FULL PAPER *Virology*

The Prevalence of a Group 2 Coronavirus in Dogs in Japan

Takashi KANESHIMA[1)], Tsutomu HOHDATSU[1)]*, Kumi SATOH[1)], Tomomi TAKANO[1)], Kenji MOTOKAWA[2)] and Hiroyuki KOYAMA[1)]

[1)]Department of Veterinary Infectious Diseases, School of Veterinary Medicine and Animal Sciences, Kitasato University, Towada, Aomori 034–8628 and [2)]Research Center for Biologicals, The Kitasato Institute, Kitamoto, Saitama 364–0026, Japan

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ABSTRACT. Canine coronavirus (CCoV) has been reported to cause acute diarrhea mainly in young pups. CCoV and feline coronavirus are classified as group 1 coronaviruses. However, it has recently been reported in the United Kingdom that the group 2 coronavirus gene, which is more closely related to the bovine coronavirus (BCoV) and human coronavirus strain OC43, has been detected in respiratory tract tissue samples from dogs with respiratory disease. In this study, we examined the prevalence of antibodies to group 2 coronaviruses in domestic dogs and cats in Japan by a neutralization test using BCoV. All 104 feline serum samples were negative (<1:5) for anti-BCoV antibodies. In contrast, of the 898 canine serum samples, 160 (17.8%) were positive for anti-BCoV antibodies, and the antibody titers ranged from 1:5 to more than 1:640, with 1:160 being the most frequent. No correlation was found between the titers of the anti-BCoV and anti-CCoV antibodies in the 198 serum samples of dogs with a known history of CCoV vaccination. We amplified, by RT-PCR, group 2 coronavirus-specific hemagglutination/esterase genes in the oral swabs of a total of 10 young pups presenting with or having recovered from respiratory signs, or having anti-BCoV antibodies, with the result that 2 pups were positive for the hemagglutination/esterase genes. These results strongly suggest that an unknown group 2 coronavirus as well as the known enteritis-causing CCoV (group 1 coronavirus) is prevalent among domestic dogs in Japan.

KEY WORDS: bovine coronavirus, canine coronavirus, group 2 coronavirus, respiratory disease.

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The causative agent of severe acute respiratory syndrome (SARS) that originated in Asia and spread all over the world in 2003 was identified as a new coronavirus; however, the route of infection and animal sources of infection have not been identified [5–7]. For many years, animal coronaviruses have been known to induce mainly digestive or respiratory disease in pigs, chickens, cattle, cats, and dogs, and preventive vaccinations have been conducted. It is known that new viruses tend to emerge from existing coronaviruses, which have RNA as genetic information, as a result of interspecies recombination or genetic deletions [11]. The coronaviruses, including the newly-emerged SARS virus, are currently classified into 4 distinct antigenic groups based on sequence analysis of their genomes and antigenic relationships.

Canine coronavirus (CCoV) is known as a causative agent of moderate to severe enteritis in young pups, and is classified as group 1 coronaviruses, together with feline coronavirus (FCoV), transmissible gastroenteritis virus (TGEV), human coronavirus strain 229E, and porcine epidemic diarrhea virus [9,10]. However, in 2003, Erles *et al*. [4] reported that, in tissue samples collected from the respiratory tract of dogs with respiratory disease in a large rehoming kennel, they detected by RT-PCR what appeared to be the group 2 coronavirus gene, which was more closely related to the bovine coronavirus (BCoV) and human coronavirus strain OC43 than to the CCoV gene. Canine parainfluenza virus (CPIV) [2], canine adenovirus type 2 (CAV-2) [3], and the bacterium Bordetella bronchiseptica [1] are the major causative pathogens of canine infectious respiratory disease. However, in the rehoming kennel, infectious respiratory disease remained prevalent in newly-introduced dogs despite vaccinations against these causative pathogens. Using respiratory tract tissue samples from dogs with infectious respiratory disease, they performed RT-PCR with conserved primers for the coronavirus polymerase gene, and demonstrated that a coronavirus was involved in canine infectious respiratory disease.

In this study, to investigate the prevalence of new coronaviruses in dogs and cats, we examined first the carrier state of antibodies to group 2 coronaviruses in domestic dogs and cats in Japan. Next, we attempted to detect viral genes by RT-PCR targeting group 2 coronavirus-specific hemagglutination/esterase genes in young pups with respiratory signs or having recovered from respiratory signs, or having anti-group 2 coronavirus antibodies.

MATERIALS AND METHODS

Cells and viruses: HRT-18G human rectal tumor cells were grown, at 37°C in a humidified atmosphere of 5% CO_2, in monolayers in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal calf serum (FCS), 100 U/m*l* penicillin, and 100 μg/m*l* streptomycin. Felis catus whole fetus (*fcwf*)-4 cells were cultured as monolayers at 37°C in Eagle's minimum essential medium (MEM) containing 50% Leibovitz's L-15 medium, 10% FCS, 100 U/m*l* penicillin, and 100 mg/m*l* streptomycin. The BCoV strain

* CORRESPONDENCE TO: HOHDATSU, T., Department of Veterinary Infectious Diseases, School of Veterinary Medicine and Animal Sciences, Kitasato University, Towada, Aomori 034–8628, Japan.

Mebus was grown in HRT-18G cells at 37°C. The CCoV strain 1–71 was grown in fcwf-4 cells at 37°C. The BCoV strain Mebus and CCoV strain 1–71 were supplied by Dr. H. Ohhori of Kitasato University, Japan and Dr. E. Takahashi of the University of Tokyo, Japan, respectively.

Serum samples: A total of 898 canine serum samples collected between 1998 and 2004 were used. These samples were submitted by private veterinarians in the Hokkaido region (Sapporo and Ishikari), the Tohoku region (Aomori, Yamagata, Miyagi, Akita, and Fukushima), the Kanto region (Saitama, Kanagawa, Tokyo, Chiba, Gunma, Tochigi, and Ibaraki), the Koshinetsu and Hokuriku regions (Niigata, Yamanashi, Toyama, Ishikawa, Fukui, and Nagano), the Tokai region (Aichi, Shizuoka, Mie, and Gifu), the Kinki region (Osaka, Kyoto, Hyogo, Shiga, and Nara), the Chugoku region (Hiroshima, Tottori, Okayama, and Yamaguchi), the Shikoku region (Tokushima, Ehime, and Kochi), and the Kyushu region (Fukuoka, Nagasaki, Kumamoto, Oita, and Miyazaki). One hundred and four feline serum samples collected in Saitama Prefecture between 2001 and 2002 were used.

Neutralization (NT) test: Anti-BCoV and anti-CCoV NT antibody titers were determined in HRT-18G and fcwf-4 cells, respectively. Serial two-fold dilutions of serum samples were mixed with an equal volume of a virus suspension containing approximately 200 $TCID_{50}$, and the mixtures were incubated at 37°C for 60 min. Each mixture was then inoculated into cell cultures in flat-bottomed microplates, followed by incubation at 37°C for 4 days in an atmosphere of 5% CO_2. All NT tests were performed in duplicate. The antibody titer was expressed as the reciprocal of the highest dilution of the test serum that completely inhibited the viral cytopathic effect.

Isolation of RNA from oral swabs: Viral RNA was extracted from oral swabs using a SepaGene RV-R (Sanko Junyaku Co., Ltd., Tokyo, Japan) according to the instructions of the manufacturer. RNA preparations were treated with 150 U of RNase-free DNase (TaKaRa Bio Inc., Kyoto, Japan) at 37°C for 20 min in the presence of 30 U of placental RNase inhibitor (PerkinElmer, Conn., U.S.A.).

RT-PCR: cDNA synthesis and PCR were performed in a one-step reaction using the RNA preparation from an oral swab as a template, Ready-To-Go RT-PCR Beads (Amersham Biosciences, UK), and a set of primers annealing to the BCoV hemagglutination/esterase (HE) gene (sense primer, positions 418–437, 5'-TATCGCAGCCTTACTTTTGT-3'; antisense primer, positions 914–896, 5'-ACCGCCGTCATGTTATCAG-3'), according to the instructions of the manufacturer. The reverse transcription of RNA preparations to cDNA was performed at 42°C for 30 min, followed by inactivation of the reverse transcriptase and the first denaturation of the template DNA at 95°C for 5 min. Subsequently, DNA amplification was performed for 35 cycles of denaturation for 1 min at 95°C, annealing for 40 sec at 50°C, and synthesis for 1 min at 72°C, with a final incubation step at 72°C for 10 min. The second PCR using the same primers was performed in a total volume of 50 μl in a 0.2-m*l* PCR tube. One microliter of PCR products was mixed with 5 μl of 10 × PCR buffer, 5 μl of 2.5 mM dNTPs, 1 μl of 50 μM primers, 0.25 μl of Recombinant Taq DNA Polymerase (5 units/μl; Takara, Kyoto, Japan), and 37.75 μl of distilled water. Amplification was performed in the same way as the first PCR. Five-microliter aliquots of the second PCR products were analyzed by electrophoresis on an 8% polyacrylamide gel. Bands were visualized by ethidium bromide staining.

Virus isolation: An oral swab was suspended in 1 m*l* of MEM with 2% FCS, and centrifuged at 2,500 rpm for 15 min. HRT-18G cells and fcwf-4 cells, cultured in 48-well plates, were inoculated with the supernatant (0.1 m*l*/well), and incubated for 1 hr at 37°C for virus adsorption. The cells were washed with MEM with 2% FCS, and cultured in maintenance medium (with 2% FCS) at 37°C for 4 days. The cultures were passaged three times, with no cytopathic effect. Subsequently, RNA was extracted from the cells, and RT-PCR of group 2 coronavirus was performed using the primers for the HE gene.

RESULTS

Detection of neutralizing antibodies to group 2 coronaviruses in domestic dogs and cats in Japan: A total of 898 canine serum samples collected in various regions of Japan and 104 feline serum samples collected in Saitama Prefecture were examined for the prevalence of antibodies to group 2 coronaviruses by a neutralization test using BCoV. All 104 feline serum samples were negative (<1:5) for neutralizing anti-BCoV antibodies. In contrast, 160 (17.8%) of the 898 canine serum samples were positive for neutralizing anti-BCoV antibodies, and the neutralizing antibody titers ranged from 1:5 to more than 1:640, with 1:160 being the most frequent (Fig. 1). By region, the antibody-positive rates were 25.0% (8 of 32) in the Hokkaido region (Sapporo and Ishikari), 26.9% (14 of 52) in the Kinki region (Osaka, Kyoto, Hyogo, Shiga, and Nara), 21.2% (102 of 481) in the Kanto region (Saitama, Kanagawa, Tokyo, Chiba, Gunma, Tochigi, and Ibaraki), and 20.2% (17 of 84) in the Tokai region (Aichi, Shizuoka, Mie, and Gifu), followed by 18.8% (9 of 48) in the Kyushu region (Fukuoka, Nagasaki, Kumamoto, Oita, and Miyazaki), 13.6% (3 of 22) in the Koshinetsu and Hokuriku regions (Niigata, Yamanashi, Toyama, Ishikawa, Fukui, and Nagano), 13.5% (5 of 37) in the Chugoku region (Hiroshima, Tottori, Okayama, and Yamaguchi), 1.7% (2 of 115) in the Tohoku region (Aomori, Yamagata, Miyagi, Akita, and Fukushima), and 0% (0 of 27) in the Shikoku region (Tokushima, Ehime, and Kochi). Neutralizing anti-BCoV antibodies were also detectable in serum samples collected in 1998 (positive rate: 5%, 3 of 60 serum samples).

Relationship between neutralizing anti-BCoV antibody titer and neutralizing anti-CCoV antibody titer: Of the 198 serum samples collected in Saitama Prefecture (including 146 samples from pups with a history of vaccination against CCoV), 75 (51.4%) were positive for neutralizing anti-

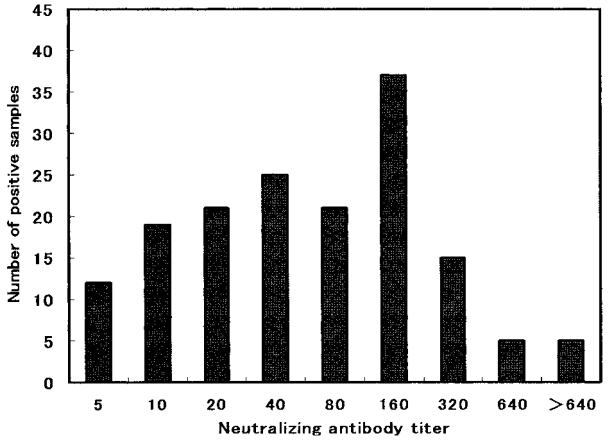

Fig. 1. Distribution of neutralizing anti-BCoV antibody titers among canine serum samples collected in Japan.

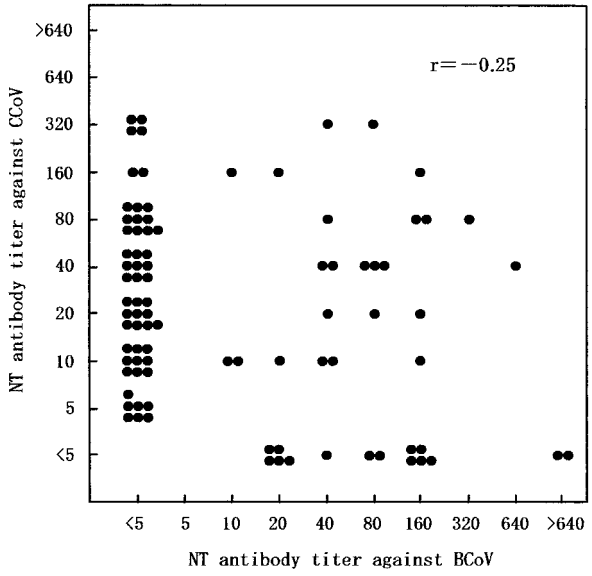

Fig. 2. Relationship between titers of neutralizing antibodies to BCoV and CCoV.

CCoV antibodies (>1:5) with titers ranging from 1:5 to 1:320, and about the same numbers (13–15) of samples had titers of 1:10, 1:20, 1:40, and 1:80, respectively. No correlation was found between the neutralizing anti-BCoV and anti-CCoV antibody titers in these 198 serum samples (Fig. 2).

Detection of coronavirus HE gene in oral swabs from young pups: We attempted to detect group 2 coronaviruses by RT-PCR with group 2 coronavirus specific-primers for the HE gene, and confirmed the specificity of the RT-PCR using the BCoV strain Mebus (group 2 coronavirus), CCoV strain 1–71 (group 1 coronavirus), and FCoV strain 79–1146 (group 1 coronavirus) as controls. As shown in Fig. 3a, the RT-PCR of the group 2 coronavirus BCoV strain

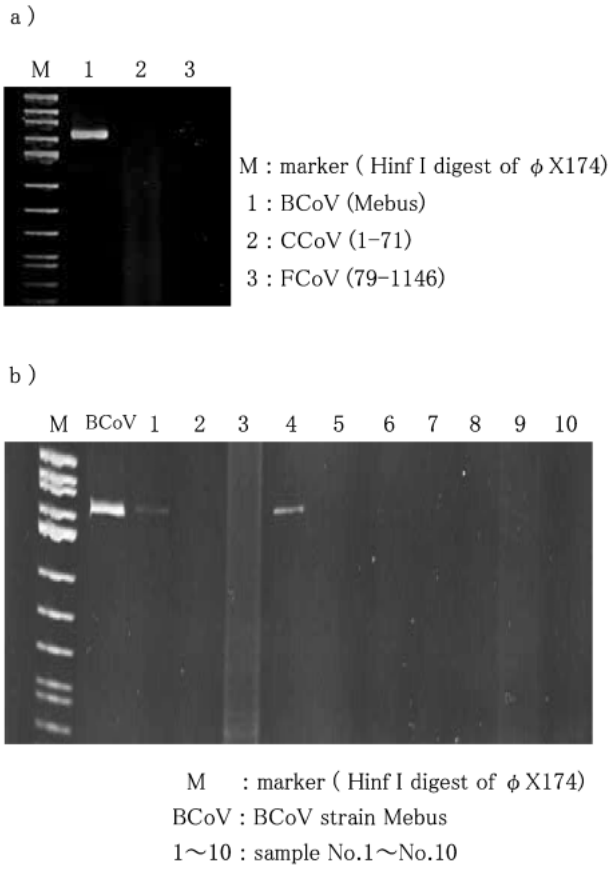

Fig. 3. Detection of coronavirus HE gene by RT-PCR. a) Specificity of RT-PCR of coronavirus HE gene for group 2 coronavirus. b) Results of RT-PCR analysis of test samples.

Mebus alone produced an expected size of 497-bp band. Thus, using this RT-PCR method, we attempted to detect the group 2 coronavirus HE gene from the oral swabs of a total of 10 young pups with respiratory signs or having recovered from respiratory signs, or having anti-group 2 coronavirus antibodies, with the result that the samples from the No. 1 and No. 4 pups were positive for the HE gene (Table 1 and Fig. 3b).

Virus isolation: Oral swab samples from the 10 dogs, used for HE gene detection, were inoculated into HRT-18G and fcwf-4 cells to isolate viruses. The cultures showed no signs of a cytopathic effect during three passages. After the third passage, RNA was extracted from the cultures. The RT-PCR of the RNA failed to detect the coronavirus HE gene.

DISCUSSION

In this study, we showed that the known group 1 CCoV, which cause enteritis, and an unknown group 2 coronavirus were prevalent simultaneously in domestic dogs in Japan.

The coronaviruses, including the SARS virus, are cur-

Table 1. Detection of coronavirus HE gene from oral swabs of young pups

Sample No.	Species	Age/Sex	Symptom[a)] (medical history)	NT antibody titer	RT-PCR HE
1	M. Dachshund	2 mon/ ♀	cough	<5	+
2	Pug	3.5 mon/ ♂	healthy (cough)	<5	–
3	M. Dachshund	6 mon/ ♂	healthy (cough)	<5	–
4	Toy Poodle	4 mon/ ♂	healthy	20	+
5	Mongrel	2 mon/ ♂	healthy (cough)	<5	–
6	Chihuahua	8 mon/ ♂	cough	5	–
7	Welsh Corgi	3 mon/ ♀	healthy (cough)	<5	–
8	M. Dachshund	3.5 mon/ ♂	sneezing	<5	–
9	Chihuahua	7 mon/ ♀	cough	<5	–
10	M. Dachshund	3 mon/ ♂	healthy (nasal discharge)	<5	–

a) Symptoms at the time of oral swabbing. Parentheses show the history of the disease.

rently classified into four distinct antigenic groups based on sequence analysis of their genomes and antigenic relationships [6, 7]. The BCoV used in this study and the HCoV strain OC43 are classified under the group 2 coronaviruses [9]. Of the 898 dogs in Japan, 160 (17.8%) had neutralizing anti-BCoV antibodies, strongly suggesting that coronaviruses resembling BCoV are widely prevalent in dogs. The spike genes of the known CCoV (group 1 coronavirus) and BCoV have low homology, and do not exhibit cross-reactivity in the neutralization test [9]. No correlation was found between the neutralizing anti-BCoV and anti-CCoV antibody titers in the 198 serum samples of dogs with a known history of vaccination (r=–0.25), suggesting that the neutralizing anti-BCoV antibodies detected in the canine sera were not produced by infection with CCoV. HE glycoprotein is found on the surface of group 2 coronaviruses alone, and not group 1 coronaviruses [8]. The HE gene was successfully detected from the oral swabs of 2 pups by RT-PCR with HE gene-specific primers, strongly suggesting the presence of infection with a group 2 coronavirus. Using HRT-18G and fcwf-4 cells, we were not able to isolate viruses from the oral swabs of any of 10 pups, including the above 2. HRT-18G cells are sensitive to BCoV, and fcwf-4 cells to FCoV, CCoV, and TGEV. Erles *et al.* [4], who showed the presence of a group 2 coronavirus in dogs, have not successfully isolated the virus. Neutralizing antibodies to BCoV were also present in the canine sera collected in 1998, indicating that a group 2 coronavirus, which was unknown in dogs, was already prevalent in Japan in 1998. This suggests the need to examine serum samples that were collected before 1998.

The 104 feline serum samples were collected in the Kanto region where the anti-BCoV antibody-positive rate of dogs was relatively high. However, all of them were negative for neutralizing anti-BCoV antibodies, suggesting that BCoV-like group 2 coronaviruses are not prevalent in cats.

In this study, we were not able to determine the relationship between group 2 coronavirus infection and the etiology. RT-PCR using oral swabs successfully detected the HE gene from a young puppy with respiratory signs and a healthy young puppy with neutralizing anti-BCoV antibodies. In the clinical setting, when young pups are observed to have respiratory disease, infection with canine CPIV, CAV-2, or Bordetella bronchiseptica is suspected first and treated. Since these infections spread rapidly but do not become serious, no detailed study has been made to determine the etiology. It is hoped that the involvement of the group 2 coronavirus in these infections and canine infectious respiratory disease will be investigated. It is also hoped that their involvement in canine digestive disease will be investigated, because BCoV is closely associated with digestive disease.

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