Genetic and Antigenic Characterization of Newly Isolated Bovine Toroviruses from Japanese Cattle

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Torovirus, a member of the Coronaviridae family, is a gastrointestinal infectious agent that has been identified in humans, cattle, pigs, and equines. Toroviruses, except equine torovirus, are difficult to propagate in cell culture; indeed, to date, only the Aichi/2004 strain of bovine torovirus (BToV) has been isolated among the human, bovine, and porcine toroviruses. In the present study, four cytopathogenic BToVs were isolated from diarrheal feces of the cattle using the HRT-18 cell line, and their genetic and antigenic properties were compared. The cytopathogenic features of BToV isolates in HRT-18 cells were similar to those of the Aichi/2004 strain. However, none of the isolates showed cytopathogenic effects in the HRT-18 cells of different origin, suggesting that one significant factor contributing to the cytopathogenicity of BToV depends on properties of the HRT-18 cells themselves. All BToVs isolated were able to agglutinate mouse, but not chicken, erythrocytes, while they lacked receptor-destroying enzyme activity. Analysis of the N terminus of the spike gene showed that three isolates, but not the Gifu-2007TI/E strain, were phylogenetically located in cluster 1 and its analogs and revealed high cross-reactivity with each other, as demonstrated by neutralization (NT) and hemagglutination inhibition (HI) assays. The Gifu-2007TI/E strain was classified close to cluster 2 and exhibited relatively low cross-reactivity with these viruses; however, the difference was not sufficient to classify BToVs into serotypes, suggesting that at least two subtypes distinguishable by the structure of the N terminus of the spike gene and that both NT and HI tests may be exist.

The family Coronaviridae consists of enveloped and positive-stranded RNA viruses, specifically coronaviruses and toroviruses (2, 3). Toroviruses identified thus far have been further classified into human, bovine, porcine, and equine toroviruses on the basis of their hosts. Several studies have established associations between human and bovine toroviruses with gastrointestinal and respiratory symptoms (1, 10, 11, 21). However, the research on toroviruses has been limited, compared to that on coronaviruses, primarily because toroviruses, with the exception of equine torovirus, are very difficult to propagate in cell culture (20). In fact, among the human, bovine, and porcine toroviruses, only one strain of bovine torovirus (BToV) has been successfully propagated in cell culture. This strain was isolated from the intestinal contents of a calf with diarrhea in 2004 using the stable cell line HRT-18, derived from a human rectal tumor (13). Although several studies on the genetic characterization of toroviruses have been published, almost all were dependent on analyses of PCR products from biomaterials (5, 9, 10, 12, 18). Thus, antigenic variation among toroviruses and relationships between their genetic and antigenic properties using culturable viruses in cell cultures remain to be investigated.

Epidemiological research conducted by our group has suggested that BToV may act as a risk factor for the induction of gastrointestinal and respiratory diseases in cattle in Japan. In addition, the sequences of coding regions for the spike (S) and hemagglutinin-esterase (HE) genes of BToV have been shown to vary among several field viruses (9, 10). Because the products of both genes in coronaviruses are known to correlate closely with their antigenic properties (4), the relationship between genetic and antigenic properties, and antigenic variation of BToV, seem to be important lines of research with respect to understanding immunological and pathogenic aspects of BToV infection and in the development of an effective vaccine.

In the present study, fecal and nasal samples positive for the BToV nucleocapsid (N) gene by reverse transcription-PCR (RT-PCR) were inoculated onto monolayers of HRT-18 cell cultures, and four cytopathogenic BToVs were successfully isolated. The purpose of the present study was to report the isolation of cytopathogenic BToVs in cell culture and their characterization, with respect to their genetic and antigenic properties.

MATERIALS AND METHODS

Samples. In total, 55 individual samples collected from Japanese cattle positive for the BToV N gene, as determined by the RT-PCR method described previously (9, 10), were used for virus isolation. These samples included some BToV-positive fecal and nasal samples that we have reported previously. The details of the samples were as follows: fecal samples were collected from 48 cattle, 47 showing enteric symptoms and 1 that was asymptomatic, raised on 20 farms located in 12 prefectures between September 2004 and May 2009; nasal samples were obtained from 7 cattle with respiratory symptoms on 6 farms located in 5 prefectures between December 2006 and June 2008. Each sample was diluted 1:10 in Dulbecco modified Eagle medium (DMEM) and centrifuged (3,000 × g, 5 min, room temperature [9]). The supernatants were collected and used for virus isolation in HRT-18 cell cultures.

Virus isolation. Virus isolation was carried out using the human rectal tumor cell line HRT-18. The reference Aichi/2004 strain of BToV was used as a positive control. The HRT-18 cells and reference virus were kindly supplied by M. Kuwabara, Central Livestock Hygiene Service Center, Aichi Prefecture, Japan.

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(13). Confluent monolayers of HRT-18 cells, grown in test tubes, were inoculated with 0.1-ml portions of the samples. At 60 min after static absorption at 37°C, all cultures received 1.0 ml of DMEM, were further incubated with rotation at 37°C for 7 days, and were observed for cytopathic effect (CPE). These procedures were further repeated three times. CPE-positive samples were cloned by two rounds of a limiting dilution method and tested for the presence of BToV, bovine coronavirus (BCoV), and bovine rotavirus, as described previously (9, 15).

**Measurement of HA and RDE activity.** The hemagglutinin (HA) and receptor-destroying enzyme (RDE) titers of isolated virus were measured by the microplate method using mouse (1.0%) and chicken (0.5%) erythrocytes, as described elsewhere (16).

**Immunization of guinea pigs.** Antisera against the isolates and reference strain were produced in guinea pigs, as described elsewhere (19). Briefly, concentrated viral suspensions were prepared from culture media by ultracentrifugation, emulsified with oil adjuvant consisting of liquid paraffin supplemented with 10% anhydrous mannitol-oleic acid ester, and used for immunization. The amount of virion contained in the final immunogens was estimated at 10^0.9 median tissue culture infective doses (TCID_50/ml). Guinea pigs were injected intramuscularly twice with 0.5 ml and subsequently intraperitoneally with 2.5 ml of the respective immunogens at weekly intervals for 3 weeks. At 2 weeks after final inoculation, sera were collected and used in the experiments.

**Genetic analyses.** The isolates were subjected to RNA extraction, followed by nested RT-PCR. The methods used for RNA extraction and nested RT-PCR amplification of the BToV S gene, and primer sets used in the reaction are described elsewhere (9). In addition, the following primers were used to amplify the full-length open reading frame (ORF) of the S gene: fl-F1, ATT TTT GCT GTT GTG TTG AAG (corresponding to nucleotides 22274 to 22294 of AY427798); fl-F2, GTT GCA AGT YTA TGA AAC ACC (corresponding to nucleotides 23155 to 23174 of AY427798); fl-F3, TGC ACT TCA ATG GAT TAT TTT (corresponding to nucleotides 23855 to 23875 of AY427798); fl-F4, AAC ATC TTT GGC TCA TTG GTA GG (corresponding to nucleotides 24917 to 24936 of AY427798); fl-R1, GCA TGG AAA CCT GAT GTA TT (corresponding to nucleotides 25053 to 25072 of AY427798); fl-R2, AAA GCC CAC ACA ACC AGG TA (corresponding to nucleotides 25155 to 25174 of AY427798); fl-R3, TGT GAA GAT GGT GAT ACA AA (corresponding to nucleotides 25605 to 25624 of AY427798); fl-R4, ATA CCT ATC GCA ATG ATC GTA CTT (corresponding to nucleotides 25639 to 25658 of AY427798). Nucleotide sequencing and phylogenetic tree analyses were performed as described elsewhere (9).

**Virus neutralization (VN) test.** Antisera were serially diluted 2-fold with serum-free DMEM and incubated at 37°C for 60 min with an equal volume of the respective viral suspension containing 200 median TCID_50/ml. Each serum-virus mixture was inoculated onto confluent monolayers of HRT-18 cells grown in test tubes. After 60 min of static absorption at 37°C, all cultures received 1.0 ml of DMEM and were incubated with rotation at 37°C for 7 days. Neutralization antibody titers were expressed as the reciprocal of the highest serum dilution that completely inhibited HA.

**HI test.** Antisera were treated with kaolin and mouse erythrocytes to remove nonspecific hemagglutinins before testing, according to methods described by Hasokazu et al. (7). Serial 2-fold dilutions of antisera were made in a volume of 25 μl and mixed with an equal volume of 8 HA units of each viral suspension. After a 60-min incubation at 37°C, 25 μl of a 1.0% mouse erythrocyte suspension was added to each suspension. Settling patterns of erythrocytes were read 90 min after incubation at 22°C, and hemagglutination inhibition (HI) antibody titers were expressed as the reciprocal of the highest serum dilution that completely inhibited HA.

**Antigenic-relatedness values.** Antigenic similarities among the BToV isolates were indicated by the percent antigenic-relatedness values (R), calculated from the results of cross-VN and HI tests using the following formula, as described previously (7, 17):

\[ R = 100 \times \frac{1}{\frac{1}{r_1} + \frac{1}{r_2}} \]

where \( r_1 \) and \( r_2 \) are the heterologous titer of strain 1/homologous titer of strain 2 and the heterologous titer of strain 2/homologous titer of strain 1, respectively.

**RESULTS**

**Virus isolation.** Four fecal samples of the 55 fecal and nasal samples investigated revealed CPE similar to that of the reference Aichi/2004 strain after serial passage in HRT-18 cell cultures. Agents causing CPE were identified as BToV by RT-PCR and immunofluorescence using antisera prepared to the reference Aichi/2004 strain of BToV (data not shown). They were designated as the Miyagi-2006TI/E, Gifu-2007TI/E, Hokkaido-2008TI/E, and Gifu-2009TI/E strains.

**HA and RDE activity.** Four BToV isolates, as well as the reference Aichi/2004 strain, showed similar HA activity, with titers ranging from 16 to 128 to mouse erythrocytes at both 34°C and 37°C (Table 2). However, none of them agglutinated chicken erythrocytes at either temperature. Moreover, all of the isolates lacked RDE activity against both mouse and chicken erythrocytes.

**Genetic analysis.** As indicated in Table 3, the similarities of the 594-bp nucleotide sequences and 197 amino acid (aa) residues of the S gene N-terminal region were ≥91.6 and ≥91.9% among the isolates and the reference Aichi/2004 strain, respectively. These similarities were comparable with the results determined previously in comparing BToVs derived from feces and nasal swabs (9, 10). Interestingly, the nucleotide sequences of Hokkaido-2008TI/E and Gifu-2009TI/E strains were completely identical, although they were derived from the clinical

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**TABLE 1. Origin and general properties of the BToV strains isolated from cattle**

<table>
<thead>
<tr>
<th>BToV strain</th>
<th>Origin of the sample</th>
<th>Material</th>
<th>Sampling period</th>
<th>Prefecture</th>
<th>Other pathogen detected</th>
<th>Body condition (symptoms)</th>
<th>Age (mo)</th>
<th>Breed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Miyagi-2006TI/E</td>
<td>Loose stool</td>
<td>Muddy diarrhea stool</td>
<td>Feb. 2006</td>
<td>Miyagi</td>
<td>BCoV</td>
<td>Gastrointestinal</td>
<td>Calf</td>
<td>Beef</td>
</tr>
<tr>
<td>Hokkaido-2008TI/E</td>
<td>Loose stool</td>
<td></td>
<td>Mar. 2008</td>
<td>Hokkaido</td>
<td>BCoV</td>
<td>Gastrointestinal and respiratory</td>
<td>1</td>
<td>Beef, female</td>
</tr>
</tbody>
</table>
specimens collected at geographically distinct areas and at different times.

Analysis of the full-length ORF of the S gene showed that the ORF of the isolates and the reference Aichi/2004 strain encoded a 1,584-aa residue protein, as did the registered sequences of the BRV-1 and B145 that were detected in the feces of cattle from North America and Europe, respectively (5, 18). Among the four isolates and the reference Aichi/2004 strain, the nucleotide similarities of the S gene ORF were $\geq 95.4\%$ for the nucleotide sequence and $\geq 96.1\%$ at the amino acid level (Table 4). In a comparison with all registered sequences, the similarities of the nucleotide sequence and amino acid residues of the S gene ORF were $\geq 91.5\%$ and $\geq 90.3\%$, respectively. Representative sequence data have been deposited in the DNA Data Bank of Japan and assigned the accession numbers AB526862 to AB526866.

Phylogenetic analysis of the 197-aa region of the S gene N-terminal revealed that Miyagi-2006TI/E, the sequence of which was identical to that of K-637, grouped into cluster 1, which also included the Aichi/2004 strain (9). The Hokkaido-2008TI/E and Gifu-2009TI/E strains were located in a position close to cluster 1 and were most closely related to rBToV-6, detected previously in a nasal swab collected from a Japanese cow (10). In contrast, Gifu-2007TI/E was distant from the other BToV isolates and close to cluster 2, which consisted of the Aichi/2004 strain (9). The Hokkaido-2008TI/E strain was located in cluster 1 and was most closely related to rBToV-6, which also included the Aichi/2004 strain (9). The Hokkaido-2008TI/E strain was also distant from the other BToV isolates and close to cluster 2, which consisted of the Aichi/2004 strain (9).

Indeed, to date, only one strain has been isolated among human, bovine, and porcine toroviruses (13). In the present study, however, cytopathogenic BToVs were isolated from fecal samples collected from four cattle between February 2006 and March 2009. They all showed intestinal symptoms and some manifested respiratory signs, a finding suggestive of that BToV played some roles as a predisposing factor.

The HRT-18 cell line used in the present study was provided by M. Kuwabara, who succeeded in first isolating a cytopathogenic BToV strain, Aichi/2004 (13). Unexpectedly, all of the BToVs isolated in the present study lacked susceptibility to HRT-18 cells maintained in our laboratory and used previously. In contrast, the BCoV strains isolated previously in the HRT-18 cells of our laboratory failed to propagate in or exhibited only weak susceptibility to those provided by M. Kuwabara. The fecal materials from which the Gifu-2007TI/E and Gifu-2009TI/E strains were isolated were coincidentally positive for BCoVs, but only BToVs were propagated after repeated passage in cell culture. This seems to indicate that BToVs were preferentially propagated under the particular conditions, including the source of the HRT-18 cells used. In addition, the virus isolation process, several bovine enteroviruses, which had been considered as being nonreplicable in HRT-18 cells, were isolated from several samples besides four BToV isolated samples (our unpublished data). Based on these
results, one significant factor that allowed the BToV isolation appears to be the particular properties of the HRT-18 cell line used. Although the cytological differences between the two lines of HRT-18 cells remain unclear, an unexpected cellular mutation that affects susceptibility to viruses might occur during long-term maintenance of the cells in the separate laboratories.

BToV, as well as certain other viruses, is known to possess HA activity to animal erythrocytes via hemagglutinin present on the surface of the viral envelope. BCoV, another member of the Coronaviridae family having similar genomic structures to BToV, is well known to show several HA and RDE reaction patterns with mouse and chicken erythrocytes (6, 7), whereas those of BToVs have not been investigated to date. In the present study, five BToVs, four novel isolates and the Aichi/2004 strain, were compared for HA and RDE reaction patterns to mouse and chicken erythrocytes, and we found that all viruses tested revealed similar HA and RDE properties to each other; they all agglutinated only mouse erythrocytes at both 4 and 37°C but lacked RDE activity. Because the number and source of BToVs tested were limited to five fecal specimens, the presence of BToV strains with different HA and RDE properties cannot be ruled out. However, that the five BToVs, including the five BToVs, tested were predominant (9). According to the S gene sequences we found that BToVs belonging to cluster 1 or its analogs, with the exception of the Gifu-2007TI/E strain, which belonged closer to cluster 2. In a previous report, we found that BToVs belonging to cluster 1 or its surroundings were predominant (9). According to the S gene sequences reported by Kirisawa et al. (12); however, 16 of 17 BToVs analyzed showed identity >99.0% with the Gifu-2007TI/E strain, suggesting that they are possibly placed around cluster 2. These findings suggest that BToVs of both genotypes and their surroundings are widespread in Japan.

The full-length S gene ORF of four isolates and foreign BToVs encoded 1,584 aa, with the exception of BRV-2, in which the ORF encoded 1,583 aa. Accordingly, BToV seems to be genetically well conserved among intestinally derived strains. To examine the relationship between tissue tropism and S gene structures of BToVs, isolating BToVs from respiratory materials and comparing their complete S gene sequences with those of intestinally derived BToVs would be of great interest.

The BToV isolates were phylogenetically classified into cluster 1 or its analogs, with the exception of the Gifu-2007TI/E strain, which belonged closer to cluster 2. In a previous report, we found that BToVs belonging to cluster 1 or its surroundings were predominant (9). According to the S gene sequences reported by Kirisawa et al. (12); however, 16 of 17 BToVs analyzed showed identity >99.0% with the Gifu-2007TI/E strain, suggesting that they are possibly placed around cluster 2. These findings suggest that BToVs of both genotypes and their surroundings are widespread in Japan.

All BToV isolates were antigenically related to each other, as revealed by both NT and HI tests, although the degree of cross-reactivity was different between the isolates. Moreover, the overall R values of both tests were well correlated with each other, although they were somewhat lower in the HI than NT tests. With respect to phylogenetic characteristics, the cross-reactivity between the Miyagi-2006TI/E and Aichi/2004 of cluster 1, and the its neighboring viruses Hokkaido-2008TI/E and Gifu-2009TI/E was remarkably high compared to the Gifu-2007TI/E strain, located around cluster 2. Unfortunately, no BToV belonging to cluster 3 was isolated in the present study, but investigating antigenicity in this context would be of interest. R values below 25% are generally regarded as an indication of a significant antigenic difference between two viruses.
and a difference greater than 20-fold in both directions (R < 5%) is used as a serotype criterion in several gastroenteric viruses (8, 17, 23). Thus, it seems unlikely that BToVs comprise multiple serotypes, at least based on the results of NT and HI tests using four viruses in the present study. BToVs seem to consist of a single serotype, although the serological properties of BToVs belonging to cluster 1 and surroundings and cluster 2 were markedly different. Nonetheless, our results suggest that at least two subtypes distinguishable by NT and HI tests, and probably related to the structures of N terminus of the S gene, may be present in BToVs. This is based on the finding that the sequence diversity between the Gifu-2007TI/E strain and the other isolates was greater in the N-terminal region than in the full-length S gene.

We believe the present study to be the first report dealing with antigenic characterization of BToVs by both NT and HI tests, and we could partially determine their serological characteristics. Antigenic and genetic investigations using more BToVs cultivatable in cell culture, especially those derived from respiratory specimens, are expected to yield more variable information for understanding serological properties of the BToVs.

Our preliminary investigation suggests that the HE gene of
the isolates consisted of a nonfunctional ORF that probably resulted from sequential insertion and/or deletion during the isolation process in tissue culture. Although the mechanism and frequency of these mutations remain unclear, the high mutability seems to be a unique characteristic for the HE gene, compared to the coding regions of the N and S genes, which revealed no or less divergence between isolated viruses and the original fecal materials (data not shown). This is not always relevant to antigenic diversity of BTV, however, because isolates classified within cluster 1 and its surroundings showed strong cross-reactivity in NT and HI tests. The details of the frequency, mechanism, and significance of these mutations in the HE gene are now under investigation.

A few studies have been conducted to determine pathogenicity and antigenicity of BTV using infected diarrheal stools (22). In the present study, we successfully isolated four novel BTV strains in tissue cultures following the report of Kuwabara et al., who first reported a cytopathogenic BTV in 2007 (13). We believe the cytopathogenic viruses isolated will provide important tools for studying the pathological role and immunology of BTVs in enteric and respiratory diseases.

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