Infection, Genetics and Evolution 38 (2016) 90–95

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

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

Mika Ito [a], Shinobu Tsuchiaka [b], Yuki Naoi [b], Konosuke Otomaru [c], Mitsuo Sato [d], Tsuneyuki Masuda [e], Kei Haga [f], Tomoichiro Oka [f], Hiroshi Yamasato [e], Tsutomu Omatsu [b], Satoshi Sugimura [g], Hiroshi Aoki [h], Tetsuya Furuya [b,i], Yukie Katayama [b], Mami Oba [b], Junsuke Shirai [b,i], Kazuhiko Katayama [f], Tetsuya Mizutani [b], Makoto Nagai [b,i,*]

[a] Ishikawa Nanbu Livestock Hygiene Service Center, Saida, Kanazawa, Ishikawa 920–3101, Japan
[b] Research and Education Center for Prevention of Global Infectious Disease of Animal, Tokyo University of Agriculture and Technology, Fuchu, Tokyo 183-8509, Japan
[c] Joint Faculty of Veterinary Medicine, Kagoshima University, Korimoto, Kagoshima 890-0065, Japan
[d] Tochigi Prefectural Central District Animal Hygiene Service Center, Hiraidekougyoudanchi, Utsunomiya, Tochigi 321-0905, Japan
[e] Kurayoshi Livestock Hygiene Service Center, Kiyotani, Kurayoshi, Tottori 683-0017, Japan
[f] Department of Virology II, National Institute of Infectious Diseases, Musashimurayama, Tokyo 208-0011, Japan
[g] Institute of Agriculture, Tokyo University of Agriculture and Technology, Fuchu, Tokyo 183-8509, Japan
[h] Faculty of Veterinary Science, Nippon Veterinary and Life Science University, Musashino, Tokyo 180-8602, Japan
[i] Department of Veterinary Medicine, Faculty of Agriculture, Tokyo University of Agriculture and Technology, Fuchu, Tokyo 183-8509, Japan

ARTICLE INFO

Article history:
Received 20 October 2015
Received in revised form 15 December 2015
Accepted 15 December 2015
Available online 18 December 2015

Keywords:
Bovine torovirus
Japan
Natural recombination
Porcine torovirus

ABSTRACT

Bovine toroviruses (BToVs), belong to the subfamily *Toroviridae* within the family *Coronaviridae*, and are pathogens, causing enteric disease in cattle. In Japan, BToVs are distributed throughout the country and cause gastrointestinal infection of calves and cows. In the present study, complete genome sequences of two Japanese BToVs and partial genome sequences of two Japanese BToVs and one porcine torovirus (PToV) from distant regions in Japan were determined and genetic analyses were performed. Pairwise nucleotide comparison and phylogenetic analyses revealed that Japanese BToVs shared high identity with each other and showed high similarities with BToV Breda1 strain in S, M, and HE coding regions. Japanese BToVs showed high similarities with porcine toroviruses in ORF1a, ORF1b, and N coding regions and the 5′ and 3′ untranslated regions, suggestive of a natural recombination event. Recombination analyses mapped the putative recombinant breakpoints to the 3′ ends of the ORF1b and HE regions. These findings suggest that the interspecies recombinant nature of Japanese BToVs resulted in a closer relationship between BToV Breda1 and PToVs.

1. Introduction

Toroviruses (ToVs), belonging to the subfamily *Toroviridae*, are members of the family *Coronaviridae*, order *Nidovirales* (Cavanagh and Horzinek, 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 (Duckmanton et al., 1997; Ito et al., 2009; Kroneman et al., 1998; Uziel et al., 1999; Vanopdenbosch et al., 1991; Woode 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 (Woode 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 (Duckmanton 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 ToV, 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

* Corresponding author at: Research and Education Center for Prevention of Global Infectious Disease of Animal, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai, Fuchu, Tokyo 183-8509, Japan.
E-mail address: m-nagai@cc.tuat.ac.jp (M. Nagai).

http://dx.doi.org/10.1016/j.meegid.2015.12.013
1567-1348/

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 Tochigi/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, Tochigi, 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 repeating 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 Ishi culture ($10^{5.3}$ $TCID_{50}$/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) with 151 paired-end reads. To obtain complete genome sequence of BToV Ishi and BToV Kago, rapid amplification of cDNA end method (RACE) (5′-Full RACE Core Set and 3′-Full RACE Core Set; TaKaRa Bio, Otsu, Japan) was employed.

2.3. Genome analysis

The sequence data were collected using the MiSeq Reporter program (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 MEGA5.22 (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 MEGA5.22. 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%), 1527 (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 were determined using the 5′ and 3′ RACE method. The complete genome length of BToV Ishi and BToV Kago excluding poly(A) were 28,309 nucleotides (nt) and 28,301 nt, respectively, which were similar to the length of published sequences of ToVs; BToV Breda1 (28,475 nt), PToV NPL/2014 (28,305 nt), and PToV SH1 (28,301 nt). The nucleotide sequence identities among complete genome sequences of BToV Ishi and BToV Breda1, PToV NPL/2014, PToV SH1, and BToV Kago were 82.3%, 86.3%, 85.3%, and 97.6%, respectively. The sequences of BToV Ishi and BToV Kago were deposited in the DNA Data Bank of Japan, DDBJ/EMBL/GenBank database under the accession numbers LC088094 and LC088095, respectively.

3.2. Pairwise nucleotide sequence identity comparison

Pairwise alignment for comparing nucleotide sequences of BToV Ishi and BToV Kago to other BToV 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, N 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 MEGA5.22 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 BToV 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. 2. A. 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 BToV corresponding to nt 27,404–27,428 of BToV Ishi showed low identities (84–88%) with those of PToVs; however, the 25-nt Japanese BToV 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 PToV determined in this study together with ToV and PToV genome sequences available in GenBank. Although the