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Original article

Genotyping and pathogenic characterization of canine distemper virus based on mutations in the hemagglutinin gene in Chinese domestic dogs

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Abstract

Canine distemper virus (CDV) infects wild and domestic *Canidae* worldwide. The hemagglutinin (H) gene has the highest genetic variation in the genome of this virus. Thus, the H gene is commonly used for lineage identification and genetic analyses. In order to study the genetic characteristics and pathogenicity of CDV strains prevalent in China, 132 samples were collected from domestic dogs with suspected CDV infection, 58 samples were confirmed to be positive, and the H gene was successfully amplified from 15 samples. The epidemic strain was identified as type Asia-1 and the novel mutations, A51T, V58I, R179K and D262N, were detected in this strain. Isolated strains, BJ16B53, BJ16B14, and BJ17B8, were used for an animal infection experiment in raccoon dogs. BJ16B53 and BJ16B14 were found to cause clinical symptoms, death, and extensive lesions in various organs. These results are expected to facilitate the development of effective strategies to monitor and control CDV infection in China.

Key words: canine distemper virus, hemagglutinin, phylogenetic analysis, animal infection

Introduction

Canine distemper (CD) is a highly contagious viral disease caused by the CD virus (CDV), and results in high morbidity and mortality in both wild and domestic carnivores. The hemagglutinin (H) gene has the highest

genetic variation in the CDV genome, and is used for lineage identification and genetic analysis of CDV strains (Iwatsuki et al. 2000). Strains in the same clade that share >95% amino acid similarity in the H protein are considered the same genotype. Based on this criterion, the America-1 and -2, Asia-1 and -2, Asia-3 and -4,

Europe-1/South America-1 (EU-1/SA-1), Arctic, European wildlife (EW), South America (SA-2), Rock-born-like (RL, Vaccine D), Africa-1, and Colombian (South America-3, SA-3) strains were identified.

The H protein is a constituent of the envelope glycoprotein spikes on the virion, and mediates the binding of the virus to the cell membrane. Antigenic and sequence variations in the H protein may affect the virulence, host range, and neutralization epitopes of CDV. H protein substitutions at residue 549 are crucial for host membrane fusion and host switches (Denzin et al. 2013). Moreover, site-specific changes in the H gene may indicate the evolutionary trajectory of CDV fitness.

The purpose of this study was to identify the genetic characteristics and pathogenicity of CDV strains prevalent in China. We investigated the epidemiology of CDV infection in domestic dogs in Beijing and identified the genetic characteristics and pathogenicity of the prevalent CDV strains. These findings will facilitate the development of effective strategies to control CDV infection in domestic and wild canids and non-canids in China.

Materials and Methods

Sample collection and sequencing of the CDV H gene

Samples were collected from dogs with suspected CDV infection at the Guan Zhong Animal Hospital in Beijing from 2016 to 2017. CDV infection was diagnosed based on clinical signs, blood examination, and a positive result on the CDV colloidal gold test strip (Bionote, South Korea). Total RNA was extracted using the Axyprep Body Fluid Viral DNA/RNA Mini-prep Kit (Axygen, USA) and reverse transcription was performed using the Prime Script II 1st Strand cDNA Kit (Takara, Japan) according to manufacturers' protocols. A commercial vaccine (Pfizer) was the positive control for RT-PCR. Primer pairs to detect the CDV N protein (491 bp) and to amplify and sequence the H gene (1,824 bp) were based on GenBank sequences KT341046 and FJ535063, respectively, and are as follows: N-F TCCCCTGGACAGTTGATCCA and N-R TTCCCTGGGGATCGTTTGAT; H-F ATGCTCTCTTACCAAGACAA and H-R TCAAGGTTTTGAACGGTTAC. Amplicons were cloned into a pEASY-Blunt Zero Cloning Vector (TransGen Biotech Company, Beijing, China), and five positive recombinant plasmids were sequenced (Invitrogen, Shanghai Biotechnology Co., Ltd., Shanghai, China).

Phylogenetic and amino acid analysis

The isolated CDV strains were compared with CDV reference strains. Phylogenetic relationships based on the amino acid sequences of the H gene were analysed using a distance-based on neighbour-joining method in MEGA 6.0 (Tamura et al. 2013). Bootstrap values were calculated from 1,000 replicates. H protein amino acids were deduced, aligned, and analysed using MegAlign (DNASTAR, USA). Possible N-linked glycosylation sites in the H protein were predicted using NetNGlyc 1.0 software.

Animal infection and histopathology

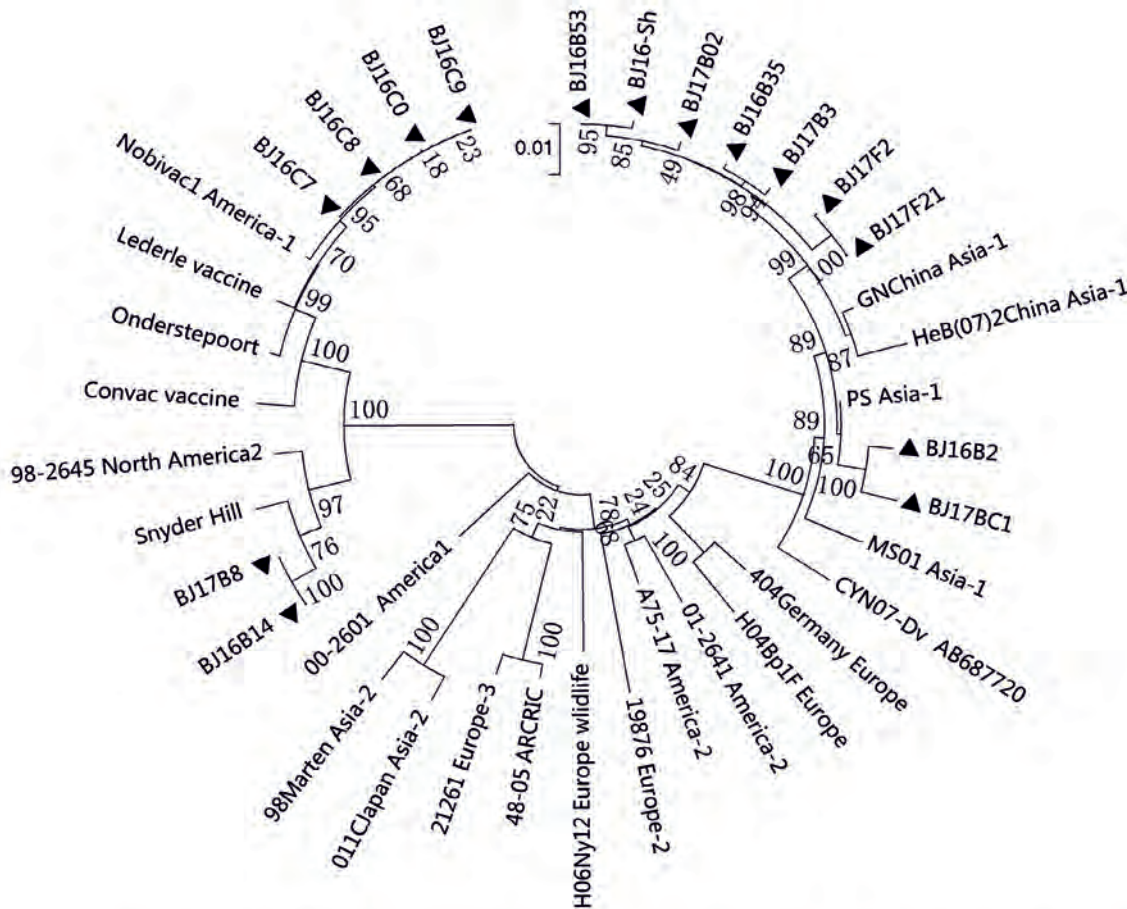
The CDV strains, BJ16B53, BJ17B8, BJ16B14, were isolated from positive samples using Vero/SLAM cells and prepared for inoculation experiments. Viruses were tested by RT-PCR, IFA, and electron microscopy. Titres were determined by a standard 50% tissue culture infective doses (TCID₅₀) assay. Ten unvaccinated 9-10 week-old healthy raccoon dogs, obtained from the Nanjing University Experimental Animal Centre, had no virological/serological evidence of parvovirus or CDV infection. The dogs were cared for according to the Guidelines for the Care and Use of Laboratory Animals. The animals were randomized into three experimental groups (3 dogs per group). The dogs in each infection group were intramuscularly challenged with 2ml and intranasally administered with 2 ml of each virus containing 2×10^5 TCID₅₀ (BJ16B53, BJ17B8, or BJ16B14). The control dog was inoculated with the same dose of sterile PBS. The animals were housed separately to avoid contact transmission, and were clinically monitored for 4 weeks. Body weight, body temperature, presence or absence of vomiting and diarrhea, and mental status were recorded twice a day. Nasal and rectal swabs and whole blood samples were collected at 0, 3, 7, 10, 14, and 21 days post-infection. All surviving dogs were humanely euthanized on day 28. Necropsy and histological examinations were performed on the most severely infected dogs. Tissue samples were fixed in 10% formaldehyde and processed for histopathology. Paraffin-embedded tissue sections (lung, spleen, liver, heart, and mesenteric lymph nodes) were obtained and stained with hematoxylin and eosin.

Results

Sequencing of the CDV H gene

A total of 132 clinical samples were collected from domestic dogs suspected of CDV infection, and 58 samples (43.9%) tested positive for CDV using RT-PCR for the N protein (491 bp). The H gene was successfully amplified from 15 of the 58 positive samples. All of the

A



B

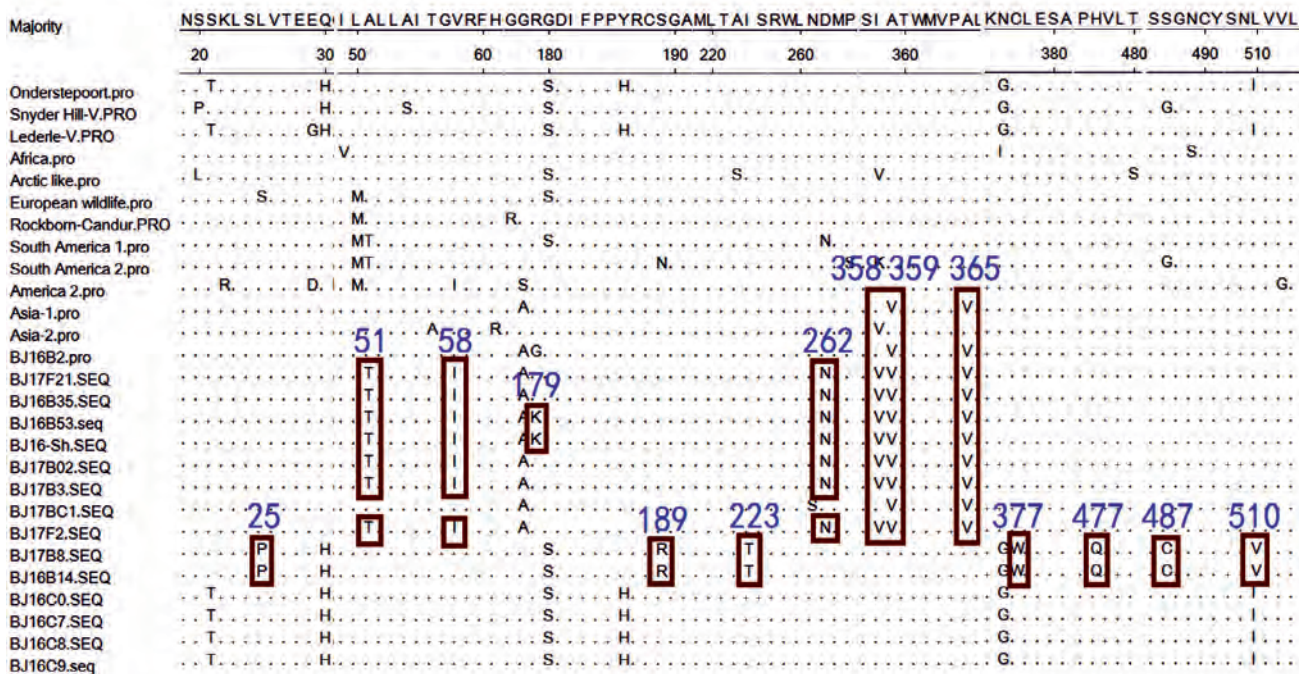


Fig. 1. A: Phylogenetic relationships of 57 CDV isolates based on amino acid sequences of the H gene. Filled triangles (▲) indicate the 15 CDV isolates obtained in this study. B: Alignment of the deduced amino acid sequences of the H protein in wild-type and vaccine strains of CDV. Abbreviations for the sequences: Onderstepoort vaccine (AF378705), Lederle vaccine (DQ903854), Snyder Hill vaccine (AF259552), European wildlife (Z47759), Africa strain (FJ461703), South America-1 strain (Z47761), South America-2 strain (FJ392651), Arctic-like (Z47760), Asia-1 strain (FJ423608), and Asia-2 strain (AB040768).

H gene sequences were submitted to GenBank (accession numbers MF926597 to MF926611).

Phylogenetic analysis of the H gene

The fifteen amplified H gene fragments were compared to CDV reference strains obtained worldwide. Figure 1a presents the phylogenetic relationships based on amino acid sequences of the H gene. Of the 15 strains, 9 clustered with the Asia-1 strains from China. BJ16B14 and BJ17B8 displayed a high identity with the Snyder Hill vaccine strain. Four strains, BJ16C0, BJ16C7, BJ16C8, BJ16C9, were closely related to the Onderstepoort vaccine strain.

Analysis of H gene amino acid sequences

The amino acid sequences of the 15 isolates, 3 vaccine strains, and 9 reference CDV strains were aligned (Fig. 1b). Gly (G) was present at the 530 site in 9 isolates, identified as Asia-1, similar to the reference Asia-1 strain; BJ17B8 and BJ16B14 had Asn (N) at 530, and four strains had Ser in position 530, similar to the Onderstepoort and Lederle vaccine strains. All of the Asia-1 isolates had Tyr (Y) at amino acid 549 and all vaccine isolates had His (H) in this position. The mutations, A51T, V58I, R179K, D262N, were first detected in Asia-1 strains. BJ18B14 and BJ17B8 had the following substitutions in the H protein: L25P, S189R, I223T, C377W, P477Q, S487C, and L510V.

The potential N-glycosylation sites in the H protein were deduced. All 9 Asia-1 strains possessed 9 potential N-linked glycosylation sites (N19, N149, N309, N422, N456, N584, N587, N603), while vaccine strains had 6 glycosylation sites, except BJ16B14 and BJ17B8 which possessed an additional site at N603.

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The titres of BJ16B53, BJ17B8, and BJ16B14, used in the dog experiment, were $10^{5.4}$ TCID₅₀/ml, $10^{4.6}$ TCID₅₀/ml, and $10^{5.1}$ TCID₅₀/ml, respectively. In the BJ16B53 group, 2 dogs showed typical symptoms, grew worse daily, and finally died on days 17 and 22, respectively. In the BJ16B14 group, 1 dog died on day 25 and 2 dogs showed transient symptoms. In the BJ17B8 and PBS-inoculated control group, no clinical signs of infection were detected.

After death, tissues were collected from dogs of the BJ16B53 group, and a histopathology examination was conducted. The tissues tested positive for CDV by RT-PCR. As shown in Fig. 2, mild histopathologic lesions in the lung, liver, spleen, and heart developed in infected dogs. In the lung, interstitial lymphoid cells with large nuclei, evidence of alveolar fusion and sep-

tum thickening, and pink dye exiting the alveolar cavity were observed (Fig. 2a). Hepatocytes were highly swollen in the superficial region, cytoplasm was diffuse, and the hepatic sinus contained abnormal erythrocytes (Fig. 2b). Lymphocytes (stained blue), small blood vessels, red blood cells, and reticular cells were observed in the spleen (Fig. 2c). Myocardial cells appeared swollen and stained, and showed erythrocyte dispersive division (Fig. 2d).

Discussion

In 1999, Mochizuki et al. revealed genetic lineages of CDV, defined as amino acid sequences with similarities higher than 4%. Pomeroy et al. (2008) revealed that the estimated mean substitution rate for the CDV H gene was 11.65×10^{-4} s/s/y. Virus species show consistently high substitution rates and decreased protein stability. Thus, despite the recent vaccination procedures adopted in China, CD remains a serious threat to the breeding of raccoon dogs and domestic dogs (Zhao et al. 2010). To examine the prevalent CDV strains circulating in northern China, clinical samples were collected in Beijing and analysed.

Of the 132 clinical samples collected, 58 samples (43.9%) tested positive for CDV by RT-PCR, indicating that CDV remains a high risk to canines in Beijing. The H gene was successfully amplified from 15 of the 58 positive samples. Among the 15 infected dogs, 4 dogs were not vaccinated against CDV and 13 dogs were below five months of age. Although most dogs were vaccinated with live attenuated vaccines, they remained susceptible to infection, especially puppies less than 5 months old. Causes of immunization failures include vaccine quality, poor immune response, inappropriate vaccination, CDV genetic diversity, and antigenic drift of wild-type CDV strains (Li et al. 2014). The remaining 74 animals, thought to be infected with CDV, tested negative twice by PCR screening. Possible reasons for this discrepancy include: the animals were truly negative for CDV, there was a high copy number threshold to detect CDV RNA/cDNA in the PCR assay, and Taq polymerase inhibitors were present in the clinical samples, including nucleases and substances such as heparin and haemoglobin.

A phylogenetic analysis based on the H gene was conducted, as shown in Fig. 1a. Nine samples were classified into the Asia-1 group, showing the highest identity with the Chinese Asia-1 strains, GN and HeB(07)2. The other six strains were similar to vaccine strains. These findings indicate that the epidemic CDV strains belong to the Asia-1 group, consistent with previous studies (Zhao et al. 2010, Tan et al. 2011, Wang et al. 2011). Explanations for the presence of vaccine stains

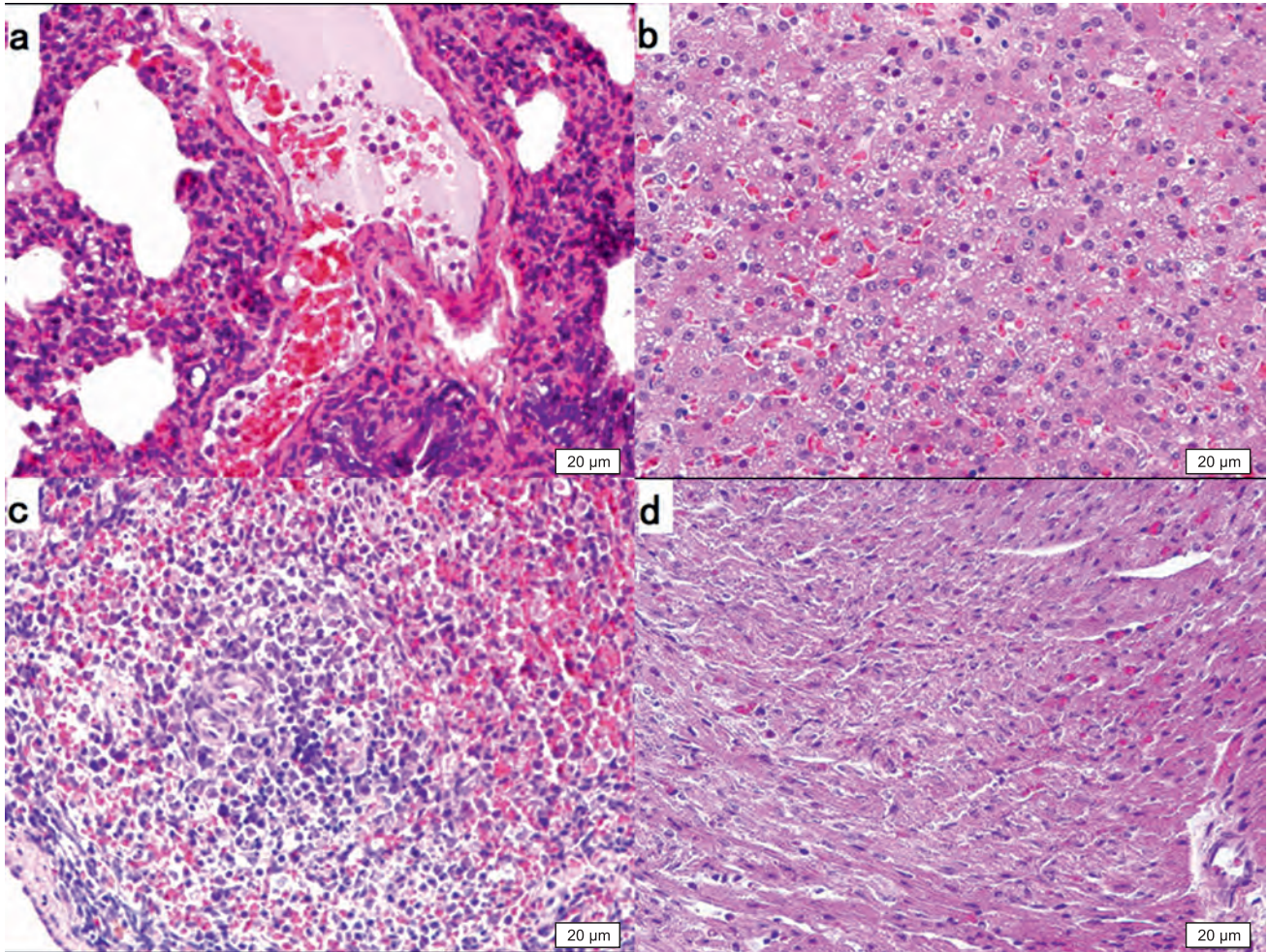


Fig. 2. Histopathology of hematoxylin and eosin-stained lung, liver, spleen, and heart tissues from dogs infected with the CDV isolate, BJ16B53. a: Lung, dilated blood vessels filled with red blood cells and small numbers of lymphoid cells were seen in the interstitium; $\times 400$; b: liver, liver cells were highly swollen with diffuse cytoplasm, and abnormal erythrocytes were observed in the hepatic sinus; $\times 400$; c: spleen, blue-dyed lymphocytes; $\times 400$; d: heart, myocardial cells were swollen and stained, and showed erythrocyte dispersive division, $\times 400$.

include antigenic escape, genetic recombination with wild-type strains, environmental adaptation, and constant evolution of the virus (McCarthy et al. 2007).

Asia-1 infections are present in many countries, including Korea, Thailand, and China, demonstrating an expanding geographic distribution (Li et al. 2014, Cheng et al. 2015). Outbreaks of Asia-1 CDV have caused losses in tigers, monkeys, and other animals (Nagao et al. 2012). The debate is ongoing regarding the role of the Y549H substitution in host switches; this change has been associated with viral adaptation to non-canid species. However, Nikolin et al. (2012) have found that domestic dogs, wild-canids, and non-canid hosts are equally likely to encounter strains with Y or H at the 549 site and are equally susceptible to both strain variants. In our study, the population was composed of domestic dogs, and the Y549H substitution was frequent (6/15, 40%) in the Asia-1 lineage, much higher than in South America (Fischer et al. 2016), indicating

that CDV strains circulating among wild canids and other carnivores are frequently transmitted to domestic dogs. The R580Q substitution can impair attachment protein surface expression and fusion efficiency, thus reducing the virulence of CDV *in vitro*. However, this substitution is infrequent (Sattler et al. 2014). In this study, all isolates had R at position 580, possibly resulting from CDV adaptation to environmental challenge. A51T, V58I, R179K, and D262N were first detected in Asia-1 strains, and were different from the vaccine strains. BJ18B14 and BJ17B8 had L25P, S189R, I223T, C377W, P477Q, S487C, and L510V substitutions in the hemagglutinin protein. The locations of these sites in three-dimensional macromolecular structures were analysed. Sites 179 and 189 are localized to the surface of the protein in the three-dimensional structure of morbillivirus H protein, suggesting the involvement of these sites in high virulence and adaptation to vaccinated canids.

N glycosylation plays an important role in the correct folding, transport, and function of other fusion and attachment glycoproteins of paramyxoviruses and an increase in N glycosylation may result in vaccine failure (Sawatsky and von Messling 2010). In addition, alterations in N glycosylation may affect neutralization by antibodies and replication in vitro. Thus, N-glycosylation sites of the 15 isolates were predicted and analysed. Compared with the seven potential sites in vaccine strains (CDV3, Convac), all 9 Asia-1 strains possessed 9 potential N-linked glycosylation sites (N-X-S/T), similar to the reference Asia-1 wild strains (Guo et al. 2013). The remaining six isolates shared the same 6 N-glycosylation sites as the reference vaccine strains, except for an additional site at N603 in BJ16B14 and BJ17B8. Vaccine strains may have undergone a distinctive mutation, to generate different antigenicity and increased N glycosylation, resulting in vaccine failure.

Disease severity depends on the susceptibility of the host, the age of the animal, and the properties of the virus strain (Jensen et al. 2015). In the infection experiment, in order to avoid cross-infection or natural infection, and be sure of the infected dose, dogs were both intramuscularly challenged with 2ml and intranasally administered with 2ml of each virus containing a total of 2×10^5 TCID₅₀. Among the infected groups, BJ16B53 group presented the most severe symptoms and typical pathological changes were observed in tissues, while the other two experimental groups showed transient, slight, or no symptoms. These results suggest that the BJ16B53 strain poses a high pathogenic threat to canines and warrants further study.

In summary, this study determined the predominant genotype of CDV and detected genetic mutations and antigenic changes in the H gene of CDV strains isolated in Beijing from 2016 to 2017. Furthermore, we showed that one strain, BJ16B53, caused extensive lesions in a variety of tissues. These findings increase our understanding of the genetic characteristics of Chinese CDV isolates and may facilitate the development of more effective strategies to monitor and control CDV in wild canids and non-canids in China.

Acknowledgements

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