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Development of CDV-specific monoclonal antibodies for differentiation of variable epitopes of nucleocapsid protein

Zhenwei Bi[a,b], Yongshan Wang[a,b,*], Qunxing Pan[a,b], Xingxia Xia[a,b], Libo Xu[c]

[a] Institute of Veterinary Medicine, Jiangsu Academy of Agricultural Sciences, Key Laboratory of Veterinary Biological Engineering and Technology, Ministry of Agriculture, National Center for Engineering Research of Veterinary Bio-products, Nanjing 210014, China
[b] Jiangsu Co-innovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonoses, Yangzhou 225009, China
[c] Zhaoyuan Bureau of Animal Husbandry and Veterinary, Yantai 265400, China

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ABSTRACT

The highly contagious canine distemper viruses (CDVs) are still a major threat to a wide range of natural susceptible hosts. The nucleocapsid (N) protein plays various roles in the virus-induced immune response. But precise mapping of epitopes and antigenic variations in N protein of CDV are still scant. In this study, two monoclonal antibodies (MAbs), designated as F8N and G3N, against the N protein of CDV were generated and characterized. The epitopes recognized by the two MAbs were mapped by truncated N protein fragments expressed in *E.coli* based on western blotting. The 470ESRYDTQ476 and 385GITKEEAQL393 were identified as the minimal linear epitopes recognized by F8N and G3N, respectively. The amino acid residues of the epitope (385–393aa) were highly conserved in a variety of CDV strains from the databases as well as five CDV strains in this study, indicating that MAb G3N can detect various CDV strains. However, MAb F8N was found not to react with an older CDV 851 strain of the five CDV strains due to both of two amino substitution (S471P and Y473H) in the epitope, whereas either single mutant S471P or Y473H did not eliminate the binding of F8N. Further, the variable epitopes existed in the N protein of six CDV strains resembling CDV3 in phylogenic tree by alignment with sequences from the databases. This is the first record of a precise epitope affecting antigenity of N protein of CDV. These results may facilitate future investigations into the function of NP of CDV and diagnostic methods for CDV infection.

1. Introduction

Canine distemper (CD) is a highly contagious and fatal infectious disease that affects a wide variety of animal families including members of *Canidae*, *Felidae*, *Mustelidae*, *Procyonidae*, *Ursidae*, *Phocidae*, *Viverridae*, *Hyaenidae*, *Ailuridae*, *Mephitidae*, *Muridae*, *Cricetidae* and *Cercopithecidae* (Martinez-Gutierrez and Ruiz-Saenz, 2016). The disease course and pathogenesis is characterized by systemic respiratory and gastrointestinal signs such as dyspnea, vomiting and diarrhea as well as a high incidence of neurological complications. The infection of CDV leads to a profound immunosuppression, directly increasing the host's susceptibility to opportunistic infection and causing the high morbidity and mortality of CDV infection (Carvalho et al., 2012). In general, live-attenuated CDV vaccines are available and they efficiently induce protective immunity. However, CDV has a remarkable ability to cross species barriers, resulting in a broad and expanding host range, these vaccines have found to be insufficiently attenuated and unsuitability for many CDV-susceptible species (Buczkowski et al., 2014). Recently, CDV in the world canine population seems to have increased in the last decades, despite the fact that most of them had been vaccinated (Simon-Martinez et al., 2008). Genetic and antigenic variation has been observed between wild-type CDV and the vaccine strains, which may be one cause of failure in vaccine protection against CD (Bi et al., 2015b).

Canine distemper virus (CDV) belonging to the *Morbillivirus* genus within the *Paramyxoviridae* family contains a nonsegmented single-stranded negative-sense RNA genome that encodes six structural proteins (nucleocapsid N, martrix M, fusion F, hemagglutinin H, phospho-P and large-L proteins) and two nonstructural proteins (C and V proteins). The N gene contained 1683 nucleotides and harbored an ORF capable of encoding 523 amino acids, which was divided into three regions, the variable N-terminus (amino acids 17–159), the variable C-terminus (amino acids 408–519) and the highly conserved middle part (Stettler and Zurbriggen, 1995). The 1–400 N-terminal residues form a globular structure and that the C-terminal region appears as a tail that extends

* Corresponding author at: Institute of Veterinary Medicine, Jiangsu Academy of Agricultural Sciences, Key Laboratory of Veterinary Biological Engineering and Technology, Ministry of Agriculture, National Center for Engineering Research of Veterinary Bio-products, Nanjing, 210014, China.
E-mail addresses: bizhenwei@126.com (Z. Bi), wangys63@126.com (Y. Wang).

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from the surface of the globular body (Masuda et al., 2006). The positions 4–11 aa and 70–77 aa of N protein were identified to be the nuclear localization (NLS) and nuclear export (NES) signals contributing to the nucleocytoplasmic transport of N protein (Sato et al., 2006). Being the most abundant viral protein and highly immunogenic in nature, the N protein get exposed to the host immune system for the significant production of antibodies (Yuan et al., 2015) and specific cell-mediated immunity (Beauverger et al., 1993). CDV N is the main regulator of the virus replication. The viral genome is encapsidated by helical nucleocapsids formed by the N protein which protects it from nuclease activities. The RNA-N protein complex are assembled into ribonucleoprotein (RNP) with the L protein and its cofactor P protein, which acts as polymerase to synthesize and transcript RNA genome (Bankamp et al., 1996). The N protein plays multiple functions being concerned in CDV pathogenicity by interaction with various host systems. For example, N protein can form characteristic intranuclear inclusion bodies in CDV-infected cells, which is correlated with persistent viral infection (Sato et al., 2006). In the host natural immune response, it is reported that N protein blocks the IFN-stimulated nuclear translocation of ISGF3 and inhibits IFN signaling (Takayama et al., 2012).

The genetic and antigenic diversity of CDV can be demonstrated by phylogenetic analysis and monoclonal antibody analysis, respectively. In the last decade, the genetic diversity of CDV has been extensively studies by phylogenetic analysis, resulting in distinct CDV lineages of geographic origin known as America-1 (vaccines), America-2, Arctic, Asia-1, Asia-2, Asia-4, Europe-1/South America-1, Europe wild-life, Rockborn-like CDVs and Africa-1 based on the least conserved H gene in the CDV genome (Bi et al., 2015a). Less than H gene, only five CDV lineages including America-1, Asia-1, Asia-2, and Asia-3 and Europe were defined according to the phylogenic tree of N gene (Yuan et al., 2017), due to the highly conservation of N protein in all of viral protein. Though CDV are of one serotype, different CDV strains have been antigenically characterized and differentiated by MAbs in N, H, F and P genes (Giraudon and Wild, 1981; Orvell et al., 1985; Sugai et al., 2009). To date, MAbs against the N protein of CDV have been produced (Masuda et al., 2006; Yi et al., 2016; Yoshida et al., 1999). However, there is little knowledge about the epitopes mapping of CDV N protein, especially precise epitopes. It is therefore important for the characterization of epitopes in CDV N protein and investigation of the antigenic variations as this knowledge contributes on identifying which N protein regions that were conserved or variable between different viruses. Some antigenic sites of N protein were also found to be involved in virulent of CDV (Hamburger et al., 1991). The aim of this study was to generate CDV-specific monoclonal antibodies using the CDV N protein expressed in *Escherichia coli* as an immunogen, and to subsequently define the B-cell epitopes recognized by the MAbs.

2. Materials and methods

2.1. Virus strains and cells

The CDV851 strain have been isolated from dogs since the 1980s in China and cultured in Vero cells long-term in our laboratory. A Chinese isolate CDV NJ01 was isolated from a dog in 2011 and passaged in Vero cells, the GenBank accession number for its N gene is JF965338 (Zhen-Wei et al., 2011a). Three Chinese field isolate CDV NJ(12)1, NJ(12)3 and NJ(12)5 were recently isolated from infected and clinically sick dogs by using Vero cells expressing SLAM, and the H genes of them were characterized in our previous study (Bi et al., 2015a). The nucleotide sequence of N genes of CDV 851, NJ(12)1, NJ(12)3 and NJ(12)5 were listed in Supplement material 1. Vero cells expressing SLAM were cultured in DMEM (GIBCO) supplemented with 10% fetal calf serum (FCS) containing 0.1 mg/ml Zeocin (Invitrogen).

Table 1
Primers set for amplification of the overlapping and truncated segments of N gene of CDV NJ01.

Fragment	Primer sequence (5′–3′)
1–523aa	CCGGAATTCATGGCTAGCCTTCTCAAGAGCCTC (*Eco*RI) CCCAAGCTTTTAATTGAGTAGCTCTCTATC (*Hin*d III)
1–190aa	CCGGAATTCATGGCTAGCCTTCTCAAGAGCCTC (*Eco*RI) CCCAAGCTTTTAGTCGGCTGCAGTATCAGGAGC (*Hin*d III)
174–360aa	CCGGAATTCATTTGGATCCTGCTAGCTAAAGCGG (*Eco*RI) CCCAAGCTTTTAAGCTGGGTCAAAGTAAGATCGACCG (*Hin*d III)
348–523aa	CCGGAATTCGGAGGGTTAAATTTCGGTCGATCTTAC (*Eco*RI) CCCAAGCTTTTAATTGAGTAGCTCTCTATC (*Hin*d III)
348–456aa	CCGGAATTCGGAGGGTTAAATTTCGGTCGATCTTAC (*Eco*RI) CCCAAGCTTTTAAAGCCTTTCGTCACTGAAGTGAATGGGG (*Hin*d III)
440–523aa	CCGGAATTCGAAAACCAGGGAGGAGACAAATAC (*Eco*RI) CCCAAGCTTTTAATTGAGTAGCTCTCTATC (*Hin*d III)
440–485aa	CCGGAATTCGAAAACCAGGGAGGAGACAAATAC (*Eco*RI) CCCAAGCTTTTAATCGTCATTTCCATCATCTTGGAT (*Hin*d III)
469–523aa	CCGGAATTCTAATTTGGGTGTCATAGCGTGACTCACT (*Eco*RI) CCCAAGCTTTTAATTGAGTAGCTCTCTATC (*Hin*d III)
350–408aa	CCGGAATTCTTAAATTTCGGTCGATCTTACTTTG (*Eco*RI) CCCAAGCTTTTATCGGATTGTCCGGTCCTCTGTT (*Hin*d III)
380–440aa	CCGGAATTCCTTGCCGCCGAGCTTGGCATCAC (*Eco*RI) CCCAAGCTTTTATTCGGACCTCTTGTTGATGGTTGGG (*Hin*d III)

2.2. Expression of full-length N protein in E.coli and insect cells

The recombinant CDV N protein as antigen for immunization of mice was produced using a prokaryotic expression system (Zhen-Wei et al., 2011b). Briefely, the full length N gene (GenBank accession: JF965338) of CDV NJ01 was amplified by PCR and subcloned in frame into expression vector pET-28a (+) with *Eco*RI and *Hin*d III. The recombinant expression vector pET-N was transformed into *E. coli* Rosetta (DE3) for the expression of N gene. After induction with 1 mM IPTG, the recombinant N protein was purified using His-Bind Purification Kit (Novagen, Germany) according to the manufacturer's instructions. The recombinant N protein was analyzed by SDS-PAGE and Western blot using CDV-positive mouse sera as the primary antibody with an Horseradish Peroxidase (HRP)-conjugated goat anti-mouse secondary antibody. In addition, the *Eco*R I and *Hin*d III fragment of the full-length N gene of CDV NJ01 was also subcloned into the same sites of the donor plasmid pFastBacHTA (Invitrogen) to generate the recombinant donor plasmid pFastBac-N, then was transformed into *E.coli* DH10Bac bacterial cells (Invitrogen) for transposition of N gene into the genetically modified baculovirus (*Autographa californica*) genome (bacmid). The recombinant bacmid DNA, designated as rBacmid-N, was isolated using the BAC/PAC DNA Kit (OMEGA) and transfected into *Spodoferafrigiperda* (Sf9) insect cells with Cellfectin® Reagent (Invitrogen) for viral particles formation. The generated recombiant baculovirus was used to express N protein for antigen production by infecting Sf9 insect cells as described previously (Zhen-Wei et al., 2011a).

2.3. Production and identification of MAbs against the N protein

BALB/c mice were injected intraperitoneally with 50 μg of the purified recombinant N protein (*E.coli*), mixed with complete Freund's adjuvant (Sigma). Two boosts were given at days 14 and 28 with 50 μg of the purified N protein with incomplete Freund's adjuvant (Sigma). Three days the last boost with 100 μg of the purified N protein, the splenocytes of the best responder animals were fused with myeloma cells SP2/0 using polyethylene glycol 4000 (Merck). The hybridomas were selected in RPMI 1640 medium supplemented with hypoxanthine, aminopterin and thymidine (HAT) (Sigma). Positive clones were screened by indirect ELISA using the recombinant N protein produced in Sf9 cells and stabilized successively 3–5 times by limiting dilution. After three times of subcloning, the hybridomas producing MAbs were established and charactered. Mice ascites were generated by injecting