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Genomic characterization of a RdRp-recombinat nebovirus strain with a novel VP1 genotype

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ABSTRACT

Nebovirus is a new genus within the family *Caliciviridae* and is a causative agent of calf diarrhea. The limited nebovirus genomic sequences that are currently available has hampered understanding of nebovirus genetic evolution. The aim of the present study was to determine the genomic characterization of strain Bo/LZB-1/17/CH, which was previously identified as being similar to the novel genotype strain Bo/DijonA216/06/FR based on partial capsid sequences. Our results show that the complete RNA genome of strain Bo/LZB-1/17/CH is 7453 nucleotides (nt) in length and shares 79.0%–83.5% nt identity with all available nebovirus genomes in the GenBank database. A phylogenetic analysis based on its complete genome sequence revealed that strain Bo/LZB-1/17/CH clustered into an independent branch. Two interesting characteristics were observed in the genome of strain Bo/LZB-1/17/CH. First, the major capsid protein (VP1) of strain Bo/LZB-1/17/CH shares 96.6% amino acid (aa) identity with strain Bo/DijonA216/06/FR but shares only 75.2%–76.8% aa identity with other nebovirus strains and has an even lower identity in the P2 domain (61.1%–65% aa identity). Second, the RNA-dependent RNA polymerase (RdRp) of strain Bo/LZB-1/17/CH is more closely related to NB-like strains than it is to strain Bo/DijonA216/06/FR, and a recombination event was identified within the 3′ end of the RdRp in strain Bo/LZB-1/17/CH. In conclusion, the results in this study indicate that strain Bo/LZB-1/17/CH may represent a novel nebovirus strain. To the best of our knowledge, this is the first description of a recombinant event in nebovirus RdRp.

1. Introduction

Caliciviruses are small non-enveloped viruses with a positive-stranded RNA genome, which widely infect humans and other animals, causing gastrointestinal disorders and even fatal bleeding (Van der Poel et al., 2000). The family *Caliciviridae* includes the genera *Vesivirus*, *Sapovirus*, *Lagovirus*, *Norovirus*, and *Nebovirus*. Noboviruses and genogroup III noroviruses (NoVsGIII) have been identified as causative agents of calf diarrhea (Otto et al., 2011; Smiley et al., 2002).

Nebovirus was officially classified as a new genus within the family *Caliciviridae* in 2010 (Carstens, 2010). In the initial study on these viruses, an analysis of the 3′ end of the RdRp region identified two distinct polymerase types, NA1-like and NB-like, but these strains only belonged to one capsid type (Oliver et al., 2006). Subsequently, noboviruses were classified into four phylogenetic lineages based on 31 complete nebovirus VP1 sequences; however, the pairwise distances between these four lineages were small, leading researchers to question whether these lineages were actually individual genotypes or subgroups within a genotype (D'Mello et al., 2009). When strain Bo/DijonA216/06/FR was detected in France in 2011, phylogenetic analyses based on its complete VP1 sequence or the 3′ end of its RdRp sequence plotted this strain on an independent branch, indicating that Bo/DijonA216/06/FR may represent a novel genotype (Kaplon et al., 2011). Unfortunately, the genome sequence of this strain is still not available in the GenBank database. At present, noboviruses can be divided into three RdRp types and two VP1 types based on the limited available sequence information (Candido et al., 2016; Hassine-Zaafrane et al., 2012; Kaplon et al., 2011).

An analysis of all four complete nebovirus genomes available in the GenBank database, including prototype strains Bo/Nebraska/80/US and Bo/Newbury1/76/UK, showed that these linear RNA genomes of 7453–7454 bp are organized into two open reading frames (ORFs) (Oliver et al., 2006; Peter et al., 2013; Smiley et al., 2002). ORF1 encodes the predicted viral nonstructural proteins and the major capsid protein (VP1), ORF2 encodes the minor capsid protein (VP2) (Oliver et al., 2006; Smiley et al., 2002). Recently, Kırklareli virus, the

Abbreviations: VP1, the major capsid protein; VP2, the minor capsid protein; RdRp, RNA-dependent RNA polymerase; nt, nucleotide; aa, amino acid; ORFs, open reading frames; UTR, untranslated region

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pathogenicity of which was unknown, was identified from a calf in Turkey; this virus is suggested to be related to neboviruses, and it shares 48%–49.9% nt identity with nebovirus strains based on the complete genome sequences (Alkan et al., 2015). The complete genome of Kırklareli virus is 7484 nt in length, and it has a 1-nt overlap between ORF1 and ORF2, in contrast to the genomes of genus Nebovirus members, which have a 1-nt interval between these two ORFs (Alkan et al., 2015; Oliver et al., 2006; Peter et al., 2013; Smiley et al., 2002). In addition, ORF1, ORF2, the 5′ untranslated region (UTR), and the 3′ UTR of Kırklareli virus all differ in length from those of nebovirus strains.

Limited sequence information is available for neboviruses, and the only one recombinant nebovirus strain reported to date has a recombination breakpoint located downstream from the highly conserved S domain of VP1 (Di Martino et al., 2011). Virus recombination may produce new virus with potentially different pathogenesis and virulence, and affect phylogenetic groupings (Bull et al., 2007; Mathijs et al., 2010), and more and more recombinant strains belonging to other genera of family Caliciviridae have been reported (Coyne et al., 2006; Di Felice et al., 2016; Forrester et al., 2008; Hansman et al., 2005; Lopes et al., 2015; Wang et al., 2005). Therefore, recombination events in neboviruses should be investigated.

In our previous study, strain Bo/LZB-1/17/CH was identified as being similar to the novel genotype Bo/DijonA216/06/FR based on 227 bp at the 5′ end of the capsid (Guo et al., 2018). However, the 5′ end of the capsid-coding region is not informative for typing the nebovirus capsid (D'Mello et al., 2009; Di Martino et al., 2011), so additional experiments remained necessary for a firm molecular characterization of strain Bo/LZB-1/17/CH. Therefore, this study aimed to acquire and analyze the full-length genome of strain Bo/LZB-1/17/CH.

2. Material and methods

2.1. Sample collection

A fecal sample was collected from a 4-month-old calf with severe diarrhea in October 2016 in Sichuan Province, China. Bloody stools and fever were observed in the calf. The sample was identified in our previous work as being positive for nebovirus strain Bo/LZB-1/17/CH based on virus metagenomics and RT-PCR (Guo et al., 2018). The fecal specimens were shipped on ice and stored at −80 °C in sterile 50-ml centrifuge tubes.

2.2. RNA extraction and cDNA synthesis

The clinical fecal samples were fully resuspended in PBS (1:5) and centrifuged at 10,000 × *g* for 10 min, followed by filtration through a 0.22-μm filter. Viral RNA was extracted from 300 μl of the fecal suspension using RNAios Plus (TaKaRa Bio Inc., Japan) according to the manufacturer's instructions. The cDNA was synthesized using the PrimeScript™ RT Reagent kit according to the manufacturer's instructions (TaKaRa Bio Inc.) and stored at −20 °C.

2.3. Full-genome amplification

In total, 11 pairs of primers were designed based on our previous virus metagenomic sequencing data [GenBank accession number SRP108885], which included partial sequences of strain Bo/LZB-1/17/CH (Table 1). The 3′ end of the complete viral genome was acquired by rapid amplification of cDNA ends (RACE) using a Smart RACE cDNA amplification kit (Clonetech, USA). All PCR products were purified using the Omega Gel kit (Omega, USA) following the manufacturer's instructions, after which they were ligated to the pMD19-T vector (TaKaRa Bio Inc.) and transformed into DH5α competent *Escherichia coli* cells (Yeasen, China). For each product, three to five colonies were selected and sequenced (Sangon, China) in both directions. The sequences were assembled using SeqMan software (version 7.0; DNASTAR Inc., WI, USA).

Table 1
Oligonucleotide sequences used for PCR amplification and genomic sequencing.

Primer name	Nucleotide sequence(5′→3′)	Amplicon (bp)	Annealing temperature (°C)
NeVs-F1	GTGATTTAATTATAGAGAGA	285	36
NeVs-R1	TCTTTGAGGCGACATAGC		
NeVs-F2	AGGGCTGGGATGACTTTT	449	51
NeVs-R2	ACGCCAGTGATGATACCG		
NeVs-F3	AGTCCCACCAAATCCACC	760	44
NeVs-R3	GTTATGATGCTCTTTACGG		
NeVs-F4	ACAACATCCCGTCCAAAG	1010	49
NeVs-R4	CAAGTGGGAGCAATCAGC		
NeVs-F5	CAGCCCATTTGTGCCTAA	1263	48
NeVs-R5	TATTCCTCATCAGACAGC		
NeVs-F6	CTATCACCACGGCTTTCACT	515	52
NeVs-R6	GCAGTTGTTGCCTTCACG		
NeVs-F7	GCTCGTGACTATCGCTCGTT	1054	53
NeVs-R7	AAATGAGCCTGCGTTTGC		
NeVs-F8	TCCCCACAGCCATACAGT	1038	53
NeVs-R8	AACGCCGAGGACCATTCA		
NeVs-F9	CAGCCCGTCTGGGTGAAT	524	54
NeVs-R9	CCAGCGTTAGCGTTCCAG		
NeVs-F10	TATGACGGGCGCTTTTAT	723	50
NeVs-R10	TGGGAGCAGGGAAAAGAA		
NeVs-F11	GTCCTCCGTATGCCTCATG	1227	53
NeVs-R11	CCTCCGAAGTTCACCACCTAT		

2.4. Sequence, recombination, and phylogenetic analyses

For the genome organization analysis, putative ORFs and their corresponding amino acids were predicted using the ORF Finder tool (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The homologies of nucleotide and deduced amino acid sequences were determined using the MegAlign program of DNASTAR 7.0 software (DNASTAR Inc.). MEGA 7.0 was used to perform a multiple sequence alignment and to subsequently build a maximum likelihood phylogenetic tree with bootstrap support (Kumar et al., 2004). Recombination events were assessed using SimPlot software (version 3.5.1) and the Recombination Detection Program (RDP 4.0) with the RDP, GeneConv, Chimaera, MaxChi, BootScan, SiScan, and 3Seq methods (Martin et al., 2015; Waters et al., 2007).

To further characterize the VP1 sequences, an amino acid alignment was performed using all 44 complete nebovirus VP1 sequences in the GenBank database. The VP1 sequence analysis was performed with reference to a homology model of the Newbury1 capsid protein produced using the Swiss-Model based on the structure of San Miguel sea lion virus (SMSV-4) (Chen et al., 2003; D'Mello et al., 2009). According to the homology model, the VP1 protein forms an icosahedral particle with two principal domains, the protruding (P) domain and shell (S) domain. The S domain resides between residues 1 and 216. The more variable, surface-exposed P domain is predicted to be located between residues 217 and 549, with the P1 domain mapped from 217 to 273 and from 452 to 549, and the P2 domain mapped from 274 to 451 of the capsid (Chen et al., 2003; D'Mello et al., 2009).

3. Results

3.1. Overview of the strain Bo/LZB-1/17/CH genome

The sequencing results revealed that the complete genome of strain Bo/LZB-1/17/CH is 7453 nt in length, with a G + C content of 56.72%, including a 75 nt 5′ UTR and a 67 nt 3′ UTR. The viral genome displays the same organization as other previously described neboviruses and is composed of two ORFs. ORF-1 is 2210 aa in length and encodes a large