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Short Communication

Molecular analysis of the S glycoprotein gene of bovine coronaviruses isolated in Japan from 1999 to 2006

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In total, 55 isolates of *Bovine coronavirus* (BCoV) were collected from cases of enteric and respiratory disease occurring between 1999 and 2006 in Japan. Phylogenetic analysis of the polymorphic region of the S glycoprotein gene of these isolates, together with those of other known strains, classified the BCoV strains and isolates into four clusters. Recent field isolates display distinctive genetic divergence from the prototype enteric BCoV strains – Mebus, Quebec, Kakegawa, F15 and LY138 – and have diverged in three different aspects over 8 years. These data suggested that the genetic divergence in the polymorphic region of the S glycoprotein has progressed considerably; thus, molecular analysis of this region should be useful in investigating the molecular epidemiology of BCoV. In addition, based on the differences in amino acids among the isolates, our study did not reveal the presence of certain genetic markers of pathogenicity and clinical symptoms in this polymorphic region.

Bovine coronavirus (BCoV) causes severe diarrhoea in neonatal calves (CD), winter dysentery (WD) in adult cattle and also respiratory-tract infections (Lathrop *et al.*, 2000; Mebus *et al.*, 1973; Saif *et al.*, 1991; Storz *et al.*, 2000). In general, each isolated virus strain is discriminated as either enteric (EBCoV) or respiratory (RBCoV) based on the symptomatic features of the disease. However, the antigenic and genetic markers that differentiate the disease types remain to be clarified, although several examinations have been reported (Hasoksuz *et al.*, 1999; Kourtesis *et al.*, 2001; Tsunemitsu & Saif, 1995).

BCoV, which belongs to the order *Nidovirales*, family *Coronaviridae* (Spaan *et al.*, 2005), contains a single-stranded, non-segmented RNA positive-sense genome that is 31 kb in length. The virion contains five structural proteins: the nucleocapsid (N) protein, the transmembrane (M) protein, the spike (S) protein, the small envelope (E) protein and the haemagglutinin–esterase (HE) protein (Lai & Cavanagh, 1997).

The coronavirus S glycoprotein forms large, petal-shaped spikes on the surface of the virion and is cleaved into S1 (N terminus) and S2 (C terminus) subunits (Abraham *et al.*, 1990; Cavanagh *et al.*, 1986). The S1 subunit is responsible for virus binding to host-cell receptors (Godet *et al.*, 1994; Kubo *et al.*, 1994), induction of neutralizing antibody (Takase-Yoden *et al.*, 1991; Yoo & Deregt, 2001) and haemagglutinating activity (Schultze *et al.*, 1991). Its sequences are variable and mutations in this region have been associated with altered antigenicity and virus pathogenicity (Ballesteros *et al.*, 1997; Fazakerley *et al.*, 1992; Hingley *et al.*, 1994). On the other hand, the sequences of the S2 subunit are conserved and responsible for membrane-fusion activity (Luo & Weiss, 1998; Yoo *et al.*, 1991).

Molecular analysis of the S gene of BCoV isolates has been performed and the results obtained were compared with those for other strains (Chouljenko *et al.*, 1998; Hasoksuz *et al.*, 2002; Jeong *et al.*, 2005; Liu *et al.*, 2006; Rekik & Dea, 1994); however, the prevalence and genetic diversity of recent BCoV cases worldwide remain unclear. This paper reports the results of a molecular analysis of Japanese field isolates collected between 1999 and 2006 in comparison with classical reference strains and other recent field strains isolated in Korea to investigate the genetic relationship among them and genetically divergent features over a relatively long period.

Faecal or nasal samples were collected from prefectures in which diarrhoea and/or respiratory symptoms were observed in cattle (see Supplementary Fig. S1, available in JGV Online). Each sample was inoculated into human rectal tumour cells (HRT-18) to isolate the virus. Some isolates were kindly provided by the Livestock Hygiene Service Center of the relevant prefecture. In total, 55 isolates were collected. RNA was extracted from the virus culture by using a High Pure viral RNA kit (Roche) according to the manufacturer's instructions. The oligonucleotide primers

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are AB277098–AB277153.

A supplementary figure showing the geographical distribution of the cases of BCoV infection in Japan is available in JGV Online.

used in RT-PCR were designed from the nucleotide sequence of the Mebus strain (GenBank accession no. U00735). The primers were (positions from the start codon of the S gene): S-S1, 5′-GATAAGTTTGCTATACCCAATGG-3′ (nt 24817–24839, sense primer); S-AS1, 5′-ACTATCATTTACTGAATTAACAG-3′ (nt 25988–26010, antisense primer). This 1194 bp amplification fragment contains a polymorphic region (nt 25006–25416, 411 bp) and an S1/2 cleavage site. RT-PCR was performed by using a Titan One Tube RT-PCR kit (Roche), followed by purification of the DNA fragments using a QIAquick PCR purification kit (Qiagen); these fragments were subsequently used for sequencing.

The sequencing reaction was performed by using a BigDye Terminator v. 3.1 cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions. The sequencing primers were designed based on the sequence of the Mebus strain in addition to S-S1 and S-AS1. The primers were: S-S2, 5′-GTAATCCTTGTACTTGCCAACC-3′ (nt 25361–25382, sense primer) and S-AS2, 5′-TTGTAAACAAGAGTCAACAGACC-3′ (nt 25400–25422, antisense primer). Sequencing was performed by using an ABI 3130 Genetic Analyzer (Applied Biosystems). In addition to the recent field isolates, the S gene of Japanese prototype EBCoV Kakegawa strain (Akashi *et al.*, 1980) was also sequenced.

Nucleotide sequence alignments were performed of the polymorphic region, i.e. aa 456–592, of the S gene (Rekik & Dea, 1994) by using CLUSTAL_W (Thompson *et al.*, 1994). A phylogenetic tree was generated by using the neighbour-joining method with CLUSTAL_W and the tree was constructed by using the TreeView program (Page, 1996). 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–AY935646) – were obtained from GenBank and analysed with those of Japanese field isolates to evaluate their relationships.

In total, 55 BCoV isolates (Table 1) were sequenced in the S1 region and these were compared with each other and with other BCoV strains. The alignment of each sequence confirmed that the polymorphic region of all field isolates collected between 1999 and 2006, and also that of the Kakegawa strain, comprised 411 bp (aa 456–592), identical to the other BCoV strains. We did not find any Japanese field isolates with insertions or deletions similar to the Brazilian strains, which have a 6 aa deletion in the polymorphic region of the S gene (Brandao *et al.*, 2006). The nucleotide and amino acid identity among these Japanese isolates was 95.9–100 and 91.2–100 %, respectively. In total, 34 nucleotide substitutions were found in the Japanese isolates compared with the Mebus strain; among these nucleotide polymorphisms, 21 amino acid changes were identified in the polymorphic region (Table 2). Based on the sequence of the polymorphic region of the S gene, a phylogenetic tree was constructed by using CLUSTAL_W. The phylogenetic tree revealed that all of the Japanese BCoV isolates, including prototype strains Mebus, Quebec and Kakegawa, RBCoV strains LSU and OK, EBCoV strains F15 and LY138 and Korean field strains were divided into four clusters (Fig. 1). Group 1 comprised the Mebus, Quebec and EBCoV F15 and LY138 strains, as well as the Japanese prototype Kakegawa strain. RBCoV OK and LSU were clustered in group 2 together with the Korean strains and the eight Japanese field isolates (IS1, -2, -3, -6, TC1, -2, YM3 and -4) that were obtained from cattle showing enteric disease. In this group, as IS1, -2 and TC1 showed more divergence among the isolates, they may form a subgroup or an entirely new group. Almost all isolates collected from Hokkaido, except for HK17 to -21, were clustered in group 3 with isolates from the other prefectures, and these sequences showed high similarity. Group 4 comprised HK17 to -21, IS5, -7, -8 and -9, WK1 to -5 and OS1 and -2, all of which were isolated after 2004. The only isolates that were collected after 2004 and were not in this cluster were HK13 to -16 (group 3) and IS6 (group 2). Among the isolates from Tochigi prefecture, TC1 and -2 (isolated in 2001) belong to group 2, and TC4 to -11, isolated in 2002 and 2003, belong to group 3. This suggests that the predominant virus changed during 2001–2002. Similar results were also observed in the isolates from Wakayama; the predominant strain changed during 2003–2005 and the isolates were classified in groups 3 and 4. More interestingly, although the isolates from Yamagata were collected during the same outbreak period in 2003, they were divided into two distinct clusters, groups 2 and 3. This suggests that two distinct virus strains existed in the same city and caused simultaneous disease outbreaks. This phylogenetic study thus reveals that the genetic properties of recently collected Japanese field isolates are distinct from those of the classical reference EBCoV strains and that they vary in at least three different aspects. Among these clusters, the isolates in group 4 may be considered the predominant lineage in Japan, because all isolates in this group were collected after 2004.

Liu *et al.* (2006) reported that BCoV strains isolated from different cattle in the same herds had identical nucleotide sequences in a 624 nt fragment (nt 23656–24279) of the S gene located upstream of the polymorphic region. However, in our study, individual nucleotide sequences with amino acid changes were observed in four isolates, i.e. TC4 to -7, collected from different cattle in the same herd, although all of the isolates belonged to the same group (Fig. 1). Similarly, TC8 has a different nucleotide and amino acid sequence from those of TC9 and -10, although they were isolated from same herd. This feature of rapid genetic diversity in isolates from the same herd has not yet been reported. It has been suggested that the amino acids in the S glycoprotein, particularly in the polymorphic region, are more sensitive to mutations than those in the other regions (Wu & Yan, 2005). Based on these results, it

Table 1. BCoV isolates in Japan

Geographical information is given in Supplementary Fig. S1 (available in JGV Online).

Prefecture/city	Type	Sample	Date	Isolate*	Group
Hokkaido					
Nemuro	EBCV	Faecal	20 November 2002	Hokkaido/1/02 (HK-1)	3
Nemuro	EBCV	Faecal	20 November 2002	Hokkaido/2/02 (HK-2)	3
Nemuro	EBCV	Faecal	20 November 2002	Hokkaido/3/02 (HK-3)	3
Nemuro	CD	Faecal	28 April 2003	Hokkaido/4/03 (HK-4)	3
Nemuro	EBCV	Faecal	29 November 2002	Hokkaido/5/02 (HK-5)	3
Nemuro	EBCV	Faecal	28 January 2003	Hokkaido/6/03 (HK-6)	3
Chitose	RBCV	Nasal	24 September 2003	Hokkaido/7/03 (HK-7)	3
Chitose	RBCV	Nasal	24 September 2003	Hokkaido/8/03 (HK-8)	3
Monbetsu	RBCV	Nasal	24 July 2003	Hokkaido/9/03 (HK-9)	3
Monbetsu	RBCV	Nasal	18 September 2003	Hokkaido/10/03 (HK-10)	3
Monbetsu	RBCV	Nasal	18 September 2003	Hokkaido/11/03 (HK-11)	3
Shizunai	ND†	Faecal	21 October 2003	Hokkaido/12/03 (HK-12)	3
Erimo	EBCV	Faecal	20 July 2004	Hokkaido/13/04 (HK-13)	3
Erimo	EBCV	Faecal	20 July 2004	Hokkaido/14/04 (HK-14)	3
Erimo	EBCV	Faecal	20 July 2004	Hokkaido/15/04 (HK-15)	3
Erimo	EBCV	Faecal	20 July 2004	Hokkaido/16/04 (HK-16)	3
Makubetsu	EBCV	Faecal	11 January 2005	Hokkaido/17/05 (HK-17)	4
Obihiro	EBCV	Faecal	8 January 2005	Hokkaido/18/05 (HK-18)	4
Obihiro	EBCV	Faecal	8 January 2005	Hokkaido/19/05 (HK-19)	4
Taiki	EBCV	Faecal	6 February 2005	Hokkaido/20/05 (HK-20)	4
Taiki	EBCV	Faecal	6 February 2005	Hokkaido/21/05 (HK-21)	4
Ishikawa					
Nakajima	EBCV	Faecal	11 October 1999	Ishikawa/1/99 (IS-1)	2
Shiga	EBCV	Faecal	27 October 1999	Ishikawa/2/99 (IS-2)	2
Uchinada	EBCV	Faecal	26 December 2001	Ishikawa/3/01 (IS-3)	2
Mattou	EBCV	Faecal	25 December 2002	Ishikawa/4/02 (IS-4)	3
Kaga	EBCV	Faecal	15 May 2004	Ishikawa/5/04 (IS-5)	4
Mattou	EBCV	Faecal	12 April 2004	Ishikawa/6/04 (IS-6)	2
Oshimizu	EBCV	Nasal	26 November 2004	Ishikawa/7/04 (IS-7)	4
Oshimizu	EBCV	Faecal	29 November 2004	Ishikawa/8/04 (IS-8)	4
Oshimizu	EBCV	Nasal	26 November 2004	Ishikawa/9/04 (IS-9)	4
Tochigi					
Kuroiso	EBCV	Faecal	10 December 2001	Tochigi/1/01 (TC-1)	2
Nishinasuno	EBCV	Faecal	27 December 2001	Tochigi/2/01 (TC-2)	2
Utsunomiya	EBCV	Faecal	13 December 2002	Tochigi/4/02 (TC-4)	3
Utsunomiya	EBCV	Faecal	13 December 2002	Tochigi/5/02 (TC-5)	3
Utsunomiya	EBCV	Faecal	13 December 2002	Tochigi/6/02 (TC-6)	3
Utsunomiya	EBCV	Faecal	13 December 2002	Tochigi/7/02 (TC-7)	3
Kuroiso	EBCV	Faecal	13 December 2002	Tochigi/8/02 (TC-8)	3
Kuroiso	EBCV	Faecal	13 December 2002	Tochigi/9/02 (TC-9)	3
Kuroiso	EBCV	Faecal	13 December 2002	Tochigi/10/02 (TC-10)	3
Tsuga	EBCV	Faecal	14 March 2003	Tochigi/11/03 (TC-11)	3
Yamagata					
Yamagata	EBCV	Faecal	17 October 2003	Yamagata/1/03 (YM-1)	3
Yamagata	EBCV	Faecal	17 October 2003	Yamagata/2/03 (YM-2)	3
Yamagata	EBCV	Faecal	5 November 2003	Yamagata/3/03 (YM-3)	2
Yamagata	EBCV	Faecal	5 November 2003	Yamagata/4/03 (YM-4)	2
Yamagata	EBCV	Faecal	9 November 2003	Yamagata/5/03 (YM-5)	3
Yamagata	EBCV	Faecal	9 November 2003	Yamagata/6/03 (YM-6)	3
Yamagata	EBCV	Faecal	1 December 2003	Yamagata/7/03 (YM-7)	3
Wakayama					
Kainan	EBCV	Faecal	25 April 2005	Wakayama/1/05 (WK-1)	4
Yuasa	EBCV	Faecal	6 June 2005	Wakayama/2/05 (WK-2)	4
Iwade	EBCV	Faecal	4 June 2005	Wakayama/3/05 (WK-3)	4

Table 1. cont.

Prefecture/city	Type	Sample	Date	Isolate*	Group
Wakayama	EBCV	Faecal	10 June 2005	Wakayama/4/05 (WK-4)	4
Kinokawa	EBCV	Faecal	28 October 2005	Wakayama/5/05 (WK-5)	4
Wakayama	EBCV	Faecal	23 May 2003	Wakayama/6/03 (WK-6)	3
Osaka					
Kishiwada	EBCV	Faecal	2 March 2006	Osaka/1/06 (OS-1)	4
Izumi	EBCV	Faecal	6 March 2006	Osaka/2/06 (OS-2)	4

*Abbreviation of virus isolates is shown in parentheses.
†Virus was isolated from non-diseased cattle.

is proposed that the polymorphic region of the S gene is useful for studying the genetic evolution of BCoV.

Studies to reveal the genetic determinants of the different clinical symptoms of BCoV (RBCoV, EBCoV, WD and CD) have proposed several amino acids as contributing to each disease type (Chouljenko *et al.*, 1998; Hasoksuz *et al.*, 2002; Jeong *et al.*, 2005; Liu *et al.*, 2006; Rekik & Dea, 1994). However, no clear genetic markers have been established and *in vivo* experiments using virus strains altered by reverse genetics are required.

Isolates HK7 to -11, obtained from nasal-swab samples of cattle showing respiratory disease, were also clustered in group 3, together with other EBCoV strains. Chouljenko *et al.* (1998) reported that five RBCoV-specific amino acid substitutions at aa 465, 510, 531, 543 and 578 can be determinants of the disease type among respiratory, enteric

Table 2. Comparison of the predicted amino acid sequences of the polymorphic region of the S glycoprotein

Underlined italics indicate amino acid sites known to be RBCV-specific and bold type indicates those known to be virulence-specific (Chouljenko *et al.*, 1998). Asterisks indicate the same amino acid as the Mebus strain.

Strain	457	458	459	465	470	484	499	501	509	510	514	525	531	543	546	547	554	563	570	571	578
Mebus	Q	F	V	V	H	S	N	P	N	S	T	H	N	S	P	Y	Y	S	D	Y	T
Kakegawa	*	S	*	*	*	T	*	*	*	*	*	*	D	*	*	*	*	*	*	*	*
Quebec	*	*	*	*	*	K	*	*	*	*	*	*	D	*	*	*	*	*	*	*	*
LSU	*	S	*	*A*	**D**	T	S	S	*	*T*	*	*	*G*	*A*	*	*	*	*	*	*	*S*
OK	*	S	*	*A*	**D**	T	S	S	*	*T*	*	*	*G*	*A*	*	*	*	*	*	*	*S*
LY138	*	S	*	*	**D**	T	S	S	*	*	*	*	*	*	*	*	*	*	*	*	*
F15	*	S	*	*	**D**	T	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
HK1, -3	*	S	*	*A*	**D**	T	*	*	T	*	*	*	D	*A*	*	*	*	*	N	H	*S*
HK2, 5, -6	*	S	*	*A*	**D**	T	*	*	T	*	*	*	*	*A*	*	*	*	*	N	H	*S*
HK4, TC5, -8	*	S	*	*A*	**D**	T	*	*	T	*	*	*	*	*A*	*	*	*	*	*	H	*S*
HK7, -8	*	S	*	*A*	**D**	T	*	*	T	*	*	*	D	*A*	*	*	*	*	*	L	*S*
HK9, -10, -11, -12, TC6, -9, -10, -11, YM1, -2, -5, -6, -7, WK6	*	S	*	*A*	**D**	T	*	*	T	*	*	*	D	*A*	*	*	*	*	*	H	*S*
HK13, -14, -15, -16, TC4	*	S	*	*A*	**D**	T	*	*	T	*	*	*	*G*	*A*	*	*	*	*	*	H	*S*
HK17, -18, -20, -21, IS5	*	S	*	*A*	**D**	T	S	S	*	*T*	*	*	D	*A*	*	H	*	*	*	H	*S*
HK19	*	S	*	*A*	**D**	T	S	S	*	*T*	*	*	D	*A*	S	H	*	L	*	H	*S*
IS1	*	S	G	*A*	*	T	S	S	*	*T*	*	Y	H	*A*	*	N	H	*	*	H	*
IS2	*	S	G	*A*	*	T	S	S	*	*T*	*	Y	H	*A*	*	N	*	*	*	H	*
IS3, TC2	*	S	*	*A*	**D**	T	S	S	*	*T*	*	*	D	*A*	*	*	*	*	*	*	*S*
IS4	*	S	*	*A*	**D**	T	K	*	T	*	*	*	D	*A*	*	*	*	*	*	H	*S*
IS6	E	S	*	*A*	**D**	T	S	S	K	*T*	*	*	D	*A*	*	*	*	*	*	*	*S*
IS7, -8, -9	*	S	*	*A*	**D**	A	S	S	*	*T*	*	*	D	*A*	*	H	*	*	*	H	*S*
TC1	*	S	G	*A*	*	T	S	S	*	*T*	*	Y	D	*A*	*	N	*	*	*	H	*
TC7	*	S	*	*A*	**D**	T	*	*	T	*	*	*	*G*	*A*	*	*	*	*	N	H	*S*
YM3, -4	*	S	*	*A*	**D**	T	S	S	K	*T*	*	*	D	*A*	*	*	*	*	*	*	*S*
WK1, -2, -3, -4, -5	*	S	*	*A*	**D**	T	S	F	*	*T*	*	*	D	*A*	*	H	*	*	*	H	*S*
OS1, -2	*	S	*	*A*	**D**	T	S	S	*	*T*	S	*	D	*A*	*	H	*	*	*	H	*S*

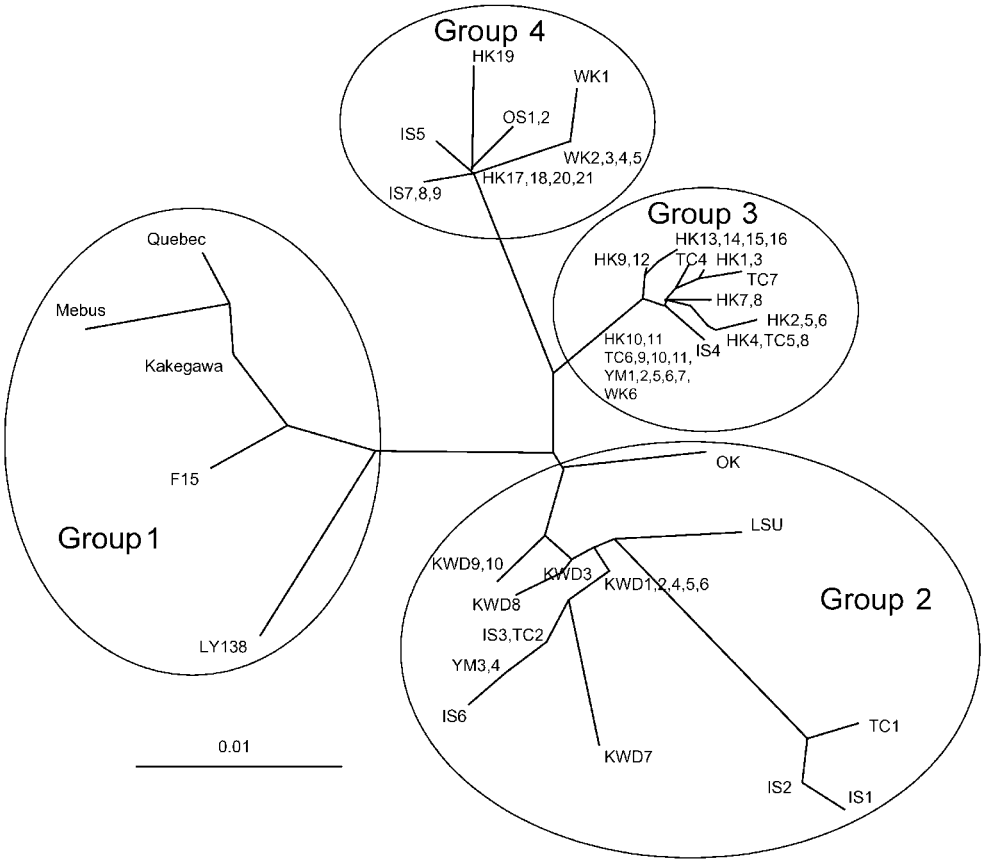

Fig. 1. Phylogenetic tree generated by neighbour-joining analysis of genetic distances in the polymorphic region (nt 1366–1776) of the S gene (Rekik & Dea, 1994).

and vaccine strains by using the data of the RBCoV strains LSU and OK. Among the Japanese field isolates, RBCoV HK7 to -11 exhibited amino acids different from those in LSU and OK at aa 510 and 531 (Table 2). Furthermore, these RBCoV-specific amino acids at aa 510 and 531 (Thr and Gly) were detected in several Japanese EBCoV isolates. At aa 465, 543 and 578, RBCoV HK7 to -11 showed the same amino acids as those in LSU and OK, but other Japanese EBCoV also showed the same amino acids at the relevant positions (all isolates have Ala at aa 465, Ala at aa 543 and Ser at aa 578 except for IS1, -2 and TC1, which have Thr at aa 578). Similar results have been shown in the Korean field EBCoV strains (Jeong *et al.*, 2005). Hence, our data also suggest that these five amino acids in the polymorphic region may not contribute to the respiratory disease type. This reasoning is applicable in the case of isolates IS7 to -9 that were obtained from nasal or faecal samples of individual cattle from herds in which severe diarrhoea was observed after the appearance of respiratory-disease symptoms. The nucleotide sequence of these isolates was identical in the polymorphic region. Further, it also suggests that there are no disease type-specific amino acids in this region.

Chouljenko *et al.* (1998) described virulence-specific amino acids in the S gene. Of these seven amino acids, aa 470 in the polymorphic region in avirulent strains Mebus and L9 was His, in contrast to the virulent strains (F15, LY138, LSU and OK) that had Asp. In our study, HK12 isolated from non-diseased cattle had Asp, whereas IS1, -2 and TC1 had His, similar to the Mebus strain. These results suggest that this amino acid at aa 470 is not independently responsible for the virulence of BCoV.

Further, it was also observed that the genetic determinants for WD and CD may not be present in this polymorphic region because HK4, isolated from a newborn calf, showed no significant difference from the other isolates and its sequence was identical to that of the EBCoV isolates TC5 and -8.

The predicted proteolytic-cleavage site at aa 763–768 with the sequence KRRSRR (Abraham *et al.*, 1990) was conserved in all Japanese field isolates (data not shown). Chouljenko *et al.* (1998) reported that the amino acid immediately after this cleavage site (aa 769) was an RBCoV-specific amino acid. However, Hasoksuz *et al.* (2002) reported that this Ser was observed in both respiratory and enteric

strains and that the Korean EBCoV strains also had Ser at aa 769 (Jeong *et al.*, 2005); they concluded that this amino acid does not appear to be a potential marker of respiratory tropism. In our study, all Japanese field isolates had Ser at aa 769 (data not shown), and our results support the observation of Jeong *et al.* (2005).

Our study demonstrates no virulence-specific or disease type (RBCoV and EBCoV)-specific sites in the polymorphic region of the S gene. However, the S glycoprotein has important roles in virus infection, such as host-receptor binding, haemagglutination and induction of neutralizing antibodies; hence, this fact led us to hypothesize that such determinants would be present in this gene. To clarify the functions of the S gene, it is necessary to analyse the remaining region. On the basis of our recent study, we hypothesize that the genetic determinants of pathogenic properties may be in another region of the BCoV genome. In *Porcine reproductive and respiratory syndrome virus*, a member of the family *Arteriviridae* in the order *Nidovirales*, together with the family *Coronaviridae*, amino acid changes in ORFs 1a, b and 6 may provide the molecular basis for the attenuated phenotype (Grebennikova *et al.*, 2004). Therefore, it is necessary to focus on other genomic regions of BCoV for investigating the genetic determinants of pathogenicity properties, if they exist in the genome.

In summary, molecular analysis of the polymorphic region of the S gene using recent Japanese field isolates and reference strains revealed that recent isolates collected between 1999 and 2006 have distinctive genetic divergence from the prototype EBCoV strains (Mebus, Quebec, Kakegawa, F15 and LY138) and have diverged in three different aspects. Over these 8 years, genetic divergence in the polymorphic region of the S gene was observed to have progressed. This suggests that molecular analysis using this region is useful for investigating the molecular epidemiology of BCoV. Our finding that the HE glycoprotein gene (1275 bp) shows no significant genetic divergence among the Japanese isolates (>99 % identity; data not shown) also supports this hypothesis. In addition, based on the differences in the amino acids among the isolates, our study did not reveal the presence of certain genetic markers of pathogenicity and clinical symptoms in this polymorphic region.

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