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# Development and Characterization of Canine Distemper Virus Monoclonal Antibodies

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Five canine distemper virus monoclonal antibodies were developed by immunizing BALB/c mice with a traditional vaccine strain Snyder Hill. Among these monoclonal antibodies, four antibodies recognized both field and vaccine strains of canine distemper virus without neutralizing ability. One monoclonal antibody, 1A4, against hemagglutinin protein of canine distemper virus was found to react only with vaccine strain virus but not field isolates, and showed neutralizing activity to vaccine strain virus. These monoclonal antibodies could be very useful tools in the study of the pathogenesis of canine distemper virus and the development of diagnostic reagents.

**Keywords:** canine distemper virus, monoclonal antibody, neutralizing antibody

## Introduction

Canine distemper virus (CDV) is a member of the *Morbillivirus* subgroup of *Paramyxoviridae* and can cause serious multisystem disease in Canidae and other Carnivore.[1] CDV has a single-stranded, nonsegmented, negative-sense RNA genome of 15,960 nucleotides that comprise six genes arranged in invariant order: N-P-M-F-H-L. The virus RNA is enclosed in a helical nucleocapsid formed by N protein. The mature ribonucleoprotein complexes also contain copies of P and L proteins. The host cell-derived lipid envelope is spiked with two transmembrane glycoproteins, H and F proteins. The extracellular domains of those glycoproteins are likely to induce protective antibody responses. In addition, the envelope is stabilized by a layer of M protein, which is the most abundant viral protein in infected cells.[2,3]

The first attenuated live vaccine of CDV was developed in the late 1950s. The cell culture adapted strains were named as Snyder Hill and Onderspoort and are available for use worldwide as live strains against CDV infection in dogs and other carnivores.[4–7] CDV vaccines have significantly reduced the incidence of disease, but infection of vaccinated dogs, mink, foxes, and raccoon dogs with CDV was frequently reported.[7–10] The antigenic shift or neutralizing epitope changes in the field CDV strains may be one of the major reasons making the current vaccines less protective. Therefore, it is very important to generate monoclonal antibodies (MAbs) with different neutralizing activities between vaccine strains and recurrent field strains for CDV antigenic alteration studies.

## Materials and Methods

### *Cell and viruses*

Sp20 myelomas were cultured in RPMI-1640 medium with 10% fetal bovine serum (FBS) (Hyclone, South Logan, UT). Vero cells and Vero/DogSLAM cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 2% FBS. Two strains of CDV isolates, HL001 and HNly150520B were used as field strains. These field strain CDVs were cultured and passaged three times in Vero/DogSLAM cells. The GenBank accession number for these two field strains are KX709880 and KU030833, respectively. Onderstepoort and Snyder Hill were cultured in Vero cells and used as vaccine strains in this study.

### *Mice immunization and MAb preparation*

Vero cells infected with CDV Snyder Hill strain were frozen and thawed when cells showed 80% cytopathic effect (CPE), then sonicated and clarified by centrifugation at 6000 rpm to prepare immunizing antigens. Seven-week-old female BALB/c mice were immunized with CDV antigen mixed with complete Freund's adjuvant. Two booster immunizations with the same CDV antigen plus incomplete Freund's adjuvant were administered on days 14 and 28. Three days after the last boost, the mice were euthanized and

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Table 1. Primers Used for Polymerase Chain Reaction Amplification

| *Name* | *Primer sequences* |
|---|---|
| CDVN-*Eco*RI-F | 5′-CCGGAATTCCAAAGTTGGCTAAGGAT-3′ |
| CDVN-*Xho*I-R | 5′-CCGCTCGAGCTGCCATTTAGAGATAGAAT-3′ |
| CDVP-*Eco*RI-F | 5′-CCGGAATTCCCCGATCAACCATTCA-3′ |
| CDVP-*Xho*I-R | 5′-CCGCTCGAGGTGAAAGCAGTTTTGAGCCT-3′ |
| CDVM-*Xho*I-F | 5′-CCGCTCGAGAAAACTGCTTTCACTATCGC-3′ |
| CDVM-*Bgl*II-R | 5′-GGAAGATCTCAGGAGCAACACCGAGACAA-3′ |
| CDVH-*Xho*I-F | 5′-CCGCTCGAGTTAGGGCTCAGGTAGTCC-3′ |
| CDVH-*Bgl*II-R | 5′-GGAAGATCTTTTAATTCAATCGTCGGTAA-3′ |
| CDVF-*Xho*I-F | 5′-CCGCTCGAGAGCCCCATGCACAGGGGAAT-3′ |
| CDVF-*Bgl*II-R | 5′-GGAAGATCTTTGGGCGGGGTCAATAGATTT-3′ |

The underlined letters are corresponding restriction enzyme sites.



spleenocytes were harvested for fusion with sp20 myelomas using polyethylene glycol 4000. Positive hybridoma clones were shown by immunofluorescence assay (IFA), cloned by the limiting dilution method, and inoculated intraperitoneally into pristane-primed BALB/c mice. The MAb isotypes were determined using a mouse monoclonal antibody isotyping kit (Sigma, St. Louis, MO) according to the manufacturer's instructions.

### *Virus neutralization assay*

The neutralization activities of MAbs were identified as previously described.[11] Serial twofold dilutions (starting from 1/2) of the purified ascites of MAbs or the mock medium (negative control) in duplicate were prepared in DMEM and then mixed with an equal volume of 100 $TCID_{50}$/mL of each CDV virus (Snyder Hill and HL001). After incubation for 1 hour at 37°C in a humidified $CO_2$ atmosphere, the MAb-virus mixture was shaken up and down every 15 minutes. Subsequently, the mixture was inoculated onto Vero cells (vaccine strain) or Vero/Dog SLAM cells (field strain) in 96-well microplates. The CPE was observed for 5–10 days and VN antibody titers were expressed as the reciprocal of the highest plasma dilution giving complete protection.

### *Immunoperoxidase monolayer assay*

CDV-infected cells in a 24-well plate were fixed with 80% cold acetone for 10 minutes at 4°C, and incubated with a 1:400 dilution ascitic fluid specifically against CDV at 37°C for 1 hour. After three washes with phosphate-buffered saline (PBS), the cells were incubated for 30 minutes at 37°C with HRP-labeled goat anti-mouse IgG (Biomedical Technologies Inc., Madrid, Spain) diluted 1:500 in PBS. The wells were washed three times with PBS, followed by incubation for 4–5 minutes at room temperature in diaminobenzidine solution (ZSGB-BIO, Beijing, China). Cell staining was examined by using a light microscope.

### *Transient expression of CDV proteins*

The field strain CDV (HL001) and vaccine strain (Onderstepoort) were lysed with reagent for total RNA extraction, respectively. Total RNAs were isolated and were reverse-transcribed with SuperScript III Reverse Transcriptase (Invitrogen, Waltham, MA). To construct a mammalian expression plasmid encoding entire N, P, M, H, F proteins, full-length N/P/M/H/F cDNA were amplified by polymerase chain reaction using CDV HL001 or Onderstepoort cDNA as templates, Ex-Taq DNA polymerase (TAKARA, Dalian, China) and primer pairs with restriction sites are shown in Table 1. Each individual full-length gene was then inserted into pCAGGS mammalian expression vector to generate pCAG-OnderN or pCAG-HL001N, pCAG-OnderP or pCAG-HL001P, pCAG-OnderM or pCAG-HL001M, pCAG-OnderF or pCAG-HL001F, pCAG-OnderH or pCAG-HL001H plasmids.

CHO cells were seeded into 12-well plates and grown until semi-confluent, and 0.8 μg of each individual plasmid DNA was transfected using lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 36 hours, cells were fixed with acetone and analyzed by indirect IFA as described below.

### *Immunofluorescence assay*

The cells were fixed with acetone for 30 minutes at room temperature, followed by incubation in a 1:1000 dilution of the ascitic fluids for 1 hour at 37°C. After washing in PBS, cells were incubated in a 1:500 dilution of fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (Sigma) for 1 hour at 37°C. After washing in PBS, fluorescence was observed with a fluorescent microscope (Olympus).

## Results

### *Production of MAbs against CDV Snyder Hill*

To obtain MAbs against CDV, mice were immunized with CDV Snyder Hill strain. The hybridoma cells were generated and screened as described above. Five MAbs (1A4, 1G5,

Table 2. Characterization of Monoclonal Antibodies by Neutralizing Assay and Immunofluorescence Assay

| | | *Neutralizing activity* | | *IFA* | |
|---|---|---|---|---|---|
| *MAb* | *Isotype* | *Wild-CDV* | *Vaccine-CDV* | *Wild-CDV* | *Vaccine-CDV* |
| 1A4 | IgG2b | <1:2 | 1:64 | − | + |
| 1G5 | IgG1 | <1:2 | <1:2 | + | + |
| 2H11 | IgG1 | <1:2 | <1:2 | + | + |
| 6D9 | IgG1 | <1:2 | <1:2 | + | + |
| 6E11 | IgG1 | <1:2 | <1:2 | + | + |

CDV, canine distemper virus; IFA, immunofluorescence assay.

2H11, 6D9, and 6E11) against CDV Snyder Hill strain detected by IFA were generated and expanded for further characterization. Isotype determination revealed that 1A4 was subclass IgG2b, whereas MAbs 1G5, 6D9, 6E11, and 2H11 were subclass IgG1 (Table 2).

### *Reactivity of CDV MAbs with CDV field and vaccine strains*

Immunoperoxidase monolayer assay was performed to determine whether five MAbs recognized both CDV vaccine strains and wild strains. The CDV vaccine strains (Onderstepoort and Snyder Hill) and field strains (HL001, HNly150520B) were inoculated onto monolayer cultures of Vero cells or Vero/Dog SLAM cells, respectively. Cells that had developed CPE were acetone-fixed and reacted with supernatants of hybridomas followed by HRP-conjugated goat anti-mouse antibody. Four MAbs (1G5, 6D9, 6E11, and 2H11) showed strong reactivity with Vero cells infected with CDV vaccine strain and Vero/Dog SLAM cells infected with CDV field strain (Fig. 1). Whereas, 1A4 MAb only reacted with CDV vaccine strain, but not to field strains (Fig. 1).

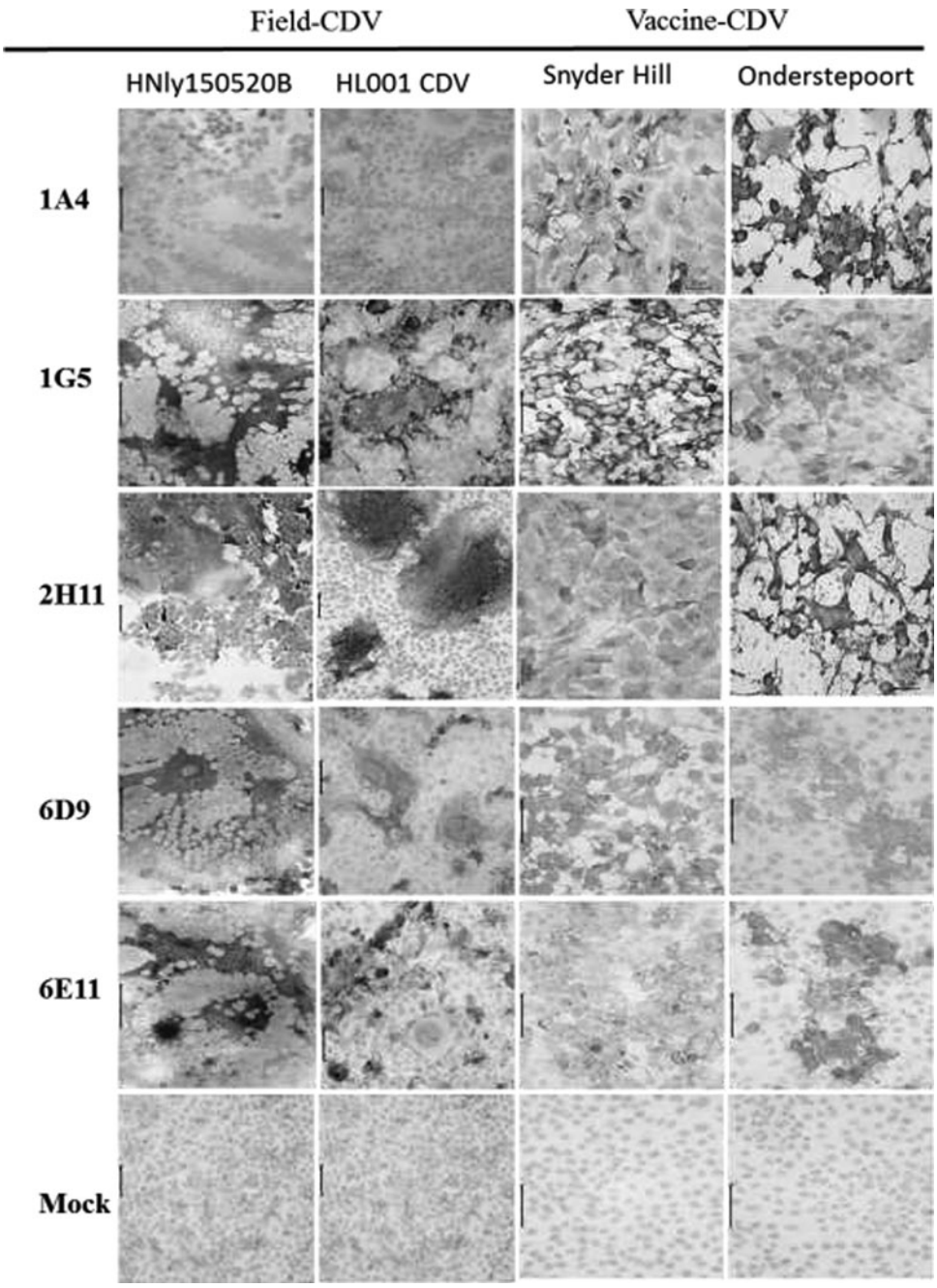


**FIG. 1.** Reactivity of MAbs with CDV field (HL001 and HNly150520B) or vaccine strains (Onderstepoort and Snyder Hill) by IPMA. Magnification ×200. CDV, canine distemper virus; IPMA, immunoperoxidase monolayer assay; MAbs, monoclonal antibodies.

*Virus proteins recognized by each MAbs*

CHO cells transfected with the recombinant plasmids pCAGGS-N/P/M/H/F from vaccine strain (Snyder Hill) and wild strain (HL001 strain) were used to analyze the MAbs by IFA. The results showed that 1A4 MAbs reacted with recombinant H protein of vaccine strain (Synder Hill), but not with H protein of field strain (HL001). 1G5 and 6E11 MAbs reacted with F protein from vaccine and field strain CDV. 6D9 recognized N protein from vaccine and wild strain CDV. 2H11 reacted to P protein of vaccine and wild strain CDV. There were no positive signals in normal CHO cells or CHO cells transfected with pCAGGS (Fig. 2).

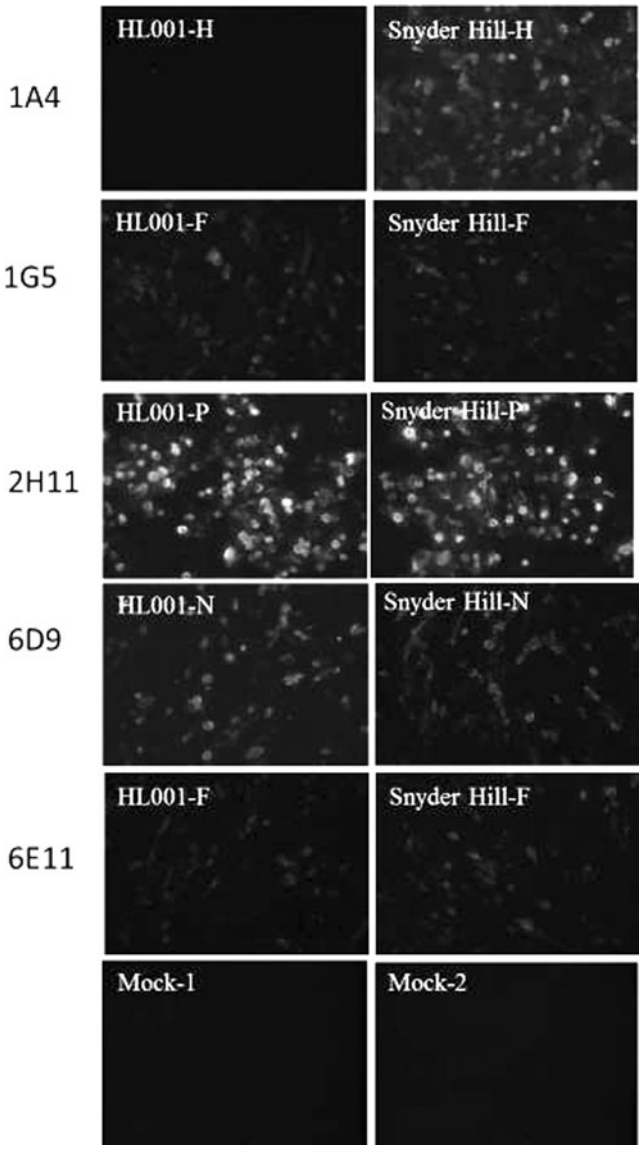


**FIG. 2.** Reactivity of MAbs with recombinant CDV N, P, H, and F proteins by immunofluorescence assay. The cells were transfected with each recombinant plasmid. At 48 hours post-transfection, the cells were fixed and reacted with five MAbs individually by immunofluorescence assay. Mock1, CHO cells transfected with the empty vector pCAGGS; Mock2, Normal CHO cells. Magnification × 200.

*Virus neutralizing abilities of each MAb*

Four MAbs (1G5, 2H11, 6D9, and 6E11) exhibited no neutralization activities against both CDV vaccine strain (Snyder Hill) and wild strain (HL001) (Table 2). By contrast, 1A4 against CDV-H showed neutralization activity to Snyder Hill and Onderspoort strain but not HL001 and HNly150520B.

## Discussion

The field strains of CDV are antigenically and phylogenetically distinct from vaccine strains by the phylogenetic analysis based on H proteins.[10,12,13] Recent studies have reported that 16 CDV field strains were isolated from vaccinated minks, foxes, and raccoon dogs in China between 2011 and 2013 that indicated the insufficient protection of current vaccines.[10] Therefore, it will be useful to develop CDV MAbs that could differentiate the field strains from vaccine strains. In this study, five CDV MAbs were successfully developed. Among them, 1A4 only reacted with CDV vaccine strain but not with field strain, which could work as a good antibody candidate to develop ELISA for virus screening.

1A4 monoclonal antibody with neutralizing activity was tested to recognize CDV H protein. Field CDV isolates (HL001and HNly150520B) only shared 89.3%–90.6% similarity with vaccine strains (Onderstepoort and Snyder Hill) based on H protein. The H protein of field CDV has undergone mutations that lead to additional potential N-glycosylation sites other than vaccine strains (Onderstepoort and Snyder Hill).[14–17] The change of N-glycosylation in H protein has been previously reported to compromise current vaccine efficacy and to be associated with the increased virulence.[14] Since 1A4 could only react with vaccine strains but not with field strains of CDV, the results indicate that a neutralizing region recognized by 1A4 in H protein had altered in current circulating field CDV strains.

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## Author Disclosure Statement

No competing financial interests exist.

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