

# First detection of Nebovirus and Norovirus from cattle in China

Zijing Guo<sup>1</sup>  · Qifu He<sup>1</sup> · Hua Yue<sup>1</sup> · Bin Zhang<sup>1</sup> · Cheng Tang<sup>1</sup>

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**Abstract** Neboviruses and genogroup III noroviruses (NoVsGIII) are causative agents of calf diarrhea. The purpose of this study was to investigate the presence of neboviruses and noroviruses in cattle in China. Twenty-eight diarrhea fecal samples collected from 5 different farms were analyzed by RT-PCR. The results showed that 3 nebovirus positive samples were detected on 2 farms, with two strains being related to Bo/DijonA216/06/FR strain and the other one clustering with NB-like strains. Meanwhile, 3 norovirus positive samples were detected on 3 farms, all of which belonged to genotype 1. Our results confirmed the presence of neboviruses and NoVsGIII in China for the first time, and supported the presence of a novel “DijonA216-like” nebovirus genotype.

**Keywords** Nebovirus · Norovirus · RT-PCR · Calf · China

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 [1]. The *Caliciviridae* family includes the genera *Vesivirus*, *Sapovirus*, *Lagovirus*, *Norovirus* and *Nebovirus*. Neboviruses and genogroup III noroviruses (NoVsGIII) have been associated with enteric disease in calves [2, 3].

*Nebovirus* was officially classified as a new genus in 2010 [4]. Experimental infection with neboviruses can cause intestinal lesions and diarrhea 2 to 5 days after infection [2, 5, 6]. Initially, the genotypes Nebraska (NB) and Newbury-1 (NA1) have been suggested for sub-classifying neboviruses [12]. Recently, in 2011, the strain Bo/DijonA216/06/FR was detected in France, which could represent a novel genotype [7]. Until now, NB-like strains have been found in the United States, the United Kingdom, France, South Korea, Italy, Tunisia and Turkey [2, 7–12], while NA1-like strains have been found in the United Kingdom and Brazil [12, 13]. “DijonA216-like” has only been detected in France, and this genotype has only one sequence in GenBank.

Bovine noroviruses, belonging to genogroup III (NoVsGIII), are divided into two distinct genotypes, genotype 1 and genotype 2. Experimental infection with NoVsGIII causes calf diarrhea of a long duration, with the symptoms of genotype 1 being more severe than genotype 2 [3, 14]. Previous reports have shown that NoVsGIII has been detected in 17 countries with various prevalence rates [7, 8, 10, 15–28]. Indeed, high prevalence rates now indicate that NoVsGIII have become the major pathogen causing calf diarrhea in the Midwestern United States [19, 29].

The purpose of this study was to investigate the presence of neboviruses and noroviruses in cattle in China. From September 2016 to April 2017, twenty-eight diarrhea fecal samples were collected from 5 different farms in the Hebei and Sichuan Provinces, China. The ages of the calves ranged from 3 to 4 months. The fecal specimens were shipped on ice and stored at -80 °C.

The clinical fecal samples were fully resuspended in PBS (1:5) and total RNA from 300 µL of the fecal suspension was extracted using Trizol<sup>TM</sup> Reagent (TaKaRa, China), following the manufacturer’s instructions. cDNA was synthesized

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✉ Cheng Tang  
tangcheng101@163.com

<sup>1</sup> College of Life Science and Technology, Southwest University for Nationalities, No.16, South 4th Section 1st Ring Road, Chengdu 610041, China

using the Primescript™ reverse transcription kit (TaKaRa, China) and stored at -20 °C.

Primers targeting the nebovirus genome were designed (using Primer 5.0 software) based on virus metagenomics sequencing data [GenBank accession number SUB2762873] obtained from a previously acquired calf diarrhea sample, which included nebovirus, as well as the nebovirus nucleotide sequences in GenBank. The primer sequences were as follows; F: 5'-CAGCCCGTCTGGGTGAAT-3'; R: 5'-CCA GCGTTAGCGTTCAG-3'. The amplified fragment was 524 bp, composing part of the RdRp (1 bp to 297 bp) and the capsid gene (298 bp to 524 bp). For NoVsGIII, primer sets CBECU-F/CBECU-R were used to amplify 532 bp of the RdRp ORF of the virus according to previous reports [30].

Reverse transcription polymerase chain reaction (RT-PCR) was performed with Premix Taq™ (TaKaRa, China), following the manufacturer's protocol. The PCR amplification products were gel-purified using a gel extraction kit (OMEGA, USA) and then cloned into the pMD19-T vector and transformed into *Escherichia coli* DH5α competent cells. The recombinant plasmids were extracted using a plasmid extraction kit (OMEGA, USA) and sent to Sangon Biotech (China, Chengdu) for sequencing.

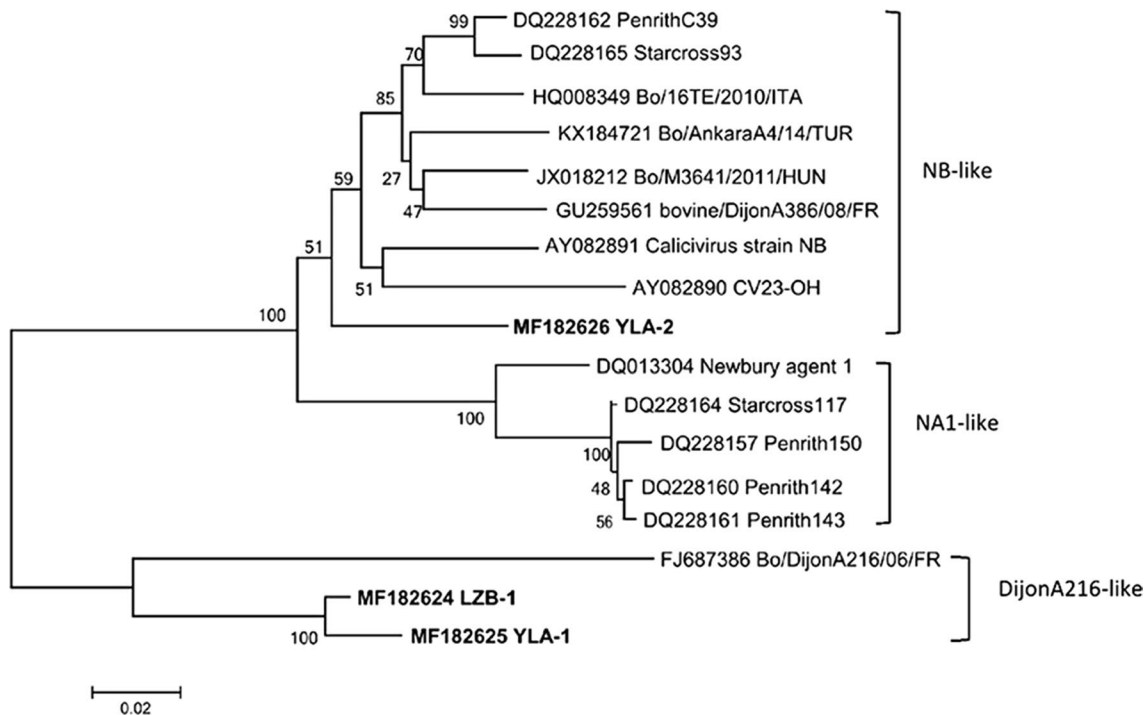
Among the 28 fecal samples from cattle, 3 diarrhea samples from 2 farms were positive for nebovirus (accession numbers in GenBank: MF182624-MF182626 for strains

LZB-1, YLA-1 and YLA-2 respectively). Meanwhile, 3 diarrhea samples from 3 farms were positive for NoVsGIII (accession numbers in GenBank: MF182621-MF182623 for strains HBC-1, SCD-1 and YLA-3, respectively). YLA-1, YLA-2 and YLA-3 were all discovered at the same farm, which indicated there was co-circulation of nebovirus and norovirus at this farm.

Diarrhea is one of the commonest diseases of calves in China, which leads to serious economic losses. Bovine rotavirus, bovine coronavirus and bovine viral diarrhea virus have been identified as major diarrhea causing viruses. However, there is little attention to neboviruses and noroviruses in terms of calf diarrhea. Although the number of samples was limited, the study confirmed for the first time the presence of neboviruses and NoVsGIII in China, which has significant implications for the diagnosis and control of calf diarrhea in China.

The partial nucleotide sequences of the neboviruses and noroviruses identified in this study were analyzed using the MegAlign software to calculate sequence identities to other strains in GenBank. MEGA 6.0 was used to perform multiple sequence alignment and subsequently to build a neighbour-joining phylogenetic tree with bootstrap support.

The 524 bp of the 3 neboviruses sequences in this study shared 88.4% to 97.7% nt identity to each other and 77.1% to 92.9% nt identity with neboviruses sequences in



**Fig. 1** Neighbour-joining phylogenetic analysis of neboviruses based on the cloned 524 bp fragments identified in this study. The numbers at the nodes represent the percentage branching following 1000 boot-

strap replicates. Bars indicate the number of inferred substitutions per site. The nebovirus sequences determined in this study are show in bold



In conclusion, this study has confirmed for the first time the presence of neboviruses and NoVsGIII in China, which will have significant implications for the diagnosis and control of calf diarrhea in China. The two nebovirus strains in this study clustered with Bo/DijonA216/06/FR, which supports the presence of a novel “DijonA216-like” nebovirus genotype in China.

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#### Compliance with ethical standards

This article does not contain any studies with human participants or animals performed by any of the authors and is in compliance with ethical standards for research.

**Conflict of interest** The authors declare no conflict of interest.

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