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Whole genome analysis of Japanese bovine toroviruses reveals natural recombination between porcine and bovine toroviruses

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1. Introduction

Toroviruses (ToVs), belonging to the subfamily Toroviridae, are members of the family Coronaviridae, order Nidovirales (Cavanagh and Horznitek, 1993). ToVs have a single-stranded positive-sense RNA genome of nearly 28 kb, containing two large open reading frames (ORFs) encoding nonstructural proteins, ORF1a and ORF1ab, and four structural proteins, spike glycoprotein (S), membrane glycoprotein (M), hemagglutinin esterase (HE), and nucleocapsid phosphoprotein (N) (Draker et al., 2006; Sun et al., 2014).

ToVs are identified in various animals and humans and are thought to cause diarrheic and respiratory diseases (Duckmantion et al., 1997; Ito et al., 2009; Kroneman et al., 1998; Uziel et al., 1999; Vanopdenbosch et al., 1991; Wood et al., 1982). Berne virus (ETOV Berne), the first isolated ToV, was isolated from a horse with diarrhea in 1972 in Switzerland (Weiss et al., 1983). In 1979, a bovine ToV (BTOV), named Breda virus, was detected in calves with diarrhea in the United States (US). BTOV Breda causes enteric disease in gnotobiotic reared calves (Wood et al., 1982). BTOVs are found throughout the world including North America (Hoet et al., 2002, 2003), Central America (Pérez et al., 1998), Europe (Haschek et al., 2006; Koopmans et al., 1991; Matiz et al., 2002), Asia (Aita et al., 2012; Ito et al., 2010; Park et al., 2008) and South Africa (Penrith and Gerdes, 1992). BTOVs have been detected in 2.9-36.4% of fecal samples obtained from cattle with diarrhea (Duckmantion et al., 1998; Hoet et al., 2003; Ito et al., 2007; Kirisawa et al., 2007; Nogueira et al., 2013; Park et al., 2008). Porcine ToV (PToV) is also prevalent in piglets worldwide; however their pathogenicity in swine remains unclear (Anbalagan et al., 2014; Shin et al., 2010; Sun et al., 2014).

So far, only three whole genome sequences of ToVs, namely, BTOV Breda1 (AY427798) (Draker et al., 2006) and PToV NPL/2014 (KM403390), which was identified in the US in 2014 (Anbalagan et al., 2014) and PToV SH1 (JQ860350), which was identified in China...
in 2010 (Sun et al., 2014), are available on the database. To gain more information about genetic diversity, relationship, and evolution of ToVs, we performed whole genome analysis of Japanese BToVs and PToV. Our data showed natural interspecies recombination events of Japanese BToVs, which originated from genetic recombination of BToV Breda1 and PToV strains.

2. Material and methods

2.1. Viruses

Four BToVs and one PToV were studied. BToV Ishikawa/2010 (BToV Ishi) was isolated using HRT-18-Aich cells (Aita et al., 2012; Kuwabara et al., 2007) from a fecal sample of a cow with diarrhea in Ishikawa Prefecture in 2010 (Ito et al., 2012). BToV Kagoshima/2014 (BToV Kago), BToV Tochi/2013 (BToV Tochi), and BToV Tokyo/2014 (BToV Tokyo) were detected in the course of metagenomics of fecal samples obtained from 18-, 12-, and 16-day-old calves with diarrhea in Kagoshima, Tochi, and Tokyo Prefecture in 2014, 2013, and 2014, respectively. PToV Tottori/2015 (PToV Tottori) was identified in the course of metagenomics in fecal samples collected from a healthy two-month-old pig in 2015 in Tottori Prefecture.

2.2. Whole genome sequencing

Since viruses could not be isolated from samples using HRT-18-Aich cells by repeated passage thrice, fecal suspensions (20% v/v in sterile phosphate-buffered saline) of BToV Kago, BToV Tochi, BToV Tokyo, and PToV Tottori were used for RNA extraction. Viral RNA was extracted from 0.25 ml supernatant of BToV Kago culture (103.3 TCID50/ml) or 0.25 ml fecal suspensions by using TRizol® LS Reagent (Life Technologies, Carlsbad, CA, USA), followed by treatment of the RNA with DNase I (TaKaRa Bio Inc., Shiga, Japan). cDNA library was constructed using NEBNext® Ultra RNA Library Prep Kit for Illumina version 2.0 (New England Biolabs, Ipswich, MA, USA), as described previously (Nagai et al., 2015). The libraries obtained were loaded onto a MiSeq cartridge (MiSeq Reagent Kit V2 (300 cycles)); Illumina, San Diego, CA, USA) and sequenced using a MiSeq bench-top sequencer (Illumina). Since viruses could not be isolated from samples using HRT-18-Aich cells by repeated passage thrice, the sequence data were collected using the Illumina MiSeq sequencing system (Illumina) to generate reads in FASTQ format. Collected reads were trimmed and assembled into contigs by de novo assembly using CLC Genomics Workbench 6.5.1 (CLC bio, Aarhus, Denmark). The sequences were aligned using ClustalW in MEGAS5.22 (Tamura et al., 2011). Pairwise sequence identity calculations were performed using CLC Genomics Workbench 6.5.1 (CLC bio). The phylogenetic tree was constructed by the maximum likelihood method statistically supported by bootstrapping with 1000 replicates by using MEGAS5.22. Recombination analysis was performed using SimPlot software v. 3.5.1 (Lole et al., 1999) and the Recombination Detection Program (RDP) v. 4.58 (Martin and Rybicki, 2000; Martin et al., 2005).

3. Results

3.1. Deep sequencing and determination of whole genome sequences

We performed deep sequencing using the Illumina MiSeq sequencing system. The total ToV sequence read counts (percentage of ToV sequence reads: ToV sequence reads/total reads) of BToV Ishi, BToV Kago, BToV Tochi, BToV Tokyo, and PToV Tottori samples were 198,526 (13.2%), 31,685 (2.4%), 492 (0.04%), 1,527 (0.4%) and 1805 (0.05%), respectively. An approximately 28-kb contig was obtained from BToV Ishi and BToV Kago samples with sufficient average sequence read depth of 1007 (maximum depth: 2384) and 162 (maximum depth: 320), respectively; however, large contigs were not obtained from BToV Tochi, BToV Tokyo, and PToV Tottori samples. Since nearly complete sequences of contigs were obtained, complete genome length of BToV Ishi and BToV Kago determined in this study together with ToV and PToV genome sequences available in GenBank. Although the

3.2. Pairwise nucleotide sequence identity comparison

Pairwise alignment for comparing nucleotide sequences of BToV Ishi and BToV Kago to other BToVs and PToVs was performed using the whole genomic region of the 5′ untranslated region (UTR), ORF1a, ORF1b, S, M, HE, N, and the 3′ UTR (Table 1). BToV Ishi shared high sequence identities (96.8–99.4%) with BToV Kago for all genomic regions analyzed. Japanese BToVs showed high identities with PToVs in 5′UTR, ORF1a, ORF1b, S and 3′UTR (86.9–95.1%) and showed low identities with PToVs in S, M, and HE (70.2–80.3%). On the other hand, Japanese BToVs showed high identities with BToV Breda1 in S, M, and HE (87.9–95.7%) and showed low identities with BToV Breda1 in 5′UTR, ORF1a, ORF1b, N and 3′UTR (68.9–83.0%). These results suggest the occurrence of recombination events between BToV Breda1 and PToVs.

3.3. Recombination analysis

To investigate the recombination events, the complete genomes of BToV Ishi, BToV Kago, PToV NPL/2014, PToV SH1, and BToV Breda1 were aligned using ClustalW program in MEGAS5.22 and standard similarity plot analysis was performed using SimPlot software v. 3.5.1 with BToV Ishi (Fig. 1) and BToV Kagoshima/2014 sequences as separate queries. Both SimPlot graph indicated that the sequences of Japanese BToVs had high similarity with those of PToVs except in the 3′ end of ORF1b, S, M, and most of the HE coding regions, which had high similarity with that of BToV Breda1. The bootscanning analysis using RDP v. 4.58 was performed to identify the presumed recombinant breakpoints. Schema of ToV genome structure is shown in Fig. 2A. Beginning and end breakpoint positions were mapped near the 3′ of ORF1b and HE regions, respectively (Fig. 2B). At the starting putative recombination breakpoint, 25 nt sequences of Japanese BToVs corresponding to nt 20,138–20,162 of BToV Ishi showed low identities (72–76%) with that of BToV Breda1, whereas 25 nt sequences of Japanese BToVs corresponding to nt 20,161–20,185 showed high identities (100%) with that of BToV Breda1. At the end putative recombination breakpoint, 25 nt sequences of Japanese BToVs corresponding to nt 27,404–27,428 of BToV Ishi showed low identities (84–88%) with those of PToVs; however, the 25 nt Japanese BToVs sequences corresponding to nt 27,418 to 27,442 showed high identities (96–100%) with those of PToVs (Fig. 2C).

3.4. Phylogenetic analysis

For further confirmation of the recombination event, phylogenetic trees of nine genomic regions were constructed using nucleotide sequences of BToVs and PToVs determined in this study together with ToV and PToV genome sequences available in GenBank. Although the
Genome sequences of BToV Tochi, BToV Tokyo, and PToV Tottori had gaps owing to insufficient sequence read counts, sequence regions with no gap and corresponding to BToV Ishi were used: 5′ UTR (161 nt, 499–659), ORF1a (1800 nt, 3396–5195), ORF1b (915 nt, 18,425–19,339), S (594 nt, 20,889–21,482 and 3863 nt, 21,693–25,548), M (304 nt, 25,752–26,055), HE (1015 nt, 26,427–27,477), N (485 nt, 27,629–28,113), and 3′ UTR (172 nt, 28,114–28,285). However, we could not obtain the sequences of regions S (20,889–21,482) of PToV Tottori, S (21,693–25,548) of BToV Tokyo, and N (27,629–28,113) of PToV Tottori. The phylogenetic trees of S and N coding regions were constructed without these strains. The four Japanese BToVs branched separately from BToV Breda1, and clustered with PToV strains in the 5′ UTR, ORF1a, ORF1b, N, and 3′ UTR phylogenetic trees (Fig. 3A–C, H and I). In addition, Japanese BToVs in this study together with other BToV strains from Japan and other countries selected from GenBank, clustered with BToV Breda1 but not with PToVs in the S, M, and HE phylogenetic trees (Fig. 3D–G). Ito et al. classified Japanese BToVs using 5′ portion of S coding region into three genotypes, Clusters 1–3 (Ito et al., 2007, 2009, and 2010). In the phylogenetic tree, the 5′ portion of S coding region of BToVs in this study showed that BToV Ishi and BToV Tochi clustered with Cluster-1 and Cluster-2 strains, respectively, while BToV Kago and BToV Tokyo clustered with Cluster-3 strain, though enough bootstrap support could not be obtained. (Fig. 3D). BToV Tochi separately clustered with BToV Ishi, BToV Kago, and BToV Tokyo in the S (21,693–25,548), M, and HE phylogenetic trees (Fig. 3E–G).

### 4. Discussion

In the present study, we performed whole genome sequencing of Japanese BToVs and PToV by deep sequencing using supernatants of virus cultures and fecal suspensions. We could obtain a sufficient number of sequence reads and contigs of nearly 28 kb from 0.25 ml of 105.3 TCID50/mL BToV Ishi cell culture supernatant. Only one sample among three 0.25-mL fecal suspension samples gave a sufficient number of sequence reads and large contigs, though the number of sequence reads from this sample was lower than that from the viral culture supernatant. Unfortunately, we could not isolate BToV from fecal sample of BToV Kago by using HRT-18-Aich cells, which are useful for BToV isolation (Aita et al., 2012; Ito et al., 2012; Kuwabara et al., 2007), possibly because the samples could not be preserved at −20 °C at the veterinary clinic. Although numerous sequence reads originating from bacterial species and hosts might interfere and reduce the number of sequence reads from fecal suspensions, the whole genome sequence

| Pairwise nucleotide identity (%) | BToV Ishikawa/2010 | BToV Kagoshima/2014 |
|--------------------------------|--------------------|--------------------|
|                                  | PToV-NPL/2014 | PToV SH1 | BToV Breda | EToV Berne | BToV Kagoshima/2015 | PToV-NPL/2014 | PToV SH1 | BToV Breda | EToV Berne |
| 5′ UTR                           | 91.7             | 92.0         | 75.4       | 63.3       | 99.4       | 91.7             | 92.3         | 75.6       | 63.4       |
| ORF1a                            | 88.7             | 86.9         | 77.0       | 67.3       | 97.3       | 88.5             | 86.9         | 76.9       | 67.2       |
| ORF1b                            | 92.7             | 91.9         | 83.0       | 81.1       | 98.2       | 92.0             | 91.8         | 82.8       | 81.2       |
| S                                | 73.4             | 73.2         | 95.7       | 75.7       | 96.9       | 73.0             | 73.4         | 95.3       | 75.6       |
| M                                | 79.6             | 80.3         | 94.3       | 84.3       | 98.6       | 78.8             | 79.5         | 94.0       | 84.5       |
| HE                               | 70.8             | 71.7         | 88.3       | 84.3       | 96.8       | 70.2             | 71.2         | 87.9       | 81.2       |
| N                                | 92.9             | 90.2         | 69.1       | 67.1       | 98.2       | 93.1             | 90.2         | 69.1       | 67.3       |
| 3′ UTR                           | 95.1             | 90.8         | 68.9       | 66.9       | 98.8       | 93.9             | 90.8         | 68.9       | 66.9       |

*The complete HE sequence of EToV Berne strain is not available.*
Japanese BToVs and PToVs (NPL/2013 and SH1) are highlighted in pink; identities between Japanese BToV and BToV Breda1 are highlighted in green.

Cut-off of the bootstrapping test.

HE coding regions. Pink boxes indicate sequence regions originating from PToV. Green boxes indicate sequence regions originating from BToV Breda1 strain.

(B) Bootscan analysis of BToV was also reported in PToVs (Cong et al., 2013; Smits et al., 2003), of ToV shares sequence homology to that of coronavirus and in compare Japanese BToVs with European BToVs. The HE coding region of 5′ common ancestor. However, owing to the lack of sequence information of S, M, HE, and N coding regions. These phylogenetic tree analyses, Japanese BToVs were found to be closely related to European BToVs. In the phylogenetic tree, suggesting that all BToVs investigated in this study, except BToV Breda1, underwent recombination at the same genomic regions. Strains genetically closely related to Japanese BToVs are distributed worldwide.

In conclusion, we identified a natural recombination event between BToV Breda1 and PToV of Japanese BToVs. The four Japanese BToVs possessed two recombination breakpoints mapped to the 3′ end of ORF1b coding region and to the 3′ end of HE coding region and nearly 76% of the genome is similar to the PToV genome. Homologous recombination events of the virus genome are crucial for their evolution, and are significant factors for changes in host range and virulence of viruses. Our current data may provide important evidence to evaluate the epidemiological basis of ToV in cattle and swine population and to understand the mechanisms underlying the evolution of BToV.
Acknowledgements

This work was supported by the Grants from the Ministry of Health, Labour and Welfare of Japan and JSPS KAKENHI Grant Number 15K07718.

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Fig. 3. Phylogenetic trees based on sequences of 5′ UTR (A), ORF1a (B), ORF1b (C), S (D, E), M (F), HE (G), N (H), and 3′ UTR (I). Phylogenetic trees were constructed using the maximum likelihood method in MEGA5.22 with bootstrap values (1000 replicates). Scale bar indicates nucleotide substitutions per site. The BToV strains are represented by green (BToV Breda1 is indicated by boldface), whereas the PToV strains are represented by red. The BToV strains analyzed in this study are shown by black open square. *: Length of nucleotide sequences using analysis. **: Nucleotide position of BToV Ishikawa/2010.
