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Identification and genetic characterization of bovine parainfluenza virus type 3 genotype c strain isolated from cattle in western China

Xiaobo WEN[1,*], Jinhua SUN[2,*], Yao ZHANG[1], Si CAO[1], Xuhua RAN[1], Hongbo NI[1,]**
[1]College of Animal Science & Veterinary Medicine, Heilongjiang Bayi Agricultural University, Daqing, P.R. China
[2]College of Animal Science & Technology, Northeast Agricultural University, Harbin, P.R. China

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Abstract: Bovine parainfluenza virus type 3 (BPIV3) is a leading respiratory pathogen of cattle and is often implicated as a causative pathogen of bovine respiratory disease complex (BRDC). Three genotypes (BPIV3a–c) have been tentatively classified based on genetic and phylogenetic analyses. A BPIV3c isolate, designated NX49, was isolated using the Madin-Darby bovine kidney cell culture from cattle in western China, where feedlot cattle with severe respiratory disease symptoms were observed. Hemagglutination assay demonstrated that NX49 had low agglutination activity against guinea pig erythrocytes, whereas SD0835, the first BPIV3c strain reported in China, showed high agglutination activity. Phylogenic analysis indicated that NX49 shared 99.3% whole genome identity with SD0835 and 97.5%–98% similarity with BPIV3c strains isolated from Japan, South Korea, and the United States. NX49 also showed 82.5% nucleotide similarity with NM09, a BPIV3a strain isolated from China. These data suggest that the BPIV3c strains circulating in China are conserved. Moreover, BPIV3a and BPIV3c might concomitantly circulate in feedlot cattle for long durations in China. Further characterizing of such viruses and continued surveillance may help to prevent BRDC caused by BPIV3 and aid in vaccine development.

Key words: Bovine parainfluenza virus type 3, cattle, phylogenetic analysis, NP gene, complete genome

1. Introduction

The bovine respiratory disease complex (BRDC) is considered a major illness in dairy and beef cattle worldwide. Bovine parainfluenza virus type 3 (BPIV3) is one of the most important pathogens associated with BRDC (1,2). In most cases where BPIV3 is implicated in disease, mild clinical signs are often observed (1), including coughing, fever, and nasal discharge (3). However, in some circumstances when animals undergo high stress, such as during transportation, BPIV3 can contribute to tissue damage and immunosuppression, resulting in severe bronchopneumonia and a high risk of secondary bacterial or viral infections (4). Therefore, BRDC caused by BPIV3 may result in a significant loss to the cattle industry worldwide.

BPIV3 (genus *Respirovirus*, subfamily *Paramyxovirinae*, order *Mononegavirales*) is an enveloped, single-stranded, negative-sense RNA virus. BPIV3 was first described and isolated from the nasal discharge of cattle with shipping fever in the United States (5,6). BPIV3 strains have been tentatively classified into three genotypes, BPIV3a–c, based on genetic and phylogenetic analyses. Numerous BPIV3a strains have been isolated in North America (6–9), one in China (10), and one in Japan (11). BPIV3b was originally isolated in Australia (12) and recently identified in the United States (8). More recently, the isolation of all three genotypes from buffalo in Argentina was reported (13). BPIV3c has also been confirmed in South Korea (14), Japan (15), the United States (8), and China (16). Although BPIV3 appears to have a wide distribution, only a few complete genome sequences have been reported, which hampers the understanding of its molecular evolution and evaluation of cross-protection of BPIV3 vaccines against newly circulating strains. Therefore, complete sequencing of an increased number of BPIV3 genomes is required to evaluate viral variation, as well as to conduct epidemic surveillance and further vaccine development. In this study, we isolated and characterized a BPIV3c strain from cattle with severe BRDC symptoms in the Ningxia Hui Autonomous Region of western China using whole-genome sequencing and phylogenetic analysis.

* These authors contributed equally to this work.
** Correspondence: nihongbo@sina.com

2. Materials and methods

2.1. Samples and virus identification

Sixteen nasal swabs from dairy cattle were collected in the Ningxia Hui Autonomous Region in 2014 and shipped to the laboratory of Heilongjiang Bayi Agricultural University for diagnosis. Each nasal swab was rehydrated with 500 µL of Eagle's minimum essential medium (MEM, Thermo Fisher Scientific, Waltham, MA, USA), and then 200 µL of solution was taken for DNA extraction using the TIANamp Genomic DNA Kit (TIANGEN, Beijing, China) or RNA extraction using TRIzol (Thermo Fisher Scientific) according to the manufacturer's instructions. Reverse transcriptase PCR (RT-PCR) amplification was conducted to test for BPIV3 (using primer pair P6; Table 1). The RT reaction was conducted using random hexamer primers (Thermo Fisher Scientific) according to the manufacturer's instructions. PCR amplification with cDNA as the template was carried out in a total volume of 25 µL in the 2X GoTaq system (Promega, Madison, WI, USA). The following conditions were used: predenaturation at 95 °C for 3 min, followed by 35 cycles of denaturing at 94 °C for 30 s, annealing at 50 °C for 25 s, and extension at 72 °C for 1 min. A final extension was performed at 72 °C for 5 min.

2.2. Virus isolation and cultivation

Positive samples identified by RT-PCR were used to inoculate Madin-Darby bovine kidney (MDBK) cells when cell monolayers reached 80% confluence, followed by incubation for 1 h at 37 °C in 5% CO_2. The inoculum was then discarded and replaced with fresh MEM supplemented with 2% fetal bovine serum, which was incubated at 37 °C. The cells were harvested by three freeze–thaw cycles when an obvious cytopathic effect (CPE) was observed. The viral culture was then subjected to plaque purification from the infected cells as previously described (7) and a single plaque was picked and amplified using MDBK cells. The cells were harvested as described above, followed by identification using RT-PCR with primer pair P6.

2.3. RT-PCR amplification and genome sequencing of BPIV3

Total RNA was extracted from cell culture supernatants using the PureLink RNA Mini Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. cDNA was synthesized using BPIV3-specific sense primers (Table 1) and PCR was conducted using high-fidelity DNA polymerase (Thermo Fisher Scientific). The following conditions were used: predenaturation at 95 °C for 3 min, followed by 35 cycles of denaturing at 98 °C for 20 s, annealing at 53 °C for 25 s, and extension at 72 °C for 0.5 kb/min. A final extension at 72 °C for 10 min was added. Each fragment was then purified by agarose gel electrophoresis and adenine was added at both 3′-ends of the amplicon using Taq DNA polymerase (TaKaRa, Dalian, China). Each amplicon was subcloned into the pMD18T vector (TaKaRa) by T/A clone strategy and then transformed into DH5α competent cells. Leader and trailer sequences were obtained using the 5′-3′-RACE kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Recombinant plasmids were extracted using the TIANamp Plasmid Mini-Prep Kit (TIANGEN). Recombinant plasmids were then sequenced and assembled using SeqMan software (DNASTAR, Inc., Madison, WI, USA) and nucleotide and putative amino acid sequences were created using DNAMAN software version 8.0 (Lynnon Biosoft Company, San Ramon, CA, USA). The complete genome sequence of the BPIV3 isolate, designated as NX49, was finally obtained (GenBank accession number: KT071671).

2.4. Hemagglutination assay

The hemagglutination assay was conducted using a 0.25% guinea pig erythrocyte suspension in 96-well V-bottom plates. Equal volumes (0.25 mL) of twofold serial dilutions of BPIV3 (in 0.01 M phosphate-buffered saline) and erythrocyte suspension were incubated at 37 °C or 4 °C to test for hemagglutination activity. A BPIV3a reference strain, BN-1, was used as a control. Endpoint titers were

Table 1. The primers used for amplification in this study.

#	Sense primers (5′-3′)	Antisense primers (5′-3′)	Position [a]
P1	ACCAAACAAGAGAAGAGACTTGT	CAGACCTGCTAAAAAATCGATCTTC	1–3007
P2	TGTATGTGTGGCGAATGTCCTAAAC	TTGTCTATATCTGCTCTTGCTTGTTTG	2941–5532
P3	AACAGTACTAGTTCCAGGAAGAAGC	GGACAGCCAGTTAAATTGCATATTAC	5396–7884
P4	TAAAGGTAAAGTTATTTTTCTCGGATATG	GCAGTGCTCAGATCCTGTTATTATTA	7801–12188
P5	GGAAGAATGATAAATGGGCTTGAAACAC	ACCAAACAAGAGAAAAACTCTGTT	12108–15474
P6	TCTATATTTGCYCTTGGWCCMTCAATAACAG	CAACATATTGTCTTGATCATACTCAAGATCAC	207–542

[a] Numbers represent the nucleotide position within the genome of BPIV3 (GenBank accession number: HQ530153).

recorded when the erythrocyte suspension in the control became completely sediment.

2.5. Tissue culture infectivity dose ($TCID_{50}$) test

Viral infectivity was quantified by estimating $TCID_{50}$ using a standard cell culture procedure. Briefly, 3×10^4 MDBK cells were seeded to each well of 96-well microtiter cell culture plates (Corning Inc., Corning, NY, USA). When cells reached about 80% confluence, eight replicates were infected with 200 µL of a 10-fold serial dilution of NX49 and then incubated at 37 °C in 5% CO_2 for 7 days. BN-1 was used as the control. Additionally, BN-1 and NX49 were incubated at 50 °C for 30 min or with chloroform at 4 °C for 10 min prior to serial 10-fold dilution to test viral stability. Infectivity titers were expressed as $TCID_{50}$/mL based on the Reed–Muench formula.

2.6. Phylogenetic analysis

Five fragments based on P1–P5 primer pairs were harvested, followed by subcloning into the pMD18 T vector, respectively. Each fragment was sequenced by BGI Science and Technology Ltd. (Shenzhen, China) with universal sequencing primer M13-47 targeting the flank region of the cloning site and subsequent primers based on a gene walking strategy. The sequence of each fragment, as well as leader and trailer sequences, were assembled using SeqMan software (DNASTAR). The complete genome sequence of NX49 was aligned with those of reference strains (listed in Table 2) using EditSeq software (DNASTAR). Phylogenetic and molecular evolutionary analyses were conducted with Molecular Evolutionary Genetics Analysis (MEGA) software version 6.0 (Hachioji, Tokyo, Japan) with bootstrap values calculated from 500 replicates. The maximum likelihood phylogenetic algorithm was used for construction of the tree.

3. Results

3.1. Confirmation of BPIV3c

MDBK cells showed obvious CPE 5–7 days after inoculation. RT-PCR was conducted and an expected band of approximately 330 bp was visualized via 1% agarose gel electrophoresis (Figure 1). The fragment was then sequenced, demonstrating high identity with reference strains within the BPIV3c cluster.

Table 2. Details of BPIV3 stains from China and other countries retrieved from GenBank and used for phylogenetic analysis.

Virus	GenBank accession	Year of isolation	Genotype	Country	Length of genome
ISU	EU439428	1992	a	USA	15480
TVMDL24	KJ647288	2008	a	USA	15480
910N	D84095	Unknown	a	Japan	15480
NM09	JQ063064	2009	a	China	15456
TVMDL60	KJ647289	2007	a	USA	15456
Kansas	AF178654	1984	a	USA	15456
Texas	EU439429	1981	a	USA	15456
SF	AF178655	1958	a	USA	15456
Q5592	EU277658	Unknown	b	Australia	15498
TVMDL15	KJ647284	2009	b	USA	15474
TVMDL17	KJ647286	2007	b	USA	15474
NX49	KT071671	2014	c	China	15474
SD0835	HQ530153	2008	c	China	15474
TVMDL16	KJ647285	2007	c	USA	15474
TVMDL20	KJ647287	2012	c	USA	15474
12Q061	JX969001	2012	c	S. Korea	15474
HS9	LC000638	2012	c	Japan	15474
P	AB012132	Unknown	Human	Japan	15462
LZ22	FJ455842	2003	Human	China	15462
JS	U51116	Unknown	Human	USA	15462

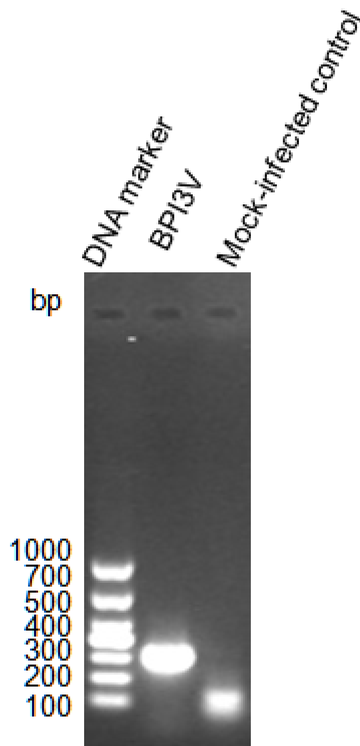

Figure 1. Identification of isolated BPIV3 by RT-PCR.

3.2. Infectivity and hemagglutination assays

NX49 showed high sensitivity to high temperature and chloroform treatment, as incubation at 50 °C for 30 min or with chloroform at 4 °C for 10 min dramatically impaired infectivity. NX49 did not demonstrate agglutination activity against guinea pig red blood cells at either 37 °C or room temperature. However, NX49 showed agglutination activity at a titer of 1:4 at 4 °C. Conversely, BN-1 demonstrated agglutination activity at a titer of 1:8 at 37 °C and room temperature.

3.3. Phylogenic analyses

The complete genome of NX49 comprised 15,474 nucleotides (nt), which was identical to all strains previously identified in the BPIV3c group and two strains in the BPIV3b group (TVMDL15 and TVMDL17). Comparison of NX49 to reference strains in the BPIV3a group, including ISU, TVMDL24, and 910N (15,480 nt), indicated an insertion of 12 nt in the open reading frame of the P gene, a deletion of 8 nt and an insertion of 2 nt in the untranslated region (UTR) between the M and fusion (F) proteins, and an additional deletion of 12 nt in the UTR between the F and HN proteins. Other available strains in the BPIV3c group shared similar genomic characteristics (data not shown). The first identified BPIV3b strain in Australia, Q5592 (15,498 nt), has the longest genome among BPIV3 strains because of two additional insertions (totaling 32 nt), a 2-nt insertion in the UTR between the M and F proteins, a 30-nt insertion in the UTR between the F and HN proteins, and a deletion of 14 nt in the UTR between the M and F proteins compared to prototype strains in the BPIV3a group.

Phylogenetic comparison of the complete genomic sequences of BPIV3 demonstrated that six strains of BPIV3c could be further divided into subgenotypes based on the degree of divergence (Figure 2). NX49 clustered with SD0835 (isolated in China) and comparatively clustered with TVMDL16 and TVMDL20 (USA), 12Q061 (South Korea), and HS9 (Japan), which formed another cluster. The full-length genomic identity between NX49 and SD0835 was 99.3%, which was higher than those among TVMDL16, TVMDL20, 12Q061, and HS9 (range: 97.5%–98%). NX49 and SD0835 shared 97.5%–98% and 97.7%–98.1% identity, respectively, with 12Q061, TVMDL20, HS9, and TVMDL16.

4. Discussion

To date, available data based on phylogenic analysis indicate that isolated strains from local geographical regions, including strains from China and the United States, share high similarity, with the exception of Australian BPIV3 (Q5592), which is distinct from all other isolates. These findings suggest that no significant genetic variations in circulating strains occur locally. However, the presence of three sublineages in the BPIV3a group was confirmed based on phylogenetic analysis (Figure 2). Additionally, TVMDL60 and TVMDL24, isolated from the United States in 2007 and 2008, clustered into different sublineages in the BPIV3a group, suggesting that genetic variation may occur within a short period of time. Although whole-genome sequencing of BPIV3c strains isolated in China (SD0835 and NX49) demonstrated no significant genetic variation, continued global epidemiological surveillance is required to monitor viral variation. This effort may also improve diagnostic detection of BPIV3 by RT-PCR methods.

NX49 showed poor agglutination activity against guinea pig red blood cells. Comparatively, Zhu et al. suggested that SD0835 (BPIV3c) can agglutinate guinea pig erythrocytes at 4 °C at titers ranging from 1:32 to 1:128 (16). This difference in agglutination activity should be further investigated. Further whole-genome sequencing of BPIV3 strains is necessary to analyze the key differences between BPIV3c and BPIV3a strains, as Haller et al. suggested that a single amino acid substitution in the viral polymerase of BPIV3 can result in attenuated virulence (17). The mutation of the differential amino acid in the HN protein of NX49 to that of SD0835 by reverse genetics and subsequent increase of agglutination activity may help answer the question above.

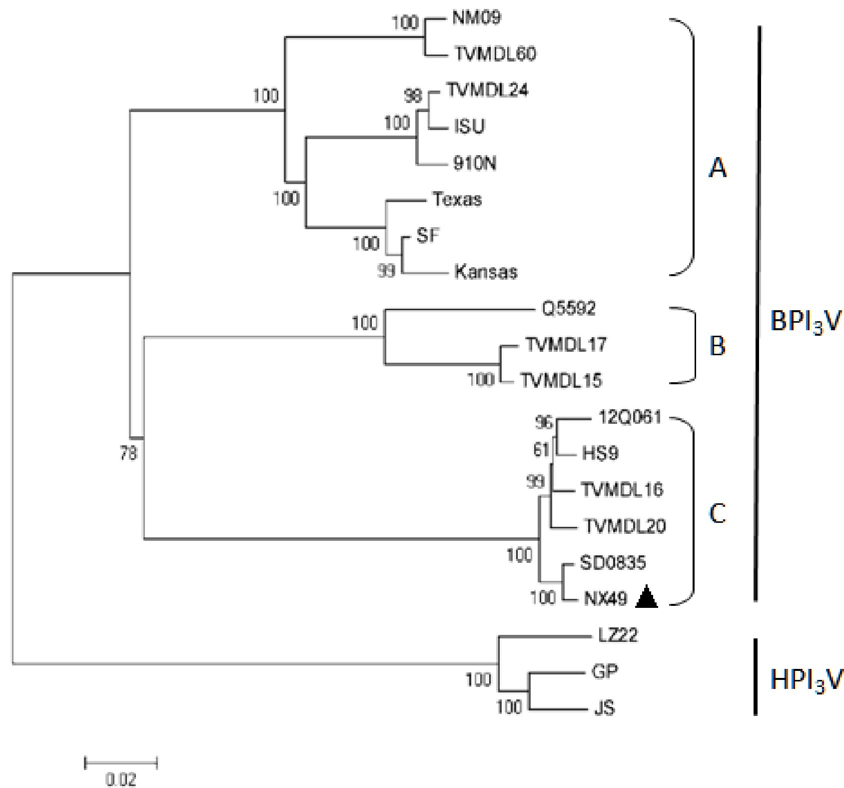

Figure 2. Phylogenetic tree created based on complete genome of BPIV3. Phylogenetic tree was prepared using the whole-genome sequences of BPIV3 isolated in this study and nineteen BPIV3 and HPIV3 reference strains retrieved from GenBank (eight BPIV3a, three BPIV3b, five BPIV3c, and three HPIV3). The tree was determined using the maximum likelihood phylogenetic algorithm and bootstrap test. Numbers over branches indicate the percentage of 500 bootstrap replicates. The solid triangle indicates the NX49 isolated in this study.

The hemagglutinin-neuraminidase (HN) gene (14,16) or matrix (M) gene (13) of BPIV3 is generally targeted for viral identification using RT-PCR. However, we selected a region within the NP gene because NP genes among different strains also share high nucleotide identity. The phylogenetic tree based on the NP nucleotide sequence was consistent with that based on whole-genome sequencing of BPIV3 (Figures 2 and 3). Thus, the NP gene might be a candidate target for BPIV3 identification or genotyping, although the HN or M genes appear to be appropriate targets for genotyping or phylogenetic reconstruction (12,16).

It is difficult to confirm the origin of circulating BPIV3 strains because of the lack of sufficient full-length genomic information. Cross-species infections have been reported in numerous instances, including BPIV3 in humans (18), sheep (19), swine (9), and Atlantic bottlenose dolphins (20). A novel parainfluenza virus type 3 was recently identified from goat herds with respiratory diseases in eastern China (21). However, the similarity of nucleotide and putative amino acid sequences of NP (partial sequence), M, F, HN, and L (partial sequence) between caprine parainfluenza virus type 3 (CPIV3) and NX49 was relatively low, ranging from 74.6% to 81.8% nucleotide identity and 83.3% to 91.8% amino acid identity (Table 3). Additionally, the import of young calves or newborn calf serum for vaccine production in China may increase the risk of introducing a novel virus strain to cattle herds. Therefore, further sequence analysis of BPIV3 isolates from various host species and investigation of the distribution of BPIV3 should be conducted to monitor its evolution and elucidate the relationship between various host strains of PIV3. NX49 shared 97.5%–98% identity with 12Q061, TVMDL20, HS9, and TVMDL16. In comparison, SD0835 shared 97.7%–98.1% identity with the strains above, respectively. This suggests that SD0835 shares a closer identity with strains isolated from countries other than China rather than with NX49, indicating that the origin of SD0835 comes with the importation of cattle. However, much more whole-genome sequencing of BPIV3 will be necessary to analyze the possible origin of NX49, as well as that of SD0835.

It is of note that the available vaccines against bovine parainfluenza have been developed based on BPIV3a strains. The cross-protection of such vaccines against emerging BPIV3c or BPIV3b strains remains unclear.

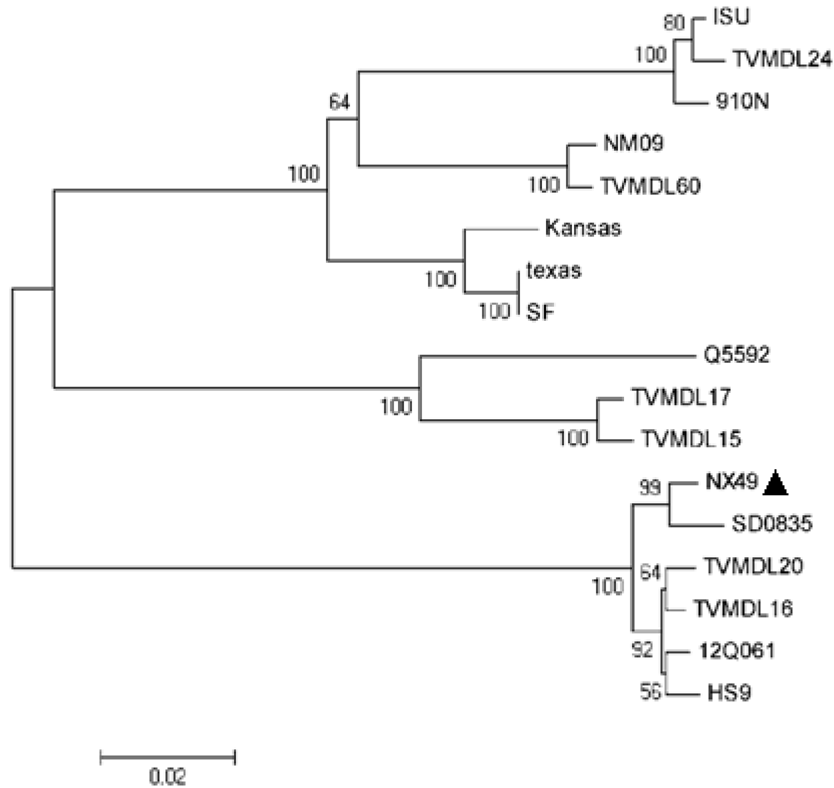

Figure 3. Phylogenetic tree created based on the NP gene of BPIV3. The tree was prepared using the full-length NP nucleotide sequences of BPIV3 isolated in this study and sixteen BPIV3 reference strains retrieved from GenBank (eight BPIV3a, three BPIV3b, and five BPIV3c). The tree was determined by bootstrap analysis using the maximum likelihood program (500 replicates). The solid triangle indicates NX49 isolated in this study.

Table 3. Identity comparison of nucleotide sequence of structural genes and their deduced amino acid sequence of NX49 with counterparts of selected reference strains.

Virus	Genotype	Selected reference strains	Nucleotide identity %						Deduced amino acid identity %					
			NP	P	M	F	HN	L	NP	P	M	F	HN	L
BPIV3	a	SF	84.7	80.8	83.7	82.3	80.4	84.9	93.4	73.3	97.7	88.1	85.8	93.0
		TexasΔ	84.7	80.8	83.7	82.3	80.5	85.1	93.4	73.3	97.7	88.1	86.0	92.8
		Kansas	84.4	81.3	83.1	82.6	80.6	84.8	93	74.7	97.2	87.4	85.5	92.9
		910N	82.5	80.9	83.4	82.1	80.1	85.0	92.0	73.2	97.2	86.9	85.5	92.7
		ISUΔ	82.7	80.8	82.5	82.3	80.3	85.0	92.0	73.0	96.0	86.7	85.5	92.8
		NM09	83.9	82.0	82.8	81.5	80.2	85.0	92.6	75.7	97.2	87.4	85.7	92.7
		TVMDL24	82.5	81.4	83.2	82.3	80.6	85.0	92.2	72.7	97.2	86.7	86.0	93.0
		TVMDL60	84.2	82.2	82.9	81.4	80.4	85.0	93.0	75.2	97.2	87.2	86.0	92.7
	b	Q5592	83.2	80.1	85.6	81.2	79.6	83.2	91.5	71.3	96.0	84.1	84.3	91.7
		TVMDL15	83.6	80.1	83.7	81.2	79.8	84.1	91.8	70.2	96.6	84.4	84.6	92.1
		TVMDL17	83.8	79.8	83.9	81.0	80.1	84.0	91.5	70.2	96.6	84.4	84.4	92.1
	c	SD0835	98.7	99.2	99.4	99.1	99.4	99.6	98.6	98.8	100.0	99.4	99.3	99.1
		12Q061	98.2	98.1	97.3	97.3	97.7	97.6	99.0	96.5	98.0	97.4	98.3	97.8
		HS9	98.1	98.4	97.5	98.0	97.6	98.4	99.0	97.2	99.1	98.3	98.4	99.1
		TVMDL16	98.3	98.5	97.4	97.8	97.7	98.5	98.8	98.3	99.4	98.5	98.4	99.2
		TVMDL20	98.1	98.0	97.7	97.7	97.7	98.3	99.2	96.5	99.4	98.5	97.9	99.1
CPIV3		JS2013	81.1p	-	77.7	74.6	76.4p	81.8p	91.8	-	88.9	77.4	83.3	94.5
HPIV3		LZ22	77.1	71.0	79.5	75.1	72.3	80.6	82.5	59.8	93.7	79.1	74.5	87.4

Δ: Swine parainfluenza virus 3; CPIV3: caprine parainfluenza virus type 3; HPIV3: human parainfluenza virus type 3; p: partial CDs.

Therefore, to evaluate the necessity of the development of alternative tests and vaccines, cross-neutralization of sera raised by BPIV3a to BPIV3c and BPIV3b circulating strains should be carried out in future studies. The genetic characterization of circulating strains based on whole-genome sequencing may facilitate the development of better diagnostic methods and efficacious vaccines to prevent and control disease due to BPIV3.

In conclusion, this study identified and characterized a new BPIV3c strain, NX49, causing BRDC. Continued efforts to characterize such viruses through whole-genome sequencing will help to prevent BRDC caused by BPIV3 and aid in vaccine development.

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