Virus Genes (2012) 45:542–547
DOI 10.1007/s11262-012-0810-1

Phylogenetic analysis of the bovine parainfluenza virus type 3 from cattle herds revealing the existence of a genotype A strain in China

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Received: 15 May 2012 / Accepted: 16 August 2012 / Published online: 26 August 2012

Abstract In 2009, a bovine parainfluenza virus (BPIV3), named as NM09, was isolated using MDBK cell culture from the nasal swabs of normal cattle in China. The NM09 isolate was characterized by RT-PCR and nucleotide sequence analysis. Its complete genome was 15,456 nucleotides in length. Similar to other sequenced PIV strains, the NM09 virus consisted of six non-overlapping genes, which were predicted to encode nine proteins with conserved and complementary 3′ leader and 5′ trailer regions, conserved gene starts, gene stops, and trinucleotide intergenic sequences. Nucleotide phylogenetic analysis of matrix and hemagglutinin-neuraminidase gene demonstrated that this NM09 isolate belonged to BPIV3 genotype A instead of the previously reported BPIV3 genotype C in China. It is implicated that the different genotypes A and C might coexist infection for a long time in China.

Keywords Bovine parainfluenza virus type 3 (BPIV3) · Cattle · Phylogenetic analysis

Introduction

Bovine parainfluenza virus type 3 (BPIV3) is an important pathogen of the known viruses associated with bovine respiratory disease complex (BRDC), which is a major health problem of cattle worldwide [1, 2]. BPIV3 is an enveloped, non-segmented negative-strand virus and belongs to the family *Paramyxoviridae*. It has been placed within the genus *Respivirus* [3]. All members of the subfamily *Paramyxovirinae* have six genes in the following order: 3′-N-P-M-F-A-L-5′, where N, P, M, F, A, and L indicate the genes for the nucleocapsid protein, phosphoprotein, matrix, fusion, attachment, and large polymerase proteins [4], respectively. The BPIV3 infection in cattle could gradually cause tissue damage and immunosuppression, and even result in severe bronchopneumonia from secondary bacterial infections in the presence of high stress, such as transportation and feedlot situations [5]. BPIV3 was first detected and isolated in the cases of "shipping fever" in USA [6, 7]. In shipping fever, the crowded calves and poor ventilation during transport facilitates the spread of infection and deteriorates the disease. Clinical signs associated with the BPIV3 infections are coughing, anorexia, pyrexia, nasal and ocular discharges, dyspnea and sometimes diarrhea.

BPIV3 is widespread among cattle around the world in three genotypes A, B, and C. Previously, the complete genome analyses of the representative BPIV3 isolates from Australian and American indicated that their viral species were classified into two distinct genotypes, BPIV3 genotype A (BPIV3a) and BPIV3 genotype B (BPIV3b) [2]. Recently, four Chinese isolates of BPIV3 were distinct

Authors' contributions Yong-Jun Wen carried out the molecular genetic studies, participated in the sequence alignment and drafted the manuscript. Xin-Chuan Shi participated in the design of the study and performed the statistical analysis. Feng-Xue Wang and Shu-Qin Zhang participated in the sequence alignment. Wei Wang, Guo Li and Ni Song took the animal samples and isolated the virus. Li-Zhi Chen, Shi-Peng Cheng, and Hua Wu conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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from other reported BPIV3 strains and classified as genotype C (BPIV3c), named SD0835, which has only been detected in China [8]. In this study, we isolated one mild BPIV3 strain using MDBK cell cultures from normal cattle herds in China. This Chinese BPIV3 strain was classified into genotype A (BPIV3a) instead of the previously reported genotype C, based on its nucleotide phylogenetic analysis.

Materials and methods

Samples and virus isolation

Hundreds of nasal swabs from cattle in an auction market were randomly collected from Inner Mongolia Province, China, in 2009. These nasal swabs being positive for hemagglutinin-neuraminidase (HN) gene detection by RT-PCR were selected for virus isolation. Madin-Darby bovine kidney (MDBK) cells, which were grown in minimum essential medium (MEM, GIBCO) supplemented with 10 % fetal bovine serum (GIBCO), were cultured in a 24-well culture plate. A 100 µl of nasal swabs was inoculated into each well of MDBK cells and incubated at 37 °C for 1 h. Subsequently, all culture medium and inoculations were discarded, and a 500 µl of MEM supplemented with 4 % FBS were added to each well. Harvest the cell cultures by freezing and thawing them for three times until CPE appeared in the monolayer of MDBK cells. The viral plaques were immunostained with a specific BPIV3 mouse monoclonal antibody conjugated with FITC (VRMD Inc., WA, USA), and fluorescence signal was observed using an Axioskop-40 fluorescence inverted microscope (Zeiss, Germany).

RT-PCR detection and HN gene sequence

Oligonucleotide primers for BPIV3 detection and identification were designed from the HN gene sequence of BPIV3 strain Shipping Fever (GenBank accession number AF178655). The RNA extraction and reverse transcription (RT) reactions were performed using standard procedures. In brief, the purified virus-infected MDBK cells were scraped into the medium and subjected to three cycles of freezing and thawing. After initial clarification at 3,000×*g* for 15 min, a polyethylene glycol 8000 solution (Sigma) was added to the cell lysate at a concentration of 10 % and then incubated at 4 °C for 4 h. The virus was then pelleted at 12,000×*g* for 60 min at 4 °C, and the viral genomic RNA was extracted from the virus pellet using a RNeasy minikit (Qiagen). The cDNA copies of the virus genomic RNAs were synthesized using the specific oligonucleotide primers and SuperScript III reverse transcriptase (Invitrogen, Beijing, China). Subsequently, the primers of HN fwd (5′-TGGAAACACACAAACAGCACAA-3′) and HN rev (5′-TTTGGAACTTCTGTTTTGAATA-3′) and Platinum *Taq* DNA polymerase (Invitrogen) were used to amplify an approximately 1,697 bp fragment from cDNA products. The cycling parameters were 94 °C for 4 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 1 min and 72 °C for 1 min 30 s, and a final 7 min extension step at 72 °C. The PCR products were purified using a Qiaquick PCR purification kit (Qiagen) and cloned using a TOPO TA cloning kit according to the manufacturer's instructions (Invitrogen). To obtain the consensus sequence, the purified PCR products and at least three independent TA cloned products were sequenced in both directions by Shanghai YingTanJieJi Biotechnological Co. Ltd (Invitrogen).

Sequence analysis of M gene and complete genome for the isolate

The complete genome sequence of the isolate was determined to further characterize and confirm the data in HN region. Twelve primer sets were designed to amplify overlapping regions of the complete BPIV3 genome. The leader and trailer sequences of the isolate were obtained using a 5′–3′ RACE kit (Takara) according to the manufacturer's instructions. The complete genome sequence was compiled from overlapping sequences of the amplified fragments. Based on the complete nucleotide sequence, a 1,067 bp product encompassing the matrix coding region was amplified from the isolate with the primers of Mfwd (5′-TCCAAAACAATGAGCATCACCA-3′) and Mrev (5′-TTTTACTGTCTGATTTTCCCGA-3′).

Phylogenetic analysis

Comparative analysis with the BPIV3 strain SD0835 genome sequence from GenBank (access NO.HQ530153) was performed to identify the coding regions of the isolate. The putative amino acid sequences of the isolate and other BPIV3 strains were created following BLASTX search routines (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Sequence analyses, nucleotide sequences editing, and prediction of amino acid sequences, were conducted using the software package DNAstar (Lasergene) with BPIV3 and HPIV3 sequences retrieved from GenBank. Phylogenetic reconstructions for the isolate was compiled using a 696 nucleotide region of Matrix (nucleotide 4,062–4,757 of BPIV3 strain genome), a 603 nucleotide region of HN (nucleotide 7,244–7,846 of BPIV3 strain genome), and the complete genome sequence of the isolate by MEGA version 4 [9] software with 1,000 bootstrap replicates.

Results

Identification of NM09 isolate

HN gene fragments of BPIV3 were detected by RT-PCR from the nasal swabs of cattle collected in Inner Mongolia Province, China. The nasal swabs from which the fragments consistent with the expected size of 1,697 bp were amplified indicated being positive for NH gene detection (data not shown). These positive nasal swabs were cultured and passaged in MDBK cells, and ultimately one virus isolate was obtained. The isolate uniformly produced CPE characteristic of BPIV3 isolates with many scattered, rounded, refractory cells and small syncytia. The spherical or pleomorphic virions with approximately 50–300 nm in diameter were observed by transmission electron microscopy in negative-stain preparations of MDBK cells inoculated with the new isolate, and the fluorescence signals were detected from the cells infected with this virus by IFA (data not shown). All results demonstrated that this new isolate, named as NM09, was BPIV3. The complete genome sequence of the NM09 isolate analyzed was deposited in GenBank under following accession number: JQ063064.

Sequence analysis of NM09 isolate

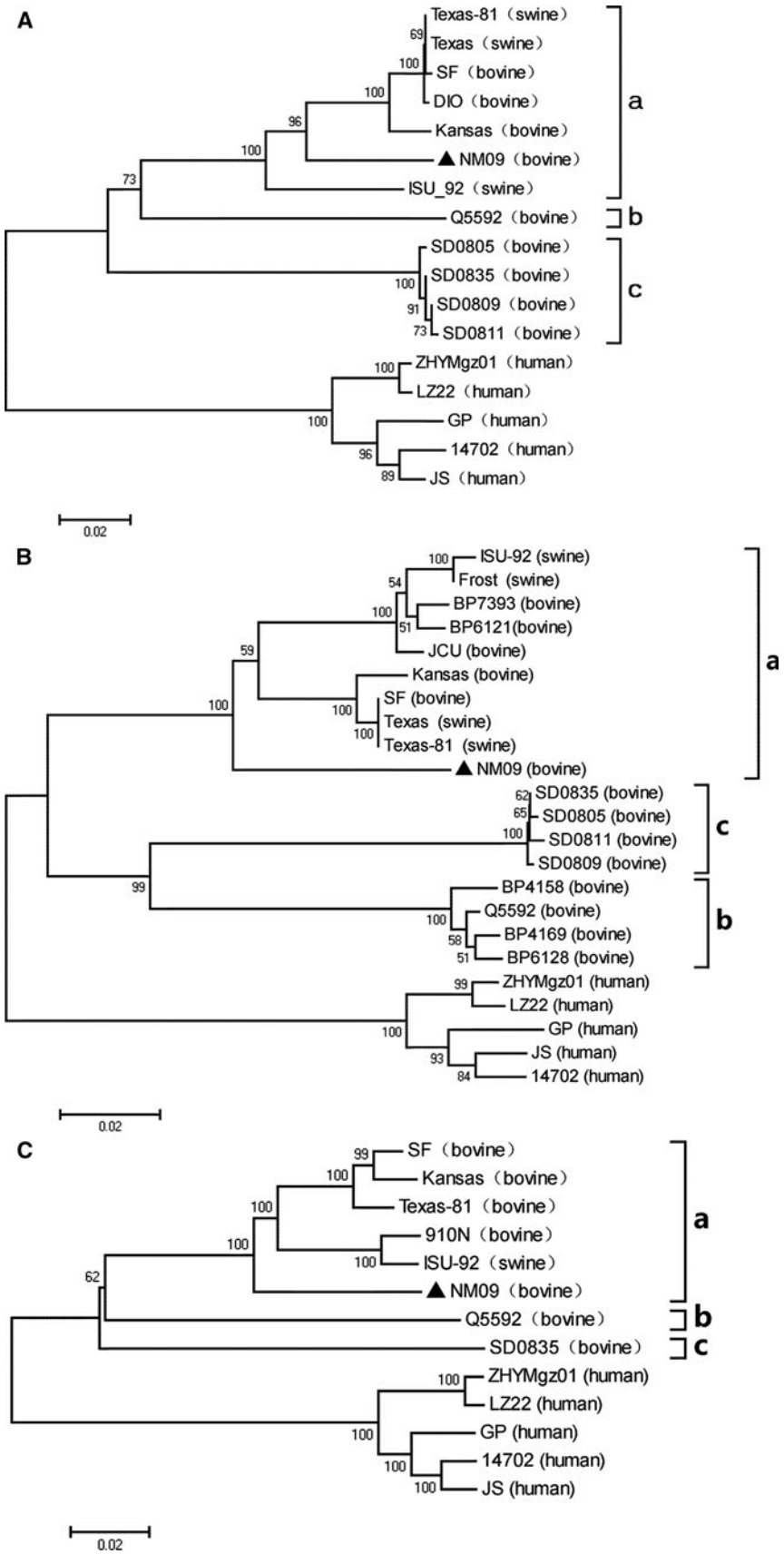
The HN gene of NM09 isolate was 1,719 nucleotides (nt) in length with a single ORF beginning at position 74 that could encode a 572-amino acid protein. The nucleotide similarity of HN gene was 74–93.1 % when compared to BPIV3 strain SD0835 and other BPIV3 strains using the DNAStar (Table 1). Comparing with HPIV3, the mild virus of NM09 indicated a 74 % identity in the HN gene nucleotide sequences and a 76.9 % identity in the deduced amino acid sequences.

In order to further reveal the variation in nucleotide sequences, the complete genome sequence for NM09 isolate was determined. The results showed that the nucleotide sequence of NM09 isolate was little different to the previously characterized BPIV3 strains in both of the non-coding and coding regions of the genome (Table 1). The complete genome of NM09 isolate was 15,456 nt) in length, which was 18 bases shorter than the previously identified BPIV3 strain SD0835 genome (15,474 nt). The NM09 isolate was a multiple of six and conforms to "the rule of six", which was consistent with the sequenced HPIV3 in previous studies [10]. The new NM09 isolate presented a universal pattern of paramyxovirus genome structure where every gene flanked with a conserved gene-start (GS) sequence and a gene-end (GE) sequence. The GS and GE sequences of matrix and HN genes were essentially similar in all BPIV3 strains. The nucleotide identity for the complete genome was 77.8–92.2 % between NM09 isolate and BPIV3 and HPIV3 strains, especially, a 92.2 % identity of NM09 isolate and BPIV3 strain SF genome. The similarity of deduced amino acids for the BPIV3 coding regions was 83.3–95.7 % between NM09 isolate and other PIV3 strains.

Table 1 The nucleotide and putative amino acid identities of NM09 (BPIV3) compared to three BPIV3 isolates (SD0835, SF and Texas, USA) and a HPIV3 isolate (LZ22 from China)

Gene region	Comparison viruses	Nucleotide alignments				Putative amino acid alignments			
	NM09	SF	SD0835	Texas	LZ22	SF	SD0835	Texas	LZ22
Leader UTR	110 nt	95.5	87.3	93.6	78.2				
Nucleoprotein (N)	1,548 nt	93.2	85.5	93.3	79.7	97.1	91.5	97.1	86.3
N–P UTR	125 nt	90.4	72.0	90.4	57.6				
Phosphoprotein (P)	1,791 nt	90.8	81.7	90.6	73.5	87.2	75.5	87.1	64.4
P-M UTR	160 nt	77.5	65.0	78.8	52.5				
Matrix (M) protein	1,056 nt	92.5	82.2	92.5	79.7	98.3	97.2	98.3	92.3
M-F UTR	275 nt	84.2	64.4	84.2	57.5				
Fusion (F) protein	1,623 nt	91.6	81.9	91.6	78.0	94.8	87.4	94.8	80.7
F-HN UTR	111 nt	80.2	65.8	80.2	58.7				
Hemagglutinin/ neuraminidase (HN)	1,719 nt	93.1	81.7	93.1	74.0	95.6	86.0	95.8	76.9
HN-L UTR	121 nt	79.3	63.6	78.5	43.5				
Large (L) protein	6,702 nt	93.4	85.0	93.0	82.0	97.4	92.3	96.9	88.6
Tailer UTR	115 nt	83.5	79.1	83.5	71.3				
Complete genome	15,456 nt	92.2	82.2	92.0	77.8	95.7[a]	89.2[a]	95.4[a]	83.3[a]

[a] Coding regions only. Nucleotide and putative amino acid sequence alignments were created using the computer program DNA Star7.0


A
Texas-81（swine）
Texas（swine）
SF（bovine）
DIO（bovine）
Kansas（bovine）
▲NM09（bovine）
ISU_92（swine）
Q5592（bovine）
SD0805（bovine）
SD0835（bovine）
SD0809（bovine）
SD0811（bovine）
ZHYMgz01（human）
LZ22（human）
GP（human）
14702（human）
JS（human）
a
b
c
0.02
B
ISU-92 (swine)
Frost (swine)
BP7393 (bovine)
BP6121(bovine)
JCU (bovine)
Kansas (bovine)
SF (bovine)
Texas (swine)
Texas-81 (swine)
▲NM09 (bovine)
SD0835 (bovine)
SD0805 (bovine)
SD0811 (bovine)
SD0809 (bovine)
BP4158 (bovine)
Q5592 (bovine)
BP4169 (bovine)
BP6128 (bovine)
ZHYMgz01 (human)
LZ22 (human)
GP (human)
JS (human)
14702 (human)
a
c
b
0.02
C
SF（bovine）
Kansas（bovine）
Texas-81（bovine）
910N（bovine）
ISU-92（swine）
▲NM09（bovine）
Q5592（bovine）
SD0835（bovine）
ZHYMgz01 (human)
LZ22 (human)
GP (human)
14702 (human)
JS (human)
a
b
c
0.02

◀**Fig. 1** Phylogenetic tree analysis of the HN and Matrix were created using the nucleotide sequences of the BPIV3 isolate (**a** HN and **b** Matrix), and phylogenetic tree analysis of the complete genome was created using the complete genome sequence of the NM09 isolate in this study (**c** the complete genome), with BPIV3, SPIV3, and HPIV3 reference strains retrieved from GenBank. The phylogenetic tree was prepared using the Neighbor-Joining method and bootstrap testing. Numbers over branches indicate the percentage of 1,000 bootstrap replicates that support each phylogenetic branch. The GenBank accession numbers of BPIV3 reference strains are as follows: Q5529 (EU277658), BP4169 (EF108223), BP6128 (EF108225), BP4158 (EF108222), JCU (EF108221),SF (AF178655), BP6121 (EF108224), BP7393 (EU266069), Kansas/15626/84 (AF178654), SD0809 (HQ530158), SD0835(HQ530153), SD0805(HQ530157), NM09 (JQ063064),SD0811(HQ530159).The GenBank accession numbers of HPIV3 reference strains are as follows: 14702(EU424062), GP(AB012132),JS(Z11575), ZHYMgz01(EU326526),and LZ22(FJ4 55842).The GenBank accession numbers of SPIV3 reference strains are as follows: 81-19252_Texas-81 (EU439429), Texas (DQ166824), Frost (DQ166823), ISU-92 (EU439428)

Phylogenetic reconstruction of NM09 isolate

The nucleotide sequences of NM09 isolate and other BPIV3 strains were analyzed for the phylogenetic reconstruction. The phylogenetic tree based on the nucleotide sequences of BPIV3 and HPIV3 complete genomes demonstrated that the NM09 isolate joined the lineage of BPIV3 and HPIV3 groups and appeared to be a genotype A (BPIV3a) with 62 % bootstrap value (Fig. 1c). Phylogenetic reconstructions of the full-length HN gene indicated the three discernible subgenotypes in BPIV3 genotype A (BPIV3a): one represented by ISU_92 virus, the second represented by Kansas and SF viruses, and NM09 strain formed the third genetic group (Fig. 1a, c). The Matrix phylogenetic tree also showed that NM09 isolate was closely related to the BPIV3a from America with 99 % bootstrap value, and formed a separate branch with 100 % bootstrap value (Fig. 1b). In addition, the phylogenetic tree created from the complete genome sequence was in agreement with the results from the Matrix and HN gene nucleotide data (Fig. 1a, b).

Discussion

The viral RNA of BPIV3 was detected in the nasal swabs from infected cattle in Inner Mongolia, China, by RT-PCR, but these cattle experienced no respiratory disease. A virus strain, named as NM09, was isolated using MDBK cell cultures from positive nasal swabs samples, and its complete genome sequence was deposited in GenBank under following accession number: JQ063064. The nucleotide sequences of NM09 isolate were analyzed for its phylogenetic reconstruction with previously sequenced BPIV3 and HPIV3. The HN protein was an immunodominant glycoprotein of BPIV3 and could provide good protection against BPIV3 challenge infection [5]. In this study, phylogenetic reconstructions with HN gene of BPIV3 and HPIV3 also provided meaningful inferences in genetic characterization of BPIV3 and HPIV3, as well; the Matrix gene of PIV3 also is an appropriate target and candidate for genotyping of PIV3. The recognition of a BPIV3 genotype is dependent on the sequence analysis of BPIV3 isolates from a range of geographical distribution. Previously, a new genotype C was detected in China [8], but this NM09 virus distinct from the reported BPIV3b and BPIV3c, was classified as BPIV3a on the basis of phylogenetic analysis of M, HN and complete genome with 100 % bootstrap value (Fig. 1). Particularly, the NM09 virus induced a very mild respiratory illness without any neurological signs in cattle, suggesting that coinfection with other infectious agents or the presence of other environmental factors may be required to precipitate the clinical disease.

Because only a small number of BPIV3 genome sequences have been reported [11, 2], it is very difficult to accurately evaluate whether the level of genetic variation presented here is typical of the BPIV3 species. Australian BPIV3 reported isolates were distinct from the other reported strains and classified as a new BPIV3b, which has only been recognized in Australia geographically removed from other cattle breeding countries. Based on the hypotheses that the BPIV3a mainly distribute in North America, we speculated that this NM09 isolate might be derived from North America. For a long time, the Chinese factory of animal health used the import newborn calf serum to develop and product vaccine for the animals, and this may be a risk to introduce the virus to animals. Nowadays, there is no quarantine inspection and regulations on BPIV3 and other BRDC relative disease during the transportation of cattle in China. Thus, a broad pathogen epidemiological investigation of BPIV3 must be carried out and serological methods would be established for detecting antibodies against BPIV3 in China. Meanwhile, the vaccine for BPIV3 might be developed using the new BPIV3 isolates of NM09. The validity of the three proposed genotypes for BPIV3 may also require further immunological validation to determine their potency for vaccine development. All of these efforts would greatly improve the research and prevention on BRDC in China.

Acknowledgments The authors would like to thank Hui Jiang for the assistance during this study. This study was supported by the Grant of National High Technology Research and Development Program 863 (2011AA10A213) funded by the Ministry of Science and Technology of China.

Conflict of interest None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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