

Prevalence of canine coronavirus (CCoV) in dog in Japan: detection of CCoV RNA and retrospective serological analysis

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ABSTRACT. We collected rectal swabs from dogs in Japan during 2011 to 2014, and canine coronavirus (CCoV) nucleocapsid gene was detected by RT-PCR. The relationship between CCoV infection and the manifestation of diarrhea symptoms was investigated, and a correlation was noted ($df=1$, $\chi^2=8.90$, $P<0.005$). The types of CCoV detected in samples from CCoV-infected dogs were CCoV-I in 88.9% and CCoV-II in 7.4%, respectively. We retrospectively investigated the seroprevalence of CCoV-I in dogs in Japan during 1998 to 2006. The sera were tested with a neutralizing antibody test. In the absence of CCoV-I laboratory strain, we used feline coronavirus (FCoV)-I that shares high sequence homology in the S protein with CCoV-I. 77.7% of the sera were positive for neutralizing anti-FCoV-I antibodies.

KEY WORDS: canine coronavirus, RT-PCR, serological prevalence, serotype

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Coronavirus is a single positive-strand RNA virus with a genome size of approximately 30kbp and has been classified into the Family *Coronaviridae*, Subfamily *Coronavirinae*. Subfamily *Coronavirinae* is further classified into 4 genera: *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus* and *Deltacoronavirus* [4]. Canine coronavirus (CCoV) of the Genus *Alphacoronavirus* and canine respiratory coronavirus of the Genus *Betacoronavirus* have been identified in dogs [4].

Since CCoV is classified into types I (CCoV-I) and II (CCoV-II) based on differences in the spike (S) protein amino acid sequence [8]. In addition, the CCoV-I genome contains a CCoV-I-specific ORF-3 gene, and CCoV-I and -II can also be distinguished based on the presence or absence of this gene [7]. Feline coronavirus (FCoV) of the genus *Alphacoronavirus* is also classified into types I (FCoV-I) and II (FCoV-II), as with the CCoV [6, 10]. The S protein sequence is highly homologous between CCoV-I and FCoV-I and between CCoV-II and FCoV-II [8]. The homology of the S protein amino acid sequence is about 80% between CCoV-I and FCoV-I, whereas it is about 50% between CCoV-I and CCoV-II/FCoV-II [8]. FCoV-I and FCoV-II/CCoV-II can be distinguished by neutralization tests [10].

CCoV-II-infected dogs develop enteritis and gastroenteritis, and the condition is aggravated by mixed infection with CCoV-II and other pathogens [8, 16]. CCoV-I viral genes have been detected mainly in pups with diarrhea symptoms

[11], but CCoV-I has not yet been successfully isolated, and many points remain unclear. CCoV infection has been observed worldwide, but no seroprevalence study of CCoV-I has been performed.

In the present study, we collected rectal swabs from dogs in Japan during 2011 to 2014, and CCoV nucleocapsid (N) gene from samples of dogs was detected by RT-PCR. We also investigated CCoV infection and its relationship with age, sex breed and clinical condition of dogs. Moreover, we retrospectively analyzed sera from dogs in Japan during 1998 to 2006.

Rectal swab samples were collected from 101 dogs between 2011 and 2014. These samples were submitted by veterinary clinics in Japan (Aomori, Tochigi, Ibaraki, Saitama, Chiba, Tokyo, Kanagawa, Osaka, Hyogo, Kochi and Okinawa). Viral RNA was extracted from rectal swabs using the High Pure Viral RNA Isolation Kit (Roche, Basel, Switzerland) following the attached instructions. cDNA was amplified by PCR using specific primers as shown in Table 1. PCR was performed using the method of Takano *et al.* [13]. ELIF-ELIR used to detect CCoV-I also detects FCoV-I. Thus, the ORF3f-ORF3r primer set which specifically detects only CCoV-I was also used. The CCoV gene was detected in rectal swab samples collected from dogs. CCoV N gene was detected in 27/101 (26.7%). CCoV-I S gene, CCoV-II S gene and CCoV-I specific ORF3 gene were detected in 21/101 (20.8%), 2/101 (2.0%) and 24/101 (23.8%), respectively. On the basis of the rate of CCoV N gene- and ORF3 gene-positive CCoV-infected dogs, the type of CCoV detected in samples from CCoV-infected dogs was CCoV-I in 88.9%. The CCoV-I S gene fragments (PCR products generated using ELIF-ELIR) were sequenced as described by Takano *et al.* [15]. Amino acid sequences were deduced from the CCoV-I S gene detected in 4 dogs with diarrhea symptoms (Nos.9, 19, 27 and 33) and 2 dogs without diarrhea symptoms (Nos. 55 and 73) (Fig. 1A). All of 6 CCoV

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Table 1. Primer sequences used for RT-PCR

Target gene	Oligonucleotide	Orientation	Nucleotide sequence	Length	Reference
CCoV N	CENP1	Forward	5'-ctcgtggycggaagataat-3'	280	[3]
	CENP2	Reverse	5'-gcaaccagamraccatc-3'		
CCoV-I S	EL1F	Forward	5'-caagtggaccgtcttattactgtag-3'	346	[11]
	EL1R	Reverse	5'-tcatatacgtaccattatagctgaaga-3'		
CCoV-II S	S5	Forward	5'-tgcattgtgtctcagactt-3'	694	[11]
	S6	Reverse	5'-3'ccaaggccattttacataag		
CCoV ORF3	ORF3F	Forward	5'-cactaaactcaaaatgttgattc-3'	628	[7]
	ORF3R	Reverse	5'-ttaaggattaaaaacatattcta-3'		

A

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No. 9      SSTLTQYTEVKASRQLAMEKVNCEVKVKSQSDRYGFCGNGTHLFLSLANAAPDGLLFLHTVLLPTEWEEVMAWSGICVND--TYAYVLKDFKSSIFSNGT
No. 27     *****
No. 55     *****
No. 19     *Q*****
No. 33     *Q*****
No. 73     *Q*****
CCoV Elmo/02 AQ*****
CCoV 982-1  AQ*****
FCoV KU-2   *Q****A*****I*L*****N*****V*S**E**F*****T*****DH*****
CCoV 1-71   *Q***RQA**R*****KD*****R***Q*F*****N*MI*F*****AY*T*T*****AS*GDR*FGL*L*VQLTL*RNLLD
    
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B

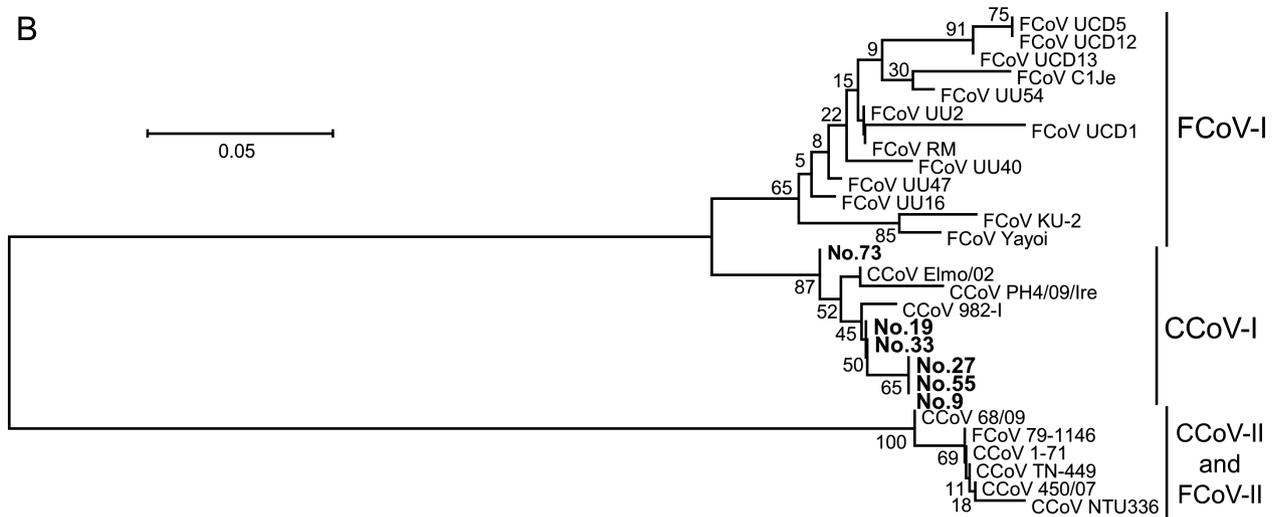


Fig. 1. Alignment and phylogenetic relationship of S protein of CCoV and FCoV. A) Alignment of the deduced amino acid sequence of the 99 amino acids of the C-terminal of S protein in CCoV strains detected in study and other CCoV and FCoV. A dot indicates conserved amino acid identity. B) A phylogenetic tree prepared based on the amino acid sequence of the C-terminal of S protein. The phylogenetic analysis is based on the deduced amino acid sequence of the 99 amino acids of the C-terminal of S protein. Phylogenetic relationships were determined using the neighbor-joining algorithm, and branching order reliability was evaluated by 1,000 replications of a bootstrap resampling analysis. Bold letters represent new CCoV detected in this study.

strains detected in this study were classified into the clade of the other CCoV-I (Fig. 1B).

We investigated CCoV infection and its relationship with age, sex, breed and clinical condition of dogs (Table 2). No significant association was found between CCoV infection and age in dogs. Sex and breed were also shown to be not related to CCoV infection. On the other hand, there was statistically significant correlation between CCoV infection and

diarrhea symptoms. When the relationship between CCoV infection and the manifestation of diarrhea symptoms was investigated using the χ^2 test, a correlation was noted ($df=1$, $\chi^2=8.90$, $P<0.005$). Similar results were obtained when the prevalence of CCoV-I infection was analyzed (Table 2, CCoV-I S and CCoV ORF3). Soma *et al.* [12] reported that dogs younger than one year old are infected with CCoV at a higher rate than one-year-old or older dogs. Based on their

Table 2. Prevalence of CCoV infection by age, sex, breed and clinical status for dogs

Clinical status	Status	No. of dogs	CCoV N		CCoV-I S		CCoV-II S		CCoV ORF3 (CCoV-I specific)		
			No. of positive	Rate (%)	No. of positive	Rate (%)	No. of positive	Rate (%)	No. of positive	Rate (%)	
Healthy	Age	<3 month	14	2	14.3	1	7.1	0	0.0	1	7.1
		3–11 month	12	1	8.3	0	0.0	1	8.3	0	0.0
		1–2 year	13	2	15.4	2	15.4	0	0.0	2	15.4
		>2 year	12	2	16.7	1	8.3	0	0.0	2	16.7
	Sex	Male	27	4	14.8	2	7.4	1	3.7	2	7.4
		Female	24	3	12.5	2	8.3	0	0.0	3	12.5
	Breed	Pure bred	45	6	13.3	3	6.7	1	2.2	4	8.9
		Mix breed	6	1	16.7	1	16.7	0	0.0	1	16.7
Diarrhea	Age	<3 month	15	7	46.7	5	33.3	1	6.7	6	40.0
		3–11 month	13	6	46.2	6	46.2	0	0.0	6	46.2
		1–2 year	9	3	33.3	2	22.2	0	0.0	3	33.3
		>2 year	13	4	30.8	4	30.8	0	0.0	4	30.8
	Sex	Male	27	12	44.4	11	40.7	0	0.0	13	48.1
		Female	23	8	34.8	6	26.1	1	4.3	6	26.1
	Breed	Pure bred	46	19	41.3	17	37.0	0	0.0	19	41.3
		Mix breed	4	1	25.0	0	0.0	1	25.0	0	0.0

report, we investigated the correlation between the age and CCoV infection in dogs with diarrhea symptoms. In these dogs, the CCoV infection rate was higher in dogs younger than one year old compared to older dogs (46.4%, of less than 1 year, and 31.8% of over 1 year, respectively; Table 2).

We retrospectively investigated the seroprevalence of CCoV-I in dogs in Japan during 1998 to 2006. Serum samples from 695 dogs collected from various regions throughout Japan were examined for the prevalence of antibodies to FCoV-I and CCoV-II by neutralization test. These samples were submitted to veterinary clinics in Japan: the Hokkaido region (Hokkaido); the Tohoku region (Aomori, Akita, Miyagi, Yamagata and Fukushima); the Kanto region (Tochigi, Ibaraki, Gunma, Saitama, Chiba, Kanagawa and Tokyo); the Chubu region (Niigata, Nagano, Toyama, Ishikawa, Fukui, Shizuoka, Yamanashi, Aichi and Gifu); the Kinki region (Osaka, Kyoto, Nara, Shiga, Mie and Hyogo); the Chugoku region (Okayama, Hiroshima, Tottori and Yamaguchi); the Shikoku region (Tokushima, Ehime and Kochi); and the Kyushu region (Fukuoka, Nagasaki, Kumamoto, Oita and Miyazaki). Of the 695 serum samples used, 405 were collected from dogs with an unclear vaccination history. The remaining 290 samples were collected from dogs previously treated with CCoV vaccine. Neutralization test was performed by using modified method based on the report described by Takano *et al.* [14]. Briefly, Serial two-fold dilutions of the test sera were mixed with an equal volume of FCoV-I KU-2 strain (isolated in our laboratory) or CCoV-II 1–71 strain (supplied by Dr. E. Takahashi of the University of Tokyo) suspension containing approximately 200 TCID₅₀/100 μ l, and mixtures were incubated at 37°C for 60 min. Each mixture was inoculated into fcfw-4 cells in 96-well microplates, followed by incubation at 37°C for 3 days in an atmosphere of 5% CO₂. The antibody titer was expressed as a recipro-

cal of the highest dilution of the test sera that completely inhibited the cytopathic effect.

540 (77.7%) of the 695 sera were positive for neutralizing anti-FCoV-I antibodies, and the neutralizing antibody titers ranged from 1:5 to 1:80. Neutralizing anti-FCoV-I antibodies were detectable in sera collected in 1998 (positive rate: 86.7%, 52 of 60 serum samples). The antibody-positive rate tended to decrease after 2001.

The neutralizing anti-FCoV-I and anti-CCoV-II antibody titers were measured in 290 serum samples from pups with a history of vaccination against CCoV-II. No correlation was found between neutralizing anti-FCoV-I and neutralizing anti-CCoV-II antibody titers in 290 of serum samples ($r=-0.196$; Fig. 2).

We herein detected CCoV gene in dogs in Japan. CCoV gene detection in dogs in Japan has been performed, similarly to our study. The CCoV gene-positive rates in our study and reported by Bandai *et al.* [1] and Soma *et al.* [12] were 26.7, 16.0 and 50.5%, respectively, showing variation among the studies, and this may have been influenced by differences in the target gene. Moreover, the age and maintenance environment of the dogs may also have influenced the results of RT-PCR. It is desirable for an epidemiological survey of CCoV infection to be performed specifying a primer set with the highest sensitivity and specificity.

We investigated the relationship between CCoV infection and differences in the age, sex, breed and clinical status of dogs. There was no correlation between CCoV infection and the age, sex or breed of dogs, but when the dogs were limited to those with diarrhea symptoms, the infection rate was higher in dogs younger than one year old, as reported by Soma *et al.* [12]. Based on this finding, CCoV-infected dogs younger than one year old are likely to develop diarrhea, whereas one-year-old or older dogs are unlikely to develop

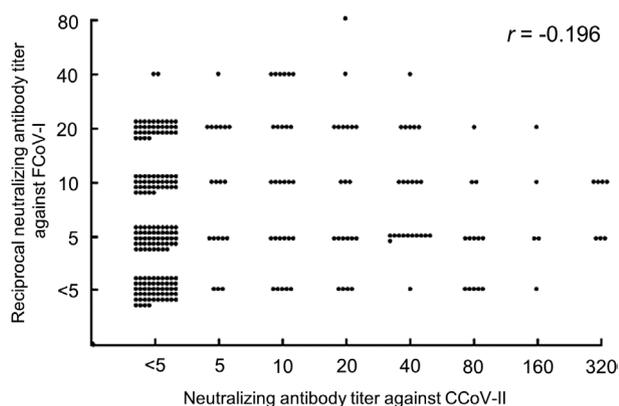


Fig. 2. Relationship between titers of neutralizing antibodies to FCov-I and CCoV-II.

diarrhea, although they are infected with CCoV. However, the relationship between the age and CCoV infection in dogs should be comprehensively judged based on the presence or absence of mixed infection with other pathogens, the host's immune condition and environment in which dogs are maintained.

The ORF 3 gene was detected in 88.9% of the CCoV-infected dogs. Since the CCoV ORF 3 gene is specific to CCoV-I [7], this finding confirmed the presence of CCoV-I in Japan, as reported by Soma *et al.* [12], and suggested that CCoV-I is dominant in CCoV-infected dogs in Japan. When the CCoV-I infection rate was compared between dogs younger than 3 months old and 3-month-old or older dogs, no difference was noted, and the CCoV infection rate in dogs younger than 3 months old was similar to that in 2-year-old or older dogs. These findings suggest that CCoV-I infects dogs regardless of the age.

The presence of CCoV-I-infected dogs was confirmed in 2007 in Japan and 2002 in Italy [11, 12], suggesting that CCoV-I-infected dogs were already present earlier than 2007 in Japan. Thus, using canine sera collected between 1998 and 2006, the presence of anti-CCoV-I antibody earlier than 2007 was investigated employing the neutralizing antibody test. However, in the absence of CCoV-I laboratory strain, we used FCov-I that shares high sequence homology in the S protein with CCoV-I. Neutralizing anti-FCov-I antibodies were detected in serum samples from dogs in Japan. It was assumed that the neutralizing anti-FCov-I antibody-positive dogs had been: i) infected with CCoV-I showing high-level genetic homology with FCov-I or ii) infected with FCov-I. It is also strongly suggested that CCoV-I-infected dogs and FCov-I-infected dogs were mixed in the dogs from which sera were collected. However, regarding ii), to our knowledge, infection of a dog with FCov-I has not been reported. In any case, it was clarified that 77.7% of dogs in Japan possess antibodies against FCov-I and/or CCoV-I. It is suggested that FCov-I and/or CCoV-I are widely prevalent in dogs in Japan since 1998. To closely investigate CCoV-I or FCov-I infection in dogs, it is necessary to perform similar tests using both CCoV-I and FCov-I. However, no assay detecting

only anti-CCoV-I antibodies is available. Development of a method to detect anti-CCoV-I antibodies is desired.

The CCoV-I infection rate may be influenced by the maintenance environment of dogs. All samples were derived from household dogs, but it was unclear whether or not they were maintained alone or in a group. Considering the presence of cats infected with CCoV-like virus, it is also necessary to investigate maintenance with a cat. It is necessary to perform an epidemiological survey of CCoV-I taking these into consideration.

In Japan, CCoV-II vaccine is administered to dogs to prevent CCoV-II infection. We investigated whether there was a correlation between neutralizing anti-FCov-I and anti-CCoV-II antibody titers in pups treated with CCoV-II vaccine, and no correlation was noted, suggesting that neutralizing anti-FCov-I antibodies in the serum samples of the dogs were not produced in response to CCoV-II infection, and CCoV-I infection cannot be prevented by the conventional CCoV-II vaccine. The rate of FCov-I and/or CCoV-I-infected dogs decreased from 2000 to 2006. However, when healthy dogs and those with diarrhea symptoms were compared, the CCoV infection rate was significantly higher in the latter. In addition, more than 90.0% of CCoV-infected dogs with diarrhea symptoms were infected with CCoV-I. Based on these facts and the findings of this study, the development of a vaccine capable of preventing CCoV-I infection is desired.

Generally, CCoV-infection has been recognized as an infectious disease causing mild diarrhea, but severe systemic disease characterized by hemorrhagic gastroenteritis and leukopenia in CCoV-II-infected dogs have recently been reported [2, 9]. At present, such serious symptoms in CCoV-I-infected dogs have not been reported, but it has been clarified that: i) coronaviral genomes readily mutate in hosts and ii) genomic recombination with another coronavirus causes large-scale mutation of coronavirus [5, 17]. On the basis of these facts, CCoV-I may acquire the pathogenicity of CCoV-II and become the novel virulent CCoV-I.

We surveyed CCoV infection and the age, sex, breed and clinical status of dogs. CCoV infection was correlated with diarrhea symptoms. In CCoV-infected dogs, the CCoV-I infection rate was very high, and the presence of CCoV-I infected dogs in Japan at least from 1998 was suggested. This study confirmed previously reported findings concerning CCoV infection in dogs in Japan and provided new information.

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