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# Development and Characterization of Monoclonal Antibodies Directed Against The Canine Parainfluenza Virus Protein

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## ABSTRACT

Four monoclonal antibodies against NP protein of canine parainfluenza virus were developed by immunizing BALB/c mice with an isolate of HeN0718. Immunofluorescence assay showed that all MAbs recognize NP protein, but did not react with M, SH, HN and P protein. Neutralization assays showed that all MAbs failed to neutralize CPIV virus. These newly established MAbs could be useful tools in diagnostic assays for CPIV.

## INTRODUCTION

Paramyxoviridae is a family of enveloped non-segmented single strand RNA viruses (NNSVs), including parainfluenza viruses 1-5 (PIV1-5), respiratory syncytial virus and measles virus [1]. This family of viruses is associated with a broad range of diseases in humans and animals, some of which lead to fatal infections [2,3].

Canine Parainfluenza Virus (CPIV) is a paramyxovirus, also known as parainfluenza virus 5 (PIV5) [4]. To date, CPIV has been found in several species, such as humans, monkeys, pigs, cats and rodents [5]. The genome of CPIV was reported to be 15,246 nucleotides in length, encoding 8 proteins [6], including Nucleocapsid Protein (NP), Phosphoprotein (P), V protein (V), Matrix protein (M), Fusion protein (F), Small Hydrophobic protein (SH), Hemagglutinin-Neuraminidase protein (HN) and Large protein (L) [7]. Paramyxoviruses infect host cells via the coordinated action of receptor binding protein (HN, H or G) and Fusion protein (F) on the viral surface [8]. HeN0718 is a CPIV strain, identified to undergo multiple nucleotide mutations in the SH gene, which led to a frame shift in the open reading frame [7].

Neutralizing Antibodies (NAbs) play a crucial role in providing lifelong protection against viral infections. Successful vaccines against viruses induce the production of NAbs. Neutralization by antibodies can be mediated by a number of different mechanisms including binding to receptors on viruses resulting in blocking of attachment to the host cells [9,10]. However, due to the fact that the antigenic targets on viruses vary greatly over time, often as a result of pressure exerted by host immune systems [11], new vaccines are essential to induce a broad range of NAbs to counteract highly antigenic viruses. As the diagnostic assays, RT-PCR was commonly used [12]. Immunohistochemistry (IHC) has been used previously to detect the CPIV

[13]. Now the product for rapid detection of clinical was seldom. The cost of laboratory diagnosis is high, and it takes a long time. Thus, it is urgent to need Rapid diagnostic product for CPIV. In this study, we describe the development and characterization of four MAbs, designated 2B7 (IgG1), 2B8 (IgG1), 4H1 (IgG1) and 6F10 (IgG2b), directed against the CPIV HeN0718 strain proteins.

## MATERIALS AND METHODS

### Cells and virus

Cell culture was carried out using aseptic technique in a class II safety cabinet. SP20 myeloma cells were cultured in RPMI1640 supplemented with 10% fetal bovine serum (FCS; Hyclone, South Logan, UT), Vero cells in Dulbecco's modified Eagle medium (DMEM, Gibco, USA) supplemented with 2% FCS. The HeN0718strainof CPIV (GenBank accession number KY114804 [7].) was propagated in cultured Vero cells.

### Mice immunization and MAbpreparation

Mice immunization and MAbs preparation were carried out as previously described (14). Vero cells infected with CPIV HeN0718 were frozen and thawed when cells showed ~80% Cytopathic Effect (CPE). Thawed cells were then sonicated and clarified by centrifugation at3000rpm to prepare the antigens for immunization. Seven week old female BALB/c mice were immunized with CPIV antigen mixed with complete Freund's adjuvant. Two booster immunizations with the same CPIV antigen plus incomplete Freund's adjuvant were administered on days 14 and 28.Three days after the last boost, the mice were euthanized and the splenocytes were harvested for fusion with SP20 myelomas using polyethylene glycol 4000. The hybridoma cells were incubated in 96-well plates at 37°C with 5% CO2 in HAT screening culture medium. Positive hybridoma clones were shown by Immunofluorescence Assay (IFA), cloned by the limiting dilution method at least three times, 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.

### Antibody titers of indirect immunofluorescence

The titers of the MAbs were also detected by IFA. In brief, Vero cells were infected with CPIV HeN0718 and cultured in DMEM at 37°C for 24 h until CPE appeared. The cells were fixed in cold acetone for 30 min after washed once with PBS. MAbs were serially diluted (starting from 1:800), then added to the cell well for 1h at 37°C. After washing three times with PBS, cells were incubated with Fluorescein Isothiocyanate (FITC)-conjugated anti-mouse IgG antibody (Sigma) for 45min followed by three washes with PBS.

### Virus neutralization assay

The virus neutralization activities were identified as previously described [14]. Serial two fold dilutions (starting from 1:2) in DMEM were prepared from the purified ascites contained MAbs or the mock medium (negative control) were. These dilutions were then mixed with an equal volume of 100 $TCID_{50}$/ml of CPIV viruses. After incubation for 1 hour at 37°C in a humidified $CO_2$ atmosphere, the MAb-virus mixture was gently mixed by inversion.Subsequently, the mixture was inoculated onto Vero cells in 96-well microplates. The CPE was observed for 5–6 days and VN antibody titers were expressed as the reciprocal of the highest plasma dilution giving complete protection. The measure was repeated three times.

### Expression of CPIV proteins

Total RNAs were isolated and were reverse-transcribed using SuperScript III Reverse Transcriptase (Invitrogen, Waltham, MA). To construct a mammalian expression plasmid encoding entire NP, P, M, SH, HN proteins, full length NP/P/M/H/F cDNAs were amplified by polymerase chain reaction using CPIVHeN0718strain 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-NP, pCAG-P, pCAG-M, pCAG-SH and pCAG-HN plasmids, respectively.

Vero cells were seeded into 12-well plates and grown until semi-confluent, 0.8 mg of each individual plasmid DNA was then 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.

### Indirect immunofluorescence assay (Ifa)

CPIV protein expressing Vero cells were fixed with acetone for 30 min at room temperature, followed by blocking with MAbs in a 1:1000 dilution for 1 hour at 37°C. Fixed cells were then washed in PBS, and then incubated in a 1:500 dilution of

Fluorescein Isothiocyanate (FITC)-conjugated anti-mouse IgG antibody (Sigma) for 45min at room temperature. Vero cells transfected with empty vector (pCAGGS) and non-transfected Verocells were used as negative controls. Reactivity of the MAbs to transfected Vero cells was observed using a fluorescent microscope (Olympus).

Table 1: Primers used for PCR amplification.

| Name | Primer sequences |
|---|---|
| CPIV-M-Xho I-F | 5'-GGCTCGAGATGCCATCCATCAGCATCC-3' |
| CPIV-M-Bgl II-R | 5'-CCAGATCTTCATTCCAGCTTCGTCAGG-3' |
| CPIV-NP-EcoR I-F | 5'-GGGGAATTCATGTCATSCGTSCTCAAGGC-3' |
| CPIV-NP-Xho I-R | 5'-CACTCGAGCTAGWTGYCGRGATCGCC-3' |
| CPIV-HN-EcoR I-F | 5'-GGGGAATTCATGGTTGSAGAAGATGCCC-3' |
| CPIV-HN-Xho I-R | 5'-GGCTCGAGTTAGSATAGTSTCACCTGACG-3' |
| CPIV-P-Xho I-F | 5'-GGCTCGAGATGSATCCCACTGATTTGAG-3' |
| CPIV-P-Bgl II-R | 5'-GGGCCAGATCTCTASATTGTACTGCGGATG-3' |
| CPIV-SH-EcoR I-F | 5'-GGGGAATTCATGCTGSCCSATCCGGAAGATC-3' |
| CPIV-SH-Xho I-R | 5'-GGCTCGAGTTATGGCAAGTGWTGGGAC-3' |

**Measurement of relative affinity of MAbs**

The relative affinity of 2B7, 2B8, 4H1 and 6F10 were determined by ELISA.

Briefly, polystyrene microtiter plates were coated with purified virus (CPIV HeN0718) at 4°C overnight. 150 µl 5% skim milk (BD Difco) in PBST was added to plates for 2h at 37°C. 100 µl of 1:5000 dilution MAbs or SP2/0 cells ascites were mixed with 100 µl double gradient dilution of CPIV HeN0718 in PBS 10649.6,5324.8,2662.4,1331.2,656.6,332.8, 83.2,41.6,20.8,10.4,5.2,2.6 ng/ml and 0 ng/ml) at 4°C for 24 h. 100 µl of this mix was added into the wells of microtiter plates for 1h at 37°C. After washing three times with PBST, HRP-conjugated goat anti-mouse (1:10000 dilution) was added to the wells at 37°C for 30 minutes. Then washing three times with PBST, the substrate solution (0.2 mg/mL of TMB and 0.2% H2O2 in 0.05 mol/L citrate buffer, pH 4.6) was added, and the colorimetric reaction was carried out for 15 minutes at room temperature away from light. And then 50 µl of 2 mol/L sulfuric acid was used to stop the reaction. The value of Optical Density (OD) was measured at 450 nm. The reactivity between antigen and antibody was evaluated by A0/Ai=1+Ka×Ii, A0 was OD of the well of 0 ng/ml, Ai was OD of the well of 13800 ng/ml, Ii was $9.2\times10^{-8}$ mol/L.

## RESULTS

**Preparation of Anti-CPIV protein MAbs**

To obtain MAbs against CPIV, mice were immunized with CPIV HeN0718 strain. The hybridomas were generated and screened as described above.Four MAbs (2B7, 2B8, 4H1 and 6F10) against CPIV were obtained in this study. Isotype determination revealed that 2B7, 2B8 and 4H1 were subclass IgG1, whereas 6F10 was subclass IgG2b (Table 2).

Table 2: Antibody titers of mAbs to CPIV (HeN0718 strain) by different methods.

| MAb | Isotype | Neutralizing activity | IFA |
|---|---|---|---|
| 2B7 | IgG1 | <1:2 | 1:12800 |
| 2B8 | IgG1 | <1:2 | 1:3200 |
| 4H1 | IgG1 | <1:2 | 1:6400 |
| 6F10 | IgG2b | <1:2 | 1:12800 |

**Antibody titers of IFA**

The antibody titers of IFA of 4 MAbs were measured. The antibody titers of IFA where the highest dilution of positive result of MAbs. As shown in Table 2, the highest titers of MAbs 2B7 and 6F10 were, 1:12800, and the lowest titer was 1:3200.

**Neutralizing activity of anti-CPIV MAbs**

The results of neutralization tests are shown in Table 2. Serum samples were serially diluted (starting from 1:2), and mixed with CPIV virus (HeN0718 strain). CPE were observed in all wells, indicating that all MAbs exhibited no neutralization activities against the CPIV (Table 2).

**Virus proteins recognized by each MAbs**

Vero cells transfected with the recombinant plasmids pCAG-NP, pCAG-M, pCAG-SH, pCAG-HN and pCAG-P were used to analyse the MAbs by IFA. The results showed that all four MAbs only reacted with NP protein but failed to bind to any other CPIV proteins (Figure 1).

**Determination of relative affinity of Mab**

Four MAbs and SP2/0 were used to determine the relative affinity. As shown in (Table 3), the relative affinity of 2B7, 2B8, 4H1 and 6F10 were $8.7\times10^{-9}$, $2.5\times10^{-9}$, $7.4\times10^{-9}$, $7.4\times10^{-}$

$^{9}$mol/L. The 2B7 and 4H1 had a higher relative affinity. While the SP2/0 ascites did not react with the virus.

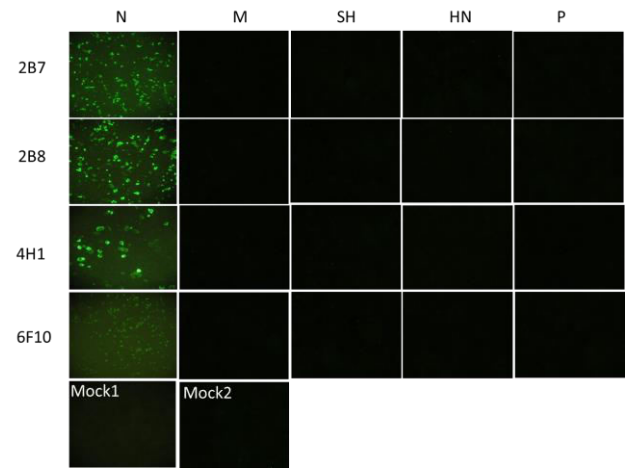



Figure 1: Reactivity of anti-CPIV mAbs to transfected cells.

Four mAbs (2B7, 2B8, 4H1 and 6F10) were incubated with transfected Vero cells expressing CPIV-NP, -M, -SH, -HN and -P proteins. MAb bindings to cells were detected with FITC-labelled rabbit anti-mouse IgG antibody. Mock1, Verocells transfected with the empty vector pCAGGS; Mock2, Normal Vero cells.

**Table 3:** Results of relative affinity of monoclonal antibodies.

| MAb | $OD_{450}$ | | relative affinity Ka(mol/L) |
|---|---|---|---|
| | 0ng/ml | 10649.6ng/ml | |
| 2B7 | 2.898 | 0.321 | $8.7\times10^{-9}$ |
| 2B8 | 2.925 | 0.882 | $2.5\times10^{-9}$ |
| 4H1 | 2.852 | 0.364 | $7.4\times10^{-9}$ |
| 6F10 | 3.144 | 0.923 | $2.6\times10^{-9}$ |

## DISCUSSION

The paramyxovirus NP protein is a component of a viral Ribonucleoprotein (RNP) complex that forms the template for viral transcription and replication, and has been shown to be important for Viral replication [6,15-16]. NP can also induce protective immunity especially at the late stage of the challenge infection [17]. In this study, four MAbsagainst the NP protein of HeN0718 isolate were generated and characterized. Immunofluorescence and neutralization assays were used to assess the reactions of four MAbs with the CPIV-HeN0718 and CPIV-NP, M, SH, HN and P proteins. The results show that,in an immunofluorescence assay, all MAbs reacted with Vero cells transfected with pCAG-NP, but did not reacted with any other CPIV proteins. Furthermore, all MAbs failed to neutralize the CPIV virus (HeN0718 strain). This result is compatible with a previous study on NP protein of Sendai virus, another member of the paramyxovirus family,which showed no virus-neutralizing activity even in the presence of complement [17]. The antibody titers of IFA of four MAbs were also measured and the result showed that the MAbs had good reactivity with CPIV. Therefore, four MAbs could be a useful tool to detected CPIV by IFA.

Since the NP gene is highly conserved between strains of CPIV and is often used for detection of CPIV, these broadly reactive MAbscould prove to be potentially very useful for diagnostic applications of CPIV.

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