Molecular Cloning and Sequence Analysis of Hemagglutinin Gene of a Novel Strain Canine Distemper Virus

Ling Wu[1]

Yuhang Sun[1]

Chuchu Xu

Changsheng Li

Cheng Xia*

College of Animal Science and Veterinary Medicine,
Heilongjiang Bayi Agricultural University, Daqing 163319, China

[1]The parallel first author: Yuhang Sun

**Corresponding author: Cheng Xia; Email: xcwlxyf@sohu.com;*
TEL: 8604596819207; FAX: 8604596819207

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ABSTRACT

Objectives

It is very important to do epidemiological survey of CDV in China and recognize the lineage, which will set the foundation for the development of efficient vaccine.

Methods

A novel strain of the canine distemper virus (CDV), designated as ZH-10, was isolated from a dog in Heilongjiang, China, and the hemagglutinin (H) gene of the CDV isolate was cloned and sequenced.

Results

The sequence analysis showed that the full-length H gene was 1,824 bp in size, and encoded 607 amino acids. The predicted H protein contained nine potential N-glycosylation sites. The nucleotide homologies between the ZH-10 strain and the Onderstepoort and Convac vaccine strains were 91.4% and 91.3%, respectively. The phylogenetic analysis showed that the ZH-10 strain and the other strains of CDV recently isolated in China were wild strains of the Asia-1 genotype.

Clinical Significance

Our results showed that variation in the amino acid sequence of the H protein among the different strains of the prevalent CDV genotype has likely contributed to the increased incidence of CD in China in recent years. Our findings support the use of regional strains of CDV for the development of more effective CD vaccines.

INTRODUCTION

Canine distemper (CD) is a highly contagious, multisystemic disease caused by the canine distemper virus (CDV). The incidences of acute and subacute CD are high worldwide (Martella et al 2007). In the absence of complications, CD-related mortality is low. However, approximately 50% of CD cases may develop pneumonia and encephalitis, among which mortality can occur at a rate of 70% or higher (Hirama et al 2003). The expanding range of naturally infected carnivore hosts via interspecies transmission has increased the incidence of devastating

CD epizootics (Harder and Osterhaus 1997).

The genome of the CDV encodes six structural proteins, including the N, P, M, F, H, and L proteins. The hemagglutinin protein plays an important role in the host immune response to CDV, and can induce the production of neutralizing antibody (Hirama et al 2003).

However, with a mutation rate that is highest among the CDV structural proteins, antigenic drift in the hemagglutinin sequence causes interstrain variation in virulence and antigenicity (Iwatsuki et al 2000, Martella et al 2006, Uema et al 2005). The hemagglutinin gene is considered to be a reliable marker for identifying the genetic relationship between CDV strains (Bolt et al 1997, Hirama et al 2004). Based on an analysis of the homology of the hemagglutinin gene sequence, previous studies have divided CDV strains into the seven following genetic lineages:

- Asia-1
- Asia 2
- American-1
- American-2
- Arctic-like
- Europe, and
- European wildlife (McCarthy et al 2007).

The currently available CD vaccines have been generated from American-1 strains (McCarthy et al 2007).

Although CDV has only one serotype, each strain exhibits differences in virulence, host range, and cell culture characteristics. Gradual adaptation to epidemiological factors and the emergence of a large number of CDV genetic variants have contributed to an increased incidence of CD worldwide (Blancou 2004, Marchesini et al 1999, Martella et a. 2006). Certain regional characteristics have also been reported among geographic CDV isolates (Calderon et al 2007, Martella et a. 2006, Simon-Martínez et al 2008).

In our current study, we performed a phylogenetic analysis of the H gene of a strain of CDV isolated in Heilongjiang Province to clarify the genetic relationship between the regional CDV strains in China. Our findings support the use of regional strains for the development of more effective CD vaccines.

MATERIALS AND METHODS

The experimental studies were performed in the school laboratory, and approved by the Animal Welfare and Research Ethics Committee of Heilongjiang Bayi Agricultural University.

Cells and Virus Strains

The Madin Darby canine kidney (MDCK) cells were provided by the Harbin Veterinary Institute Laboratory (Harbin, China). The CDV strain analyzed in our study, which we designated as ZH-10, was isolated in our laboratory from the cerebellum of a dog with CD in Heilongjiang Province, in which the presence of the virus was confirmed using polymerase chain reaction (PCR) and the colloidal gold test.

Reagents

The Trizol LS reagent, Opti-MEM, SuperScript II first-strand cDNA synthesis kit, RNaseOUT RNase inhibitor, and Lipofectamin 2000 were obtained from Invitrogen (Carlsbad, CA, USA). The restriction endonucleases, LA Taq DNA polymerase, random hexamer primers, T4 DNA ligase, dNTPs, DNA markers, isopropyl-β-D-1-thiogalactopyranoside (IPTG), and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) were obtained from Takara-Bio (Shiga, Japan). The plasmid DNA miniprep kit and the agarose gel DNA extraction kit were obtained from Hua Shun Biological (Shanghai, China). The hemagglutinin gene-specific oligonucleotides were synthesized by Shanghai Biological Engineering (Shanghai, China).

Cell Culture of the ZH-10 Strain of CDV

The MDCK cells were cultured in Opti-MEM to approximately 70% confluence, and the culture medium was discarded. The culture medium was reduced to one-tenth the original volume, and the cells were inoculated with the virus at 37°C for 2 h with

gentle shaking at 20-min intervals. After adding additional culture medium, the cells were incubated at 37°C in an atmosphere of 5% CO2. The cells were observed daily. When more than 80% of the cells exhibited a cytopathic effect (CPE), the cells were frozen and thawed three times, and centrifuged at 2,000 rpm for10 min at 4°C to remove the cellular debris. The viral supernatant was collected, and stored at -70°C in 1-mL aliquots.

Viral RNA Isolation

Total RNA was extracted from the viral supernatant. A 1-mL aliquot of Trizol was added to 200 µL of viral supernatant. The solutions were mixed by inversion 10 times, and incubated at room temperature for 10 min. The mixture was centrifuged at 14, 800 × g for 5 min. The precipitate was discarded, and 200 µL of chloroform was added to the supernatant. The solutions were mixed gently, and incubated at room temperature for 15 min. The mixture was centrifuged at 14, 800 × g for 15 min at 4°C, and the upper aqueous layer containing both the cellular and viral RNAs (approximately 600 µL) was carefully transferred to a clean microcentrifuge tube. The RNA was precipitated by the addition of 500 µL of isopropanol, followed by gentle mixing and incubation at room temperature for 10 min. The mixture was centrifuged at 14, 800 × g for 10 min at 4°C, and the RNA pellet was washed with 1 mL of 75% ethanol. After a final centrifugation at 7,600 × g for 5 min at 4°C, the supernatant was discarded. The RNA pellet was dried at room temperature for 10 min.

First-strand Complementary DNA Synthesis, PCR, and Molecular Cloning

For reverse transcription, the total RNA was denatured by incubation at 65°C for 5 min, and placed immediately on ice for 1 min. An aliquot of denatured RNA was combined with 4 uL of 5 × first-strand synthesis buffer, 2 µL of 0.1M DTT, and 1 µL (40 U) of RNaseOUT in a total volume of 19 µL. Following incubation at 25°C for 2 min, 1 µL (200 U) of SuperScript reverse transcriptase was added, and the sample was incubated an additional 10 min at 25°C. First-strand synthesis was performed at 42°C for 50 min, and the reaction was terminated by heating at 70°C for 15 min.

The HF 5'-GGGGTACCGCCAC-CATGCTCTCTTACCAAGACAAG-3') and HR (5'-AAGCGGCCGCTACCAT-CAAGGTTTTGAAC-3') primers were designed using the Oligo, version 6.0, software to amplify the full-length sequence of the H gene from the CDV genome by reverse transcription (RT), and PCR. The HF and HR primers incorporated a KpnI restriction site (GGTACC) flanking a Kozak sequence (for eukaryotic expression) and a NotI restriction site (GCGGCCGC) at the 5' and 3' ends of the H gene sequence, respectively, during RT-PCR.

The H gene cDNA was amplified during PCR using the LA Taq DNA polymerase, according to the manufacturer's instructions. Thermal cycling was performed using an initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealment at 59.2°C for 30 s, and extension at 72°C for 100 s, with a final extension at 72°C for 10 min. The PCR products were stored at 4°C. The PCR products were subjected to electrophoresis in a 1.2% agarose gel. The band corresponding to the expected size of the H gene was excised from the gel under ultraviolet light, and the H gene cDNA was recovered from the gel slice using the agarose gel DNA extraction kit.

For ligation, 1 µL of the recovered PCR product, 4 µL of pMD-18T plasmid, and 5 µL of Solution I were combined, and incubated at 16°C for 3 h. The ligation mixture was used to transform competent DH5α Escherichia coli. The transformed bacteria was spread on Luria-Bertani (LB) agar plates containing 8 µL of IPTG, 40 µL of X-gal, and ampicillin. The plates were incubated at 37°C for 16 h. A single white colony was used to inoculate 3 mL of LB broth medium containing ampicillin, and the bacteria were cultured at 37°C for 12 h with shaking. The plasmid DNA was extracted

from the 3-mL culture using the plasmid DNA miniprep kit. A 5-µL aliquot of plasmid DNA was digested using NotI or NotI and KpnI in combination, and the digested DNA was subjected to electrophoresis in a 1% agarose gel to confirm the size of the recombinant pMD18-H plasmid.

Structural and Phylogenetic Analysis

For assembly and analysis of the sequence, three pMD18-H clones were preserved in 20% sterile glycerol, and the nucleotide sequence of the H gene was determined by a commercial service provider (Invitrogen, Shanghai). The nucleotide sequence of the H gene of the CDV was downloaded from GenBank (KJ848781.1). The Seqman program (DNAStar, Madison,WI, USA) was used to assemble the sequencing reads, and the amino acid sequence of the H protein of the ZH-10 strain was deduced using the EditSeq program.

The Blast online computational tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to perform multiple sequence alignments, and homology analyses of the nucleic acid and amino acid sequences were performed using the Clustal W program. Potential glycosylation sites were identified using the N-glycoside web utility (http:// hiv.lanl.gov/content/sequence/GLY CO SITE/glycosite.html).

Figure 1. *Identification of a recombinant pMD18-H plasmid by restriction endonuclease digestion. Lane M1: DNA molecular weight marker (DL2000); lane M2: DNA molecular weight marker (DL15000); lane 1: Recombinant plasmid pMD18-H; lane 2: Recombinant plasmid digested using KpnI and NotI (fragments approximately 2692 and 1824 bp); and lane 3: Recombinant plasmid digested using NotI only (fragment approximately 4500 bp).*

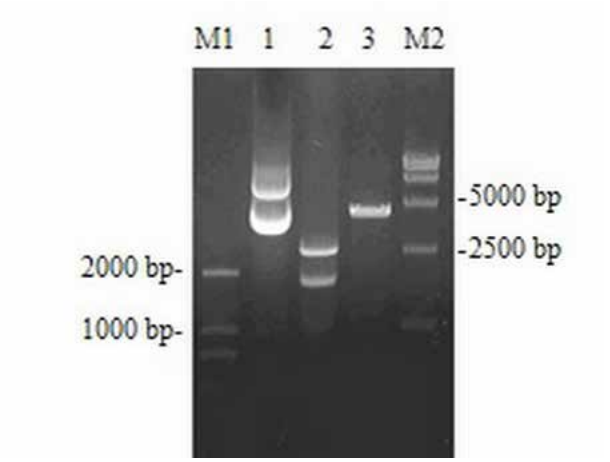
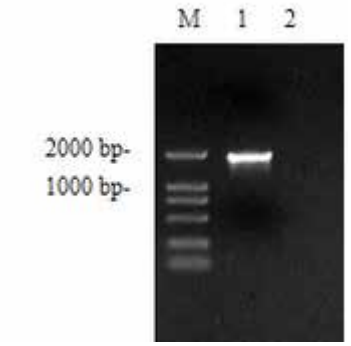


Figure 2. *Identification of a recombinant pMD18-H plasmid by PCR amplification of the H gene cDNA. Lane M: DNA molecular weight marker (DL2000); lane 1: PCR product of pMD18-H (about 1824 bp); and lane 2: Negative control.*



RESULTS

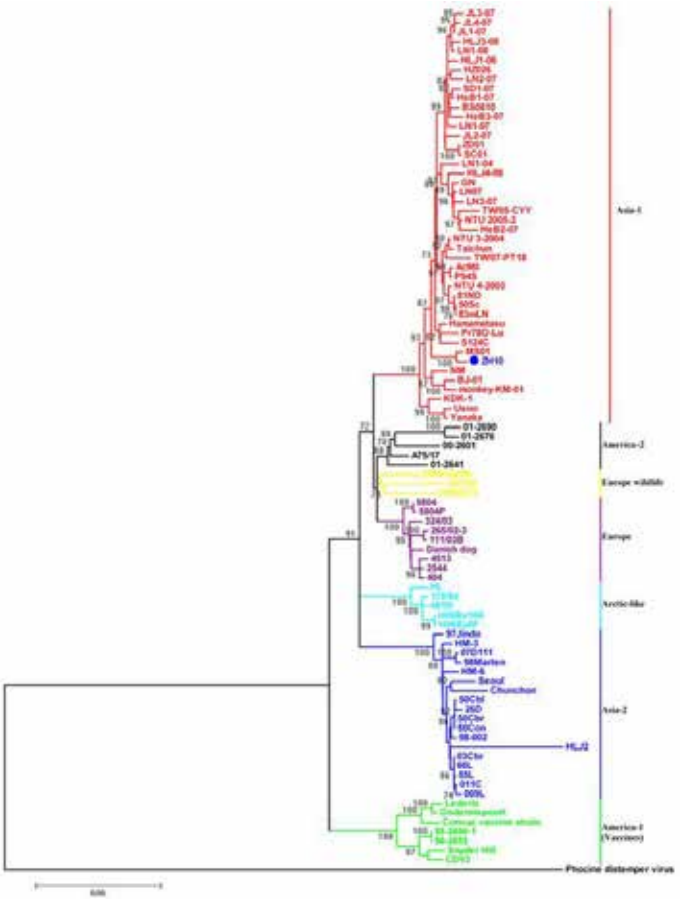
CPE of the ZH-10 Strain of the CDV

An obvious CPE was visible in the cultured MDCK cells at 48 h post-infection. The ZH-10-infected cells grew slowly, were swollen, and fused to form syncytia. The control cells formed a single layer of cells, and exhibited no signs of abnormal growth.

Characterization of H Gene cDNA

An 1,824-bp DNA fragment was amplified from the ZH-10 genomic RNA template by RT-PCR, which was consistent with the expected size of the H gene of the CDV. The digested recombinant plasmid was subjected to electrophoresis, and the size of the fragments were consistent with those of the nucleotide sequence (Fig. 1). The presence of the H gene in the recombinant pMD18-H

Figure 3. *Phylogenetic tree showing the relationships between the ZH-10 strain and various other strains of CDV.*



plasmid was also confirmed by PCR using the HF and HR primers. The PCR product was subjected to electrophoresis in a 1% agarose gel, and the size of the DNA fragment was consistent with that of the H gene (Fig. 2).

H gene Sequence Analysis

The H gene of the ZH-10 strain of CDV contained one open reading frame that encoded 607 amino acids, with a start codon (ATG) at nucleotide positions 21 to 23 and a stop codon (TGA) at positions 1,842 to 1,844.

The nucleotide homology analysis showed that the ZH-10 strain was most closely related to the LN (08) 1 and Hamamatsu strains, with homologies of 99.4% and 98.7%, respectively. The ZH-10 strain shared the lowest levels of homology with the Onderstepoort, Convac, CDV3, and Snyder Hill vaccine strains, with homologies ranging from 91.2% to 91.4%.

The phylogenetic analysis showed that, based on the H protein sequence, the various CDV strains clustered into seven genotypes (Fig. 3). The America-1-derived Onderstepoort, Convac, CDV3, and Snyder Hill vaccine strains and the Asia-1, Asia-2, America-2, Europe, European wildlife, and Arctic-like wild strain lineages were significantly different.

The ZH-10 strain clustered with wild strains of the the Asia-1 genotype. Using the porcine distemper virus (Genbank accession no. AF479274.1) as the root ancestor, a phylogenetic tree based on the amino acid sequence of the H protein was constructed using the ZH-10 strain, Ac96I strain of the Asia-1 genotype, the 007Lm strain of the Asia-2 genotype, the Onderstepoort vaccine strain, and 32 Chinese CDV isolates (Fig. 3). The results showed that the CDV strains isolated in China belonged to the Asia-1 genotype. The greatest levels of variability in accessibility, hydrophilicity, and antigenicity occurred at amino acid positions 239 to 251 and 384 to 410, with the ZH-10 strain exhibiting significant differences in these parameters, compared with those of the Onderstepoort, Convac, and 5,804P strains.

DISCUSSION

As a major structural protein of CDV, the H protein is a key antigen for the host immune response. It can elicit the production

of neutralizing antibody, and has antigenic epitopes that stimulate cytotoxic T cell effects (Sixt et al 1998). Previous studies have shown that the H protein is prone to antigenic drift, which contributes to variation in CDV virulence and antigenicity, leading to immune failure (Iwatsuki et al 2000, Martella et al 2006, Uema et al 2005). The nucleotide sequence alignment showed that the ZH-10 strain belonged to a lineage of wild strains of the Asia-1 genotype. This result was consistent with the findings of a previous study by McCarthy et al. (McCarthy et a. 2007), but differed from the findings of Hirama et al(Hirama et a. 2004).

The vaccine strains currently used in China, which include the Snyder Hill, Lederle, Convac, Rockbom, and Onderstepoort strains, were derived from the America-1 lineage. The nucleic acid sequence homology between the ZH-10 strain and the Hamamatsu strain isolated in Japan was 98.7%, whereas the levels of homology between ZH-10 and the Onderstepoort, Convac, CDV3, and Snyder Hill vaccine strains ranged from 91.2% to 91.4%. The amino acid sequence analysis of the H protein of ZH-10 showed that it differed significantly from those of the Onderstepoort, Convac, and 5804P strains at amino acid positions 239 to 251 and 384 to 410. Overall, our results suggest that variation in amino acid sequence among wild strains of CDV in Asia might contribute to immune failure in dogs immunized with vaccine strains derived from the America-1 lineage.

CONCLUSION

The ZH-10 strain is a wild strain of the Asia-1 genotype that is highly similar to strains responsible for recent CD epidemics in China. The differences in amino acid sequence between the ZH-10 strain and the various currently available vaccine strains may have contributed to immune failure in vaccinated dogs in Heilongjiang Province and other areas of China. Therefore, vaccines derived from regionally prevalent CDV strains are likely to be more effective for preventing CD.

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