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First detection of neboviruses in yak (*Bos grunniens*) and identification of a novel neboviruses based on complete genome

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ABSTRACT

Neboviruses (NeVs) is an important causative agent of calf diarrhea. Here, 354 diarrhoeic samples were collected from yak on 55 farms in the Qinghai-Tibet Plateau, China. 22.0% of the diarrhoeic samples were detected as NeVs-positive by RT–PCR assay. Phylogenetic analysis of 78 NeVs RdRp fragments showed that 69 strains were closely related to NB-like strains, and the remaining 9 strains were clustered into an independent branch, which may represent a novel RdRp genotype. Two complete NeVs genomes (YAK/NRG-17/17/CH and YAK/HY1-2/18/CH) were successfully sequenced with 7459 nt and 7460 nt in length, respectively. The genomes of the two strains only shared 68.1%–69.3% nt identity with all six known NeVs genomes, and phylogenetic trees based on its genome, VP1, RdRp, VP2, P34, NTPase, P30, VPg and 3CLpro proteins suggested that the two strains may represent a novel NeVs strain with novel VP1 genotype and novel RdRp genotype. Notably, 11.5% NeVs strains were screened as the novel NeVs strains based VP1 and RdRp sequences. These novel NeVs strains were detected from 6 farms in two counties, indicating that the novel NeVs has spread in local region. To best of our knowledge, this is the first detection of NeVs in yak. Moreover, a novel NeVs strain was identified based on complete genome. These results contribute to further understand the prevalence and genetic evolution of NeVs.

1. Introduction

Viruses of the genus *Nebovirus* belong to the family *Caliciviridae* and are important agents of calf diarrhoea (Cho et al., 2018; Smiley et al., 2002). To date, Neboviruses (NeVs) has been detected in 13 countries with a wide geographical distribution (Candido et al., 2016; Di Martino et al., 2011; Guo et al., 2018a; Turan et al., 2018). Unlike other genera *Norovirus* and *Lagovirus* in family *Caliciviridae* (Kroneman et al., 2013; Le Pendu et al., 2017), a system of dual nomenclature based on the partial RdRp and complete VP1 sequences has not yet been established in NeVs. Thus, the molecular characteristics of NeV are still described based on RdRp or VP1, respectively. Unlike noroviruses and lagoviruses (Le Pendu et al., 2017; Zheng et al., 2006), there is still no clear definition for NeVs genotype classification due to the lack of sufficient NeVs sequences to calculate the genetic distance cutoffs. NeVs can be subclassified into two RdRp genotypes (NB-like and NA1-like) and two VP1 genotypes (genotype 1 and genotype 2) based on sequence and phylogenetic analysis (Guo et al., 2018c). The VP1 genotype 1 can be further subdivided into four lineages (D'Mello et al., 2009; Guo et al., 2018b). Currently, NB-like strains detected in 12 countries have been the most common genotype in circulation worldwide, and NA1-like strains have been detected in 4 countries (Hassine-Zaafrane et al., 2012; Park et al., 2008; Pourasgari et al., 2018). The VP1 genotype 1 strains have been detected in 9 countries, and genotype 2 strains only have been detected in France and China (Guo et al., 2018c; Kaplon et al., 2011).

Analysis of all six complete NeVs genomes available in the GenBank database showed that these linear RNA genomes of 7453–7454 bp are organized into two open reading frames (ORFs) (Oliver et al., 2006; Smiley et al., 2002). ORF-1 is 2210 amino acids (aa) in length and encodes a large nonstructural polyprotein comprising a P34, NTPase, P30, VPg, 3CLpro and RdRp protein, contiguous with the major capsid protein (VP1). ORF-2 is located at the 3′ end of the complete genome and encodes a minor capsid protein (VP2) composed of 225 aa (Guo et al., 2018b; Oliver et al., 2006). The VP1 is the major structural component of NeVs and is involved in the binding of ABO histo–blood group antigen (HBGAs) (Cho et al., 2018). Moreover, the VP1 of other caliciviruses has been identified to be involved in host specificity and immunogenicity (Chen et al., 2006; Smith et al., 2019; Song et al., 2017). Although the precise functions of NeVs RdRp has not been determined, the RdRp of other caliciviruses is a key enzyme responsible

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for transcription, replication of the viral genome and accurate initiation of RNA synthesis (Deval et al., 2016; Lee et al., 2017).

The yak (*Bos grunniens*) belong to the genus *Bos* within the family *Bovidae* and is a unique long-haired bovine species in the Qinghai-Tibet Plateau (Guo et al., 2014). There are over 14 million yaks in the world, which are distributed in high-altitude regions of China, India, Nepal, Pakistan, Kyrgyzstan, Mongolia and Russian Federation, and mainly distributed in the Qinghai-Tibet Plateau (above 3000–5000 m) in China (Mi et al., 2013). Yak is an essential animal of production and livelihood for local Tibetan people, providing meat, milk, skins, transport and fuel (faces) (Ding et al., 2010; Guo et al., 2014). Diarrhea is a common disease in yak, which cause serious economic losses (Chen et al., 2015; Gong et al., 2014). Our previous studies confirmed that NeVs has circulated widely in dairy cows in China (Guo et al., 2018a, c). In this study, we sought to determine if NeVs infects yak. Unexpectedly, a novel NeVs with novel RdRp genotype and novel VP1 genotype was identified based on its complete genome, and this novel NeVs has spread in local province.

2. Material and methods

2.1. Specimen collection

A total of 354 diarrhoeic samples of yak (the ages ≤3 months) were collected from 55 farms in China from January 2017 to July 2018 (altitude from 3500 to 4500 m). These farms were located in the Tibet autonomous region (Ngari prefecture: N33°42′, E80°11) and Sichuan province (Hongyuan county: N32°47′, E102°32′; Norgay county: N34°10′, E102°32′) and Yunnan province (Shangri-la: N27°19, E99°56′). These regions are the main yak-producing areas in China, and the geographical distance between the two farthest farms is > 1,500 km. The number of the collected samples ranged from 2 to 13 animals per farm. All specimens were shipped on ice and stored at −80 °C.

2.2. RNA extraction and cDNA synthesis

Approximately 2 g 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 0.45-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.). The cDNA synthesis was conducted in a 20 μl reaction volume containing 4 μl 5 × Prime Script Buffer, 1 μl Prime Script RT Enyme MixI, 2 μl Random 6 mers, 9 μl RNase Free H2O, and 4 μl RNA. The mixtures were followed by 37 °C for 15 min, 85 °C for 15 s, 16 °C for 10 min, and stored at −20 °C.

2.3. Detection of NeVs

NeVs were detected by a specific RT-PCR assay established in our laboratory. The specificity and reproducibility of the RT-PCR assay has been validated, and the detection limit is 2.1×10^2 copies/μL. Briefly, a pair of primers (NeVs396-F: 5′-MYCCAACMGCTCCWGAYAAR-3′, NeVs396-R: 5′-GGAYRCCATAAAASCGCCCRT-3′) was used to amplify a 396-bp fragment of the RdRp gene (position 4615–5010 bp of the Bo/LZB-1/17/CH genomic sequence, GenBank accession number MG599036.1). The amplification was conducted in a 25-μl reaction volume containing 0.05 μM forward primer, 0.05 μM reverse primer, 2 μl of cDNA, 12.5 μl of EmeraldAmp PCR Master Mix (2× Premix) (TaKaRa Bio Inc.), and an appropriate volume of double-distilled water. The amplification products were analyzed by 1.5% agarose gel electrophoresis, and were further sequenced directly on both directions by Sangon Biotech (Chengdu, China), and the sequences were used for genetic evolution analysis.

Table 1
The co-infected rates of NeVs with other common enteric pathogens.

Enteric pathogens	The positive rate (%)
NeVs(only)	12.8%(10/78)
NeVs + BRV	32.1%(25/78)
NeVs + BCoV	14.1%(11/78)
NeVs + BVDV	7.7%(6/78)
NeVs + BRV + BCoV	25.6%(20/78)
NeVs + BRV + BVDV	3.8%(3/78)
NeVs + BRV + BCoV + BVDV	3.8%(3/78)

2.4. Screening for co–infection with major bovine enteric pathogens

To investigate co–infection with Bovine rotavirus (BRV), Bovine coronavirus (BCoV), and Bovine viral diarrhea virus (BVDV), all NeVs positive samples were subjected using specific RT–PCR assays for these viruses. The detection of BRV and BCoV were following our previous report (Guo et al., 2018c), and BVDV was following the previous report (Gong et al., 2014).

2.5. Complete genome amplification

The complete genome sequence of strains Yak/HY-17/17/CH and Yak/NRG-12/18/CH with novel potential RdRp genotype were amplified by combining RT–PCRs with a primer walking strategy and 3′ RACE (rapid amplification of cDNA ends) protocols, and the 9 pairs of primers sequences information as shown in supplementary Table 1. Additional, 8 pairs of primers were used to verify the complete genome sequences of the two strains, the sequences information as shown in supplementary Table 2. 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, Palo Alto, USA). All PCR products were purified using the Omega Gel kit (Omega), cloned into the pMD19-T simple vector (TaKaRa Bio Inc.), and sequenced (Sangon Biotech) in both directions.

2.6. Screening for novel NeVs by RT-PCR

A pair of primers was designed based on the VP1 sequences of strains Yak/HY-17/17/CH and Yak/NRG-12/18/CH to further screen novel NeVs strains. The primer sequences are as follows: NeVs366-F, 5′-CTGTCACCTGGAACGCTAA-3′; NeVs366-R, 5′-GGTTGATGAGCGAG GTCAT-3′. The amplified fragment was 366 bp of the VP1 S domain located at positions 5260–5625 bp of the Bo/LZB-1/17/CH genomic sequence, GenBank accession number MG599036.1. The reaction volume was identical with that of detecting NeVs strain. All PCR products were purified using the Omega Gel kit (Omega), cloned into the pMD19-T simple vector (TaKaRa Bio Inc.), and sequenced (Sangon Biotech) in both directions, and the sequences were used for genetic evolution analysis.

2.7. Sequence, phylogeny, and recombination analyses

The sequences were assembled using SeqMan software (version 7.0; DNASTAR Inc., WI, USA). 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 analysis were used SimPlot software (version 3.5.1) and Recombination Detection Program 4.0 (RDP 4.0, version 4.96) with the RDP, GeneConv, Chimaera, MaxChi, BootScan, SiScan, and 3Seq methods (Martin

Table 2
Amino acid identities of strains YAK/NRG17/17/CH and YAK/HY1-2/18/CH compared with all known six NeVs genomes.

Strain	Amino acid identities (%)								
	P34	NTPase	P30	VPg	3CLpro	RdRp	VP1	P2 domain	VP2
Newbury-1	63.4	78.4	83.6	86.2	72.8	74.4	79.5	65.7	80.4
	61.8	**78.7**	**82.5**	**86.2**	**72.8**	**74.6**	**80.4**	**66.3**	**79.6**
NB	62.4	78.1	82.5	86.2	70.7	75.3	79.1	66.3	78.7
	61.1	**77.6**	**81.1**	**86.2**	**70.7**	**75.9**	**80.0**	**66.9**	**78.7**
HUN	62.1	78.1	82.1	86.2	71.2	75.1	78.9	66.3	80.9
	60.8	**78.1**	**80.7**	**86.2**	**71.2**	**75.7**	**80.0**	**67.4**	**80.0**
TCG	63.4	77.0	82.5	86.2	72.3	74.8	79.1	66.9	79.6
	60.8	**77.0**	**81.1**	**86.2**	**71.7**	**75.5**	**80.4**	**68.5**	**78.7**
LZB-1	63.7	78.1	82.1	86.2	70.1	74.8	76.7	67.4	80.0
	62.4	**77.6**	**80.7**	**86.2**	**70.1**	**75.5**	**78.2**	**70.2**	**79.1**
YLA-2	62.4	77.6	81.8	86.2	70.1	75.1	79.3	66.3	80.9
	61.1	**77.0**	**80.4**	**86.2**	**70.1**	**75.9**	**80.5**	**68.0**	**80.0**

Note: Amino acid identities between strains YAK/HY1-2/18/CH and other all known NeVs strains were shown by **bold** and underline; in contrast, the amino acid identities between strain YAK/NRG-17/17/CH and other NeVs strains were not shown.

et al., 2015).

3. Results

3.1. NeVs detection and co–infections

22.0% (78/354) of the diarrhoeic samples were detected as NeVs-positive by RT-PCR, 38 out of the 55 farms were positive for NeVs. Notably, NeVs was found in diarrhoeic feces samples from all three provinces, and the detection rates were 35.48% (11/31), 19.0% (50/263), and 28.33% (17/60) in the Tibet, Sichuan and Yunnan provinces, respectively (Fig. 1). Out of 78 NeVs–positive diarrhoeic samples, 68 samples were found co–infections with BRV, BCoV, and/or BVDV, as shown in Table 1.

3.2. Phylogenetic analysis of partial RdRp gene

Phylogenetic tree based on 78 RdRp fragment sequences showed that yak NeVs strains could be divided into two large branches. 69 strains belong to NB-like genotype, which were most related to NeVs strains determined in Chinese dairy cows, but them are located in a unique small branch. Interestingly, the remaining 9 strains from

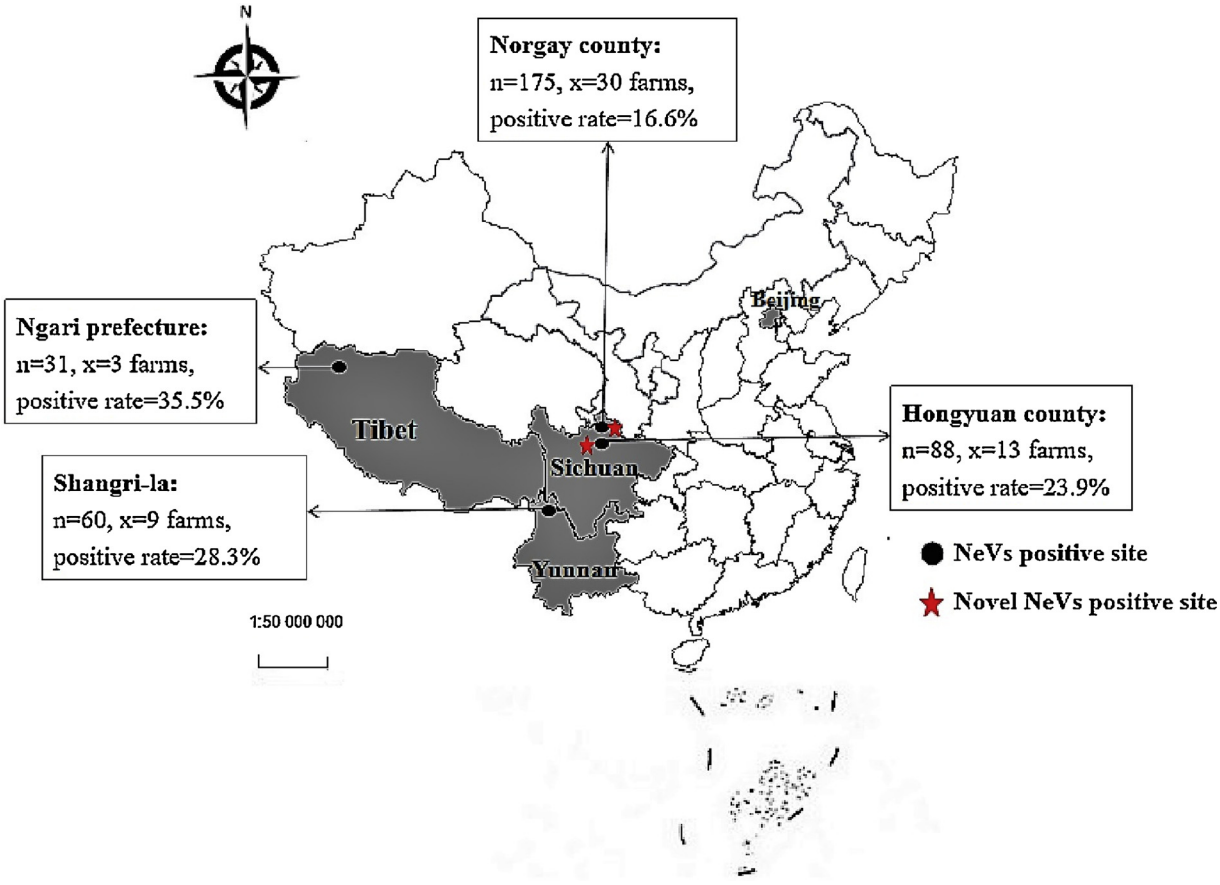

Fig. 1. Number of yak from three regions around the Qinghai Tibetan Plateau, China, 2017–2018. The three regions that were sampled was indicated in grey. n values indicate the total number of samples in each region, x values indicate the total number of farms in each region, positive rate indicate the NeVs positive rate.

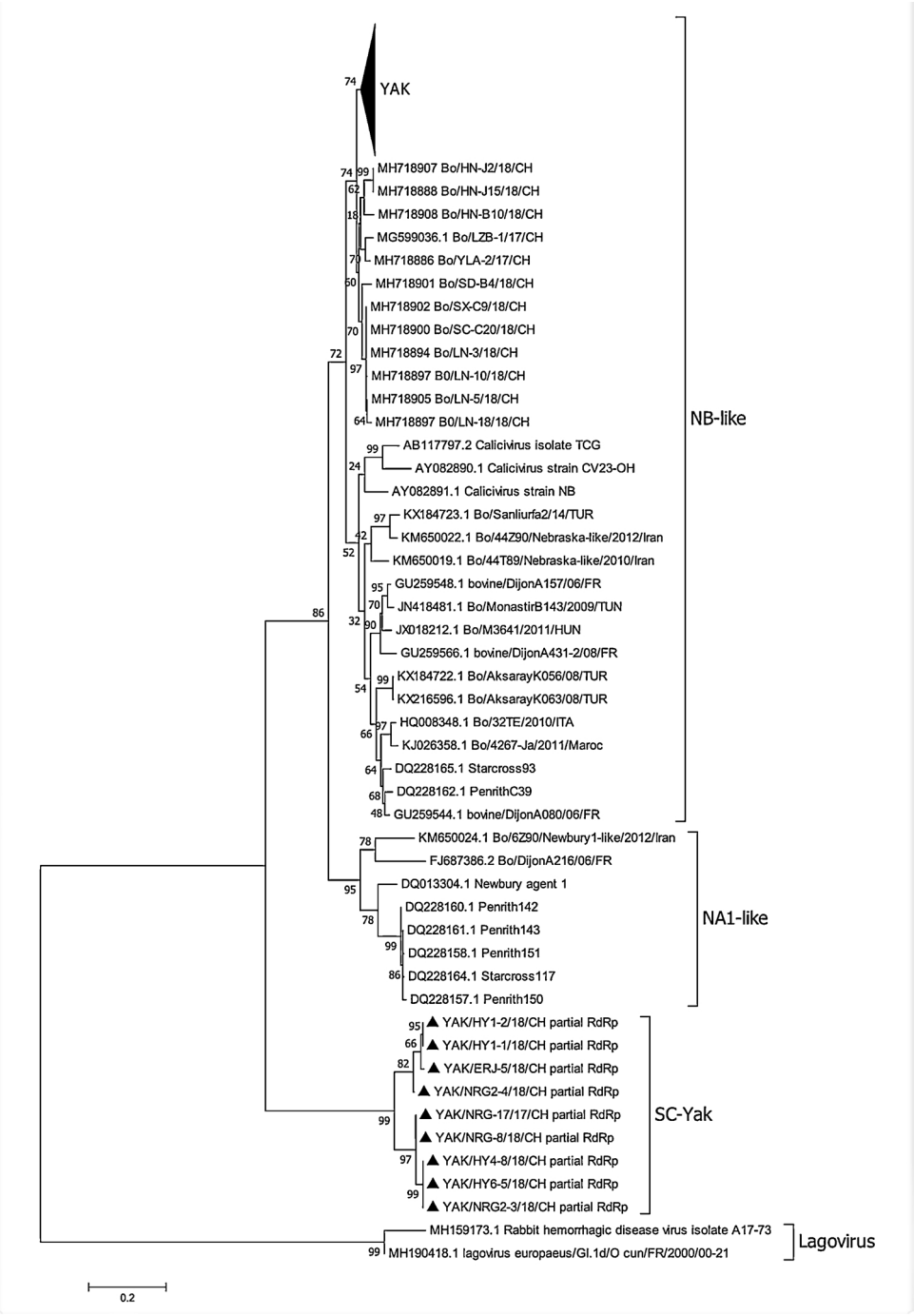

Fig. 2. Phylogenetic tree based on the RdRp fragments (396-bp). Sequence alignments and clustering were performed by ClustalW in MEGA 7.0 software. The tree was constructed by the maximum likelihood method with bootstrap values calculated for 1000 replicates. "YAK" represents 69 yak NeVs strains from this study, and black triangle also represents NeVs strains from this study. All 78 NeVs partial RdRp sequence from this study were deposited in the GenBank database under accession nos. MK452014–MK452074, and MK452079–MK452095.

Sichuan province were cluster into an independent large branch distinct from known strains of genotypes NA1-like and NB-like, which may represent a novel RdRp genotype (Fig. 2).

The 69 NB-like strains determined in this study shared 91.2%–100% nt identity compared to each other, and shared 89.5%–98.0% nt identity with 24 NeVs strains determined in Chinese dairy cows. Interestingly, compared with all 24 Chinese NeVs strains with partial RdRp sequences available in the GenBank database, all 69 yak NeVs strains of NB-like genotype had three identical nucleotide nonsense mutations (G173 T, T176C, and C188 T) in the 396-bp sequences at the 3′ end of the RdRp region. The remaining 9 strains determined in this study shared 87.1%–100% nt identity compared to each other, and shared 63.1%–66.7 nt identity with NB-like strains, and shared 61.6%–64.9% nt identity with NA1-like strains in the GenBank database.

3.3. Genomic characteristics of a novel NeVs strain

Two NeVs strains with potential novel RdRp genotype from Hongyuan and Norgay counties were selected to amplify complete genome sequence. The results showed that the complete genomes of YAK/NRG-17/18/CH and YAK/HY-12/17/CH are 7459 nt and 7460 nt

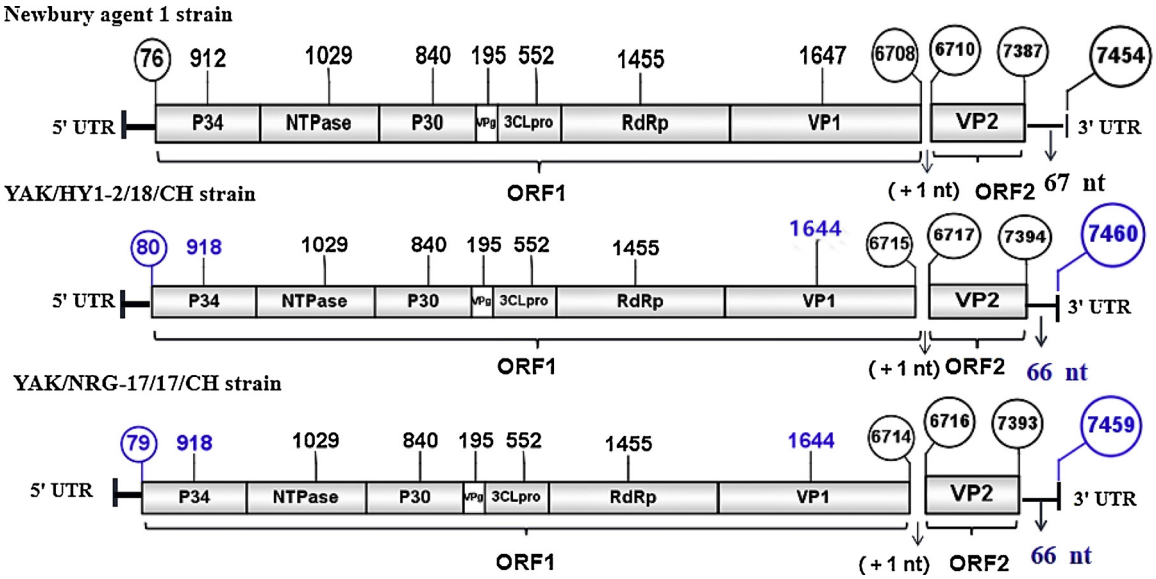

Fig. 3. Genomic organization of two NeVs strain from this study and NeVs prototype strain Newbury agent 1 (GenBank accession no. DQ013304.1). The numbers in the circle represent the location in the genome, and the remaining numbers represent the length of each gene. The length differences between two strains from this study and prototype strain Newbury 1 were shown by blue circle/numbers (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

in length with G + C content of 52.53% and 52.87%, respectively. The 5′-UTR of strains YAK/NRG-17/18/CH and YAK/HY-12/17/CH are 78 and 79 nt long, respectively. The 3′-UTR of them are 66 nt long. ORF-1 is 2211 aa in length and encodes a large nonstructural polyprotein comprising a P34, NTPase, P30, VPg, 3CLpro and RdRp proteins, contiguous with the VP1. ORF-2 is located at the 3′ end of the complete genome and encodes VP2 composed of 225 aa. ORF2 is separated from ORF1 by a single nucleotide (Fig. 3). Strains YAK/HY-17/18/CH and YAK/NRG-12/17/CH were deposited in the GenBank database under accession nos. MK452013 and MK452012, respectively.

Compared to other all six available NeVs genome, the lengths of the 5′-UTR and P34 of the two strains were 3–5 and 6 nucleotides more, respectively, while the lengths of the 3′-UTR and VP1 of the two strains were 1 and 3 nucleotides less, respectively (Fig. 3). Notably, the 5′-UTR of the two strains shared 22 identical nucleotide variations as compared to all six available 5′-UTR of NeVs, including 5 nucleotide insertion at positions 51, 56, 76–78. The 3′-UTR of the two strains shared 5 identical nucleotide variations as compared to all six available 3′-UTR of NeVs, including one nucleotide deletion at position 29 and one nucleotide insertion at position 56. The P34 protein of the two strains shared 92 out of 306 identical amino acid variations as compared to all six available P34 of NeVs, including 2 amino acid insertion at positions 154 and 155.

The two complete genomes of the yak NeVs strains shared 90.2% nt identity with each other, and only shared 68.1%–69.3% nt identity with six known complete genomes of NeVs strains, and shared 51.6% nt identity with Kirklareli virus, which is also found in calves and may be related to the genus *Nebovirus* (Alkan et al., 2015). A phylogenetic analysis of all NeVs genomes revealed that strains YAK/NRG17/17/CH and YAK/HY1-2/18/CH were clustered into an independent large branch, which indicated that the two strains may represent a novel NeVs strain (Fig. 4). Furthermore, phylogenetic trees based on its VP1, RdRp, VP2, P34, NTPase, P30, VPg and 3CLpro proteins showed that the two strains were also clustered into an independent branch. The aa identities of the individual proteins also supported that the two strains represent a novel NeVs strain that is distinct from other known NeVs strains (Table 2). No recombination events were identified in the complete genome of the two NeVs strains in this study.

The complete VP1 of strains YAK/NRG17/17/CH and YAK/HY1-2/18/CH is predicted to be 548 aa long, one amino acid shorter than other known NeVs complete VP1 sequences. The VP1 of the two strains shared 96.4% aa identity (91.2% nt identity) between each other, and shared 73.3%–81.1% aa identity (68.0%–73.6% nt identity) with all known 60 VP1 sequences of genotype 1, and shared 76.7%–78.5% aa identity (68.3%–70.0% nt identity) with all two known VP1 sequences of genotype 2 strains. Sequence analysis showed that the complete VP1 of strains YAK/NRG-17/17/CH and YAK/HY1-2/18/CH shared 80 and 106 identical amino acid variations as compared with VP1 sequences of genotype 1 and genotype 2, respectively (Supplementary Fig. 1). Interestingly, compared to all 60 VP1 sequences of genotype 1, the two VP1 sequences in this study and all two VP1 sequences of genotype 2 shared one amino acid deletion (384) in P2 domain. Compared to all VP1 sequences of genotype 2, the two VP1 sequences in this study and all VP1 sequences of genotype 1 shared one amino acid deletion (400) in P2 domain. Phylogenetic analysis based on all known 64 complete VP1 (including two complete VP1 in this study) showed that strains YAK/NRG17/17/CH and YAK/HY1-2/18/CH were clustered into an independent large branch differed from all known NeVs strains of genotype 1 and genotype 2 (Fig. 5).

Phylogenetic analysis of complete RdRp sequences showed that strains YAK/NRG17/17/CH and YAK/HY1-2/18/CH were clustered into an independent large branch. The complete RdRp sequence of strains YAK/NRG17/17/CH and YAK/HY1-2/18/CH shared 96.7% aa identity (90.0% nt identity) between each other, shared 74.8%–75.9% aa identity (66.1%–67.6% nt identity) with other known five NB-like strains, and shared 74.4% and 74.6% aa identity (66.1% and 66.9% nt identity) with NA1 strain in the GenBank database, respectively. Further analysis revealed that strains YAK/NRG17/17/CH and YAK/HY1-2/18/CH share 106 identical amino acid variations in the complete RdRp region (489 amino acid), compared with other all six available complete RdRp.

3.4. Screening for novel NeVs in yak

The 366-bp VP1 sequences of the 9 strains with potential novel RdRp genotype shared 91.8–100% nt identity with each other, 73.9–80.7% nt identity with other NeVs strains in the GenBank database. Phylogenetic tree based on 366-bp VP1 sequences indicates that the 9 strains were cluster in an independent branch (Fig. 6). Compared to all known 62 VP1 sequences, the 9 VP1 sequences in this study

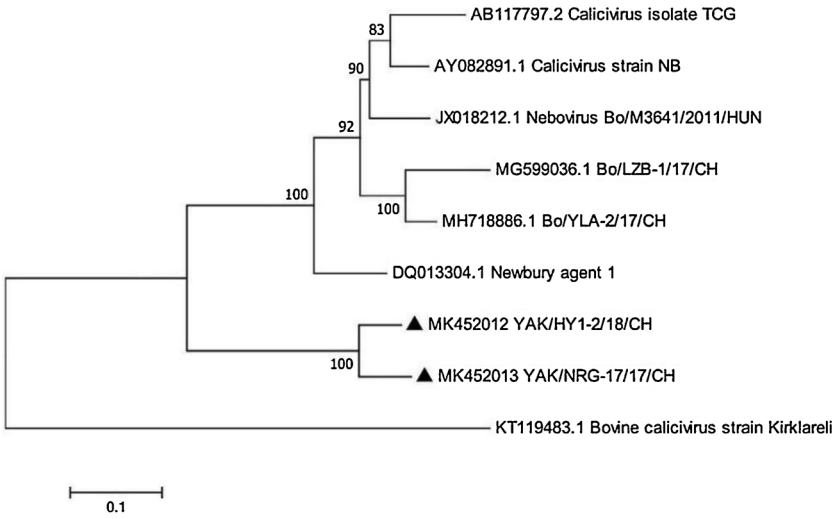

Fig. 4. Phylogenetic tree based on the complete genomic nucleotide sequence of NeVs and Kirklareli virus. Sequence alignments and clustering were performed by ClustalW in MEGA 7.0 software. The tree was constructed by the maximum likelihood method with bootstrap values calculated for 1000 replicates. Kirklareli virus (Kirklareli strain) was found in calves and may be related to the genus *Nebovirus*. Two yak NeVs strain from this study were marked with a black triangle.

shared 17 identical nucleotide variations. All 9 NeVs partial VP1 sequence from this study were deposited in the GenBank database under accession nos. MK452096–MK452104.

Therefore, 11.5% (9/78) NeVs strains including YAK/NRG17/17/CH and YAK/HY1-2/18/CH were screened as novel NeVs strains with novel VP1 genotype and novel RdRp genotype. Interestingly, all 9 NeVs positive samples screened as novel genotypes were detected from 6 farms in Hongyuan and Norgay counties in Sichuan province, and NeVs strains of two RdRp genotypes (NB-like and SC-YAK) were co-circulated in 4 out of 6 farms.

4. Discussion

4.1. Prevalence of NeVs in yak in China

Yak is a unique free-grazing bovine species in the Qinghai-Tibet Plateau (Ding et al., 2010; Guo et al., 2014). In this study, 22.0% (78/354) diarrhoeic samples of yak were detected as NeVs-positive by RT-PCR, and the virus was detected in 38 out of the 55 farms located across the three main yak-production areas in China, and the geographical distance between the two farthest NeVs positive farms is > 2000 km. It indicates that NeVs have been circulating among yak with wide geographical distribution in China. It has major implications for the diagnosis and prevention of yak diarrhea. To best of our knowledge, this is the first detection of NeVs in yak. Furthermore, a high co-infection rate of NeVs with BRV, BCoV and BVDV was found in this study, which is similar to that of NeVs in dairy cows reported in China (Guo et al., 2018c). It may lead to an increase in clinical severity and difficulty in diagnosing and controlling yak diarrhea.

Phylogenetic analysis based on RdRp fragment sequences showed that NeVs in yak showed two RdRp genotypes, as mentioned above. Interestingly, the 69 strains in NB-like were most related to NeVs strains determined in Chinese dairy cow (Guo et al., 2018c). It is possible that NeVs in yak was transmitted from NeVs in dairy cows. Moreover, these 69 strains from distinct geographic regions were located at a unique branch due to three identical nucleotide nonsense mutations (G173 T, T176C, and C188 T), instead of clustered into distinct branch based on geographic region, indicating that NeVs of NB-like genotype in yak showed unique evolutionary characteristics. The unique evolution of NeVs in yak may be related to the host species and special geographical environment of the Qinghai-Tibet Plateau, i.e., the high-altitude, low oxygen, low temperature, and low atmospheric pressure. In fact, the unique evolutionary trend has been observed previously in dairy cows in China and South Korea (Guo et al., 2018c; Park et al., 2008). Although the biological significance of the nonsense mutation at the 3′ end of the RdRp region in NeVs is still unclear, the influence of the mutation in this region on NeVs detection is worthy of attention, since this region is used as the target of molecular detection for NeVs (Park et al., 2009, 2006; Smiley et al., 2003).

4.2. Strains YAK/NRG17/17/CH and YAK/HY1-2/18/CH could represent a novel NeVs strain

The complete genomes of strains YAK/NRG-17/18/CH and YAK/HY-12/17/CH are the longest NeVs genome until now (Guo et al., 2018b; Oliver et al., 2006). Five significant characteristics were observed in the two strains: 1) The genomes of two strains only shared 67.7%–68.9% nt identity with all six available NeVs genomes, and phylogenetic trees of the complete genome or individual proteins showed that the two strains were clustered into an independent large branch; and 2) The 5′-UTR of the two strains shared 22 identical nucleotide variations, including 5 nucleotide insertion; and the 3′-UTR of the two strains shared 5 identical nucleotide variations, including one nucleotide deletion and one nucleotide insertion, which may be involved in replication, translation, and encapsidation of this novel NeVs strains (Alhatlani et al., 2015); and 3) The length of 5′-UTR, 3′-UTR, P34 and VP1 proteins of the two strains were different from other all NeVs strains, which resulted in the genome length of two yak NeVs strains being longer than all six available NeVs genome (Oliver et al., 2006; Guo et al., 2018b); and 4) The VP1 of the two strains showed significant heterogeneity differed from known strains of genotype 1 and genotype 2, which may represent a novel VP1 genotype; and 5) The RdRp of the two strains showed significant heterogeneity differed from known strains of genotypes NA1-like and NB-like, which may represent a novel RdRp genotype. Together, these characteristics suggested that strains YAK/NRG-17/18/CH and YAK/HY-12/17/CH may represent a novel NeVs strains. It is interesting to further study the origin and evolution of the novel NeVs strain.

4.3. VP1 of strains YAK/NRG17/17/CH and YAK/HY1-2/18/CH could represent a novel VP1 genotype

Currently, NeVs can be divided into two VP1 genotypes (genotype 1 and genotype 2) based on amino acid sequence and phylogenetic analysis (Guo et al., 2018c; Kaplon et al., 2011). Analysis of all available NeVs complete VP1 sequences showed that all 60 complete VP1 of genotype 1 showed ≥37.7% nt divergence (≥28.1% aa divergence) with all 2 complete VP1 of genotype 2. Previous study suggested that 14.3%–43.8% of the pairwise distances divergence was suggested for a new genotype in noroviruses based on the complete VP1 amino acid

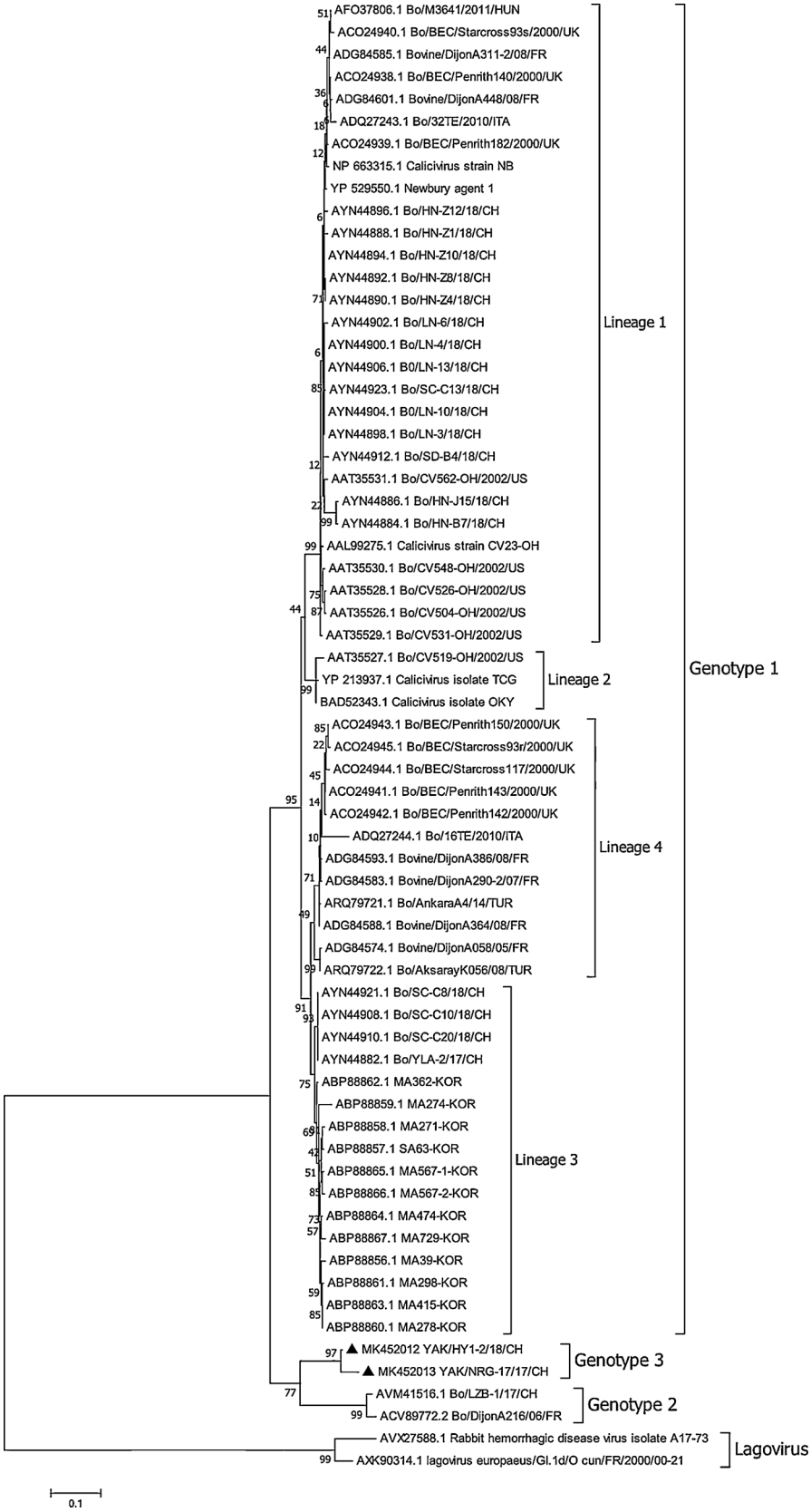

Fig. 5. Phylogenetic tree based on the complete major capsid (VP1) amino acid sequence. Strains YAK/NRG-17/17/CH and YAK/HY1-2/18/CH from this study were marked with a black triangle.

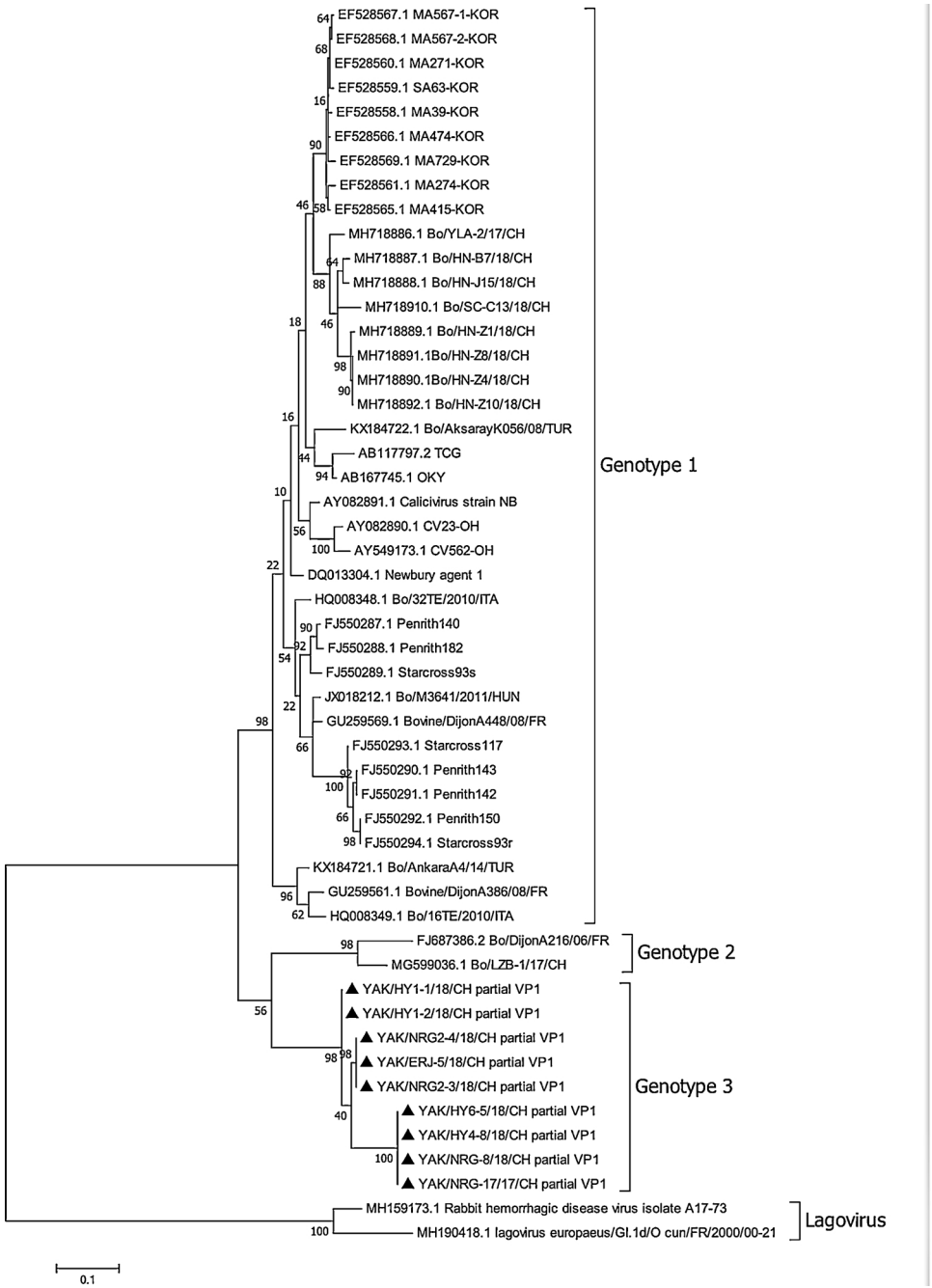

Fig. 6. Phylogenetic tree based on the 366–bp VP1 sequences. Sequence alignments and clustering were performed by ClustalW in MEGA 7.0 software. The strains in this study were marked with black triangle.

sequence (Zheng et al., 2006). For lagoviruses, 15%–30% of the genetic distances was suggested for a new genotype based on the complete VP60 nucleotide sequence (Le Pendu et al., 2017). In this study, the VP1 of strains YAK/NRG-17/18/CH and YAK/HY-12/17/CH showed > 31.9% nt divergence (> 21.7% aa divergence) and > 36.9% nt divergence (> 25.2% aa divergence) with VP1 genotype 1 and genotype 2 strains, respectively. Thus, the VP1 of strains YAK/NRG-17/17/CH and YAK/HY1-2/18/CH may represent a novel VP genotype, proposed to be genotype 3.

The VP1 is the major structural component of caliciviruses and is involved in host specificity and immunogenicity (Chen et al., 2006), and the hypervariable P2 domain is involved in the binding of ABO histo–blood group antigen (HBGAs) (Hansman et al., 2011; Tan and Jiang, 2011). Previous studies suggested that the histo-blood group antigen interactions vary among genotypes in noroviruses, for example, GII.4 viruses seem capable of binding to a wider range of histo-blood group antigens compared with other noroviruses genotypes and therefore have a larger susceptible population to infect (White, 2015). Recently, nebovirus-like capsid particles (strain Bo/BNeV/MA415/04/KR of genotype 1.3) were shown to interact with a wide spectrum of histo-blood group antigens (Cho et al., 2018). Strains YAK/NRG-17/17/CH and YAK/HY1-2/18/CH has many identical amino acid variations in VP1, especially in the P2 domain. These variations in VP1 may influence the HBGAs interaction, and it is worthy to further study the interaction between different NeVs VP1 genotypes and HBGAs.

4.4. RdRp of strains YAK/NRG17/17/CH and YAK/HY1-2/18/CH could represent a novel RdRp genotype

To date, NeVs can be divided into two RdRp genotypes (NA1-like and NB-like) based on sequences and phylogenetic analysis (Guo et al., 2018c). In this study, the complete RdRp of strains YAK/NRG17/17/CH and YAK/HY1-2/18/CH shared 74.2%–75.7% aa identity (66.1%–67.6% nt identity) with all known six NeVs complete RdRp. Phylogenetic analysis based on complete RdRp showed that the two strains were clustered into an independent large branch. Moreover, strains YAK/NRG17/17/CH and YAK/HY1-2/18/CH share 106 identical amino acid variations in the complete RdRp region (485 amino acid), compared with other all six available complete RdRp. These results suggested that the RdRp of strains YAK/NRG17/17/CH and YAK/HY1-2/18/CH could represent a novel RdRp genotype, proposed to be SC-YAK.

Until now, the precise functions of NeVs RdRp has not been reported. However, RdRp of the other caliciviruses plays a critical role in the replication (Deval et al., 2016; Lee et al., 2017). The rotation of the central helix in the thumb domain and displacement of the C-terminal tail at the active site are essential to bind RNA and substrates (Lee et al., 2017). In this study, strains YAK/NRG17/17/CH and YAK/HY1-2/18/CH share 8 identical amino acid variations in the C-terminal region (35 amino acid), compared with other all six available complete RdRp, which may have influence the virus replication of the novel NeVs strain. RdRp-driven genetic diversity is vital for viral fitness (Deval et al., 2016). In pandemic noroviruses strain, the RdRp displays reduced replication fidelity, which results in higher mutation rate and rate of viral evolution compared to the non-pandemic and less frequently detected strains (Bull et al., 2010). Currently, NB-like genotype of NeVs has been detected in 12 countries; and NA1-like strains have been detected in 4 countries (Park et al., 2008; Hassine-Zaafrane et al., 2012; Pourasgari et al., 2018; Turan et al., 2018). It seems that NeVs of different RdRp genotypes are different in prevalence. It is worthy to further monitor the prevalence of the novel RdRp genotype.

4.5. Prevalence characteristic of the novel NeVs

11.5% (9/78) NeVs were screened as the novel NeVs strains with novel VP1 genotype and novel RdRp genotype. Notably, these 9 novel NeVs strains were detected from 6 farms in two neighboring counties (the geographical distance between the two farthest farms in the two counties is more than 200 km), indicating that the novel NeVs has spread this local region. Notably, NeVs strains of two RdRp genotypes (NB-like and SC-YAK) were co-circulated in 4 out of 6 farms. Recombination at RdRp-capsid junction in caliciviruses is common (Bull et al., 2005; Coyne et al., 2006; Lopes et al., 2015), and recombination at RdRp and VP1 of NeVs also have been determined (Di Martino et al., 2011; Guo et al., 2018b). Co-circulation of different RdRp genotypes NeVs strains in a farm may increase the chance for producing new recombinant strains, involving the evolution and prevalence of the virus.

5. Conclusion

The study confirmed NeVs has been widely circulated among yak in Qinghai-Tibet Plateau, China. Two complete genomes with a novel VP1 genotype and a novel RdRp genotype were determined, which may represent a novel NeVs strain. Notably, this novel NeVs strain has spread in local provinces in China. These findings contribute to the understanding the prevalence and genetic evolution of NeVs.

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Compliance with ethical standards

This study did not involve animal experiments besides the fecal sampling of diarrhea yak that visited farm for clinical treatment.

Declaration of Competing Interest

All authors declare that they have no conflict of interest regarding the publication of this article.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.108388.

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