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Analysis on the complete genome of a novel caprine parainfluenza virus 3

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ABSTRACT

Parainfluenza virus type 3 (PIV3) is one of the most important viral respiratory pathogens for humans and for many animals. One unique caprine PIV3 (CPIV3) strain named JS2013 was isolated in Chinese goat flocks with respiratory diseases in 2013. Now, the complete genome sequence of the strain JS2013 had been determined. A total of 15 overlapping DNA clones, covering the entire genome of the virus, were obtained by primer walking RT-PCR. The sequences of the 3′ and 5′ termini of the viral genome were amplified by 3′ and 5′ RACE. The viral genome was 15,618 nucleotides (nt) in length, which was consisted of six genes in the order 5′-leader-N-P/C/V-M-F-HN-L-tailer-3′. The junction sequences between two genes were highly conserved gene start and stop signal sequences, and trinucleotide intergenic regions (IGR) similar to those of other reported PIV3 strains. Phylogenetic analysis based on the complete genomes of JS2013 with other strains of genus *Respirovirus* demonstrated that the JS2013 obviously differed from HPIV1, Sendai virus, HPIV3 and other reported BPIV3 genotypes. Further analysis of HN genes of JS2013 along with two more CPIV3 strains isolated later indicated that CPIV3 strains formed a separate cluster. The results presented here suggested that CPIV3 is a new member of the genus *Respirovirus*.

1. Introduction

PIV3 is an enveloped, single-stranded negative-sense RNA virus within the *Respirovirus* genus of the family *Paramyxoviridae*, and could cause respiratory diseases in many host species (Pringle et al., 1993; Wen et al., 2012). The genus *Respirovirus* includes human parainfluenza virus types 1 and 3 (HPIV1 and HPIV3), bovine parainfluenza virus type 3 (BPIV3) and Sendai virus (Wen et al., 2012). HPIV3 is responsible for significant morbidity and mortality in children, especially for less than 1 year of age (Crowe, 1995; Wen et al., 2012). 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 (Mahony et al., 2002). Three BPIV3 genotypes, A (BPIV3a), B (BPIV3b) and C (BPIV3c) have been described, and BPIV3a and BPIV3c infections have been found in Chinese cattle (Zhu et al., 2011). However, the reports about PIV3 infections in goats/sheep are limited and the genetic characteristics of the virus have not been understood (Elazhary et al., 1984; Lamontagne et al., 1985; Lyon et al., 1997; Taylor et al., 1975).

In 2013, the goats in Jiangsu and Anhui provinces of China suffered severe respiratory diseases, one unique caprine PIV3 (CPIV3) strain named JS2013 was firstly detected and isolated in diseased goat flocks (Li et al., 2014). Here, we determined the complete genome sequence of JS2013 strain, analyzed the genome component and protein-coding regions, and compared its nucleotide sequences with other strains of genus *Respirovirus*, which clearly demonstrated JS2013 obviously differed from other reported viral members in genus *Respirovirus*.

2. Materials and methods

2.1. Cells and viruses

Madin–Darby bovine kidney (MDBK) cells, which were grown in Dulbecco's modified eagle medium (DMEM, GIBCO) supplemented with 10% fetal bovine serum (GIBCO), were cultured at 37 °C in 5% CO_2. The virus JS2013, JSHA2014 and AHQJ2015 were isolated from positive clinical nasal swab samples and purified with three-passages of the viral plaque assays in MDBK cells.

2.2. RNA extraction and RT-PCR

The purified virus-infected MDBK cells were subjected to three cycles of freezing and thawing, and centrifuged at 12,000 ×*g* for 10 min. The total RNA was extracted from the supernatant using TRIzol reagent (Invitrogen) according to the manufacturer's instruction. The virus was sequenced using a primer walking strategy based on the consensus sequences of the complete genomes of different strains of PIV3 and the sequencing data of JS2013 strain obtained during the preliminary

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phase of this study (GenBank accession No. M: KJ850331, HN: KJ767525; N: KJ843145; F: KM279938; L: KJ843144). 15 pairs of the primers were designed to synthesize overlapping regions of the complete JS2013 genome as Table 1. RT-PCR was carried out in a 50 μL reaction mixture containing 1 × RT-PCR buffer (Takara Bio, Inc.), 20 pM of each primer, 2 U of one-step Enzyme Mix (Takara Bio, Inc.) and 4 μL of RNA. The reaction was run in a thermocycler (MJ Mini, BIO-RAD) according to the following program: reverse transcription at 50 °C for 30 min, denaturation at 95 °C for 5 min, 35 cycles at 94 °C for 30 s, 54–59 °C for 30 s, 72 °C for 1–2 min, and a final extension at 72 °C for 7 min. PCR products were detected by electrophoresis in 1.2% agarose gels.

2.3. 3′- and 5′-rapid amplification of cDNA ends (RACE)

The sequences of the 3′-and 5′-termini of the viral genome were amplified with 3′-and 5′-RACE kits (both from Takara Bio, Inc.) according to the manufacturer's instructions with a few modification. For 3′-end analysis: a poly (A) tail was added to the 3′-end of the viral RNA using poly (A) polymerase and dATP, and the first-strand cDNAs were synthesized by using M-MLV reverse transcriptase with an oligo (dT)-anchor primer. The cDNAs were then used as templates for nested PCR with the specific primers 3′ inner prime and 3′ outer prime (Table 1), which were designed from sequences near the JS2013 strain genomic termini, outer and inner primers (provided by 3′-RACE kit), which are complementary to anchor primer. For 5′-end analysis, an adaptor was ligated to the 5′-end of the viral RNA by T4 RNA ligase. The ligated products were purified and used as a template for reverse transcription with the random primer. Nested PCR was conducted using the specific primers 5′ inner prime and 5′ outer prime (Table 1) and the primers in 5′-RACE kit. PCR products were separated on 1.2% agarose gels.

2.4. Cloning and sequencing

RT-PCR products were purified using with a purification kit (Axygen Bio, Inc.), cloned into pMD18-T vector (Takara Bio, Inc.), and transformed into *Escherichia coli* DH5a competent cells. Three positive clones for each RT-PCR product were sequenced using the appropriate PCR primers for correct check. Each sequence was determined in both directions.

2.5. Sequence and phylogenetic analysis

The nucleotide sequences and corresponding predicted amino acid sequences were edited by Editseq (DNASTAR Inc., Madison, WI) and subjected to MegAlign (DNASTAR Inc., Madison, WI) and BLAST analyses.

Multiple sequence alignment of viral genome and HN gene was done by using Clustal X 1.83, together with partly reference sequences (Fig. 2) of the identified BPIV3, HPIV1, HPIV3 and Sendai virus strains. After determining the percentages of sequence identity among different strains of genus *Respirovirus*, phylogenetic trees were generated with the distance-based neighbor-joining (NJ) method by using MEGA 4.0.2 software. The robustness of the phylogenetic trees was determined by bootstrap resampling analysis carried out on 1000 replicates.

3. Results

3.1. Characteristics of the genome sequence

To confirm the specificity of JS2013, the entire M and F gene coding regions, HN, 5′-UTR-N and L gene fragments were amplified and sequenced in our previous work (GenBank accession No. M: KJ850331, HN: KJ767525; N: KJ843145; F: KM279938; L: KJ843144). Based on

Table 1
Primers used in the amplification of 17 segments covering the genome of JS2013 strain.

Primers	Sequences (5′–3′)	Location	Expected product size	Annealing temperature (°C)
F1[a]	AGGTRAGGGRGAAGARATCCT	78–98	881	54
R1[a]	AGAGCTGCCATTCTRGTYTCAAT	936–958		
F2	AGATGCTGGTCTTGCTTCCT	893–912	935	56
R2	TCCCAAGAATCCATGATTTTA	1807–1827		
F3	ATGAGCAAGAGAACATCAGAGAAAG	1516–1540	1536	59
R3	TCTACATTGTTTAGGACATTCGCC	3028–3051		
F4	TCTTGGTGTAATCCAATCTGCAG	2965–2987	930	59
R4	GTGGTTCCAAATCACCATTCTCAG	3871–3894		
F5[a]	AGCATCASMARCTCYRRAATMT	3825–3846	1053	52
R5[a]	CTATTGYYTRATYTTYCCGACYCCT	4853–4877		
F6[a]	CTCATTACCTGGTGAATTCA	4808–4827	1545	53
R6[a]	ACAAATGCATATCKTGGYAC	6333–6352		
F7[a]	ACTTCRACAGTTGAYCAATA	5949–5968	1297	53
R7[a]	GTCTTGTGTTTATTCCYGAYTG	7224–7245		
F8	GCCAATACAGAGAATGACTCATGA	7367–7390	1722	59
R8	TATCATTGGACATTTCTGGATATCT	9064–9088		
F9	ATACGATTATGAGCTTACCCCAA	8894–8916	1610	58
R9	CTCTGCTAATACTTGAGCAGCTCT	10,480–10,503		
F10	CTATATGAAAGACAAAGCATTATCC	10,206–10,230	1190	55
R10	CCGATCTAGTTTCATCTATTATTGT	11,371–11,395		
F11[a]	AGAGARGTBATGGATGATCTD	11,218–11,242	545	54
R11[a]	GAYTCRCCTGGTTCYTGATTC	11,742–11,762		
F12	CTGCTAGTGTGGGAGGTTTTAATT	11,597–11,620	1240	59
R12	TCAAAAGTGCTCAGCCCTGTTA	12,815–12,836		
F13	AGCTATGATATACACTTGGGCAT	12,525–12,547	1127	56
R13	CTAAGCAACACACAAAAGCAAG	13,630–13,651		
F14	AATGGCTTAATGGGTCTTCACT	13,538–13,559	922	57
R14	CATTCCATATTTCCTATCCATGTT	14,436–14,459		
F15	CCCATCAGAAGTATCTCTTGTTG	14,343–14,365	1086	56
R15	ATTCCTAATAACTTGACTCCTCCTA	15,404–15,428		
5′inner primer	CATGATTGATCCGATCTGTTCTA	754–776		
5′outer primer	AAGCAAGACCAGCATCTCTAATAT	886–909		
3′inner prime	TGAAAGGTTTGAAAGTAGAGCAC	15,237–15,259		
3′outer primer	GGGAGAGAGATTACGGGATTAT	14,903–14,924		

[a] Primers published in our previous work (Li et al., 2014).