NOTE *Virology*

Phylogenetic Studies of Bovine Coronaviruses Isolated in Japan

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ABSTRACT. Molecular analysis of the polymorphic region of the bovine coronavirus (BCoV)-S gene using recent Japanese field isolates and reference strains revealed that the 148 isolates collected from 1999 to 2008 from 13 prefectures, covering all regions of Japan (Hokkaido, Tohoku, Kanto, Chubu, Kinki, Chugoku, Shikoku, and Kyusyu region) and divided into 3 clusters, show distinctive divergence from the prototype enteric BCoV strains. Almost all isolates after 2005 were clustered into group 4, and there was no regional specificity in these clusters. To differentiate the genotypes without sequencing, a simple technique–reverse transcriptase-polymerase chain reaction/restriction fragment length polymorphism analysis (RT-PCR/RFLP)–was developed. The availability of a simple and easy diagnostic assay will enable larger epidemiological studies of BCoV.
KEY WORDS: bovine coronaviruses, phylogenetic analysis, RFLP.

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Bovine coronavirus (BCoV) causes severe diarrhea in neonatal calves (CD), winter dysentery (WD) in adult cattle, and also respiratory tract infections [11, 12, 14, 17]. In general, affected animals rarely die; however, dramatic reductions in milk production in dairy herds and loss of body condition in both calves and adults are observed, resulting in severe economic losses.

BCoV is a member of the order *Nidovirales*, family *Coronaviridae* [16], which possesses a single-stranded, non-segmented RNA positive-sense genome that is 31 kb in length. The virion contains 5 structural proteins: the nucleocapsid (N) protein, transmembrane (M) protein, spike (S) protein, small envelope (E) protein, and hemagglutinin-esterase (HE) protein [10].

The coronavirus S glycoprotein forms large, petal-shaped spikes on the surface of the virion and is responsible for virus binding to host cell receptors [5, 9], induction of neutralizing antibody [19, 20], and hemagglutinating activity [15]. Its sequences are variable, and mutations in this region have been associated with altered antigenicity and virus pathogenicity [1, 3, 7].

Recently, we have examined the results of a molecular analysis of the S glycoprotein gene of BCoV isolates in Japan from 1999 to 2006. Phylogenetic analysis of the polymorphic region of this gene of the isolates, together with those of other known strains, classified the BCoV strains and isolates into 4 genetic groups [8]. This study revealed that recent Japanese field isolates have distinctive genetic divergence from prototype enteric BCoV (EBCoV) strains (Mebus, Quebec, Kakegawa, F15 and LY138) and that they have diverged in 3 different aspects over this 8-year period. In addition, our study did not reveal the presence of certain genetic markers of pathogenicity and clinical symptoms in this polymorphic region, although some studies have suggested that several amino acids may contribute to each of the two disease types, that is, enteric (EBCoV) and respiratory (RBCoV) [2, 4, 6, 13].

In the present study, we have extended the molecular analysis of the polymorphic region of the S gene of Japanese BCoV isolates collected from all areas of Japan to clarify the molecular epidemiology of BCoV infection in this country and developed the reverse transcriptase-polymerase chain reaction/restriction fragment length polymorphism analysis (RT-PCR/RFLP), which differentiates the 4 genetic groups without sequencing.

Fecal or nasal samples were collected from cattle suffering from diarrhea and/or showing respiratory symptoms for BCoV infection; this was followed by isolation of viruses using human rectal tumor cells (HRT-18G). In total, from 1999 to 2008, 148 isolates were collected from 13 prefectures covering all regions (Hokkaido, Tohoku, Kanto, Chubu, Kinki, Chugoku, Shikoku and Kyusyu region) of Japan (Table 1). RNA extraction, RT-PCR and sequencing were performed as described in a previous report [8]. Based on the 411-bp (aa 456–592) sequence of the polymorphic region of the S gene, a phylogenetic tree was constructed using ClustalW. It revealed that the Japanese BCoV isolates with the prototype strains Mebus, Quebec, and Kakegawa; vaccine strain 66/H [18]; RBCoV strains LSU and OK; EBCoV strains F15 and LY138; and Korean field strains (KWD1–10) were divided into 4 clusters as reported in a previous study [8] (Fig. 1). The sequence of vaccine strain 66/H was identical to that of Kakegawa in this polymorphic region. The 84 isolates collected after 2005 were clustered into group 4, except for isolates IS14, HK22, and HK23, which were isolated in 2005. There were no regional specificities in these clusters. This suggested that the isolates in group 4 are a predominant lineage and are widespread

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Table 1. BCoV isolates in Japan

Prefectures and isolates	Year of isolation
Hokkaido	
HK1, –2, –3, –5	2002
HK4, –6, –7, –8, –9,–10, –11, –12	2003
HK13, –14, –15, –16	2004
HK17, –18, –19, –20, –21, –22, –23, –24, –25, –26, –27, –28	2005
HK29, –30, –31, –32, –33	2006
HK34, –35, –36	2007
HK37, –38, –39, –40	2008
Yamagata	
YM1, –2, –3, –4, –5, –6, –7	2003
YM8, –9, –10, –11, –12, –13	2007
Ibaraki	
IB1	2004
IB2	2005
IB3	2006
IB4, –5, –6	2007
Tochigi	
TC1	2001
TC2, –4, –5, –6, –7, –8, –9, –10	2002
TC11	2003
Toyama	
TY1	2008
Ishikawa	
IS1, –2	1999
IS3	2001
IS4	2002
IS5, –6, –7, –8, –9	2004
IS10, –11, –12, –13, –15, –16, –17, –18, –19, –20	2006
IS14	2005
IS21, –22	2007
IS23, –24, –25	2008
Gifu	
GF1, –2	2006
Osaka	
OS1, –2	2006
Wakayama	
WK1, –2, –3, –4, –5	2005
WK6	2003
WK8, –9, –10	2007
WK11	2008
Shimane	
SM1, –2, –3, –4	2000
SM5	2001
SM6, –7	2002
SM8, –9, –10, –11, –12, –13	2003
SM14, –15, –16, –17, –18, –19	2005
SM20, –21, –22	2006
SM23	2007
Kochi	
KO1, –2, –3, –4	2004
KO5, –6	2005
KO7	2006
Kumamoto	
KM1, –2	2007
Kagoshima	
KG1, –2, –3, –4, –5, –6, –7	2007

The nucleotide sequences of isolates HK1-21, YM1-7, TC1-11, IS1-9, OS1-2, WK1-6, were determined in a previous report [8].
The GeneBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are AB450825-AB450917.

throughout Japan. The reason why viruses of the other groups (2 and 3) have not been isolated since 2006 remains unknown, but group 4 viruses could spread rapidly to all the regions of Japan. Further, since HK40 isolated in 2008 showed more genetic divergence among the other isolates, it may form a new cluster. Because the S gene sequence is associated with antigenicity of BCoV [19, 20], our findings suggest that genetic diversity in the polymorphic region might also be influencing their antigenicity. To elucidate their correlation, an analysis for the antigenic properties of isolates in each genetic group is in progress.

Based on the epidemiological information of each case, we have explored the route of virus transmission in the field. It is believed that migration of cattle carrying subclinical and persistent BCoV infection is the major factor for transmission. However, our data reveal that there are many cases with no correlation between the introduction of cattle and disease onset, even in the case of the first occurrence of the disease by BCoV on the farm (data not shown); therefore, the role of cattle migration remains unknown. However, isolates HK12 and HK29 were isolated from nondiseased cattle and a calf, respectively (data not shown). These results suggested that if BCoV could readily be a subclinical and persistent infection in cattle and calves, it could play an important role in the epidemiologic factors of BCoV infection in susceptible animals.

As described in a previous report, no obvious genetic differences were detected in the polymorphic region between the isolates from the enteric cases and respiratory symptoms (data not shown). This supports the conclusion that there are no disease type (EBCoV and RBCoV)-specific sites in the polymorphic region of the S gene.

Using the alignment sequence data of the isolates, we developed the RT-PCR/RFLP technique for differentiating 4 genetic groups of BCoV. The oligonucleotide primers used in this analysis were designed from the nucleotide sequence of the Kakegawa strain (GeneBank accession no. AB354579). The primers were (position from the start codon of the S gene) as follows: SL1, 5'-GCAGGTTTAATCCTTCTACTTGGA-3' (nt 24962–24985, sense primer); SR2 5'-CACCAAGAATTATGTCTGTGTTTGA-3' (nt 25504–25528, antisense primer). This 567-bp amplification fragment contains a polymorphic region (nt 25006–25416, 411 bp) for phylogenetic analysis. RT-PCR was performed using a Titan One Tube RT-PCR Kit (Roche Diagnostics K.K., Japan), and the conditions of cycle reaction were as follows: 30 min at 50°C; followed by 35 cycles of denaturation at 94°C for 10 sec, annealing at 55°C for 30 sec, and extension at 68°C for 45 sec; and completion of amplification with a 7-min extension step at 68°C. The expected sizes of the DNA fragments were successfully amplified from all genetic groups of BCoV isolates, and the fragments were then purified using a High Pure PCR Product Purification kit (Roche Diagnostics K.K., Japan). For restriction endonuclease analysis, we selected two enzymes, namely, *Ava*II and *Eco*O65I (Takara, Tokyo, Japan). The restriction profiles of the RT-PCR products of the 4 genetic

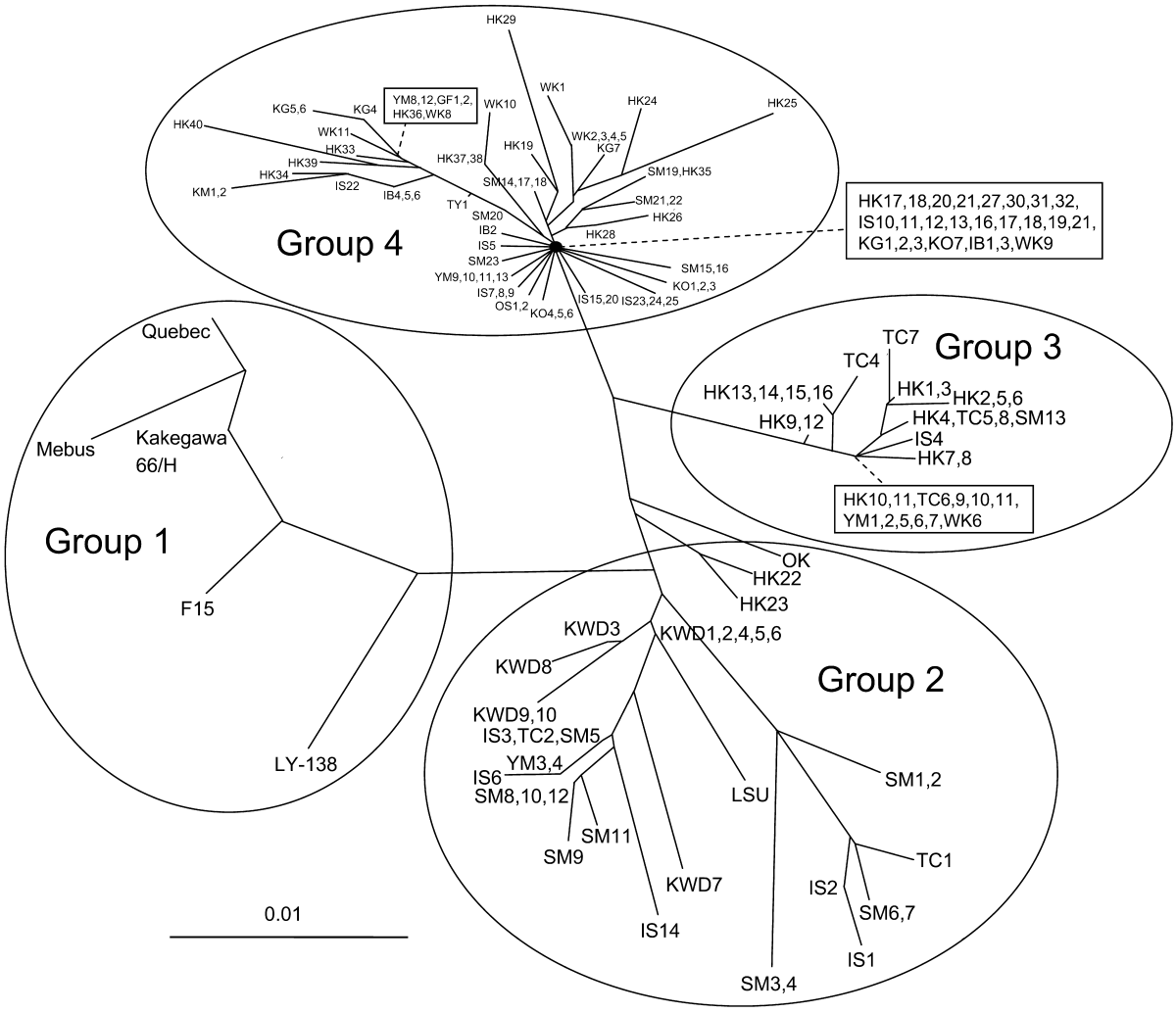

Fig.1. Phylogenetic tree generated by neighbor-joining analysis of genetic distance in the polymorphic region (nt 1366–1776) of the S gene (Rekik and Dea, 1994 [13]). The isolates were designated based on the name of the prefecture of origin: HK, Hokkaido; YM, Yamagata; IB, Ibaraki; TC, Tochigi; TY, Toyama; IS, Ishikawa; GF, Gifu; OS, Osaka; WK, Wakayama; SM, Shimane; KO, Kochi; KM, Kumamoto; KG, Kagoshima. The sequences of the reference strains of BCoV-Mebus (GenBank accession no. U00735); Quebec (AF220295); RBCoV (respiratory bovine coronavirus): LSU (AF058943) and OK (AF058944); EBCoV (enteric bovine coronavirus): F15 (D00731) and LY138 (AF058942); and the Korean strains (AY935637–935646)–were obtained from GenBank, and the sequences of Kakegawa (AB277098) and 66/H (AB451543) were determined in the present study.

Table 2. RFLP patterns of the RT-PCR products of the four genetic groups of BCoV isolates in Japan

Genetic group	Length of RT-PCR products	Sizes of the fragments *Ava*II	*Eco*O65I
1	567[a)]	177, 390[b)]	Not digested
2	567	Not digested	Not digested[c)]
3	567	177, 390	168, 399
4	567	Not digested	168, 399

a) Base pairs including the length of the primers.
b) Except LY-138, which was not digested by *Ava*II.
c) Except OK, HK22 and HK23, which were digested into 168- and 399-bp.

groups in this study are shown in Table 2 and Fig. 2. *Ava*II digested the RT-PCR products of group 1 (except LY-138) and group 3, but not those of groups 2 and 4, into 177- and 390-bp fragments. *Eco*O65I digested the RT-PCR products of groups 3 and 4, but not those of groups 1 and 2 (except OK, HK22, and HK23, which were digested into 168- and 399-bp fragments), into 168- and 399-bp fragments.

The present study revealed that 4 genetic groups of BCoV could be differentiated by a simple restriction endonuclease analysis. Although sequencing is required to obtain precise genetic information for each BCoV isolate, restriction endonuclease analysis is a rapid, simple, and inexpensive technique for primary characterization in routine diagnosis and

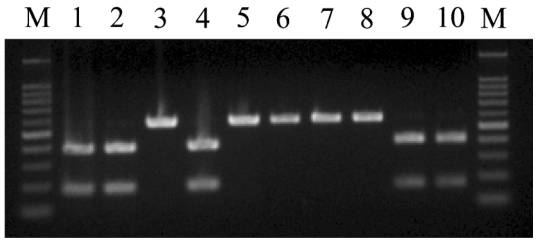

Fig. 2. Restriction endonuclease analysis of the PCR products from the four representative genetic groups of BCoV in Japan. The 567-bp PCR products were digested with *Ava*II (1–5) and *Eco*O65I (6–10). M, 100-bp DNA ladder marker; 1 and 6, Mebus (group 1); 2 and 7, Kakegawa (group 1); 3 and 8, IS2 (group 2); 4 and 9, HK12 (group 3); 5 and 10, KM1 (group 4).

is suitable for use by the local livestock hygiene service centers of prefectures in Japan for investigating the epidemiology of BCoV.

In summary, molecular analysis of the polymorphic region of the *S* gene using recent Japanese field isolates and reference strains revealed that the 148 isolates collected from 1999 to 2008 from 13 prefectures, which cover all regions of Japan and were divided into 3 clusters, show distinctive divergence from the prototype enteric BCoV strains. To differentiate the genotypes without sequencing, the RT-PCR/RFLP assay was developed. The availability of a simple and easy diagnostic assay will enable epidemiological studies of BCoV on a larger scale.

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