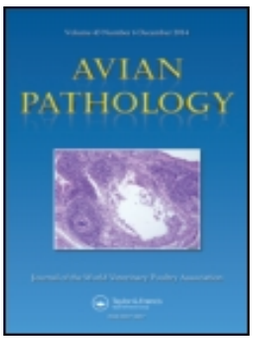

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Identification of a common conserved neutralizing linear B-cell epitope in the VP3 protein of waterfowl parvoviruses

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ABSTRACT

Waterfowl parvoviruses (WPVs) including goose parvovirus (GPV), novel goose parvovirus-related virus (NGPV) and Muscovy duck parvovirus (MDPV) cause significant economic losses and epizootic threat to the waterfowl industries and little is known about the B-cell epitope of WPVs. In this study, a monoclonal antibody (mAb) 5B5 against the VP3 protein of NGPV was used to identify the possible epitope in the three kinds of WPVs. The mAb 5B5 had neutralizing activities to the three viruses, and reacted with the conserved linear B-cell epitopes of ^{438}LHNPPP^{443} in VP3 protein of GPV, NGPV and MDPV. To the author's best knowledge, this appears to be the first report on identification of the common conserved neutralizing linear B-cell epitope on VP3 protein of three different WPVs, which would facilitate the development of a novel immunodiagnostic assay for rapid detection of WPVs infection.

Keywords

Goose parvovirus; novel goose parvovirus-related virus; Muscovy duck parvovirus; monoclonal antibody; VP3 protein; epitope

Introduction

Waterfowl parvoviruses (WPVs) are classified into goose parvovirus (GPV), novel parvovirus-related virus (NGPV) and Muscovy duck parvovirus (MDPV) (Chang et al., 2000; Poonia et al., 2006; Li et al., 2017). However, there are significant differences between the three WPVs in the infection period and the infected host. GPV infects both goslings and Muscovy ducklings (Francois et al., 2016), causing Derzsy's disease with high mortality and morbidity (Glávits et al., 2005; Jansson et al., 2007; Yu et al., 2012; Yu et al., 2016). MDPV infects Muscovy ducklings only, while NGPV infects Cherry Valley ducks and mule ducks and illustrates a high rate of infection with low mortality (Li et al, 2018a). Since 2015, Short beak and dwarfism syndrome (SBDS) caused by NGPV leads to serious economic losses to waterfowl industry in East China (Chen et al., 2015; Wan et al., 2019; Chen et al., 2016; Xiao et al., 2017; Li et al., 2018b) and is a major concern to waterfowl industries.

After serial experiments, phylogenetic and gene alignment analyses, the etiological agent of SBDS was confirmed to be a variant of GPV designed as NGPV (Bian et al., 2019). The NGPV shares more than 92.2% nucleotide similarity to GPV genome (Chen et al., 2015; Ning et al., 2017; Li et al., 2018a). As a GPV-derived virus, NGPV belongs to *Anseriform dependoparvovirus 1* (Li et al., 2016). It contains a small non-enveloped, linear single-stranded DNA genome of approximately 5.1kb in length (Deng et al., 2014) with two open reading frames (ORFs), designed as left ORF and right ORF (Ji et al., 2010; Shen et al., 2015). The left ORF encodes two nonstructural proteins (NS1 and NS2), involving in viral replication and regulatory functions, while the right ORF encodes three capsid proteins (VP1, VP2 and VP3) which are reported to be virulence determinants and immunogenic proteins playing important roles in pathogenesis (Zádori et al., 1995; Li et al., 2017). It is noteworthy that VP3 is the most variable and abundant of the three capsid proteins, inducing

neutralizing antibodies and provides protective immunity to waterfowl against WPVs infection (Le Gall-Reculé and Jestin, 1994; Ju et al., 2011).

To date, biosecurity measures and vaccination are effective means to prevent WPVs infection in waterfowl. In the prophylaxis, live attenuated or inactivated vaccines are being widely used (Kisary et al., 1978; Fournier and Gaudry, 1992). Although live attenuated vaccines confer immunity, but it has a drawback of reverting back to virulence and spread infection to susceptible unprotected flocks. On the other hand, the inactivated vaccines were found to have suboptimal cell mediated immunity. Therefore, identifying novel diagnostic antigens and alternate vaccine candidates are crucial for prompt diagnosis and prevention of disease.

The mechanisms of virus evolution and host adaption are not completely understood till date (Parrish et al., 2008). In spite of the fact that the molecular and biochemical features of WPVs have been well characterized, less is known about their antigenic structure. Monoclonal antibodies (mAbs) have become vigorous tools for detecting epitope and designing specific diagnostic protocol owing to the characteristics, such as high diagnostic specificity, reproducibility and ease in bulk production of mAbs. In this study, we identified a highly conserved neutralizing linear B-cell epitope within the VP3 protein of three WPVs, and characterized using mAb-5B5. This study not only helps to explore the antigenic structure of B-cell linear epitope, but also delivers a rapid immunodiagnostic assay utilizing the developed B-cell linear epitope and mAb-5B5.

Materials and methods

Viruses, cells and antibodies

NGPV virulent strain SDLY1602 (GenBank accession no. MF441222) was isolated in Shandong province of China in 2016 (Li et al., 2018a). GPV strain AH1101, MDPV strain JS1206, duck hepatitis A virus type 1 (DHAV-1) strain LY0801 (accession no. FJ436047), duck hepatitis A virus

type 3 (DHAV-3) strain SD1101 (accession no. JQ409566), avian influenza virus (AIV/H9N2), Newcastle disease virus (NDV), duck plague virus (DPV), Tembusu virus (DTMUV), and duck reovirus (DRV) were maintained in our laboratory. The VP3 gene of NGPV SDLY1602 strain was cloned into the prokaryotic expression vectors pET-32a (+) (Novagen, Darmstadt, Germany) and pGEX-6p-1 (Novagen, Darmstadt, Germany) to generate recombinant histidines tagged VP3 (His-VP3) and glutathione S-transferase tagged VP3 (GST-VP3). The purified His-VP3 protein was used to immunize BALB/c mice. The hybridoma cell line producing mAb 5B5 was produced by fusion of B-lymphocytes from immunized mice with mouse myeloma cells. The ascites titer of mAb 5B5 was determined to be 1: 1024000 by indirect ELISA. Subtype identification revealed that mAb 5B5 was of the IgG1/kappa type, determined by mouse immunoglobulin isotyping kit (Biodragon, Beijing, China) according to the manufacturer's instructions.

Horseradish peroxidase (HRP) labeled goat anti-mouse antibody and fluorescein isothiocyanate (FITC) labeled goat anti-mouse antibody were purchased from KPL (Maryland, USA). The positive anti-NGPV sera were obtained from five mice immunized with NGPV VP3 protein and stored in our laboratory.

Duck embryo fibroblast (DEF) and goose embryo fibroblast (GEF) cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Hyclone, Thermo scientific, USA) in humidified 5% CO_2 atmosphere at 37 °C. All culture media were supplemented with 10% heat-inactivated fetal bovine serum (FBS, PAA, Somerset, UK) and antibiotics (0.1mg/mL of streptomycin and 100IU/mL of penicillin). All of the primers were synthesized by Sangon Biotech Co., Ltd (Shanghai, China).

Specificity analysis of the mAb 5B5

To identify the specificity of the mAb 5B5, IFA was used as previously described (Said and

Osterrieder, 2014). Primary DEF cells were grown to approximately 80% confluency and inoculated with NGPV strain at a multiplicity of infection (MOI) of 1 for 2 h, then the virus inoculum was removed and replaced by cell maintenance medium with 2% FBS, which maintains cell growth at a lower rate and prevents overgrowth. At 48 h post-infection (hpi), the cells were fixed with an icy mixture of acetone-formaldehyde (1:1 v/v) for 20 min and followed by permeation with 0.2% Triton X-100 in PBS for 3 min. Nonspecific antigen was blocked with 5% non-fat milk at 37 °C for 1 h. After three washes with PBST (PBS containing 0.01% Tween-20), the cell culture plates were incubated with mAb 5B5 (1:1000 dilutions) for 1 h at 37°C. NGPV-positive mouse serum (1:1000 dilutions) and healthy mouse sera (1:1000 dilutions) were used as the positive control and negative control, respectively. Then FITC-conjugated goat anti-mouse IgG (1:500; KPL, Gaithersburg, MD, USA) was added as secondary antibody at 37 °C for 1 h. After three washes with PBST, the immunofluorescent staining was observed under a fluorescent microscope (Leica AF6000) (Feng et al., 2018). The protocols for immunolabeling of B-cell linear epitope with mAb-5B5 were same for other common waterfowl viruses included in this study.

Neutralization assay for mAb 5B5 against WPVs

The neutralizing activity of mAb 5B5 was determined using a virus-based neutralization assay as described previously (Gough, 1984). Briefly, 100 μL of serial diluted mAb 5B5 (10-fold dilution) were incubated with 100 μL GPV, NGPV and MDPV (10^6 copies/100 μL) at MOI of 1 for 2 h at 37 °C, respectively. Then, the virus-mAb mixture (200 μL) was added in DEF cells or GEF cells in a 96-well plate. Uninfected healthy mouse serum was diluted in PBS and used as a negative control. Uninfected DEF cells or GEF cells also served as controls. At 48 hours post infection (hpi), cells were observed and analyzed by IFA.

Truncated expression of VP3 polypeptide protein

A series of overlapping and truncated fragments of the VP3 gene were amplified and cloned into the pGEX-6P-1 vector and expressed in *Escherichia coli* (*E. coli*) BL21 (DE3) as fusion proteins. After two rounds of truncations, the reactive polypeptide to mAb 5B5 was shortened to 10 aa. In the final round, the 10 aa peptide was truncated with one by one residue from both ends, cloned into the pGEX-6P-1 vector, and expressed in *E. coli* BL21 (DE3). Primer sequences are listed in Table 1. The epitope sequence was finally determined when the smallest binding peptide recognized by the mAb 5B5 was identified. Complementary primers used for synthesizing mutant epitope fragments are shown in Table 2.

Identification of the precise epitopes using dot blot assay

The reactivity of truncated and mutant fragments with the mAb 5B5 was tested using dot blot assay. Briefly, the NC membrane was cut into squares of 1 cm × 1 cm size and placed in a box. The purified fractionated protein was diluted to 1 μg/μL in PBS. About 2 μg of each purified fusion proteins were spotted onto the nitrocellulose membrane (Millipore HATF00010) at the center of the grid. After blocking the membrane with 5% skimmed milk at 37 °C for 2 h, the membrane was washed three times with TBST buffer (20 mM Tris-HCl, 150 mM NaCl, 0.01% Tween-20, pH7.5). Then the membrane was incubated at 37 °C for 1 h with mAb. After washing three times with TBST, the membrane was incubated with HRP-conjugated goat anti-mouse IgG (1:4000; KPL, Gaithersburg, MD, USA) at 37 °C for 1 h. After washing three times with TBST, the membrane was colored in diaminobenzidine (DAB, TianGen PA110) liquid.

Alignment of epitope sequences of the VP3 protein

To analyze the conservation of the identified epitope, the VP3 protein sequences of 42 strains of WPVs and other parvoviruses were retrieved from GenBank (http://www.ncbi.nlm.nih.gov). The parvoviruses include NGPV, GPV, MDPV, canine parvovirus (CPV), feline parvovirus (FPV),

porcine parvovirus (PPV), mink enteritis virus (MEV), lapine parvovirus (LPV) and bovine parvovirus (BPV). We performed sequence alignment using alignment using the DNAStar Clustal W program (DNAStar Inc., Madison, WI, USA).

Results

Specificity analysis of the mAb 5B5

IFA assay revealed that the mAb 5B5 could react with GPV, NGPV and MDPV, but not with DPV, DTMUV, AIV/H9N2, NDV, DRV, DHAV-1 and DHAV-3 (Figure 1). These finding emphasis that the mAb 5B5 specially react with the B-cell linear epitopes on VP3 protein of WPVs.

Neutralization activity of mAb 5B5

The neutralizing activity of mAb 5B5 was determined by using an *in vitro* neutralization assay with DEF and GEF. The results showed that mAb 5B5 can neutralize all of the three kinds of WPVs (Figure 2).

Mapping of the epitopes of VP3 protein

According to the full-length gene of VP3 and the prediction of secondary structure, VP3 was divided into two major segments (VP3-A and VP3-B), and the approximate range of the epitope was identified (Figure 3A). Then totally six overlapping GST-fused fragments (VP3-1, VP3-2, VP3-3, VP3-4, VP3-5 and VP3-6) spanning the NGPV VP3-B protein were expressed and subjected to dot blot analysis. As demonstrated by dot blot assay, VP3-5 and VP3-6 showed reactivity with 5B5 (Figure 3B). Finally, the liner epitope recognized by 5B5 was preliminary speculated as ^{436}FGLHNPPPQV^{445} in the NGPV VP3 protein.

Subsequently, a panel of six shortened peptides were generated by deleting amino acids individually, at either the amino or the carboxy terminus in sequence of the peptide ^{436}FGLHNPPPQV^{445}. Dot blot hybridization results showed that the peptides ^{437}GLHNPPPQV^{445},

^{438}LHNPPPQV^{445}, ^{438}LHNPPPQ^{444} and ^{438}LHNPPP^{443} were recognized by 5B5, whereas ^{439}HNPPPQV^{445}, ^{439}HNPPP^{443} and ^{438}LHNPP^{442} showed no reactivity with 5B5 (Fig. 3C). Based on the dot blot analysis results, the accurate position of the epitope recognized by the 5B5 was deduced at ^{438}LHNPPP^{443} in the NGPV VP3 protein (Figure 4).

Homology analysis of the linear epitope to 5B5 in different parvoviruses

To determine whether the ^{438}LHNPPP^{443} epitope is conserved in the VP3 protein of WPVs and other parvoviruses, we aligned 42 strains of different WPVs including NGPV, GPV, MDPV, and other parvoviruses such as CPV, FPV, PPV, MEV, LPV and BPV available in GenBank. The homology alignment result showed that the amino acid sequences in the motif region were identical in all WPV strains, but they significantly differed from mammalian parvovirus strains (Figure 5), indicating that the epitope recognized by the mAb 5B5 was a highly conservative and specific motif within VP3 protein of WPVs.

Discussion

Waterfowl parvoviruses infection gives rise to high mortality and morbidity in both geese and Muscovy ducks, leading to great losses to the waterfowl industries. However, in recent years, a novel parvovirus-related virus (NGPV) has been largely isolated in the domestic diseased Cherry Valley ducks and mule ducks. Its infection period lasts longer and the infected host is more extensive (Wang et al., 2016). The elimination rate of the affected duck is as high as 80%, which causes the Chinese duck industry to suffer serious economic losses. Detection and control of the disease is essential, and it is extremely urgent to establish stable, low-cost and high-specific detection methods.

In previous studies, some molecular detection methods for GPV have been established, such as duplex semi-nested PCR, real-time quantitative PCR and quantitative loop-mediated isothermal

amplification assay (Niu et al., 2016; Li et al., 2017; Yang et al., 2017). Although these methods have the advantages of high sensitivity and good specificity, they are expensive, laborious, time-consuming, and require special instruments. Detection methods based on mAb have the advantages of stable, high specificity, rapid and convenient disease diagnosis which can help to control further spread of disease.

With high specificity to bind epitope, mAbs often are used to map antigenic epitopes (Zhang et al., 2015; Ti et al., 2017). The identification of epitopes is chiefly based on understanding the nature of protein antigens and is closely related to the immune regulation network in the biological system. In recent years, epitope-based vaccine and specific diagnostic tools have received extensive attentions. Since parvoviruses have relatively short genome and simple structure of capsid, so it is easier to screen different antigenic components. At present, there are several methods available for screening B-cell epitope which include peptide scanning technique, phage peptide library presenting technology, eukaryotic expression presenting system and prokaryotic expression presenting system. The method of prokaryotic expression system is simple and easy to operate and does not require highly sophisticated instruments and technologies (Sun et al., 2012). In this study, a mAb 5B5 against NGPV VP3 protein was prepared and evaluated for its diagnostic ability. After screening by dot blot with a series of truncated proteins expressed in prokaryotic expression system, an accurate linear B-cell epitope on VP3 protein that could react and form immune complex with in house prepared mAb was identified. To the best of our knowledge, this is the first WPVs common conserved B-cell linear epitope identified on the VP3 protein of NGPV.

The identified epitope against mAb 5B5 was finally decoded as ^{438}LHNPPP^{443} in NGPV VP3 protein. The alignment results showed that the amino acid sequences in the epitope region were identical in all WPV strains and were significantly different from those of the mammalian

parvovirus strains (Figure 5), indicating that the epitope against mAb 5B5 was a group-specific epitope conserved among WPVs. IFA assay further revealed that the mAb 5B5 could react with WPVs, but not with DPV, DTMUV, AIVH9/N2, NDV, DRV, DHAV-1 and DHAV-3. Owing to good specificity to WPVs VP3 protein, the developed mAb 5B5 can be a potent immunodiagnostic mAb, and can be utilized in antigen-capture assay to detect of WPVs infections.

To conclude, the mAb 5B5 showed high specificity to VP3 protein of three different WPVs. Utilizing the mAb 5B5, a common conserved neutralizing linear B-cell epitope ^{438}LHNPPP^{443} in the VP3 protein of WPVs was successfully identified for the first time. This study explored the antigenic structure and functional motif of B-cell linear epitope and assessed its utility in diagnosis of WPVs infection. As the primary functional unit in immune response, the identified conserved neutralizing epitope might have potential application in the design of epitope-based protective vaccine as well as further analysis of the replication and pathogenesis of WPVs.

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Conflict of interest

We declare that we have no conflicts of interest to this work. We also declare that we do not have any commercial or associated interest that represents a conflict of interest in connection with the work submitted.

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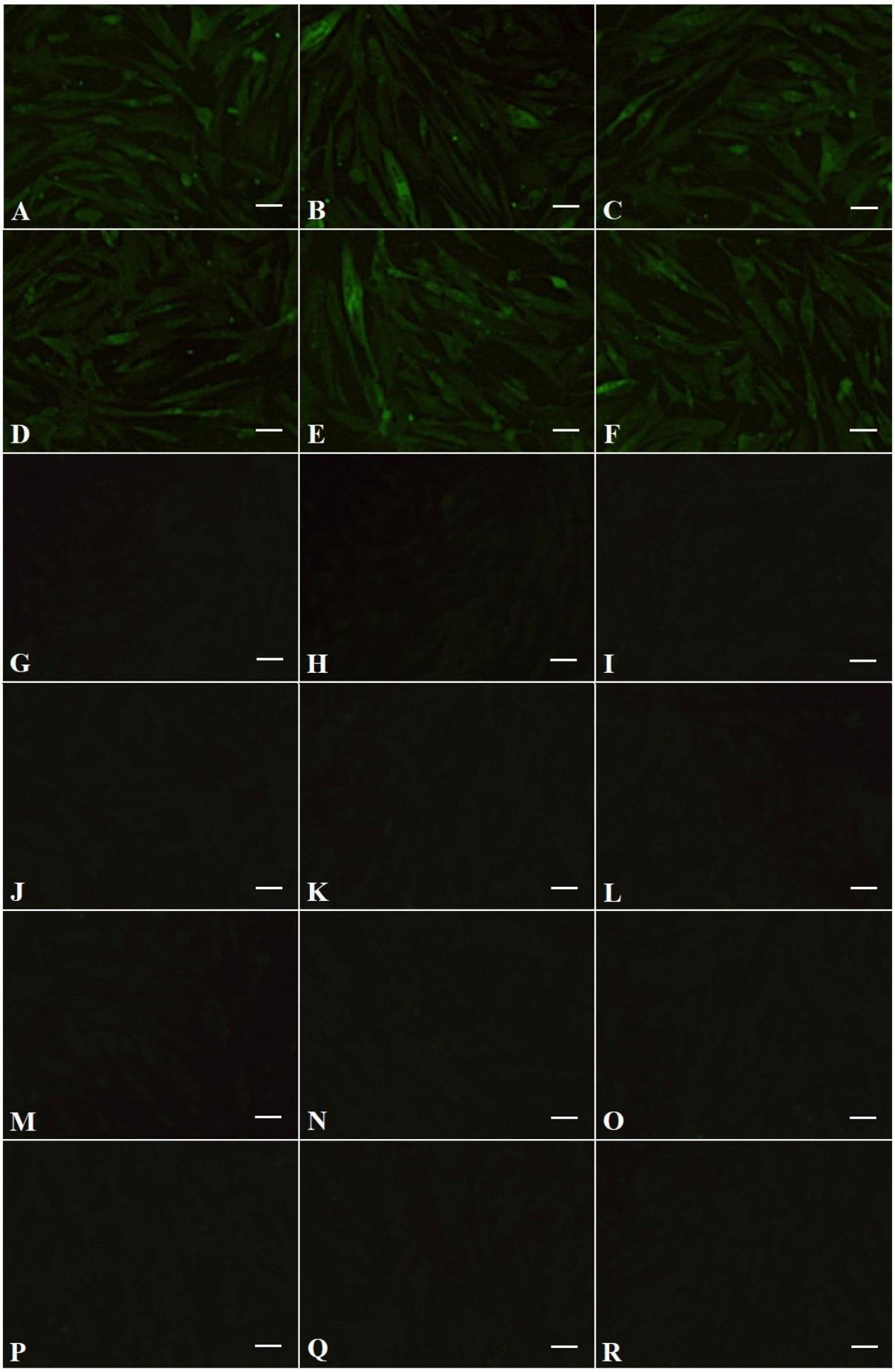
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Q
R

Figure 1. Specificity of mAb 5B5 against WPVs was detected by IFA. (A) NGPV infected DEF cells detected by anti-NGPV positive sera; (B) GPV infected GEF cells detected by positive anti-GPV sera; (C) MDPV infected DEF cells with positive anti-MDPV sera; (D) NGPV infected DEF cells detected by mAb 5B5; (E) GPV infected GEF cells detected by mAb 5B5; (F) MDPV infected DEF cells detected by mAb 5B5; (G) DPV infected DEF cells detected by mAb 5B5; (H) DTMUV infected DEF cells detected by mAb 5B5; (I) AIV/H9N2 infected DEF cells detected by mAb 5B5; (J) NDV-infected DEF cells detected by mAb 5B5; (K) DRV infected DEF cells detected by mAb 5B5; (L) DHAV-1-infected DEF cells detected by mAb 5B5; (M) DHAV-3-infected DEF cells detected by mAb 5B5; (N) NGPV-infected DEF cells detected by healthy mouse sera; (O) GPV-infected GEF cells detected by healthy mouse sera; (P) MDPV-infected DEF cells detected by healthy mouse sera; (Q) Normal DEF cells detected by mAb 5B5; (R) Normal GEF cells detected by mAb 5B5. Bars, 100 μm.

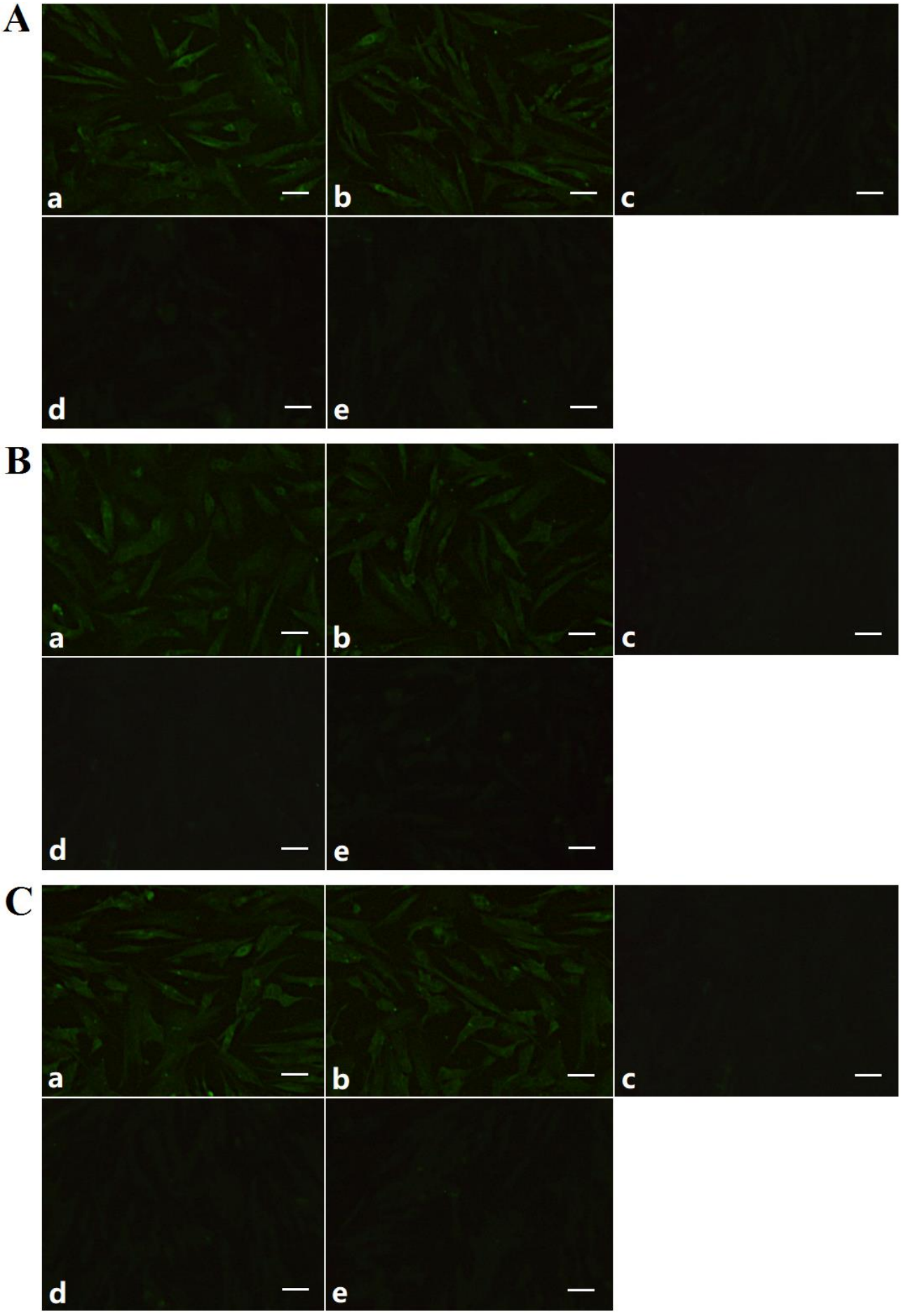
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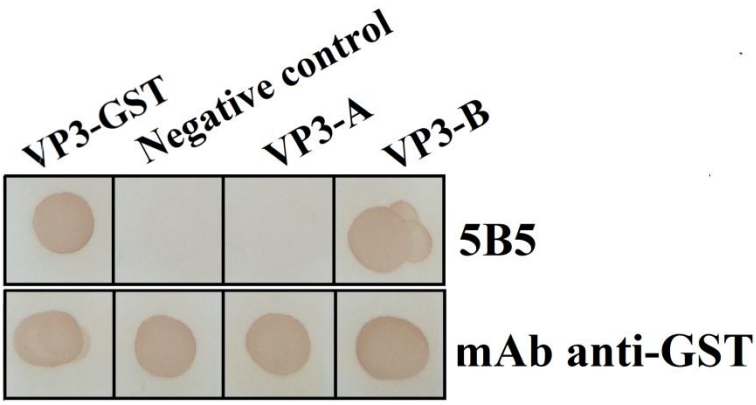
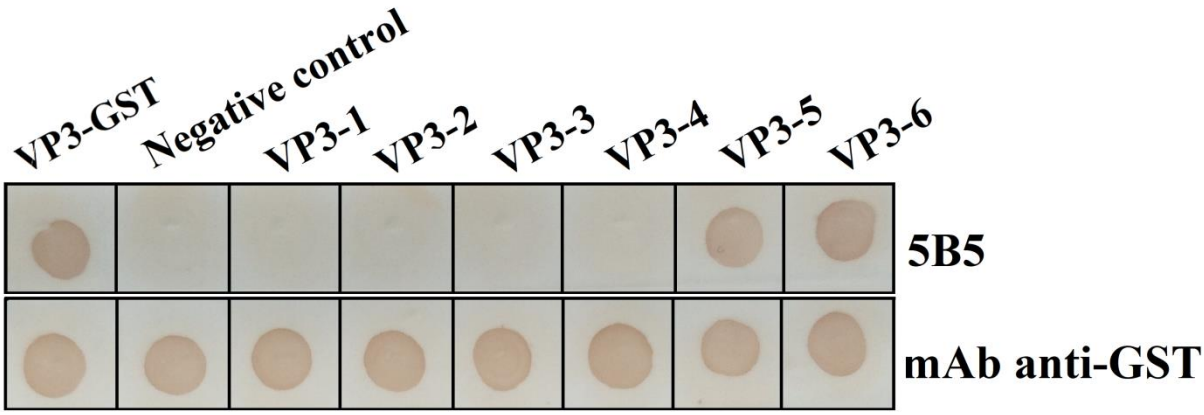
Figure 2. Neutralizing activity of mAb 5B5 to three kinds of WPVs was detected by IFA with mAb 5B5. (A) Neutralizing activity of mAb 5B5 to NGPV. a) DEF cells infected by NGPV; b) NGPV-infected DEF cells neutralized with healthy mouse sera; c) NGPV-infected DEF cells neutralized with mAb 5B5; d) NGPV-infected DEF cells neutralized with anti-NGPV positive sera; e) Uninfected DEF cells. (B) Neutralizing activity of mAb 5B5 to classical GPV. a) GEF cells infected GPV; b) GPV-infected GEF cells neutralized with healthy mouse sera; c) GPV-infected GEF cells neutralized with mAb 5B5; d) GPV-infected GEF cells neutralized with anti-GPV positive sera; e) Uninfected GEF cells. (C) Neutralizing activity of mAb 5B5 to MDPV. a) DEF cells infected by MDPV; b) MDPV-infected DEF cells neutralized with healthy mouse sera; c) MDPV-infected DEF cells neutralized with mAb 5B5; d) MDPV-infected DEF cells neutralized with anti-MDPV positive sera; e) Uninfected DEF cells. Bars, 100 μm.

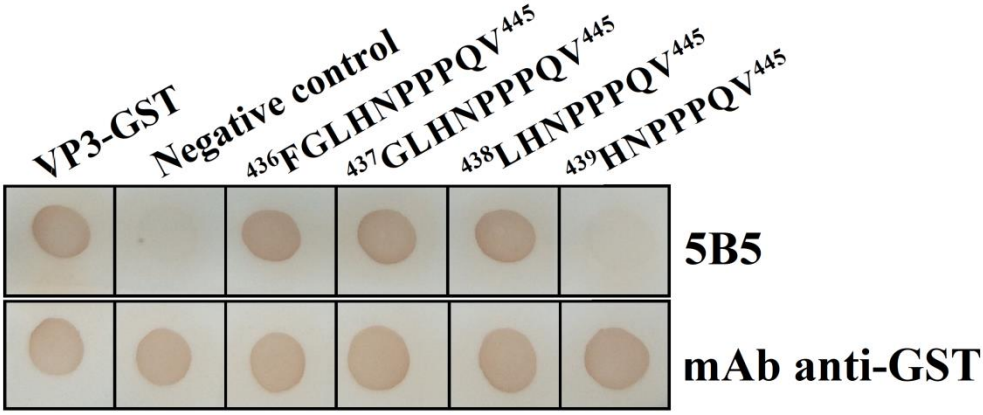
A


VP3-GST
Negative control
VP3-A
VP3-B
5B5
mAb anti-GST

B


VP3-GST
Negative control
VP3-1
VP3-2
VP3-3
VP3-4
VP3-5
VP3-6
5B5
mAb anti-GST

C


VP3-GST
Negative control
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437GLHNPPPQV445
438LHNPPPQV445
439HNPPPQV445
5B5
mAb anti-GST

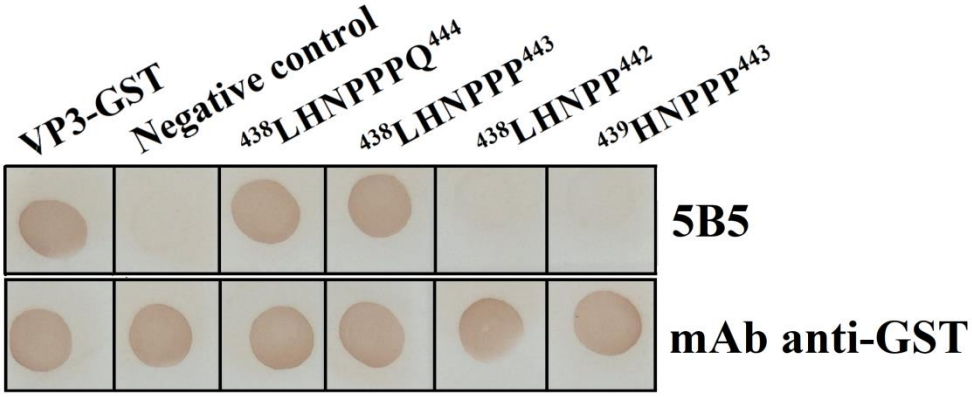
VP3-GST
Negative control
438LHNPPPQ444
438LHNPPP443
438LHNPP442
439HNPPP443
5B5
mAb anti-GST

Figure 3. Analysis of the expressed various NGPV VP3 fusion proteins and truncated versions of the peptide ^{436}FGLHNPPPQV^{445} in *E. coli* Rosetta (DE3) cells by dot blot assay using mAb 5B5 and mAb anti-GST.

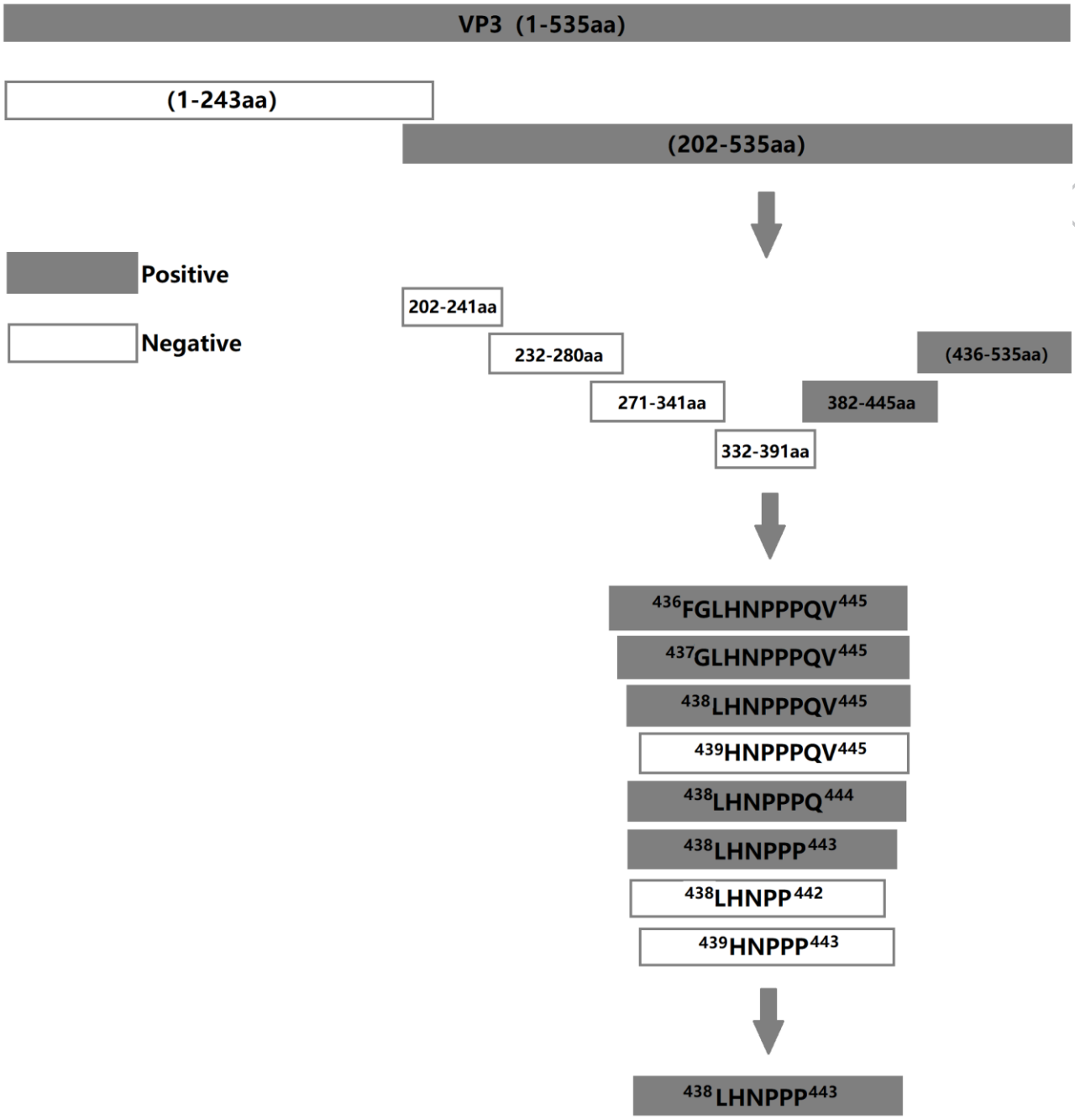

Figure 4. The accurate position of the epitope recognized by mAb 5B5 was deduced at ^{438}LHNPPP^{443} in the NGPV VP3 protein.

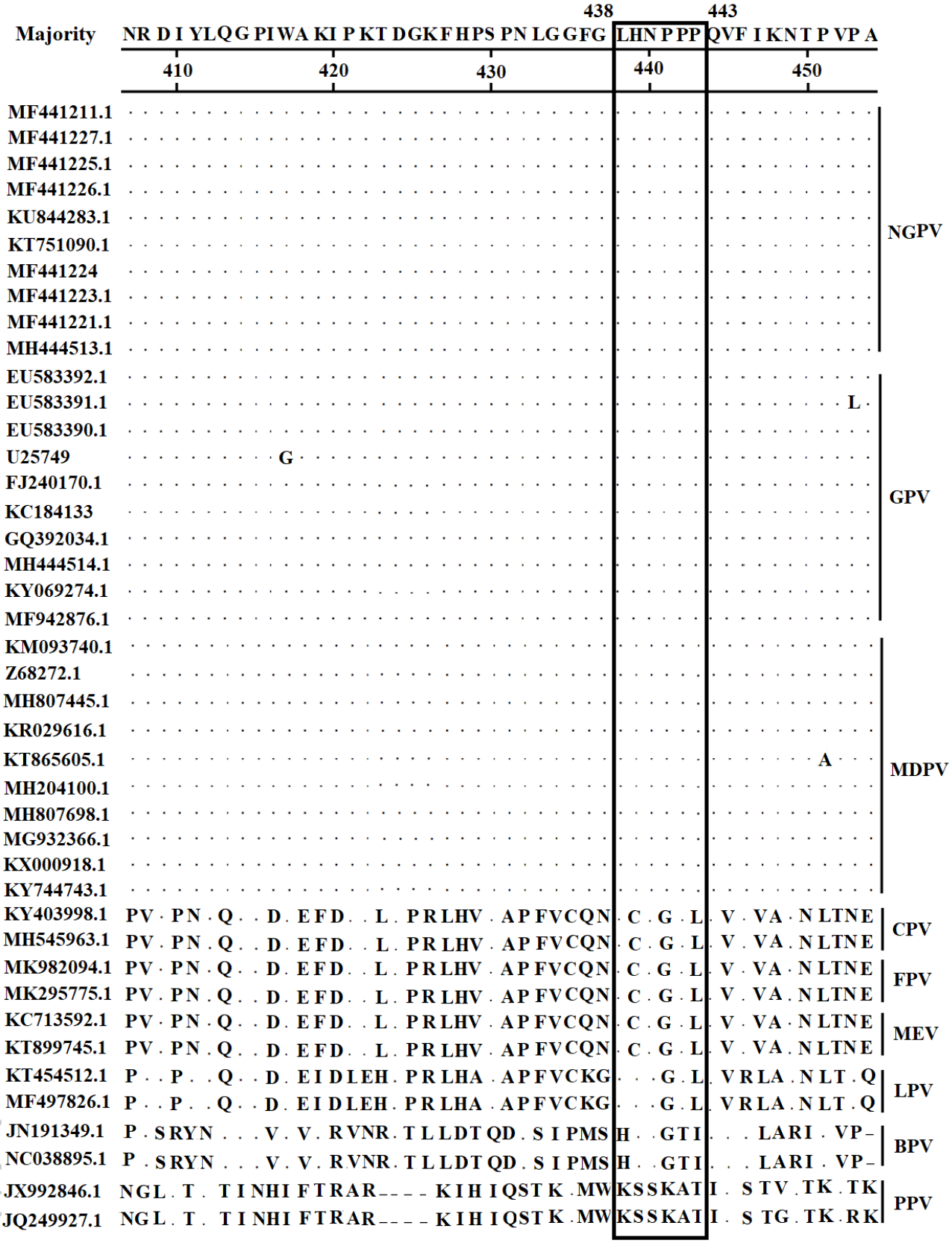

Figure 5. Sequence alignment of parvovirus strains around epitope-coding region of the VP3 protein. Major sequences are shown at the top; dots indicate identical amino acids. The identified epitope is in box.

Table 1. Primers for identification of the linear epitope in NGPV VP3

Primers	Sequence (5'→3')	AA position inVP3
VP3-AF	GGATCCTGGCAGAGGGAGGAGGCGGA	1-243
VP3-AR	CCGCTCGAGGAGGTATTGATCCACTA	
VP3-BF	GGATCCCAGATGCTGAGAACAGGT	202-535
VP3-BR	CCGCTCGAGTTACAGATTTTGAGTTAG	
VP3-1F	GGATCCCAGATGCTGAGAACAGGT	202-241
VP3-1R	CCGCTCGAGTTGATCCACTAGG	
VP3-2F	GGATCCCAGGCTTATGAACCCCCTA	232-280
VP3-2R	CCGCTCGAGATCCAGGGATTTAGGT	
VP3-3F	GGATCCTTGGCTGCCGGGACCTA	271-341
VP3-3R	CCGCTCGAGAGCTATCCCTAAAAT	
VP3-4F	GGATCCCCAGCTCAGAATATTTTA	332-391
VP3-4R	CCGCTCGAGTGAACTTGTAAGGA	
VP3-5F	GGATCCAACACTACTACAGCTCCT	382-445
VP3-5R	CCGCTCGAGGACCTGCGGTGGTGGA	
VP3-6F	GGATCCTGGCCTGCACAATCCACCA	436-535
VP3-6R	CCGCTCGAGTTACAGATTTTGAGTTAG	

Note: *BamH* I and *Xho* I restriction enzyme sites were introduced at the 5' end of the primers with underlined letters.

Table 2. The primers used in the present study for producing epitope peptides

Primers	Sequence (5'-3')	Coding motif
436-445-F	ggatccTTTGGCCTGCACAATCCAaat	^{436}FGLHNPPPQV^{445}
436-445-R	tcgagttaCGGTGGTGGATTGTGCg	
437-445-F	ggatccGGCTGCACAATCCACCAaat	^{437}GLHNPPPQV^{445}
437-445-R	tcgagttaCGGTGGTGGATTGTGCg	

438-445-F	ggatccTGCACAATCCACCAaat	^{438}LHNPPPQV^{445}
438-445-R	tcgagttaCGGTGGTGGATTGTGCg	
439-445-F	ggatccCACAATCCACCACCGaat	^{439}HNPPPQV^{445}
439-445-R	tcgagttaCGGTGGTGGATTGTGCg	
438-444-F	ggatccTGCACAATCCACCAaat	^{438}LHNPPPQ^{444}
438-444-R	tcgagttaCGGTGGTGGATTGg	
438-443-F	ggatccTGCACAATCCACCAaat	^{438}LHNPPP^{443}
438-443-R	tcgagttaCGGTGGTGGAg	
438-442-F	ggatccTGCACAATCCACCAaat	^{438}LHNPP^{442}
438-442-R	tcgagttaGACCTGCGGTGGTg	
439-443-F	ggatccCACAATCCACCACCGaat	^{439}HNPPP^{443}
439-443-R	tcgagttaCGGTGGTGGAg	

Note: Introduced bases (to form termination codon and overhanging ends of *BamH* I and *Xho* I after annealing of the two complementary oligonucleotides) are shown in lowercase letters.