I yielded the restriction fragment pattern expected from sequence data, showing that this assay works as coronavirus specific (data not shown). Sensitivity of the RT-nPCR assay was determined by comparing it to the cell culturing method with CCV 1–71 strain. Virus quantities as low as $10^{0.2}$ $TCID_{50}$ were detected with the assay (data not shown). In the survey of CCV prevalence in 100 diarrheal stool samples by means of the assay, CCV RNA was detected in 16 samples (16%). On the other hand, virus isolation by the cell-culturing method with fcwf-4 cells was successful in only two samples. CPV was detected in 44 of the same 100 samples by the cell-culturing method with Madin-Darby canine kidney cells (unpublished data), and nine cases (9%) were diagnosed as a mixed CCV and CPV infection.

DISCUSSION

A previous study had shown cross-reaction of CCV strains by using sera of cats with feline infectious peritonitis [5], but only three strains were compared. In this study we compared the antigenic relationships by using eight CCV strains newly isolated from Japanese dogs and two known North American CCV strains, and they were regarded as the same serotype with only a little antigenic difference: 1) some strains did not react with the MAbs (Table 2), suggesting that there was an epitope lacking, and 2) some strains, especially fc9 and fc100 strains, did not react with hyperimmune sera as other strains did, but there may be some differences between fc9 and fc100 strains in antigenic structures (Table 3). The latter novel result may be related to the fact that these strains seem to lack an antigenic epitope defined by the MAb 5–6–2. Another possibility is that the epitope is denatured for these strains. Further analysis with more isolates, therefore, might lead to a different conclusion, i.e. that CCV is divided into multiple serotypes, because coronaviruses are generally regarded as causing mutation and recombination more occasionally than ever anticipated. For example, FCoV can be divided into serotypes I and II [23] and it has been considered that serotype II FCoVs, is generated from recombination between serotype I FCoV and CCV in nature [26].

We could not reproduce previous results in which strain 1–71 caused diarrhea in neonatal dogs [17, 25], but fc97–022 and TN449 strains did cause diarrhea as well as other clinical abnormalities in experimentally inoculated 5-month-old dogs. This may be due to several factors such as the age of the animals used in this and other studies, passage history *in vitro* of virus inoculum or route of inoculation. In particular, the use of younger dogs may result in causing more severe clinical conditions. In the present study, only four CCV strains were studied for their pathogenic potency and the experiment itself was not fully designed, so that it was not possible to say which factor most influenced the pathogenicity of CCV.

Widespread CCV infection in dogs was detected in the U.S.A. by electron microscopic examinations of feces as well as seroepidemiological methods [2, 7, 8, 11, 19, 24], but in Japan few such studies have been published, and recent situation concerning CCV infection is also obscure. In this study we found that a high percentage (44.1%) of dogs had antibodies to CCV, in accordance with the results of a study done in 1983 which found a percentage of 46.2% out of 13 dogs [28]. These results suggest that CCV has been widespread in dogs in Japan.

Although diagnostic tests based on a RT-nPCR assay for

FCoV infection in cats have been described [10, 12, 18], no such molecular method for the diagnosis of CCV infection had previously been developed. The results obtained here indicate that the present modified method can also be applied to CCV infections. On examining rectal swabs of 100 diarrheic dogs, evidence of CCV in 16% of these specimens were found. A previous study reported that CCV was isolated by the cell-culturing method from only 1.5% of diarrheic dogs in Japan [20], and 2% of the same specimens as for RT-nPCR were positive in the present study. These results suggest that the RT-nPCR assay is superior to the cell-culturing virus isolation method as to sensitivity for the detection of CCV. The results obtained by means of the RT-nPCR method also indicated that CCV might be more frequently associated with canine diarrhea than had been previously assumed.

It has been generally considered that CPV causes fatal disease in dogs, and CCV causes a self-limiting and non-fatal disease [2–4, 8, 9], but during the recovery phase of a CCV infection rapidly dividing crypt cells would provide ideal replication sites for CPV, enabling an aggravated CPV infection [1]. This scenario would make CCV infection a far greater threat to the animal's health. The present study demonstrated that mixed infections with CCV and CPV are indeed common in the field, as expected previously [28]. This suggests that it may be as important to protect dogs against infection with CCV as it is to vaccinate against CPV.

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