

Isolation and genomic characterization of a canine parainfluenza virus type 5 strain in China

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Abstract A canine parainfluenza virus type 5 strain was isolated from a lung sample from a diseased dog. The genome sequence of this isolate, named HeN0718, was determined and compared to those of other previously reported canine parainfluenza viruses. Unlike previously reported viruses, the HeN0718 strain contained several nucleotide mutations in the SH gene that led to a frame shift in the open reading frame. Phylogenetic analysis based on the complete virus genome and the P, F, and HN genes showed that HeN0718 was genetically closest to D277, a Korean strain that was isolated in 2008.

Introduction

Parainfluenza virus type 5 (PIV5) is a non-segmented negative-strand RNA virus that belongs to the genus *Rubulavirus* of the family *Paramyxoviridae* [1]. The family *Paramyxoviridae* currently contains seven genera including *Respirovirus*, *Rubulavirus*, *Avulavirus*, *Morbillivirus*, *Aquaparamyxovirus*, *Ferlavirus*, and *Henipavirus* [2].

PIV5 was first isolated in 1956 from primary monkey kidney cells [3]. Since then, PIV5 has been isolated from a wide range of species including humans, pigs, cats, and rodents [4, 5]. It is a natural causative agent of the respiratory illness kennel cough in dogs, and as a consequence, it is often referred to as “canine parainfluenza virus” in the veterinary field [6]. PIV5 is the main infectious cofactor of a canine respiratory disease complex, and the main clinical symptoms of this disease included running nose, fever and cough [7, 8].

PIV5 has a nucleocapsid surrounded by a lipid envelope [9]. The genome of PIV5 is 15,246 nucleotides in length and is composed of seven genes (N, V/P, M, F, SH, HN and L) flanked by a 3' leader region and a 5' trailer region. The PIV5 genome encodes eight proteins from seven genes. From the 3' end, the virus genome encodes the nucleocapsid protein (N), phosphoprotein (P), V protein (V), matrix protein (M), fusion protein (F), small hydrophobic protein (SH), hemagglutinin-neuraminidase protein (HN), and large protein (L) or RNA polymerase [10, 11].

In this study, the novel PIV5 strain HeN0718 was successfully isolated in Vero cells from a lung sample from a diseased dog in Henan province in China. Genome-wide analysis showed that HeN0718 distinguished itself from other PIV5 isolates by several amino acid differences in the SH protein.

Materials and methods

Virus isolation

Lung tissue of the diseased dog was homogenized in Dulbecco's modified Eagle medium (DMEM, Gibco, USA) and filtered through a 0.22- μ m membrane. The filtrates

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Table 1 Primers used for genome sequencing in this study

Primer name	Primer sequence	Location/nt	Product /bp
CPIV-1-F	5'-ACCAAGGGGAAAATGAAGTGGT-3'	1-1170	1170
CPIV-1-R	5'-CCTATCCCATAGCATAGCTATATAGCA-3'		
CPIV-2-F	5'-GGCATTATACCAGACCCCTTGGTGAGCA-3'	1051-2310	1260
CPIV-2-R	5'-GCGATAGGATTCTCTCTGGGTTCCCTCG-3'		
CPIV-3-F	5'-AACATTACCATCAGGATCCTATAAG-3'	2209-3453	1245
CPIV-3-R	5'-CCAGGACATTCCCTAATGATGGTAGA-3'		
CPIV-4-F	5'-GACATTGTAATAACCTATGGATTC-3'	3299-4742	1444
CPIV-4-R	5'-GCTTGAAATTGATGTTATATTACATCCA-3'		
CPIV-5-F	5'-ATTCCAACAAATGTCCGGCAACT-3'	4620-5806	1187
CPIV-5-R	5'-GCCAATTGAGTGATGGTGAATCT-3'		
CPIV-6-F	5'-TGACATGTACAAATGTGTGAGTCTGCAGC-3'	5744-6922	1179
CPIV-6-R	5'-CCACGAGCAGTCTGTTCTAGCT-3'		
CPIV-7-F	ACCAGTGACAAGCTAGAACAGAAGTGC-3'	6890-8255	1366
CPIV-7-R	5'-ATGGGACAATCTGAAATTGTCCTAAG-3'		
CPIV-8-F	5'-ATCAAGCGGTCAAGAAGCAGCAT-3'	8128-9496	1369
CPIV-8-R	5'-ACAGAGCAATTCAGCCGTAAGATC-3'		
CPIV-9-F	5'-AGTTTCACCCAGGATGAATTAAGAAC-3'	9404-10439	1036
CPIV-9-R	5'-TTGCTGGTACCTCCATTGTAAACA-3'		
CPIV-10-F	5'-GATGACTTTGAATTGGCAGCATCTT-3'	10364-11490	1127
CPIV-10-R	5'-GTACTGAGTTCATCAGAGCTTGT-3'		
CPIV-11-F	5'-CTCAATCAATATAGAGTATCAATACC-3'	11410-12600	1191
CPIV-11-R	5'-GGTACTGTAATATGTGGCTTGACT-3'		
CPIV-12-F	5'-GTGCATCATGTTGTGTCCGACCT-3'	12522-13728	1207
CPIV-12-R	5'-TGGTGCGATGGAGCATTCAACTT-3'		
CPIV-13-F	5'-CCTGAGGATGATTCCAATTTCCAT-3'	13676-15246	1571
CPIV-13-R	5'-ACCAAGGGGAAAACCAAGATTAATCC-3'		

were subsequently used to inoculate Vero cells for 1 h, which were then maintained with DMEM supplemented with 2% fetal bovine serum (Invitrogen, USA) at 37 °C under 5% CO₂. The culture supernatants were harvested when a cytopathic effect (CPE) was observed in 80% of the cells and were stored at -80 °C as virus stock until use. To further identify the virus, an indirect immunofluorescence assay (IFA) was carried out using the mouse monoclonal antibody (4B10); (made in-house and stored at National Research Center for Veterinary Medicine) against the PIV5 F protein and rabbit anti-mouse-specific secondary antibodies labeled with FITC fluorescent dye.

RT-PCR, DNA cloning and sequence analysis

To determine the full-length genomic sequence of the above PIV5 isolate, designated PIV5-HeN0718, primers were designed based on published sequences in GenBank (accession no. JQ_743318.1). Thirteen overlapping fragments covering the whole viral genome were amplified using the primers shown in Table 1. Total RNA was extracted from 200 µL of virus-containing culture

Table 2 Reference strains of parainfluenza virus with sequences in the GenBank database

Host	Isolate	Accession number
Canine	PIV5-08-1990	KC237063.1
	PIV5-H221	JQ743323.1
	PIV5-78524	JQ743319.1
	PIV5-D277	KC237065.1
	PIV5-1168-1	KC237064.1
Human	PIV5-MEL	JQ743325.1
	PIV5-MIL	JQ743326.1
	PIV5-RQ	JQ743327.1
	PIV5-DEN	JQ743322.1
	PIV5-LN	JQ743324.1
	PIV5-AGS	KX060176.1
Macaque	PIV5-W3A (cl)	AF052755.1
	PIV5-W3A	JQ743318.1
Pig	PIV5-SER	JQ743328.1
	PIV5-KNU-11	KC852177.1
Calf	PIV5-PV5-BC14	KM067467.1
Lesser panda	PIV5-ZJQ-221	KX100034.1

Fig. 1 Identification of PIV5 HeN0718. (A) CPE in Vero cells after infection with PIV5-HeN0718 was photographed at 48 hpi using an inverted microscope at a magnification of 200. (B) Immunofluorescence staining of Vero cells after infection with PIV5 HeN0718. A green color indicates positive staining of PIV5 HeN0718 (200×). (C and D) Mock-infected controls. Uninfected Vero cells served as a negative control

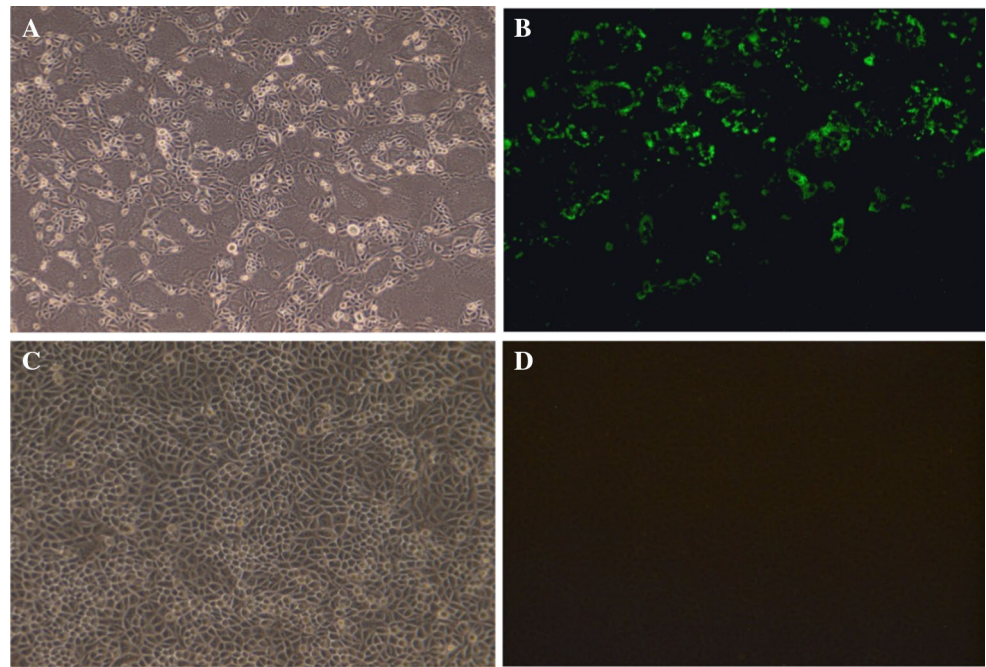


Table 3 Nucleotide and amino acid sequence identity (%) in each protein-encoding gene between PIV5 HeN0718 and the prototype PIV5 strain (PIV5-W3A)

Virus	N		V		P		M		F		SH		HN		L	
	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa
PIV5	96.8	98.6	96.9	97.3	96.8	96.9	95.5	96.3	95.7	95.5	83.0	71.4	96.8	95.6	97.7	98.9

supernatant using a Viral Nucleic Acid Extraction Kit II (Geneaid, Taiwan, China) according to the manufacturer's directions. The purified RNA was used as template in a one-step RT-PCR (TransGen Biotech, China). The RT-PCR products were purified using a TIANgel Midi Purification Kit (TIANGEN, China) and cloned into the plasmid vector pEASY-T1 (TransGen Biotech, China) according to the manufacturer's instructions. After transformation of Trans1-T1 Phage Resistant Chemically Competent Cell (TransGen Biotech, China), 3-5 positive clones were identified by colony PCR and subjected to sequencing. The complete sequences were constructed using the Seqman program in Lasergene (DNASTAR Inc, Madison, USA) and deposited in GenBank under accession number KY114804.

Multiple alignments and phylogenetic analysis

For phylogenetic analysis, 17 complete genome sequences of PIV5 from different species were obtained from GenBank and used in this study (Table 2). Multiple sequence alignments were performed and nucleotide sequence divergence was calculated using MEGA 5.10 software

[12]. The neighbor-joining method was used to construct phylogenetic trees from aligned nucleotide sequences.

Results and discussion

Similar to other parainfluenza viruses, Vero cells inoculated with PIV5 HeN0718 showed considerable CPE, with distinct cell fusion and giant cell formation observed after 48 hours postinfection (dpi) (Fig. 1A). As shown in Fig. 1B, IFA revealed strong and specific positive staining in cells infected with HeN0718, but not in mock-infected cells. To determine the complete genome sequence of HeN0718, 13 pairs of primers were used in RT-PCR. RACE experiments were performed to determine the termini of the HeN0718 genome. The genome of HeN0718 is 15,246 nucleotides long and consists of a 55-nt 3' leader region, a 14,071-nt protein coding region, and a 31-nt 5' trailer region. All PIVs, including PIV5, have a single-stranded, nonsegmented, negative-sense RNA genome of approximately 15,000 nucleotides (or 15,246 in the case of PIV5) [9]. The genome of HeN0718 contains seven genes in the order 3'-N-V/P-M-F-SH-HN-L-5'. Full-length

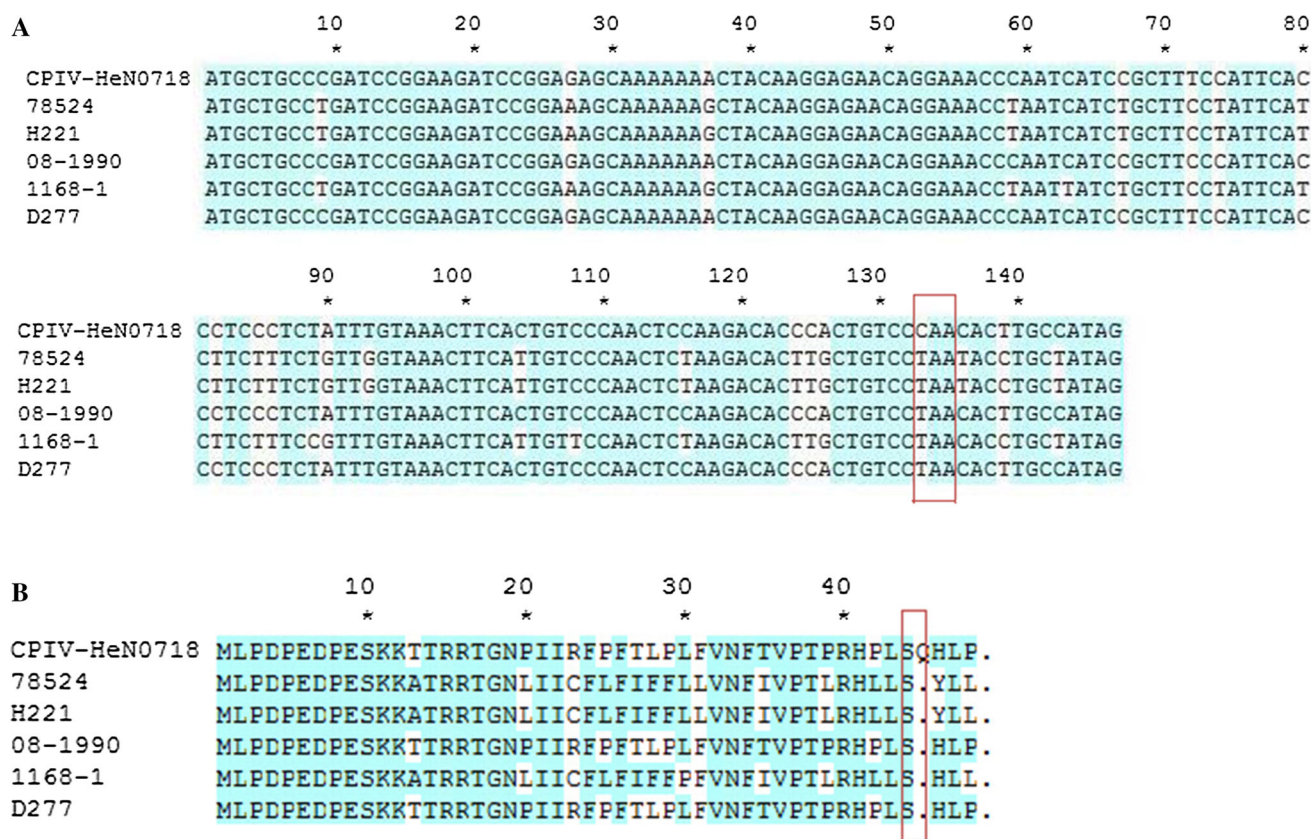


Fig. 2 Nucleotide (A) and amino acid (B) sequence alignment of SH genes of HeN0718 with those of previously reported PIV5 isolates

sequence analysis showed that the genome of HeN0718 is 96.5% identical to that of the prototype PIV5 strain (PIV5-W3A, GenBank accession no. JQ743318.1) at the nucleotide level. Comparison of the deduced amino acid sequences revealed that the predicted N, V/P(V), V/P(P), M, F, SH, HN, and L proteins of HeN0718 exhibited 98.6%, 97.3%, 96.9%, 96.3%, 95.5%, 71.4%, 95.6%, and 98.9% amino acid sequence identity, respectively, to those of the prototype PIV5 strain (Table 3).

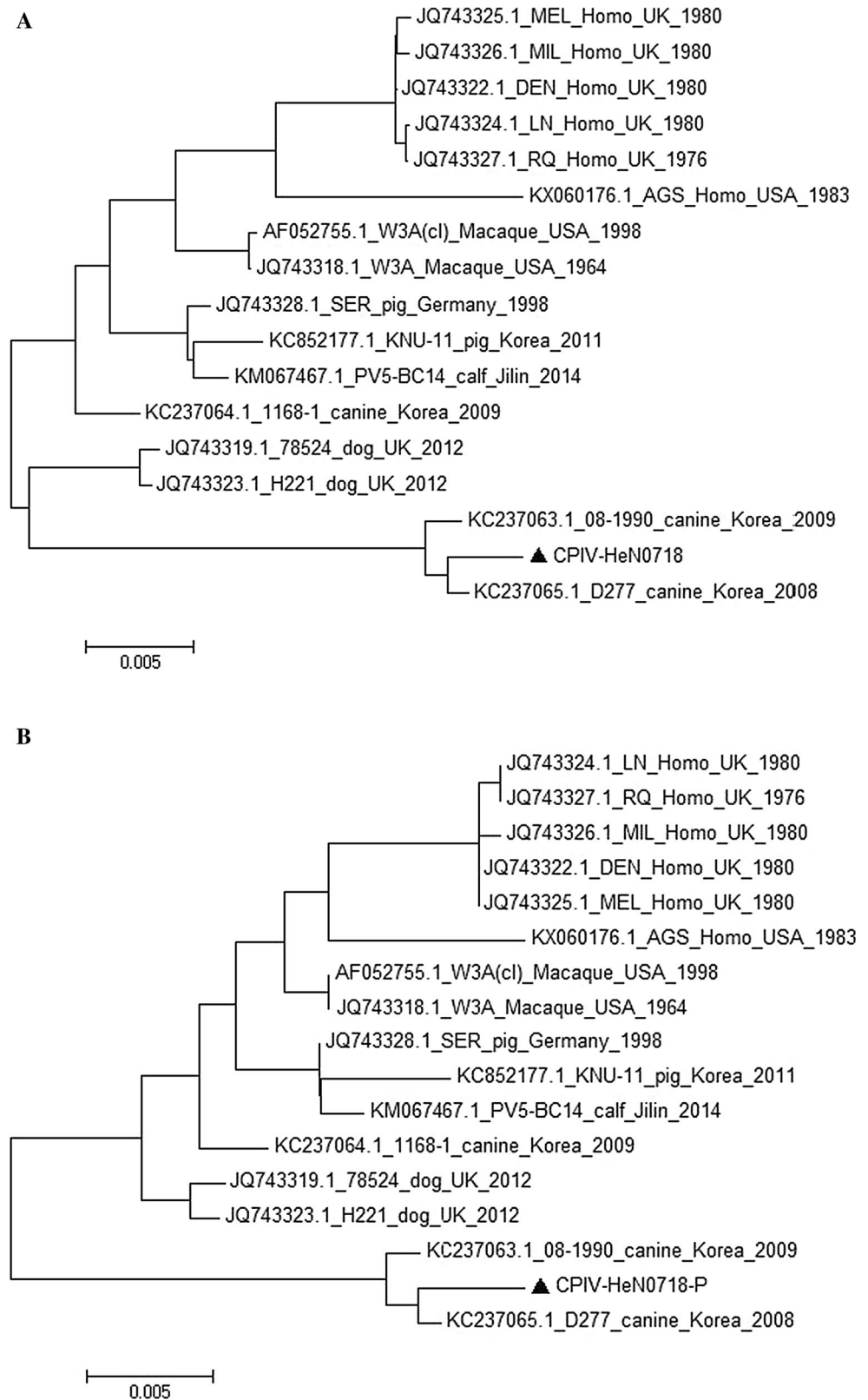
The nucleoprotein (N) gene of HeN0718 was 1732 nt in length and shared 96.8% nucleotide sequence identity with PIV5-W3A. Consistent with previous reports, HeN0718 has the conserved motif ³²³FAAANYPLLYSYAM³³⁶ in the central domain, like other PIV5 isolates [13]. The V/P gene of HeN0718 is 1304 nt in length, encoding both the V and P proteins due to a specific RNA editing mechanism that is a common feature of paramyxoviruses. The first open reading frame (ORF) is 669 nt long, encoding the V protein, and the second ORF is 1177 nt long, with the editing process yielding the phosphoprotein [14]. The V protein has a high degree of sequence conservation among PIVs, whereas the P protein is more variable [15]. The phosphoprotein (P protein) in paramyxoviruses plays multiple roles in the virus proliferation cycle. It functions as a polymerase subunit together with L and is involved in

assembly, together with NP, during genome replication [16–18]. The function of the P protein in PIV5 is not clear and needs to be investigated further.

The matrix (M) gene of HeN0718 is 1370 nt in length, encoding the M protein, which is the most abundant and conserved protein and plays a pivotal role in virion assembly and release. HeN0718 shares 95.5% and 96.3% sequence identity with PIV5-W3A at the nucleotide level and the amino acid level, respectively. The fusion (F) gene of HeN0718 is 1718 nt in length, with a major 1656-nt ORF encoding a 551-aa protein. Sequence identities between the F gene of HeN0718 strain and other reported strains ranged from 95.3% to 99.5% at the nucleotide level and 95.1% to 98.7% at the amino acid level. HeN0718 contained the F7L, C12Y and I502V changes, which were not found in any other isolates. The F protein of paramyxoviruses mediates fusion of viral and cellular membranes for virus entry and it is activated by cleavage of the F₀ protein precursor into a disulfide-bonded subunit structure (F₂-s-s-F₁) [19–21]. Since the F protein is the major antigen protein, the above amino acid changes in the HeN0718 F protein have the potential to compromise the efficacy of current PIV5 vaccines.

The SH gene of HeN0718 is encoded by a single ORF with 147 nucleotides. Previous studies showed that the

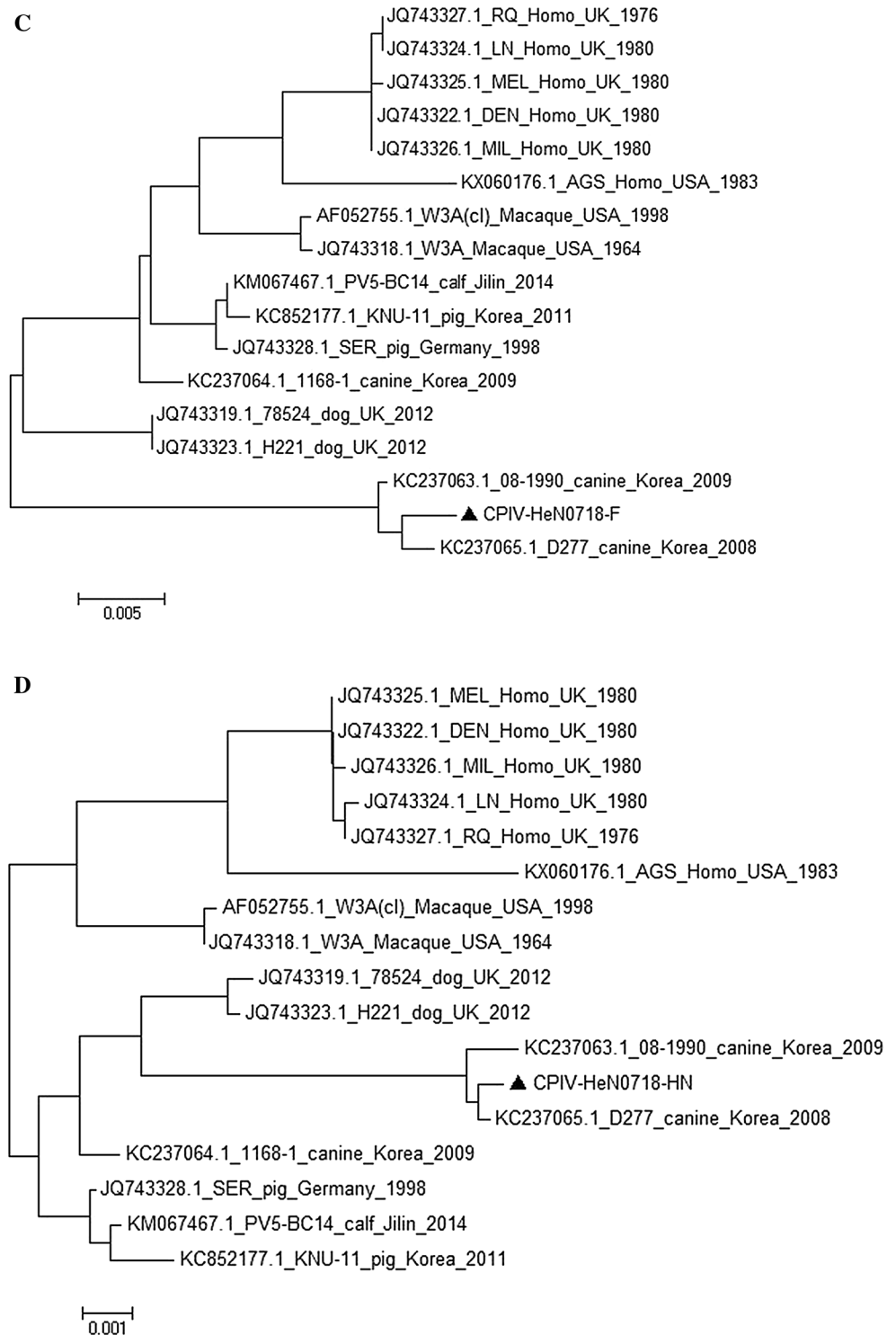
Fig. 3 Phylogenetic trees of PIV5-HeN0718 strain and related reference viruses based on genome (A), P gene (B), F gene (C), and HN gene (D) sequences. The black triangle indicates isolate PIV5-HeN0718. The scale bar indicates the number of substitutions per site



PIV5 SH protein is not present in all paramyxoviruses [2]. A unique feature of PIV5 that distinguishes it from other mammalian PIVs is the presence of a seventh gene, the SH

gene, which is located between the fusion (F) and hemagglutinin-neuraminidase (HN) genes [9]. HeN0718 shared only 83.0% and 71.4% sequence identity with PIV5-

Fig. 3 continued



W3A at the nucleotide level and the amino acid level, respectively. One distinct difference between HeN0718 and other PIV5 isolates is the critical natural nucleotide mutation at position 133 (T¹³³C), which replaces the stop codon of the SH gene and extends the ORF by four more amino acids (Fig. 2A). Therefore, the HeN0718 SH protein

is predicted to be four amino acids longer than those of other PIV5 isolates (Fig. 2B). The SH gene of this non-primate strain showed a high level of T-C substitutions, consistent with an earlier report [26]. Previous studies showed that the SH protein is not essential for virus growth but plays an important role in PIV5 pathogenesis and

inhibition of apoptosis induced by tumor necrosis factor alpha (TNF- α) [22–25]. Therefore, the affects of the mutations in the HeN0718 SH gene on virus pathogenesis and apoptosis merit further investigation.

The hemagglutinin-neuraminidase protein (HN) gene of HeN0718 is 1876 nt long and encodes a protein of 565 amino acids. The HN protein is a type II membrane glycoprotein that has been shown to be important for virus entry and induction of conformational changes in the F protein that are involved in the process of cell membrane fusion [27–29]. The conserved N-linked glycan sites at N110, N139, N267, N497 and N504 were also observed in HeN0718. The large polymerase (L) protein of HeN0718 is 6810 nt in length. HeN0718 shares 97.7% and 98.9% sequence identity with PIV5-W3A at the nucleotide level and the amino acid level, respectively. As one of the major components of the RNA polymerase, the L protein has six highly conserved domains that are involved in nucleotide polymerization, mRNA capping and methylation, and viral mRNA polyadenylation [2]. The HeN0718 L protein does not contain any sequence variations within these six conserved domains.

The complete genome sequence of PIV5 HeN0718 was aligned with 17 sequences of PIV5 strains that were isolated from different species, including humans, macaques, dogs, pigs, a calf and a lesser panda. The results showed that PIV5 HeN0718 shared similarities with other viruses, with sequence identity ranging from 95.5% to 99.5% at the nucleotide level and 79.8% to 98.8% at the amino acid level. It is notable that while PIV5 HeN0718 showed 97% sequence identity at the nucleotide level and 92.6% at the amino acid level with a PIV5 strain from a lesser panda (ZJQ-221), which was also isolated in China, the amino acid sequences of the SH genes were only 72% identical, and the HeN0718 SH protein was longer by four amino acids. Phylogenetic trees were constructed based on nucleotide sequence alignments of the complete genome and the P, F, and HN proteins of PIV5-HeN0718 and 16 PIV5 isolates (Fig. 3A–D). Our data showed that HeN0718 was closely related to strain D277, (KC237065.1), a virus isolate from a dog in Korea.

In conclusion, in this study, we report the genome sequence of PIV5 HeN0718, which was isolated from a dog in China. Different from other reported PIV5 isolates, HeN0718 had several nucleotide mutations in the SH gene, resulting in four additional amino acids in the SH protein.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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