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Survey of Dogs in Japan for Group 2 Canine Coronavirus Infection

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Specimens obtained from 96 dogs with respiratory and enteric clinical signs in Japan were retrospectively examined for group 2 coronavirus by reverse transcription-PCR. Two dogs were found to be positive. Phylogenetic analysis of the spike gene indicated that they were most probably related to the canine respiratory coronavirus recently described in the United Kingdom.

Canine coronavirus (CCoV) is an enteric pathogen for dogs (23) and is a member of the group 1 species of the genus *Coronavirus* in the family *Coronaviridae*. Previous epidemiological surveys demonstrated that CCoV is prevalent in dogs worldwide, including Japan (1, 3, 19, 25). Until recently, CCoV was the only coronavirus species of dog origin. However, in 2003 a novel coronavirus provisionally named canine respiratory coronavirus (CRCoV) was detected in dogs with kennel cough, also called canine infectious respiratory disease (CIRD), in the United Kingdom (10). The spike (S) and polymerase gene properties of this CRCoV indicated that the virus was genetically similar to both bovine coronavirus (BCoV) and human coronavirus (HCoV) strain OC43, which belong to the group 2 coronaviruses, but was distinct from the group 1 member CCoV. The virological properties of United Kingdom CRCoV isolate 4182, which was recently isolated in cell culture (24), has not been published yet.

Preliminary seroepidemiological studies of general dog populations in the United Kingdom, North America, and Japan were performed with cultivable BCoV strains as substitutes for CRCoV. In the United Kingdom, 30.1% of the serum samples obtained from the dogs on the day of entry into a rehoming kennel (9) and 55.4% of the dog serum samples from across the United States and Canada (24) were antibody positive by enzyme-linked immunosorbent assay. In Japan, anti-BCoV neutralizing antibodies were found in 19.8% of the dogs tested (12). These results suggest that some group 2 coronaviruses are prevalent among dogs throughout the world. In this context, virological evidence of CRCoV infection was explored in clinical specimens obtained from dogs brought to animal hospitals in Japan.

A total of 124 clinical swab specimens from 59 respiratory sites (oropharynx, conjunctiva, and nasal cavity) and 65 rectal sites were taken from 96 dogs manifesting respiratory and/or enteric signs. These samples were tested for general viral pathogens by animal hospitals in 12 prefectures from northern Hokkaido to southwestern Fukuoka, Japan, between February 1999 and August 2005. They were cryopreserved after primary examination and retrospectively reexamined for CRCoV in the present study.

Total RNA was obtained from 140 μl of clarified swab extract with a QIAamp Viral RNA Mini Kit (QIAGEN Science). cDNA was synthesized by using a random 9-mer primer (Takara, Tokyo, Japan) and avian myeloblastosis virus reverse transcriptase for reverse transcription-PCR (Transcriptase XL; Takara).

Detection of CRCoV was performed according to PCR methods described previously (10). The hemagglutinin-esterase (HE) gene found only in group 2 coronavirus was targeted. The first PCR was performed by using previously described primers HE1 and HE2 (10), and then a nested PCR was performed with newly devised primers. All primers were designed from an alignment of the HE gene of BCoV strain LY-138, and the nucleotide sequences of the nested PCR primers are as follows: HE3, 5′-GCACAATCTACAGCTCTTTG-3′ (sense orientation; located on the BCoV HE gene at positions 481 to 500); HE5, 5′-AGACAGATTGCTTTCGTAGGA-3′ (anti-

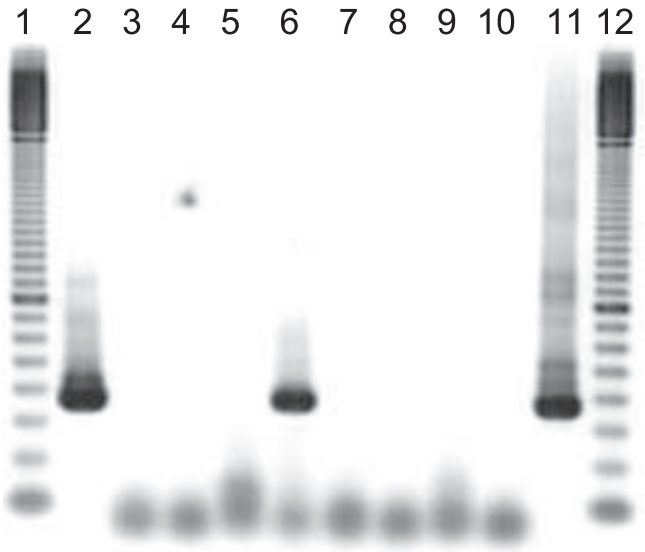

FIG. 1. Detection of group 2 coronavirus by PCR in clinicalspecimens. A fragment of 365 bp from the HE gene was expected for the virus-positive samples. Lanes: 1 and 12, 100-bp ladder size markers; 2 and 6, swab extracts of representative positive clinical samples 02/005 and 04-009, respectively; 3 to 5 and 7 to 9, swab extracts of representative negative clinical samples 02/006, 02/007, 02/008, 04-007, 04-010, and 04-011, respectively;10, negative reference group 1 CCoV strain 1-71; 11, positive reference group 2 BCoV strain Mebus.

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TABLE 1. HE gene-positive clinical specimens

Specimen (origin)	Yr, place of collection (Japan)	Breed; age (mo); clinical signs	Other viral agents detected
02/005 (nasal)	2002, Chiba	Welsh corgi; 2; coughing, nasal discharge	None[a] (HE gene not detected in oropharyngeal specimen)
04-009 (rectal)	2004, Chiba	Miniature dachshund; 1; low activity, anorexia, nausea, dehydration, eye discharge, vomiting, diarrhea	CCoV, CPIV[b] (in rectal and oropharyngeal specimens)

[a] Neither canine adenovirus, group 1 CCoV, canine distemper virus, canine parainfluenza virus, nor type 2 canine parvovirus was detected.
[b] CPIV, canine parainfluenza virus.

sense orientation; located at positions 845 to 825). The expected size of the nested PCR product was 365 bp. BCoV strain Mebus (17) and strain 1-71 of CCoV (3) were used as positive and negative controls, respectively.

Two swab specimens, no. 02/005 and 04-009, were found to be positive for the HE gene (Fig. 1), an overall dog positivity rate of 2.1%. The profiles of these clinical specimens are summarized in Table 1. These two specimens were then inoculated into cell cultures of *Felis catus* whole fetus 4 (fcwf-4) (22) and human rectal tumor 18 (HRT-18) (27) cells for virus isolation. Both cell types are highly susceptible to CCoV (1), feline coronaviruses (5), and BCoVs (2, 28), and BCoV strain Mebus grew in both cell cultures, showing a distinct cytopathic effect (data not shown). However, no virus was recovered from specimens 02/005 and 04-009.

Sequencing of the HE and S genes isolated from both specimens was performed. The nested PCR product from the HE gene was separated on an agarose gel, purified with a QIA-

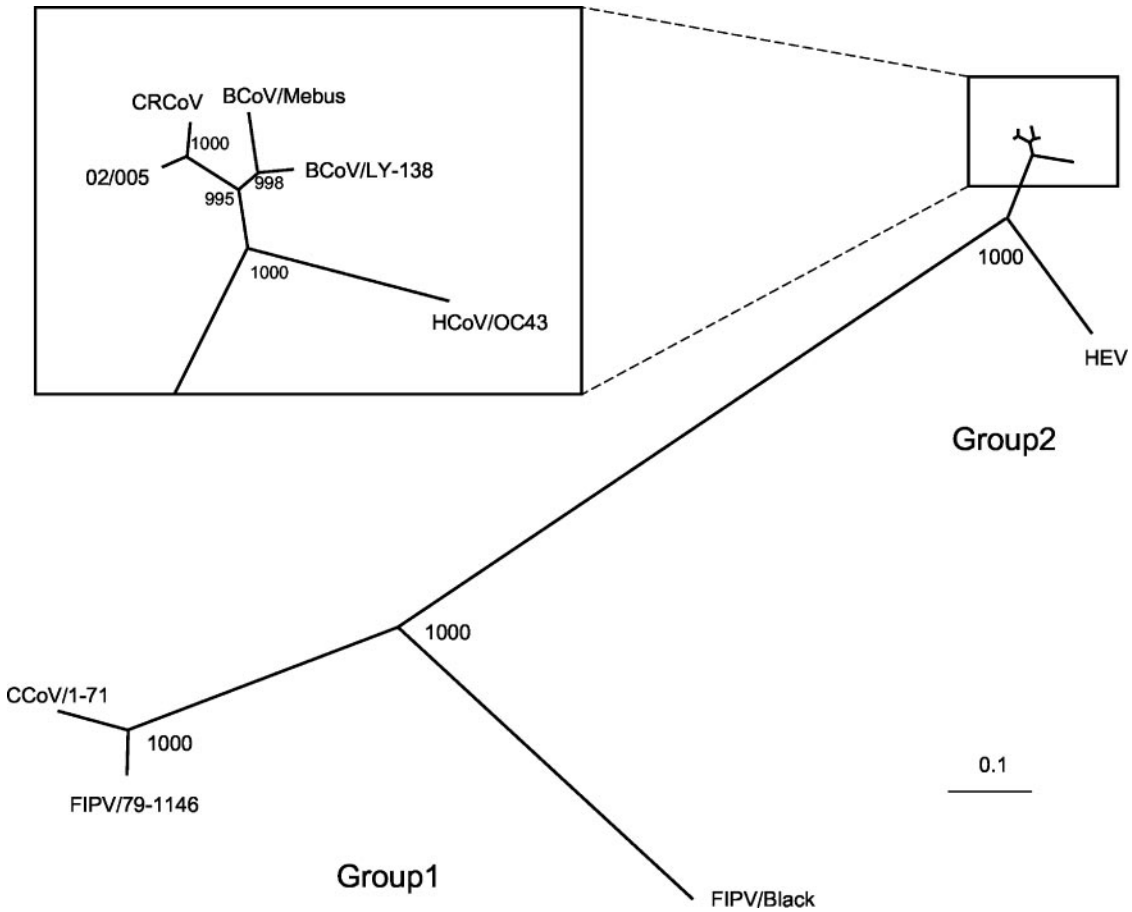

FIG. 2. Phylogenetic analysis of the nucleotide sequences of coronavirus S genes. The tree was generated with ClustalW version 1.83 (26) without outgroup specification but with bootstrap analysis and was displayed by TREEVIEW (20). Each bootstrap value indicates the number of times that each branching was found in 1,000 bootstrap analyses. Branch lengths are proportional to genetic distances. Strain 02/005 (accession no. AB242262) was generated in this study. Other sequences were from the database for the following strains (S gene accession numbers are in parentheses): BCoV strain LY-138, reference 11 (AF058942); BCoV strain Mebus, reference 17 (BCU00735); canine coronavirus strain 1-71, reference 2 (AY796289); CRCoV, reference 10 (AY150272); feline infectious peritonitis virus (FIPV) strain Black, reference 4 (AB088223), feline infectious peritonitis virus strain 79-1146, reference 16 (DQ010921), porcine hemagglutinating encephalomyelitis virus (HEV) strain 67N, reference 18 (AF481863), HCoV strain OC43, reference 15 (L14643).

quick gel extraction kit (QIAGEN GmbH, Hilden, Germany), and directly sequenced with a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) on an ABI PRISM 310 genetic analyzer (Applied Biosystems). For sequencing of the complete S gene, previously described primer sets Sp1-Sp8 and SpF-SpR (10) were used. Nucleotide sequences were analyzed by Genetyx-Win version 4.0 (Software Development Co., Tokyo, Japan). Comparative analysis with the sequences of known coronaviruses was performed by the FASTA program (21) at the DDBJ web site. ClustalW version 1.83 (26) was used to produce multiple alignments and phylogenetic trees from the sequences obtained. The phylogenetic tree was displayed by TREEVIEW (20) without outgroup specification but with bootstrap analysis.

The HE gene partial sequences (365 bp) of specimens 02/005 and 04-009 were compared with those of other group 2 coronavirus species and found to be most closely related to the sequences of CRCoV strain T101 (10) and BCoV strains LY-138 and Mebus. The nucleotide sequence identities of specimens 02/005 and 04-009 for CRCoV strain T101 were 99.7% and 98.0%, respectively. The coronavirus showing the second highest identity was HCoV strain OC43, but the homology was less than 97%.

The complete S gene sequence was successfully obtained from specimen 02/005 but not from specimen 04-009. The S gene of specimen 02/005 was 4,092 nucleotides long, representing 1,364 predicted amino acids. These were identical to those previously reported for CRCoV strain T101, BCoV strain LY-138, and BCoV strain Mebus. The nucleotide (amino acid) sequence identities of the specimen 02/005 S gene for those of CRCoV strain T101, BCoV strain LY-138, and BCoV strain Mebus were 99.1% (98.5%), 97.7% (96.6%), and 97.3% (95.7%), respectively. It was determined that homologies of the S gene nucleotide sequences of specimen 02/005 to other coronavirus species belonging to group 2, such as murine hepatitis virus strain A59, porcine hemagglutinating encephalomyelitis virus strain N67, and HCoV strain OC43, were lower (60.7 to ~92.9%), and those to group 1 coronaviruses were lower still (47.6 to ~47.9%).

A phylogenetic tree based on the S gene nucleotide sequence alignments is shown in Fig. 2. The closest relationship was again obviously to CRCoV strain T101. The tree showed a distinct branch for strain 02/005 together with CRCoV strain T101, BCoVs, and HCoV strain OC43, suggesting a close genetic relatedness between strain 02/005 and such bovine and human group 2 coronavirus species.

The present data clearly demonstrated that a coronavirus genetically similar to CRCoV strain T101 recently discovered from dogs in the United Kingdom (10) had also infected dogs in Japan, although the virus was not recovered in a cell culture. A recent retrospective pathological study in Saskatchewan demonstrated two CRCoV infection cases in 1996 (8), suggesting that the emergence of CRCoV was not recent. The etiological agents considered to be involved in kennel cough are complex and include canine adenovirus type 2, canine parainfluenza virus, and *Bordetella bronchiseptica*. Recently, in different dog populations in Florida and England, type H3N8 equine influenza virus was described as a novel virus pathogen for CIRD (6, 7). The preliminary epidemiological reports on CRCoV so far have indicated the possibility that it is also a pathogen involved in CIRD. However, the virus was detected from the rectal swab in the present study. It is well known that a new human group 2 coronavirus, severe acute respiratory syndrome virus, also infects the alimentary tract and the viscera, e.g., the kidneys and liver (13). Thus, it may be worthwhile to know whether CRCoV is also a pathogen for the alimentary system of dogs.

Nucleotide sequence accession numbers. The references for and the nucleotide sequence accession numbers in the GenBank database of the indicated strains used in this study are as follows: BCoV strain LY-138, reference 11 (HE gene, M84486; S gene, AF058942); BCoV strain Mebus, reference 17 (HE and S genes, BCU00735); canine coronavirus strain 1-71, reference 3 (S gene, AY796289); CRCoV, reference 10 (HE gene, AY423274; S gene, AY150272); feline infectious peritonitis virus strain Black, reference 4 (S gene, AB088223); feline infectious peritonitis virus strain 79-1146, reference 16 (S gene, DQ010921); porcine hemagglutinating encephalomyelitis virus strain 67N, reference 18 (HE gene, AY078417; S gene, AF481863); HCoV strain OC43, reference 15 (HE gene, M76373; S gene, L14643); murine hepatitis virus strain A59, reference 14 (S gene, NC_001846). The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases under accession no. AB242262 and AB242263 for the S and HE genes of strain 02/005, respectively, and no. AB242264 for the HE gene of strain 04-009.

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