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Bovine torovirus (BToV), a member of the Coronaviridae family, was first detected in the United States in 1982 during an outbreak of diarrhea in cattle (Woode et al., 1982). Since then, BToV has been established as a causative agent of diarrhea in cattle (Duckmanton et al., 1998a; Koopmans et al., 1991; Liebler et al., 1992; Pohlenz et al., 1984; Scott et al., 1996). The tropism of BToV for the intestinal tract and its genomic structure are similar to those of another member of the Coronaviridae family, bovine coronavirus (BCV); both viruses contain genes encoding the following proteins: RNA polymerase, spike (S), hemagglutinin-esterase (HE), and nucleocapsid (N) (Draker et al., 2006).

BCV is capable of infecting epithelial cells in both the intestinal and respiratory tracts; consequently, oral and nasal routes are etiological risk factors for developing BCV infections in cattle (Cho et al., 2001; Park et al., 1996). Similarly, the affinity of BToV for the tissues of the respiratory tract has been reported (Vanopdenbosch et al., 1991, 1992), although these finding are still unclear due to possible contamination of the reagents used (Cornelissen et al., 1998). In addition, a study conducted at a single farm in the United States showed the presence of BToV in both fecal and nasal swabs from feedlot cattle, suggesting that BToV infections may occur in cattle via the nasal route (Hoet et al., 2002). However, no surveys concerning with the relation between respiratory symptoms and the detection of BToV have been conducted in wide range.

Viral isolation via cell culture is the most desirable method for obtaining such epidemiological data, but no reports have described the detection of BToV in cultured cells except for that made by Kuwabara et al. (2007). Similarly, the affinity of BToV for the tissues of the respiratory tract has been reported (Vanopdenbosch et al., 1991, 1992), although these finding are still unclear due to possible contamination of the reagents used (Cornelissen et al., 1998). In addition, a study conducted at a single farm in the United States showed the presence of BToV in both fecal and nasal swabs from feedlot cattle, suggesting that BToV infections may occur in cattle via the nasal route (Hoet et al., 2002). However, no surveys concerning with the relation between respiratory symptoms and the detection of BToV have been conducted in wide range.

Viral isolation via cell culture is the most desirable method for obtaining such epidemiological data, but no reports have described the detection of BToV in cultured cells except for that made by Kuwabara et al. (2007) in which cytopathogenic BToV was isolated from HRT-18 cells derived from a human rectal adenocarcinoma. Amplification of the N gene by nested reverse transcription-polymerase chain reaction (RT-PCR) has shown a high degree of conservation among BToV strains (Ito et al., 2007).
In this study, we analyzed 311 nasal samples collected from Japanese cattle (107 from dairy cattle and 204 from beef cattle) for BToV and BCV by nested RT-PCR to amplify the N gene. Additionally, isolation of infectious bovine rhinotracheitis virus (IBRV), bovine viral diarrhea virus (BVDV) and bovine respiratory syncytial virus (BRSV) was performed using bovine testis and bovine kidney cells. Subsequently, the S and HE gene regions of BToV, which have been shown to be correlated with the antigenic properties of coronavirus (Clark, 1993; Gallagher and Buchmeier, 2001; Jackwood et al., 2005; Phillips et al., 2001; Yoo and Deregt, 2001), were amplified from the BToV-N gene-positive samples, and their nucleotide sequences were compared to investigate the level of genetic diversity among the BToVs detected in the nasal samples with one another and against previously collected fecal samples (Draker et al., 2006; Duckmanton et al., 1998b; Ito et al., 2007; Kuwabara et al., 2007; Smits et al., 2003). Nasal swabs were collected from 205 cattle showing respiratory symptoms and 106 asymptomatic cattle raised on 42 farms located in 16 prefectures between March 2006 and June 2008; 264 of the cattle were less than 12 months old.

The nasal swabs were diluted 1:10 in Dulbecco’s modified Eagle’s medium and centrifuged at 3000 \( \times g \) for 5 min at room temperature; the supernatants were subsequently collected and subjected to RNA extraction followed by nested RT-PCR. The methods used for extraction of the RNA and for amplification of the N, S, and HE genes from BToV and the N gene from BCV by nested RT-PCR, and the primer sets used to target the N and S genes of BToV and the N gene of BCV are described elsewhere (Ito et al., 2007). The primers used in the first round of PCR and for nested RT-PCR to target the BToV HE gene were as follows: first step forward, 5’-GGG CAA CAC CAG TAA CAC CAT-3’; first step reverse, 5’-TAA CTA AAA CTA ATA ACA CC-3’; nested forward, 5’-GAT TGG GTG GGG TTT GGT GA-3’; and nested reverse, 5’-ATA TGC AGA GGA GGT TAC ATC-3’. The expected product was 1094 bp long, corresponding to nucleotides 26,564–27,657 of AY427798. Nucleotide sequencing and phylogenetic tree analysis were performed as described previously (Ito et al., 2007).

The N gene of BToV and BCV was detected in 2.3% (7/311) and 10.9% (34/311) of the nasal samples, respectively (Table 1a). Seven BToV-N-positive samples, referred to as rBToV-1 through -7, were identified in six herds from five prefectures (Table 1b). Compared to the report from the U.S. feedlot, in which BToV was detected in almost all of the cattle during the first 21 days after arrival (Hoet et al., 2002), the frequency of BToV-positive cattle in this study was significantly lower. The reason of this difference is unclear at present as the sampling condition (population size, nature of farms, sampling period) between Hoet et al. and our study differ considerably. Supporting this idea, Hoet et al. (2002) reported that newly arrived feedlot calves became infected with BToV soon after arrival and shed BToV in their feces and nasal secretions; however, secretion of the virus disappeared according to the calves acquired immunocompetence against BToV. Compared to the detection situation of BCV among the 311 samples, the detection of BToV was restricted to symptomatic calves. In addition, the detection rate was less than half that for BCV; however, our results support the notion that BToV is shed in nasal secretions as well as in feces, and that like BCV, the virus is able to infect its host via oral and nasal routes. Among the animals that developed clinical symptoms, BToV, BCV, IBRV, BVDV and BRSV was detected in 3.4% (7/205), 15.1% (31/205), 0.5% (1/205), 1.0% (2/205) and 2.0% (4/205), respectively.

### Table 1

#### (a) Incidence of viral pathogens in 311 nasal samples

| Respiratory symptom | Age         | Total | BToV | BCV | IBRV | BVDV | BRSV |
|---------------------|-------------|-------|------|-----|------|------|------|
| *                   | 1–12 month  | 174   | 7 (4.0%) | 19 (10.9%) | 0 (0.0%) | 1 (0.6%) | 4 (2.3%) |
|                     | over 12 month | 31    | 0 (0.0%) | 12 (38.7%) | 1 (3.2%) | 1 (3.2%) | 0 (0.0%) |
|                     | Total       | 205   | 7 (3.4%) | 31 (15.1%) | 1 (0.5%) | 2 (1.0%) | 4 (2.0%) |
| –                   | 1–12 month  | 90    | 0 (0.0%) | 3 (3.3%) | 0 (0.0%) | 2 (2.2%) | 0 (0.0%) |
|                     | over 12 month | 16    | 0 (0.0%) | 3 (18.8%) | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) |
|                     | Total       | 106   | 0 (0.0%) | 3 (2.8%) | 0 (0.0%) | 2 (1.9%) | 0 (0.0%) |
| Total               | 311         |       | 7 (2.3%) | 34 (10.9%) | 1 (0.3%) | 4 (1.3%) | 4 (1.3%) |

#### (b) The general condition of the BToV-positive cattle upon the collection of the nasal samples

| Sample name | Sampling month | Prefecture | Age | breed | Other detected pathogens | BToV nested RT-PCR result |
|-------------|----------------|------------|-----|-------|--------------------------|--------------------------|
| rBToV-1     | 2006. Dec      | Gifu       | 8 month | beef | Bovine coronavirus | + | + | – |
| rBToV-2     | 2007. Apr      | Gifu       | 7 month | beef |              | + | + | + |
| rBToV-3     | 2007. Jun      | Akita      | 2 month | beef |              | + | + | – |
| rBToV-4     | 2008. Jan      | Aichi      | 1 month | dairy |              | + | + | + |
| rBToV-5     | 2008. Jan      | Aichi      | 2 month | dairy | Mycoplasma bovis | + | + | + |
| rBToV-6     | 2008. Mar      | Nara       | 5 month | beef |              | + | + | + |
| rBToV-7     | 2008. Jun      | Chiba      | 6 month | beef |              | + | – | – |

* No. (%) of positive cattle.
and K-684 (AB270919). (AJ575388 and AJ575379), B150 (AJ575380), B155 (AJ575381), K-640 and K-644 (AB270909), K-674 (AB270914), K-664 (AB270916), K-683 (AB270918), and K-684 (AB270919).

The reference bovine toroviruses (BToVs) used in the comparison and their accession numbers are as follows: BRV-1 (AY427798), BRV-2 (AF076621), B145 (AJ575388 and AJ575379), B150 (AJ575380), B155 (AJ575381), K-640 and K-644 (AB270909), K-674 (AB270914), K-664 (AB270916), K-683 (AB270918), and K-684 (AB270919).

The BToV S gene was detected in all of the N gene-positive samples except rBToV-7. Regarding the S coding region, the respiratory tract-derived BToVs showed over 91.1% nucleotide similarity and more than 91.4% amino acid similarity with one another. Compared to the previously identified fecal tract-derived BToVs, rBToV showed 89.6–99.2% nucleotide similarity and 89.3–99.5% amino acid similarity (Table 2a). The BToVs used for the comparison were BRV-1 and -2 from North America; B145, B150, and B155 from Europe; and K-640, K-644, and Aichi/2004 from Japan (see Table 2 for the relevant accession numbers). Among the respiratory tract-derived samples, rBToV-1, -2, and -3 were completely homologous despite the fact that the samples were obtained from cattle raised in distinct areas and during different seasons. These tests were performed one or two times to confirm the initial results.

Phylogenetic tree analysis of the region revealed that rBToV-1, -2, and -3 were closely related to BToV cluster 2 (K-674 and K-676), which was classified among the fecal-derived BToVs from Japan (Ito et al., 2007) (Fig. 1a). In contrast, rBToV-4 and -5 were distant from rBToV-1, -2, and -3 compared to B145 and BRV-1. rBToV-6 was clustered with K-640 and K-644 (cluster 1), as was Aichi/2004. Although two calves corresponding to rBToV-4 and -5 samples were born in same farm and the nasal samples were collected at the same time, they were clustered on a separate branch; rBToV-4 was closer to
K-683 and K-684 (cluster 3) than rBToV-5. The origin of this difference remains unclear until now.

In contrast to the N and S genes, HE was not detected in rBToV-1, -3, or -7. Regarding the HE coding region, the respiratory tract-derived BToVs showed over 90.8% nucleotide similarity and more than 91.1% amino acid similarity with one another. Compared to the fecal tract-derived BToVs, rBToV showed 73.5–99.0% nucleotide similarity and 72.0–99.1% amino acid similarity (Table 2b).

Fig. 1. Neighbor-joining phylogenetic tree showing the relationships for (a) the deduced spike and (b) hemagglutinin-esterase amino acid sequences from Japanese bovine torovirus and the reference strains described in Table 2. The numbers represent the distance to the nearest node.
As shown by phylogenetic tree analysis of the HE region, rBToV-2 exhibited significant diversity, especially for BRV-1 compared to S, but it still retained a high correlation with K-674 and K-676 compared to B145 and the other domestic BToVs (Fig 1b). Similar to S, divergence has also been detected between rBToV-4 and -5 but additionally, they were clustered on a separate major branch; rBToV-4 was clustered with K-683 and K-684, while rBToV-5 was clustered with K-640 and K-644. rBToV-6 was distant from the other rBToVs. Representative sequence data have been deposited in the nucleotide database (DNA Data Bank of Japan) and assigned the following accession numbers: AB371899–AB371906, AB448741–AB448746.

As no comparative analysis of respiratory tract-derived BToVs has been performed, we cannot compare our results with those for other respiratory tract-derived BToVs. On the other hand, previous research of the S region in domestic fecal BToVs showed greater than 99% homology among the samples collected from a single herd at the same time (Ito et al., 2007). Accordingly, the existence of two distinct viruses in a single herd, as in the case of rBToV-4 and -5, has not yet been reported. rBToV-1, -2, and -3, however, were identical despite the data having been collected during different seasons. These results suggest diversity among domestic rBToVs in terms of their geographic and genetic characteristics. In contrast, Smits et al. (2003) reported a case of intertypic recombination between BToV and porcine torovirus within the HE region that was observed in B150 and B155. However, although HE exhibited a somewhat higher degree of diversity compared to S, none of the domestic BToVs were overwhelmingly diverse as the variety observed in B150 and B155 (Table 2b).

Among the members of the Coronaviridae family, porcine coronavirus is a well-known example of a virus with affinity for both the intestinal and respiratory tracts (porcine transmissible gastroenteritis virus [TGE] and porcine respiratory coronavirus [PRCV]). The two viruses are antigenically related to each other, and PRCV is believed to have originated from TGEV by the deletion of the HE coding region, the BToVs possesses dual tropisms (to both the intestinal and respiratory tracts), similar to BCV. In conclusion, while we confirmed the possibility of respiratory tract transmission for BToV and a possible association with respiratory disease in young cattle, its etiological importance in cattle still remains obscure. Further investigation into the interactions between BToV and various tissues, viral persistence, and the immunological responses of infected cattle are needed to clarify the relationship between intestinal and respiratory disease and BToV infection. Isolating BToVs capable of being propagated by cell culture (preferably cytopathogenic) and investigating the immunological responses of infected cattle as well as their antigenic properties are of the utmost importance.

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