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The first complete genome sequence and genetic evolution analysis of bovine norovirus in Xinjiang, China

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Abstract

Introduction: Viruses are among the main pathogens causing diarrhoea in calves. The current study found that bovine norovirus (BNoV) is one of the principal viruses causing diarrhoea in calves in Xinjiang, China. **Material and Methods:** A total of 974 calf faecal samples from six regions in Xinjiang were tested for BNoV using reverse-transcriptase PCR. The genomic characteristics of BNoV and the genetic evolution of the VP1 gene, protein three-dimensional structure characteristics and amino acid variation were analysed using bioinformatics methods. **Results:** Epidemiological survey results showed that the infection rate of BNoV was 19.82%, and all samples tested positive in five regions. The results of the genetic evolution analysis showed that BNoV strains from Tacheng of northern Xinjiang and Kashgar of southern Xinjiang both belonged to the GIII.2 genotype of BNoV but were not on the same cluster of evolutionary branches. Additionally, the amino acid variation of the VP1 protein was not observed to significantly affect its spatial structure. **Conclusion:** This study is the first to report the genetic characteristics of the BNoV complete genome sequence in Xinjiang and provides a scientific basis for BNoV vaccine development and pathogenesis research.

Keywords: calf diarrhoea, bovine norovirus, complete genome, VP1.

Introduction

Diarrhoea is a common disease in calves characterised by an insidious nature and prolonged transient infections (22). It is a major cause of low productivity and economic loss for cattle producers worldwide (11). Previous research has shown that within the sub-group of pre-weaning calves which die, the cause of death for 50% of them is diarrhoea (16, 17, 19). Many factors can cause diarrhoea in calves, including bacteria, parasites and viruses. The symptoms of calf diarrhoea caused by different pathogens vary (5, 26). Usually, a virus is the main factor that causes it (6). The most common bovine diarrhoea viruses are bovine rotavirus (BRV), bovine viral diarrhoea virus (BVDV), bovine coronavirus (BCoV) and bovine norovirus (BNoV) (10). At present, most studies on calf diarrhoea mainly focus on BCoV, BRV and BVDV, and vaccines against these viruses have been developed and commercialised (21). However, because of the frequent coinfection of BNoV with other diarrhoea viruses, the role of BNoV in calf diarrhoea was often overlooked in the past. In addition, the pathogenic mechanism of BNoV is unclear because it is difficult to culture BNoV *in vitro* (24). The imperfect understanding of the pathogenicity limits the development of vaccines against BNoV (14).

In recent years, BNoV has attracted much attention as an important pathogen causing diarrhoea in calves (10). In China, BNoV positivity has been reported successively since 2017, and the complete genome sequence of BNoV was first obtained nationally in 2019 in Shijiazhuang, Hebei (31). Chen *et al.* (8). reported BNoV-positive findings in Yunnan in 2020. In 2021,

Wang *et al.* (37) detected BNoV infections in Beijing, Tianjin, Hebei, Anhui, Heilongjiang and Inner Mongolia in China. In 2018, Yu *et al.* (38) reported a BNoV-positivity rate of 11.11% (2/18) in Changji and 26.47% (18/68) in Shihezi in Xinjiang.

Bovine norovirus belongs to genogroup GIII of NoV, which includes two genotypes, GIII.1 and GIII.2 (25). These correspond to two distinct antigenic types or serotypes (13). The virus has three open reading frames (ORFs). Open reading frame 1 encodes six non-structural proteins (P48, NTPase, P22, VPg, Pro and RdRp) (20), ORF2 mainly encodes a major capsid protein (VP1) and ORF3 encodes a minor capsid protein (VP2) (7). The major capsid protein – which consists of the interior shell (S) and exterior protrusion (P) major domains – has the functions of immune recognition and host receptor binding (9, 27). The S domain is the most conserved region, the P1 subdomain is a moderately well-conserved region, and the P2 subdomain is the most highly variable region (36).

The aims of this study were to survey BNoV epidemiologically in Xinjiang in China, amplify the virus' genome sequence, and analyse it phylogenetically and in respect of the amino acid variations and protein three-dimensional structure of the VP1 gene. The results are intended to supply a reference for the prevention of calf diarrhoea, enrich the BNoV genome database, and provide a theoretical basis for the prevention and control of BNoV and development of a vaccine.

Material and Methods

Sample collection and processing. A total of 974 calf faecal samples were collected in Xinjiang, China, from July 2020 to July 2023. The seasons and regions of sample collection were clearly recorded. The calves from which samples were taken were under three months of age. The faecal samples were diluted with 1× phosphate-buffered saline solution at a ratio of 1:3. After being shaken on a vortex oscillator, the samples were subjected to freezing at −20°C and thawing three times. All animal procedures were conducted following the guidelines in document 2020025 approved by the Animal Ethics Committee of Xinjiang Agricultural University.

Reverse-transcriptase PCR (RT-PCR). Viral RNA was extracted from the samples using the TRIzol/chloroform stratification method. The reverse transcription was carried out with a Hifair III 1st Strand cDNA Synthesis Kit (gDNA digester plus) (Yeasen, Wuhan, China). The primer pairs were designed according to an epidemic strain reference sequence in the GenBank database with accession number MN122335.1. The primer sequences used for detection are shown in the Supplementary Table S1.

Amplification of the BNoV genome. The complete genome of BNoV was amplified using RT-PCR. The primer sequences used for the amplification of the BNoV complete genome designed by Primer Premier 5 (Premier Biosoft International, San Francisco, CA, USA) are shown in Supplementary Table S1. The amplified products were inserted into the pGEM-T vector (Promega, Madison, WI, USA) and sequenced by Sangon Biotech (Shanghai, China). The obtained BNoV sequence fragment results were assembled using SeqMan in DNAstar version 7 (DNASTAR, Madison, WI, USA).

Homology and phylogenetic analysis. The obtained gene sequences of BNoV were aligned with the reference sequences published in GenBank using the Clustal W algorithm. Subsequently, a phylogenetic tree was constructed using the maximum-likelihood method in MEGA version 7. The tree reliabilities were tested with 1,000 bootstrap replicates to yield a majority consensus tree.

Amino acid variation of VP1. The sequences were aligned using MEGA version 7, and multiple sequence alignment was performed by ESPript 3.0 (28).

Spatial structure analysis of the VP1 protein. A model was constructed using SWISS-MODEL with three-dimensional models of the VP1 proteins of BNoV (34). Sequence identity greater than 30% indicated successful model construction, and global model quality estimate values ranged from 0 to 1, with larger values indicating more reliable model quality. The three-dimensional model files were viewed using the Mol* 3D Viewer tool of the Research Collaboratory for Structural Bioinformatics Protein Database (30).

Results

Epidemiological survey of BNoV in Xinjiang, China. Infection with BNoV was detected by RT-PCR in 193 out of 974 calf faeces samples from six regions in Xinjiang (Table 1). The overall results showed that the infection rate of BNoV was 19.82% (193/974), and that positive cases were recorded in all six regions. The highest infection rate was noted in autumn (43.98%, 73/166) (Table 1).

Table 1. Rate of infection with bovine norovirus among calves under three months old in Xinjiang, China

Season				Region						Total (n=974)
Spring (n=274)	Summer (n=311)	Autumn (n=166)	Winter (n=226)	Tacheng (n=15)	Bortara (n=153)	Ili (n=188)	Kashga (n=424)	Urumuq (n=44)	Changji (n=150)	
25 (9.12%)	64 (20.58%)	73 (43.98%)	31 (13.72%)	9 (60%)	20 (13.07%)	51 (27.13%)	92 (21.70%)	2 (4.55%)	19 (12.67%)	193 (19.82%)

Table 2. Sequence similarity comparison of reference sequences of bovine norovirus with the isolated Bo/XJ-TC/01/CHN strain

	N/AA (%)	MN122335.1	MK159169.1	OK032546.2	EU794907.1	MN480761.1	MZ573179.1	NC029645.1	AF097917.5	AY126474.2
ORF1	Complete genome	93.48/96.78	92.40/90.57	86.60/90.64	84.88/95.06	92.81/97.35	92.74/97.60	86.36/95.42	85.45/95.41	84.97/95.38
	P48	95.03/96.66	93.62/96.05	95.04/96.96	80.79/88.75	93.41/96.96	93.11/97.26	83.79/93.31	81.19/90.58	81.26/90.58
	NTPase	93.55/98.34	93.28/97.79	95.30/98.62	84.16/97.51	93.74/98.62	93.46/98.34	85.36/97.24	85.45/97.79	84.44/98.07
	p22	91.85/97.28	93.48/98.37	94.20/97.28	85.71/97.80	89.05/94.57	92.21/98.37	84.31/96.17	84.22/97.80	85.90/96.70
	VPg	94.81/97.54	94.26/97.54	93.44/96.72	82.51/93.44	94.54/98.36	91.53/95.90	88.80/95.87	83.84/94.26	83.29/93.39
	Pro	94.09/98.89	95.03/99.45	94.11/99.45	85.58/98.34	94.48/99.45	94.11/99.45	85.56/97.24	87.43/98.34	87.29/98.34
	RdRp	94.80/99.20	95.13/99.20	94.40/98.41	87.17/97.61	93.87/99.20	93.81/99.00	88.47/98.80	86.77/97.61	85.51/97.41
ORF2	VP1	92.22/97.89	79.37/71.32	69.02/70.94	85.85/95.79	91.78/96.74	91.20/97.13	86.93/94.44	87.76/94.83	86.62/95.98
ORF3	VP2	91.55/94.44	69.30/64.81	67.88/65.74	85.25/91.20	91.72/94.91	91.86/95.37	86.33/90.28	84.49/92.06	85.43/92.59

ORF – open reading frame

Sequence characteristics. The complete genome sequences of two BNoV strains from two regions were obtained with two replicates per region. One strain, from Tacheng in northern Xinjiang, was named Bo/XJ-TC/01/CHN, and the other strain, from Kashgar in southern Xinjiang, was named Bo/XJ-KS/02/CHN. Their complete genome sequences have been uploaded to the GenBank database and assigned accession numbers OM991839 and OM992315. The complete genome sequence length of OM991839 was 7,295 nucleotides, with a guanine/cytosine value of 57.5%, and that of OM992315 was 7,316 nucleotides, with a guanine/cytosine value of 57.4%.

Homology and phylogenetic analysis. The results of the phylogenetic tree showed that the obtained BNoV belonged to the GIII genogroup and GIII.2 genotype. Interestingly, the OM991839.1 Bo/XJ-TC/01/CHN strain from Tacheng and OM992315.1 Bo/XJ-KS/02/CHN strain from Kashgar belonged to the same branch. OM992315.1 and OM991839.1 clustered with three Chinese strains, the former being closest in affinity to the MZ573179.1 and MN480761.1 strains from China, and the latter being closest in affinity to the MN122335.1 strain from China. It is most distantly related to the Belgian FJ946859.1 strain and the British AY126468.2 strain (Fig. 1).

The nucleotide identity between the two BNoV strains was greater than 92%, and the amino acid homology was greater than 90%. The nucleotide identity and amino acid homology of genogroup GIII of BNoV was analysed, and Bo/XJ-TC/01/CHN was found to be closely related to MN122335.1, with a nucleotide identity of 93.48%. Further analysis showed that the ORF1 gene and VP1 gene of Bo/XJ-TC/01/CHN were most similar to those of MN122335.1, while the VP2 gene of this strain was most similar to that of MZ573179.1 (Table 2). The highest nucleotide identity between the other strain and a reference strain was between it and MZ573179.1, at 93.46%. The genes of Bo/XJ-KS/02/CHN which were most similar to their counterparts in MZ573179.1 were ORF1 and VP1, and the VP2 gene of Bo/XJ-KS/02/CHN was most similar to that of MK159169.1 (Table 3).

Unexpectedly, the amino acid homology between Bo/XJ-TC/01/CHN and MZ573179.1 was 97.6%. Further analysis showed that the genes of Bo/XJ-TC/01/CHN which had the highest amino acid homology with MZ573179.1 were ORF1 and VP2, whereas the VP1 gene of this strain had the highest homology with MN122335.1 (Table 2). In addition, the amino acid homology between Bo/XJ-KS/02/CHN and MZ573179.1 was 98.37%. In this instance the ORF1 gene was not one of the pair with the highest homology with the same genes in with MZ573179.1, but rather the VP1 and VP2 genes were that pair. The ORF1 gene of Bo/XJ-KS/02/CHN had the highest homology with this gene in strain MN122335.1 (Table 3).

Amino acid mutation of VP1. Mutations in the amino acids of the norovirus VP1 protein are important factors in the evolutionary mechanism of norovirus (18). Therefore, the amino acid sequences of the Bo/XJ-TC/01/CHN and Bo/XJ-KS/02/CHN VP1 proteins were compared with the VP1 proteins in the respective reference sequences with the highest amino acid homology: MN122335.1 and MZ573179.1. Differences between the Bo/XJ-TC/01/CHN sequence and consensus sequence of the other subbranch (Bo/XJ-KS/02/CHN and MZ573179.1) were revealed at one site in the S domain, two sites in the P1 subdomain and nine sites in the P2 subdomain. Between the Bo/XJ-KS/02/CHN sequence and the consensus sequence of the other subbranch (Bo/XJ-TC/01/CHN and MN122335.1), there were one site of difference in the S domain, five sites in the P1 subdomain and four sites in the P2 subdomain. Interestingly, among all variations, 11 sites of difference in the same subbranch were found: 5 sites in the subbranch of Bo/XJ-TC/01/CHN and MN122335.1 and 6 sites in the subbranch of Bo/XJ-KS/02/CHN and MZ573179.1. The differences among the four strains are shown in detail in Fig. 2, which reveals the rapid evolution of the novel BNoV genocluster.

Spatial structure analysis of VP1. The possible three-dimensional structures of the structural proteins of the representative epidemic strains (MN122335.1 and MZ573179.1) were predicted and compared with those of the Bo/XJ-KS/02/CHN and Bo/XJ-TC/01/CHN strains. The superposition of Bo/XJ-KS/02/CHN and Bo/XJ-TC/01/CHN protein structures showed that the α-helix at 419–421 amino acids (aa) and 50–54 aa of Bo/XJ-TC/01/CHN had been replaced by a random coil (Fig. 3a).

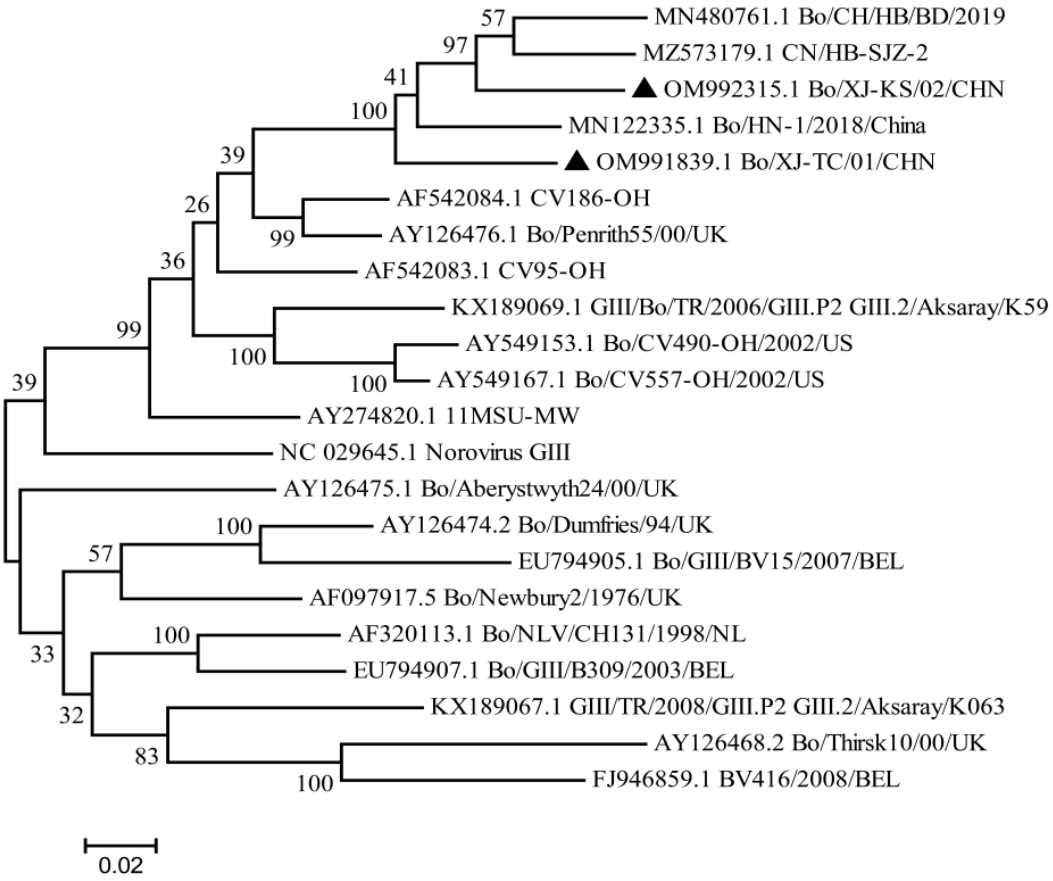

Fig. 1. Phylogenetic tree of bovine norovirus. Black triangle – isolate obtained in this study; scale bar – nucleotide substitutions

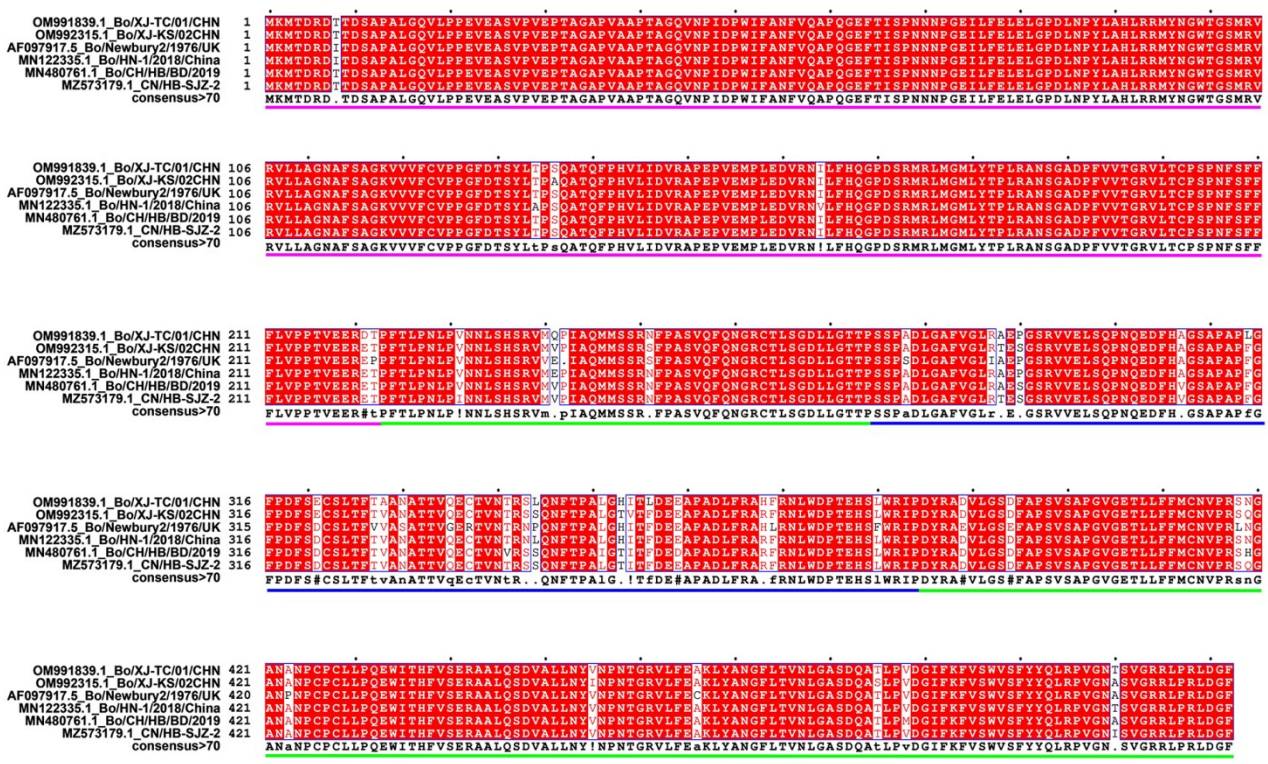

Fig. 2. Amino-acid mutation of the VP1 protein of bovine norovirus
blue – P2 subdomain; green – P1 subdomain; pink – S domain

Table 3. Sequence similarity comparison of reference sequences of bovine norovirus with the isolated Bo/XJ-KS/02CHN strain

	N/AA (%)	MN122335.1	MK159169.1	OK032546.2	EU794907.1	MN480761.1	MZ573179.1	NC029645.1	AF097917.5	AY126474.2
ORF1	Complete genome	93.06/98.19	86.25/94.83	85.84/90.56	84.97/95.50	93.46/98.19	93.70/98.37	86.6/95.940	85.46/95.76	85.33/95.57
	P48	93.81/96.96	93.01/96.35	94.02/97.26	81.68/89.97	94.22/97.26	93.52/97.57	84.40/93.31	81.05/91.19	82.14/91.19
	NTPase	93.74/99.17	93.65/99.17	94.20/99.45	84.90/98.62	94.57/100	94.66/99.72	86.00/98.62	87.38/98.90	85.82/99.17
	p22	91.85/97.83	92.21/98.91	92.39/97.83	85.35/97.25	89.41/94.57	93.12/97.83	86.31/96.72	84.22/97.25	86.28/97.25
	VPg	95.08/99.18	94.54/98.36	94.81/97.54	83.88/94.26	93.72/100	92.90/97.54	88.71/95.87	85.21/95.08	84.25/94.21
	Pro	94.27/99.44	94.29/99.45	93.92/100	85.27/98.34	97.24/100	95.21/100	86.74/97.79	86.56/98.34	85.82/98.34
	RdRp	94.66/99.41	94.73/99.41	93.74/98.62	86.76/97.63	94.66/99.41	94.66/98.81	89.53/98.81	85.97/97.63	86.17/97.43
ORF2	VP1	91.39/96.74	69.78/70.36	68.39/70.36	85.59/94.83	92.10/97.51	92.93/98.28	85.91/94.25	86.93/94.25	86.11/94.83
ORF3	VP2	91.69/96.76	92.77/96.62	66.62/63.43	84.62/93.06	91.54/96.76	91.69/97.22	84.92/92.13	84.92/93.46	85.56/92.13

ORF – open reading frame

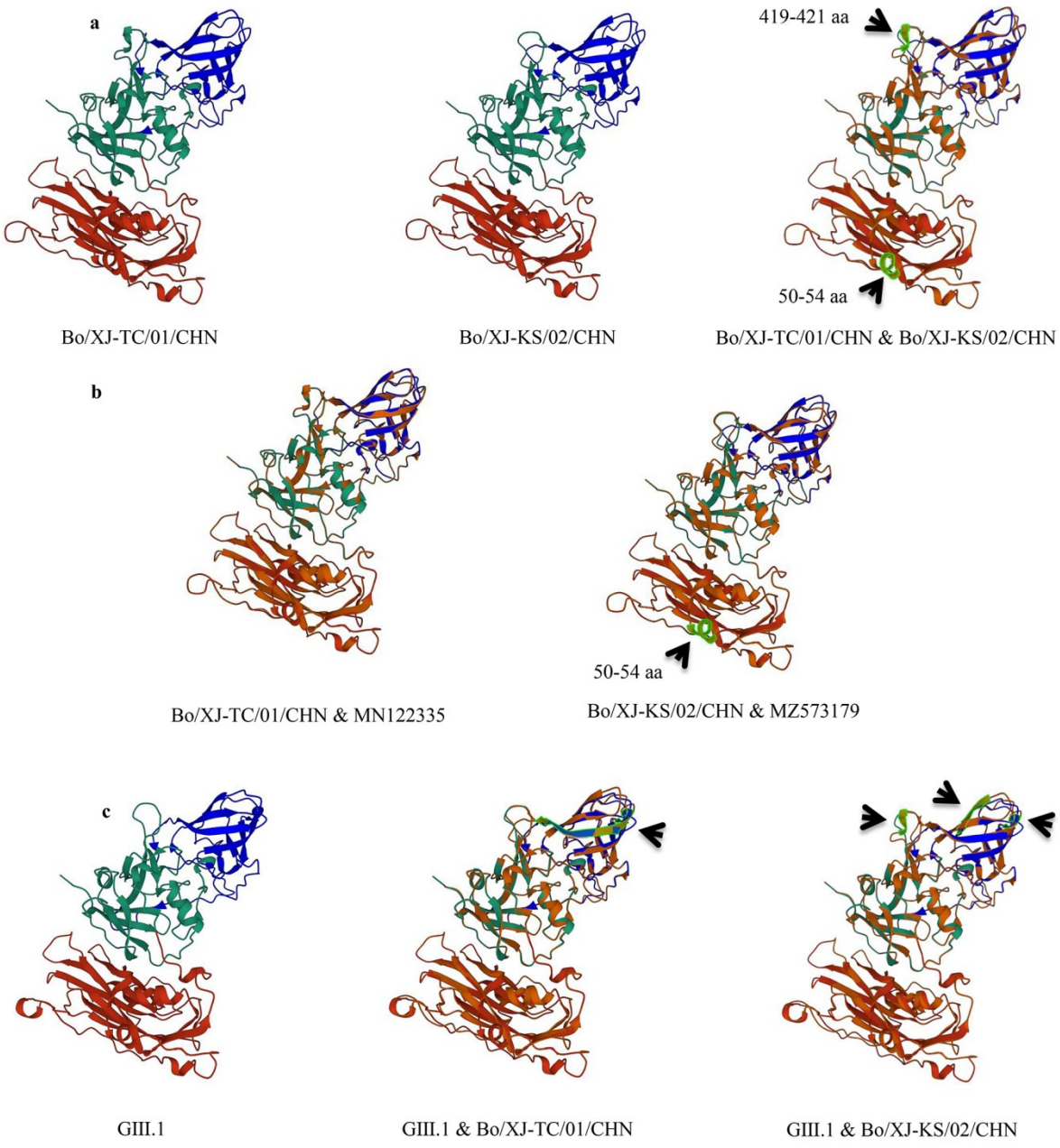

Fig. 3. Bovine norovirus VP1 protein 3D model
a – VP1 protein 3D models obtained in this study and superposition of the Bo/XJ-TC/01/CHN isolate VP1 protein on that of Bo/XJ-KS/02/CHN; b – superposition of the Bo/XJ-TC/01/CHN VP1 protein on that of MN122335, and of the Bo/XJ-KS/02/CHN VP1 protein on that of MZ573179; c – GIII.1 genotype VP1 protein, superposition of the Bo/XJ-TC/01/CHN VP1 protein on that of the GIII.1 genotype, and superposition of the Bo/XJ-KS/02/CHN VP1 protein on that of the GIII.1 genotype; mid-blue – P2 subdomain; mid-green – P1 subdomain; brown – S domain; black arrows – structural differences

The Bo/XJ-TC/01/CHN and MN122335.1 proteins had the same three-dimensional structures. Unique to Bo/XJ-KS/02/CHN was the random coil at 50–54 aa in the P1 subdomain (Fig. 3b). Comparing the VP1 structures of the Xinjiang strains with the VP1 structure of GIII.1 BNoV, there were significant differences in the P2 subdomain, which also verified the finding of a previous study (23) that the NoV genetic variation can cause differences in the P2 subdomain. (Fig. 3c). It was shown that amino acid mutations in the P structural domain affect the ability of the virus to bind to histo-blood group antigen, in addition to affecting the virus' propensity to develop gastroenteritis in the host (when the mutations are in the P2 subdomain (4).

Discussion

Since the first identification of BNoV infection in humans in 1976 (35), such infection has been reported in many countries. In China, it was first reported in 2018 (15). However, the complete genome sequence of BNoV in China was obtained and analysed for the first time in 2019 (31). In this study, we mainly conducted epidemiological investigations on BNoV and obtained the first complete BNoV genome sequences from two regions in Xinjiang, China.

The BNoV-positive rate was 19.82% (193/974), and the virus had a wider regional prevalence in Xinjiang. Previous studies have shown that BNoV was on all continents (12, 27, 29, 36) and was detected in Sichuan, Henan, Hebei, Liaoning, Ningxia, Shaanxi, Shandong and other provinces in China (15, 32, 33). The findings in this study further confirmed that BNoV is widely distributed in Xinjiang and is one of the most important pathogens involved in calf diarrhoea. The epidemiological survey results for different seasons showed that the infection rate of BNoV was highest in autumn. Previous studies have also reported that low temperatures are more conducive to the growth and transmission of pathogens, resulting in a higher incidence of diarrhoea in autumn and winter (2, 3). The peak infection rate of BNoV occurring in autumn was related to the large temperature difference between day and night, the changeable climate and the higher rainfall in autumn in Xinjiang.

The results of the genetic evolution analysis showed that the two BNoV strains obtained in this study belonged to genotype GIII.2. Previous studies and the GenBank database showed that the GIII.2 genotype has a more widespread prevalence than GIII.1 (25). Despite the two BNoV strains from Xinjiang being in the same genotype, they were not in the same clade. Through the comparison of nucleotide similarity and amino acid homology, it was found that the new strain with the highest similarity to Bo/XJ-TC/01/CHN was not Bo/XJ-KS/02/CHN, and the nucleotide identity and amino acid homology between the two strains were lower than those between one or the other strain and GIII.2 BNoV strains from other regions. The reason for this interesting finding may be that Bo/XJ-TC/01/CHN and Bo/XJ-KS/02/CHN were obtained from the northern and southern regions of Xinjiang, that is Tacheng and Kashgar, respectively. This also indicates that regional differences may be one of the factors causing genetic mutations during BNoV transmission, which, if proven, would provide a certain scientific basis for the prevention and treatment of BNoV in different regions.

Previous studies have shown that the VP1 protein is an important capsid protein involved in BNoV's recognition of receptors and triggering of antibody production (13). This study further analysed the VP1 protein, and the amino acid variation comparison revealed that it was greater in the P2 subdomain than in the P1 subdomain and S domain. Further, the three-dimensional visualisation model of the VP1 protein also verified that there was a correlation between the alteration of the P2 subdomain and the alteration of the NoV genotype, which verified the outcomes of previous studies (1, 18). However, the amino acid variants in the P2 subdomain of these two strains did not cause any changes in the spatial conformation in our study. Interestingly, the amino acid variants in the P1 subdomain of Bo/XJ-KS/02/CHN strains did cause subtle changes in the spatial conformation. Further studies are needed to elucidate these variations.

In recent years, the presence of BNoV has been identified across multiple continents, demonstrating its widespread prevalence across the globe. Epidemiology can provide useful data for studying epidemic trends and strain evolution. This study reveals the prevalence and epidemic strains of BNoV in some regions of Xinjiang, enriches epidemiological survey data, and offers guidance for BNoV prevention and control. In addition, it reports the complete genome sequence of BNoV in Xinjiang for the first time, which expands the complete genome sequence data on BNoV.

Conclusion

The results of this study can be used for future genetic studies on BNoV isolates in China and for further research on BNoV diversity, and may provide the basis for development of a vaccine against the diseases caused by this virus.

*These authors contributed equally to this work.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

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