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Characterization of a new dog isolate of canine distemper virus from China

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Summary. – Canine distemper virus (CDV) is a highly contagious pathogen of dogs. Vaccination is an effective way to protect dogs from CDV infection, but occasionally fails. In the present study, a wild type (wt) CDV, named XJ2, was isolated from a dead vaccinated dog. The hemagglutinin (H) gene of the XJ2 was amplified and analyzed for the molecular characteristics including N-glycosylation sites, phylogenesis, hydrophobicity and epitopes. The data indicated that XJ2 was a genetic variant strain of CDV. CDV-sero-negative dogs were inoculated intranasally with XJ2, developed severe clinical symptoms and died, suggesting high virulence.

Keywords: canine distemper virus; hemagglutinin gene; virulence; Chinese isolate; dogs

Introduction

Canine distemper (CD), an acute and highly contagious disease caused by CDV, is one of the serious epidemic diseases encountered in the canine farming, fur animal farming industry, and wildlife conservation. CDV (the genus *Morbillivirus*, the subfamily *Paramyxovirinae*, the family *Paramyxoviridae*, the order *Mononegavirales*) is a negative single-strand RNA virus with a capsule membrane, which contains 6 main structural proteins: nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin protein (H), and large protein (L) (Orvell, 1980). Of these, the F and H glycoproteins are the protective antigens that induce humoral antibodies (Sato *et al*., 1981; Wild and Buckland, 1997; Hirama *et al*., 2003). They also determine the host specificity of viral infection (Yanagi *et al*., 2006a,b).

Since CDV can cause serious damage in dogs, conventional vaccines such as inactivated vaccine and live attenuated vaccine have been widely applied in the prevention of the disease (Taylor *et al*., 1991; Wild *et al*., 1993; Sixt *et al*., 1998; Welter *et al*., 1999, 2000; Griot-Wenk *et al*., 2001; Dahl *et al*., 2004; Premenko-Lanier *et al*., 2004). In recent years, however, despite the fact that the wide use of vaccines has greatly reduced the number of CD outbreaks, it was reported that immunized dogs could still occasionally develop CD (Dorsey, 2005; Calderon *et al*., 2007; Simon-Martinez *et al*., 2008). In 2007, we successfully isolated a field strain of CDV from the lungs of a dead dog that had been previously immunized with an inactivated CD vaccine (Onderstepoort strain). In order to investigate the origin of this isolated strain, from the vaccine strain or from an epidemic wt strain, the hemagglutinin gene of this isolate was cloned, sequenced and analyzed for its genetic evolution. The virulence was evaluated by animal regression assay.

Materials and Methods

Virus isolation. An 8-month-old German shepherd dog from a farm in Xinjiang, China died 15 days post immunization with 2.6×10^7 $TCID_{50}$ of inactivated CD vaccine, Onderstepoort strain. This dog showed anorexia, tachypnea, diarrhea, and severe dehydration prior to death. The blood test, RT-PCR and immunofluorescence (IF) results indicated that this dog died from CD. Therefore, the lungs were harvested, minced and homogenized in normal

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Abbreviations: CD = canine distemper; CDV = CD virus; CPE = cytopathic effect; H = hemagglutinin; IF = immunofluorescence; wt = wild type; XJ2 = the CDV XJ2 isolate

saline for virus isolation. Following centrifugation at 1000 × *g* for 10 mins, the supernatant was treated with penicillin and streptomycin (both at 2,000 IU/ml) overnight, and then used to inoculate Vero cell monolayer. The inoculated cells were subsequently cultured in minimal essential medium containing 8% fetal bovine serum (Invitrogen) at 37°C with 5% CO_2 for 5 to 7 days. Five rounds of blind passage were carried out prior to virus isolation. The cultures were monitored daily for the cytopathic effect (CPE) and harvested for virus preparation by 3 freeze-thaw cycles. The isolated virus was named the CDV XJ2 isolate (XJ2 thereafter).

Electron microscopy. Negative-stain electron microscopy was used to verify the identity of XJ2. Briefly, 100 µl of the culture supernatant was fixed with 10 µl fixative (10% formaldehyde and 5% glutaraldehyde in deionized water) to give final concentrations 1% formaldehyde and 0.5% glutaraldehyde in the virus suspension. The fixed supernatant was then observed under a Hitachi H600 transmission electron microscope (Tokyo, Japan).

RT-PCR. The *H* gene of XJ2 was amplified by RT-PCR. The primer set was designed based on published CDV *H* gene sequence (GenBank Acc. No. Z47760) to include full length ORF as follows: fp: 5′-ACC**GGATCC**GGTAGTCCAACAATGCTC-3′ and rp: 5′-ACC**CTCGAG**TCAAGGTTTTGAACGGTTAC-3′. *Bam*HI and *Xho*I adapters (bold and underlined in primer sequences) were respectively introduced into the 5′ end of both primers for cloning purpose. Viral RNA was isolated from the culture supernatant using a total RNA isolation kit (Qiagen) according to the manufacturer′s instructions. cDNA was synthesized from 50 ng total RNA in a 20-µl reaction mixture comprising 4 µl of 5× RT buffer, 4 µl of 2.5 mmol/l dNTP, 1 µl of RNase inhibitor (10 U/µl), 2.5 U (1 µl) of enzyme Superscript TM II RNase H- Reverse Transcriptase (Invitrogen) and 5 pmoles (0.5 µl) of oligo(dT). The mixture was incubated at 42°C for 15 mins, 95°C for 5 mins, and 5°C for 5 mins. Fifty microliters of the PCR reaction mixture contained 2 µl of the reverse transcription product as a template, 5 µl of 10× PCR buffer, 1 µl of 25 pmol/µl primers, 4 µl of 2.5 mmol/l dNTP, and 0.3 µl of 5 U/µl Taq plus DNA polymerase. The PCR conditions were 96°C for 200 secs to denature the RNA/cDNA hybrid, then 36 cycles of 94°C for 45 secs, 54°C for 50 secs, and 72°C for 150 secs, and a final extension at 72°C for 10 mins. The PCR products were examined on 1% agarose gel and visualized under UV light after ethidium bromide staining.

Cloning and sequencing. The PCR products were purified with DNA gel Extraction Kit (Takara) and then cloned into a pGEM-T Easy vector (Promega) using T4 DNA ligase. *Escherichia coli* JM109 competent cells were transformed with the resultant ligation products, plated onto the agar plates containing 50 µg/ml ampicillin and incubated at 37°C for 12 hrs. The recombinant plasmids were extracted and identified by PCR and restriction enzyme digestion. Three randomly selected recombinant plasmids pTH were sent to Takara for DNA sequencing using primer walking method.

Sequence and phylogenetic analyses. Sequences were aligned by CLUSTAL/W. Phylogenetic trees of the *H* gene sequences were constructed by the neighbor-joining method using average linkages of Molecular Evolutionary Genetics Analysis (MEGA, version 3.1) after comparison with the CDV data from GenBank. The cysteine residues, glycosylation sites, hydrophobicity, and antigenic epitopes of *H* gene-encoded protein were also analyzed using online software (http://www.expasy.org/tools).

Infection of dogs. Four CDV-seronegative two-month-old dogs were used (Lanzhou Veterinary Research Institute, China). Two dogs were inoculated intranasally with 0.5 ml of viral cultures containing 1×10^4 $TCID_{50}$/ml XJ2, and other two dogs were inoculated intranasally with equivalent volume of PBS as control. All dogs were examined for clinical signs of CD until they were euthanized 30 days post infection. Necropsies were carried out on all animals, then brain, lung, urinary bladder and thymus were examined by RT-PCR. Frozen sections were prepared from lungs and incubated with CDV mAb (Thermo Scientific Pierce) followed by staining with FITC-conjuncted goat anti-mouse IgG (Sigma) and observed under fluorescence microscopy.

Results

Viral isolation

After 3 rounds of blind passages, the virus caused CPE in Vero cells. The cells were rounded up and integrated into multinucleated giant cells 72 hrs post inoculation. With the incubation moving on, the cells began to disaggregate and formed a fish net-like structure (Fig. 1a and 1b). The culture medium from the fourth passage was used to inoculate the cells for virus preparation, showing stable CPE. In the supernatant of the culture, irregularly shaped viral particles with an approximate diameter of 150–300 nm and with a dense layer of spikes on the envelope were observed through negative staining electron microscopy (Fig. 1c).

Cloning, sequencing and sequence analysis of the H gene of XJ2 isolate

The DNA electrophoresis results showed that the RT-PCR product of XJ2 was approximately 1.8 kbp, which is consistent with what we expected (Fig. 2). The results from restriction enzyme digestion and DNA sequencing indicated that the XJ2 *H* gene was successfully inserted into the plasmid pTH (Fig. 3 and data not shown). The DNA sequence was submitted to the GenBank (Acc. No. EU684265). The full-length ORF of the XJ2 *H* gene was 1824 bp long and deduced amino acid sequence was 607 amino acids long, which is the same size as the *H* gene of the attenuated CDV strain Convac, but 3 aa longer than the *H* gene of the Onderstepoort vaccine strain. When compared with the known *H* gene of CDV strains in the GenBank, the nucleotide sequence identity was found to be within the range of 86.2% to 98.4%, whereas the deduced amino acid sequence identity was between 88.5%

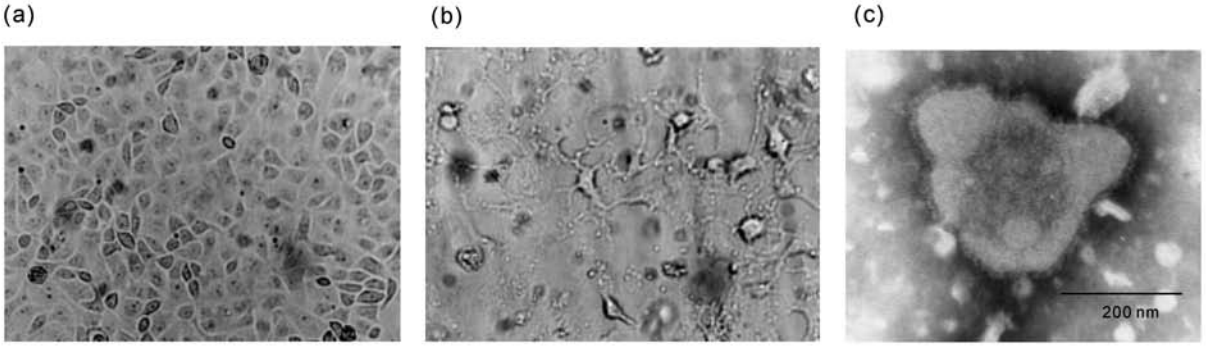

Fig. 1

Virus isolation on Vero cells

Non-infected cells (a), and infected cells (b) 96 hrs post infection. Virus particles as observed by EM (c).

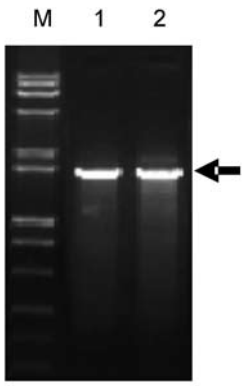

Fig. 2

RT-PCR of the H gene of the XJ2 isolate

Agarose gel electrophoresis. DNA size markers (lane M), the Onderstepoort strain (lane 1), and the XJ2 strain (lane 2).

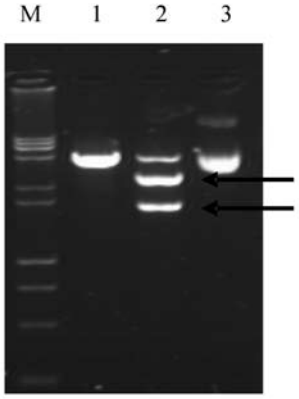

Fig. 3

Restriction analysis of the recombinant plasmid pTH

DNA size markers (lane M), pTH digested with *Bam*HI (lane 1), pTH digested with *Bam*HI + *Xho*I (lane 2), and undigested pTH (lane 3).

and 98.7%. The positions of all 12 cysteine residues found in the H protein of XJ2 were conserved in the Onderstepoort vaccine strain. The H protein of Onderstepoort vaccine strain contained 6 potential N-linked glycosylation sites, whereas the deduced amino acid sequence of XJ2 included 9 potential N-linked glycosylation sites (aa19–22, 149–152, 309–311, 391–393, 422–425, 456–458, 584–586, 587–589, and 603–605), four of which were identical to those in Onderstepoort strain and 5 of which were identical to those in Convac strain. However, two potential N-linked glycosylation sites, located at aa 309–311 and 584–586, were unique to XJ2 (Fig. 4).

H gene-based phylogenetic analysis of the XJ2 isolate

Different CDV strains can be divided into 2 genetic lineages: lineage I (virulent strains) and lineage II (attenuated strains). The nucleotide sequence identity between the 2 lineages is approximately 86.2%. The genetic lineage I comprises three different genotypes. The XJ2 showed a closer phylogenetic relationship to the epidemic strains TN, HLJ1-06, SD (07), and HLJ2-07 from China and to the Hama virulent strain, sharing more than 97% similarity in nucleotide and deduced amino acid. In contrast, it was distantly related to the American and Jave strain and separated with the farthest genetic distance from Onderstepoort and Convac vaccine strains of lineage II (Fig. 5). These results indicated that XJ2 might be a virulent CDV strain.

The difference in hydrophobicity and H protein epitopes between the XJ2 isolate and Onderstepoort vaccine strain

Online software analysis revealed that the hydrophobicity of the deduced H protein of XJ2 was different from that

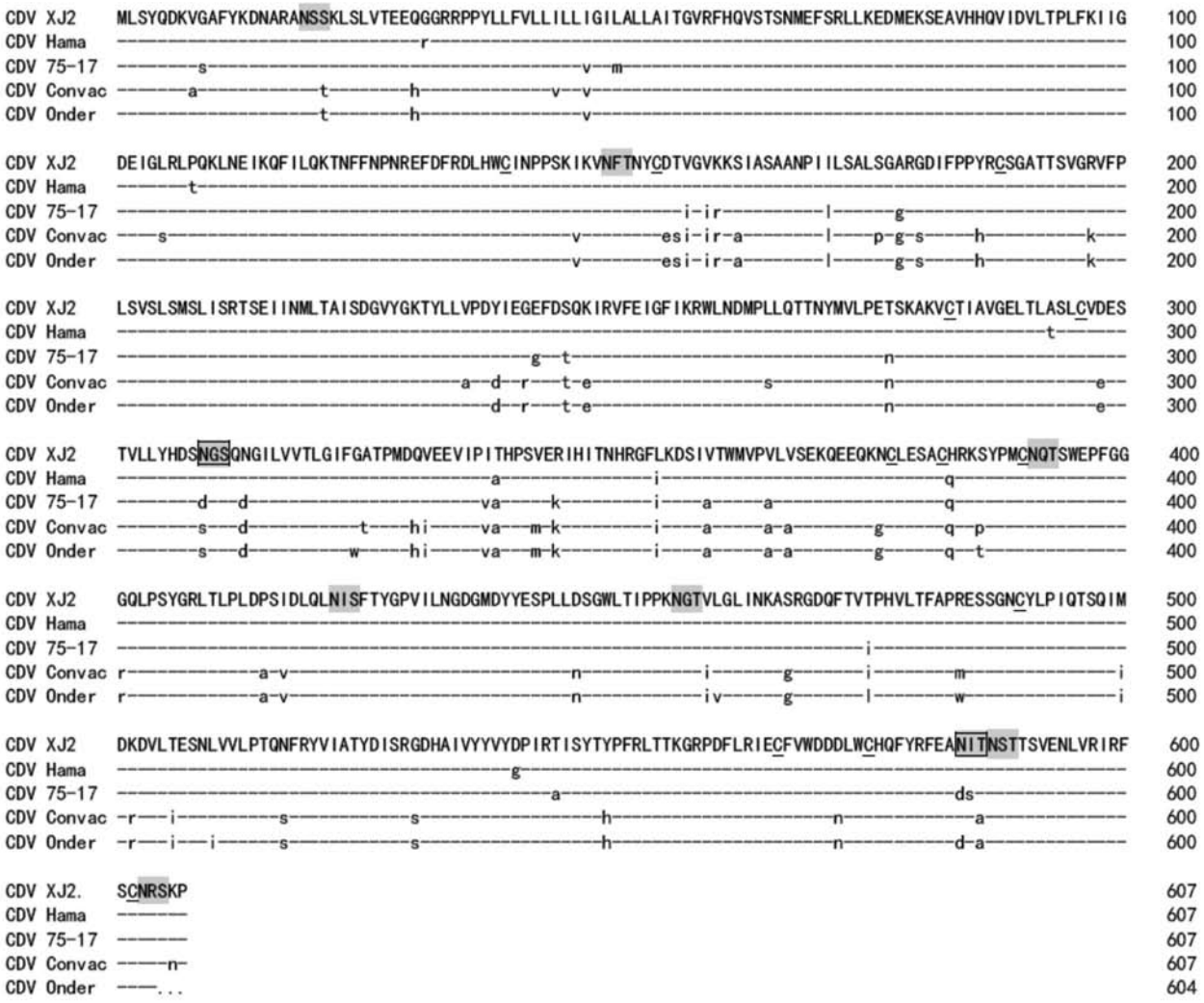

Fig. 4

Multiple alignment of amino acid sequences of *H* genes of the XJ2 isolate and selected CDVs

The sequences of selected CDVs were retrieved from the GenBank database. Potential N-glycosylation sites in the XJ2 H protein are boxed.

of the Onderstepoort vaccine strain (Fig. 6). Data from the Jameson-Wolf dominant epitope prediction showed that there were significant differences in the epitope of aa 157–169 between XJ2 and the Onderstepoort attenuated strain (Fig. 6, arrow).

Pathogenesis of the XJ2 isolate in dogs

The two dogs inoculated with XJ2 developed CD with symptoms of fever, loss of appetite, and mild eye inflammation. These symptoms became more severe and noticeable with the disease progress. Vomiting and diarrhea, dehydration, excessive salivation and coughing were also observed. Sixteen days after inoculation, these two dogs died, while the control dogs remained healthy during the whole observation period. The CDV *H* gene was detectable in brains, lungs, urinary bladders and thymuses by RT-PCR. CDV antigen was also found in these tissues by IF assay (Fig. 7).

Discussion

The H protein, an important component of CDV (Iwatsuki *et al.*, 1997; Kapil *et al.*, 2008), plays a role in virus adsorption to the cells and is prone to mutation under the immune pressure (Bolt *et al.*, 1997; von Messling *et al.*, 2001; Martella *et al.*, 2006; Singethan *et al.*, 2006). To date, it has been widely used to analyze the genetic variation of

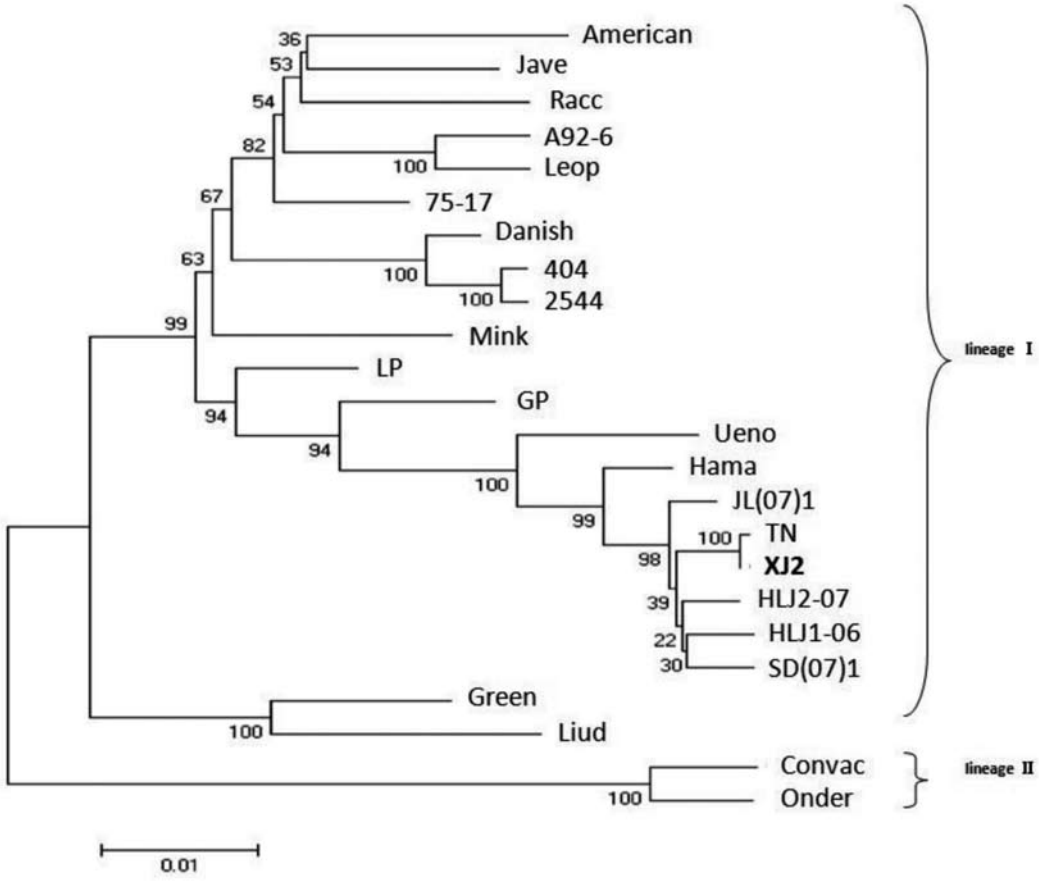

Fig. 5

***H* gene-based phylogenetic analysis of CDVs**

The sequences of CDVs were retrieved from the GenBank database. 1000 replicates were bootstrapped. The scale bars represent the numbers of substitutions per 100 positions.

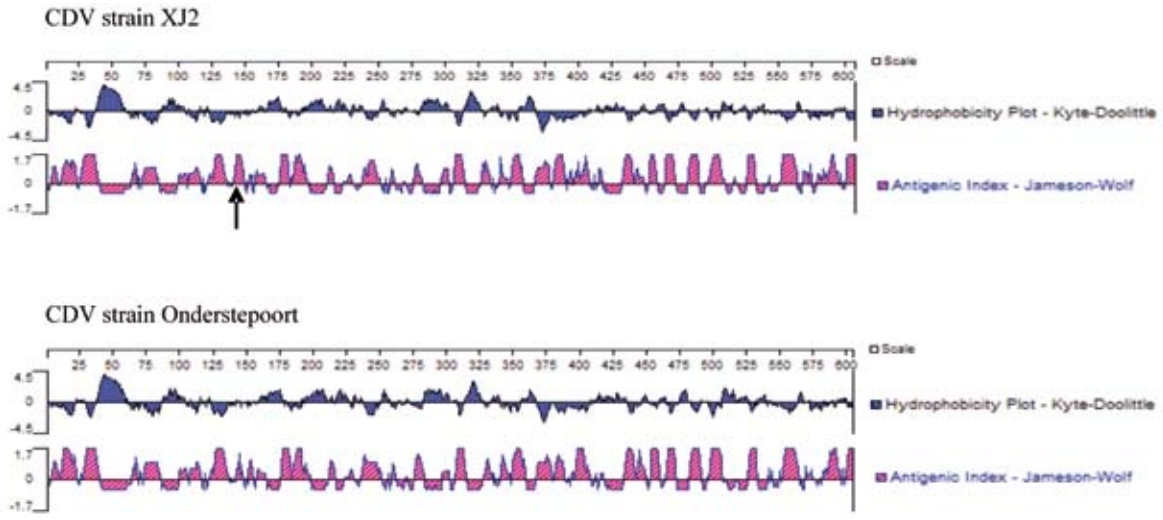

Fig. 6

The hydrophobicity of and epitopes on the H proteins of XJ2 isolate and Onderstepoort strain

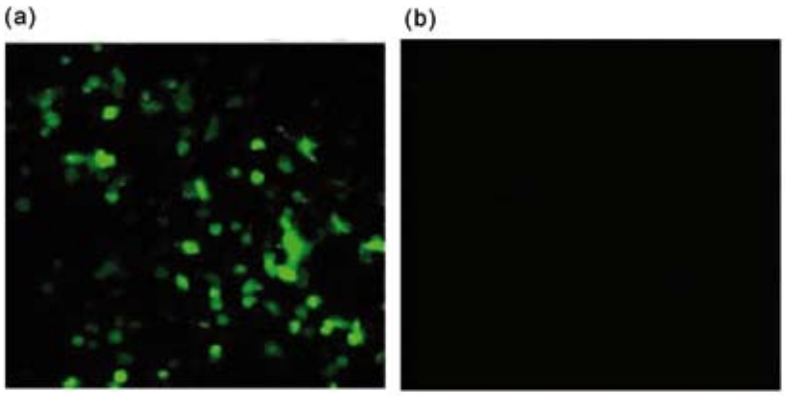

Fig. 7

Immunofluorescence assay of lung from dogs infected with XJ2 isolation

Frozen sections were prepared from lungs and incubated with CDV MAb followed by staining with FITC-conjungated goat anti-mouse IgG. (a) XJ2-infected lung; (b) mock-infected lung.

the CDV wt strain (Mamaev *et al.*, 1995; Haas *et al.*, 1997; Hirama *et al.*, 2004; Demeter *et al.*, 2007). In this study, we successfully isolated CDV XJ2 from a dead dog and identified it by RT-PCR and DNA sequencing. Sequence analysis of *H* gene showed that the XJ2 was a genetic variant strain, which had obvious difference from the vaccine strain. Phylogenetic analysis showed that the XJ2 had a close phylogenetic relationship to the epidemic strains in China [TN, HLJ1-06, SD (07), HLJ2-07] and the highly virulent Hama strain, suggesting that XJ2 strain might be derived from the same ancestor as domestic epidemic strains. In contrast, the XJ2 is separated by a farther genetic distance from Onderstepoort and Convac vaccine strains, indicating that XJ2 was not from vaccine strains. Animal inoculation test showed that the XJ2 is a highly virulent CDV strain, which coincided with the results of *H* gene genotyping.

Some studies have indicated that the CDV wt strain continues to mutate to form different groups of genes (Harder *et al.*, 1996; Iwatsuki *et al.*, 1997; Hirama *et al.*, 2004; Pardo *et al.*, 2005; Uema *et al.*, 2005; Demeter *et al.*, 2007; Han *et al.*, 2008). Uema *et al.*, for example, found that CDV wt strains in Japan have 3 different genetic groups (Uema *et al.*, 2005), but Martella *et al.* (2006, 2007) demonstrated that geographically different CDV epidemic strains could be divided into 5 genetic lineages: American-1, Europe, Asia-1, Asia-2, and Arctic (Martella *et al.*, 2006, 2007). In recent years, some studies have identified differences between many wt strains and the Onderstepoort vaccine strains throughout the world (Lan *et al.*, 2005; Pardo *et al.*, 2005; Demeter *et al.*, 2007).

The Onderstepoort strain, originally isolated in 1930s from an infected fox during a CD outbreak in North America, is a live attenuated vaccine strain. During the passage procedures, many factors such as the environment, the CDV adaptation to animal epidemic factors and the large number of interspecies transmissions may cause the virus mutate. As a result, mutations accumulated from the long-term passage may make the vaccine strain differ from the original virus. Consequently, the CDV Onderstepoort vaccine strain may not offer complete protection against all the CDV epidemic strains. In this study, we compared the hydrophobicity and H protein epitopes of the XJ2 isolate and the Onderstepoort vaccine strain and found differences between them in the epitope of aa 157–169. Since the hydrophobicity of the amino acid sequence of a protein reflects its antigenic determinants (Jameson and Wolf, 1988), the variation of the epitope aa 157–169 may be related to the failure of the protection of the dog after the CDV Onderstepoort vaccination. Surveys conducted in 2 animal hospitals in Tokyo, Japan revealed that 2/3 of vaccinated dogs were diagnosed with CD, and most of them had high neutralization antibody titers against the Onderstepoort strain, but very low titers against wt strains, suggesting that the antigenic epitopes on the envelope of vaccine strain are different from wt strains (Ohashi *et al.*, 1998; Iwatsuki *et al.*, 2000).

Moreover, Iwatsuki believed that different N-linked glycosylation sites could affect the immunogenicity of the H protein (Iwatsuki *et al.*, 2000). In our study, we found that the H protein of the XJ2 had 9 potential N-linked glycosylation sites, 4 of which were identical with those in the Onderstepoort vaccine strain, but 2 of which were unique to XJ2. Since the immunogenicity of the H protein is closely related to the glycosylation sites, the variance of N-linked glycosylation sites and positions in H protein may make the genetic variant strain gain the capability to break through the protection derived from the Onderstepoort vaccine. Serological cross-immunity test

should be employed to investigate whether the N-linked glycosylation sites variant of H proteins affects the immunogenicity of CDV isolates from different geographic distribution. Our previous study showed that another virulent strain, CDV TN strain, had 8 N-linked glycosylation sites, all of which were also found in XJ2. However, XJ2 only shared 4 N-linked glycosylation sites with the attenuated Onderstepoort vaccine strain. Whether or not the N-linked glycosylation sites are related to the CDV virulence remains to be further investigated.

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