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Antibody to VP4 protein is an indicator discriminating pathogenic and nonpathogenic IBDV infection

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ABSTRACT

Infectious bursal disease virus (IBDV) causes an acute, highly contagious immunosuppressive disease—infectious bursal disease (IBD) in poultry. It is known that VP4 is a non-structural protein and a viral protease encoded by IBDV. Currently, little is known about VP4 characteristics during pathogenic and nonpathogenic IBDV infection. Here, we investigated the expression profiles of VP4 during pathogenic and nonpathogenic IBDV infection. By IFA and ELISA, using VP4 protein respectively expressed in Vero cells transfected with VP4 gene and in *Escherichia coli* as antigens, we firstly confirmed serum anti-VP4 antibodies in pathogenic IBDV-infected rather than nonpathogenic IBDV-infected chickens. Kinetic analysis of anti-IBDV antibody shows that in the pathogenic IBDV-infected chickens, the antibody to VP4 was later detectable than anti-VP3 antibody and virus neutralizing antibody. Immunohistochemistry further demonstrates that VP4 antigen can be detected mainly in the cortex of lymphoid follicles of bursa of Fabricius infected with pathogenic IBDV. These data first suggest that VP4 antibody is an indicator discriminating pathogenic and nonpathogenic IBDV infection in chickens.

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1. Introduction

Infectious bursal disease virus (IBDV), a member of the *genus Avibirnavirus* in the *family Birnaviridae*, causes an acute, highly contagious and immunosuppressive disease—infectious bursal disease (IBD) (Bakacs and Mehrishi, 2004; Muller et al., 2003; Sharma et al., 2000), IBD is one of the most important viral diseases affecting the poultry industry worldwide (Dobos et al., 1979). Bursa of Fabricius is the target organ of IBDV (Hirai et al., 1981; Kaufer and Weiss, 1980; Rodenberg et al., 1994). IBDV replicates in Bursa and destroys follicular lymphocytes and leads to immunosuppression (Panigrahy et al., 1982; Sharma and Fredericksen, 1987; Sharma et al., 2000).

The IBDV genome consists of two segments (A and B), encodes five viral proteins (VP1–5). The smaller segment B encodes VP1 protein, an RNA-dependent RNA polymerase (RdRp) for IBDV genome replication (Morgan et al., 1988; Muller and Nitschke, 1987; Spies et al., 1987; von Einem et al., 2004; Zheng et al., 2006). The large segment A contains two partly overlapping open reading frames, encoding a precursor polyprotein VP243 and a non-structural protein VP5 respectively (Mundt et al., 1995). VP243 polyprotein was cleaved autoproteolytically to give rise to the viral structural proteins VP2, VP3, and a viral protease, VP4. VP2 of IBDV can elicit the neutralizing antibodies (Fahey et al., 1989), VP3 is a group-special and major immunogenic protein of IBDV (Birghan et al., 2000; Jagadish et al., 1988; Kibenge et al., 1997), VP5 is a non-structural protein, no evidence shows that VP1 and VP5 proteins could elicit antibodies in exposed chickens (Mundt et al., 1997); studies on VP4 of IBDV mainly focused on its protease function (Birghan et al., 2000; Feldman et al., 2006; Kibenge et al., 1997; Lejal et al., 2000; Sanchez and Rodriguez, 1999) and found it combining with type II tubule (Granzow et al., 1997). Except for these, little was known about the properties of VP4 protein. In the present study, we investigate the VP3 and VP4 proteins in epidemiology during pathogenic and nonpathogenic IBDV infections.

2. Materials and methods

2.1. Virus, cells and serum

The pathogenic IBDV NB strain ($10^{6.2}$ BLD_{50}/0.1 ml, GenBank accession no. EU595667), and nonpathogenic IBDV vaccine strain NB (GenBank accession no. EU595672) were identified and stored in our laboratory (Shi et al., 2008; Zhou et al., 2005c). Vero cells were

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Table 1
The summary of the primers used in this study.

Host	Gene	Designation	Nucleotide sequence of primers
Prokaryotic cell	VP3	Forward	5′-CGTCGTCATATGGCATCAGAGTTCAAAGAGACC-3′
		Reverse	5′-CGTCGTGAATTCTTACTCAAGGTCCTCATCAGAGACG-3′
	VP4	Forward	5′-CGTCGTCCATGGCCGACAAGGGGTACGAGGTAGTC-3′
		Reverse	5′-CGTCGTCTCGAGCATGGCAAGGTGGTACTGGCGTCC-3′
Eukaryotic cell	VP3	Forward	5′-CGTCGTGAATTCATGGCATCAGAGTTCAAAGAGACC-3′
		Reverse	5′-CGTCGTGTCGACCTACTCAAGGTCCTCATCAGAGACG-3′
	VP4	Forward	5′-CGTCGTGCTAGCATGGCCGACAAGGGGTACGAGGTAGTC-3′
		Reverse	5′-CGTCGTGAATTCCTAAGCCATGGCAAGGTGGTACTGGCG-3′

Underlined text represents the restriction enzyme digestion site.

raised in modified Eagle's medium (MEM, Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, GibcoBRL Life Technologies, Grand Island, NY) at 37 °C with 5% CO_2. Primary chicken embryo fibroblasts (CEF) were prepared from 10-day-old embryonated specific pathogen free (SPF) chicken eggs (obtained from the Beijing Merial Vital Laboratory Animal Technology, Beijing, China) and maintained in MEM supplemented with 10% FBS.

2.2. Construction of expression plasmids of VP4 and VP3 gene

For prokaryotic and eukaryotic expressions of VP3 and VP4 genes of IBDV, the specifically amplifying primers were designed according to the cDNA sequence of segment A of IBDV NB strain (GenBank accession no. EU595672) and summarized in Table 1. The total RNA extracted from CEF infected with the nonpathogenic IBDV NB strain was used as a template for reverse transcription PCR. Using the primers shown in Table 1, PCR was performed at 95 °C for 5 min, 95 °C for 30 s, 63 °C for 30 s and 72 °C for 1 min for 30 cycles, with a final elongation step for 5 min at 72 °C. Then the PCR products were digested and inserted into the vector pCI-neo (Promega, USA) or pET-28a (Novagen, Madison, WI) respectively. The resulting plasmids pET-28-VP4, pCI-VP4, pET-28-VP3 and pCI-VP3 were confirmed by restriction enzyme digestion and DNA sequencing. Then the plasmids pET-28-VP4 and pET-28-VP3 were transformed into *E. coli* BL21 (DE3) strain.

2.3. Expression and purification of VP4 and VP3 proteins

The recombinant *E. coli* BL21 (DE3) strains containing pET-28-VP4 or pET-28-VP3 were grown at 37 °C for approximately 3 h. Afterward, the bacteria were induced by isopropyl-β-D-thiogalactopyranoside (IPTG) with a final concentration of 1 mM at 37 °C, and harvested sequentially at 0.5 h intervals by centrifugation at 5000 × *g* for 10 min (Zhou et al., 2005b). The harvested bacteria were then subjected to SDS-PAGE to analyze the optimal conditions for VP4 or VP3 expression. Finally, the expressed fusion proteins were confirmed by Western blot analysis using mouse anti-His antibody (Amersham, Buckinghamshire, UK). The recombinant VP4 and VP3 proteins were purified with nickel affinity column (Novagen, Madison, WI) according to the manufacture protocol. The eluted VP4 and VP3 proteins were identified by SDS-PAGE and Western blot assays. Total protein concentration was determined by the Bradford assay using bovine serum albumin (BSA) as a reference.

2.4. Preparation of monoclonal antibodies to VP4 and VP3 proteins

Procedure was manipulated essentially as described previously (Zhou et al., 2005a). Briefly, ten 8-week-old female SPF BALB/c mice were immunized subcutaneously (S/C) with the purified rVP4 or rVP3 (50 μg per mouse) emulsified in complete Freund's adjuvant (CFA, Sigma–Aldrich, St. Louis, MO). The mice were boosted with rVP4 or rVP3 (50 μg per mouse) emulsified in incomplete Freund's adjuvant (Sigma–Aldrich) 2 weeks after the first immunization. Two weeks after the second immunization, the vaccinated mice were injected intraperitoneally with a dose of 100 μg/mouse rVP4 or rVP3 again. The mice were subsequently euthanized 3 days later and the spleen cells were harvested. Spleen cells from immunized mice were fused with the SP2/0 myeloma cell line. Hybridomas were selected, and supernatants were screened by ELISA using purified VP4 or VP3 protein and lysates of IBDV-infected CEF as coated antigens. Hybridomas reacted with recombinant proteins were cloned three times by limiting dilution. The subtype analysis of each monoclonal antibody (mAb) was performed with standard procedures illuminated by the protocol of SBA Clonotyping System/HRP (Southern Biotechnology, Birmingham, AL). The reactivity of these mAbs were further identified using the cells transfected with the genes VP4 or VP3 of IBDV.

2.5. In vitro transfection and immunofluorescent assay

For confirming the reactivity of the mAbs to the eukaryotically expressed VP3 and VP4 proteins and detecting anti-VP4 antibodies of IBDV-infected chickens, the Vero cells were seeded in 96-well plates and grown to 80–90% confluence. After washing with Opti-MEM medium (GibcoBRL), the cells were transfected with a mixture of recombinant plasmid pCI-VP4 or pCI-VP3 and Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Each well contained 0.2 μg DNA and 0.5 μl Lipofectamine 2000 in 50 μl medium. To identify the binding ability of anti-VP3 and VP4 mAbs to the native VP3 and VP4 proteins of IBDV, CEF monolayer was inoculated with IBDV (100 $TCID_{50}$/0.1 ml) (Zhou et al., 2005c). At 36 h post transfected and inoculated, the VP4 and VP3 proteins was analyzed by immunofluorescent assay as previously stated (Shang et al., 2009; Zhang et al., 2008; Zheng et al., 2006). Mock-infected CEF, and the vector pCI-neo-transfected Vero cells were used as negative controls.

2.6. Animal inoculation and sampling

Eighty 25-day-old SPF chickens were divided randomly into eight groups with ten chickens each. The mock-infected chickens were injected intraocularly (i.o.) with normal saline. The chickens in four pathogenic IBDV-infected groups were inoculated i.o. with the pathogenic IBDV at a dose of 0.1 ml (100 BLD_{50}/0.1 ml). The chickens (20 chickens) in the nonpathogenic IBDV-primed group were immunized i.o. with the nonpathogenic IBDV at a dose of 0.1 ml (5000 $TCID_{50}$/0.1 ml), in which the half (IBDV-boosted chickens) was boosted i.o. with the nonpathogenic IBDV at a week interval. Bursa of fabricius (BF) were collected from the mock-infected, pathogenic IBDV-infected and nonpathogenic chickens at 24 h, 48 h, 72 h, 96 h post infection/immunization (h.p.i.) for detecting IBDV antigen. Serum samples were collected at 0, 4, 7, 10, 14, 17, 20,

Table 2
Characteristics of monoclonal antibody to VP3 and VP4 of IBDV.

Antigen	mAb no.	Indirect ELISA		IFA		
		VP3	VP4	IBDV-infected CEF	VP3-transfected Vero cell	VP4-transfected Vero cell
VP3 protein	1B1	+	–	+	+	–
	2B12	+	–	+	+	–
	2D6	+	–	+	+	–
	2D12	+	–	+	+	–
	3F12	+	–	+	+	–
	9H1	+	–	+	+	–
	2A2	+	–	–	–	–
	2H12	+	–	–	–	–
	4C6	+	–	–	–	–
	4G11	+	–	–	–	–
	9B4	+	–	–	–	–
VP4 protein	4C3	–	+	+	–	+
	6A4	–	+	+	–	+
	6H8	–	+	+	–	+
	1G7	–	+	–	–	–
	1H9	–	+	–	–	–
	4D11	–	+	–	–	–
	4E10	–	+	–	–	–

23, 27, 30, 35, 42, 49 and 60-days post infection/immunization from the pathogenic IBDV-infected, nonpathogenic IBDV-primed and IBDV-boosted chickens for detecting anti-IBDV antibody. SPF Leghorn chickens were housed in negative-pressure isolator cages with HEPA-filtered air, and maintained with food and water ad libitum.

2.7. Enzyme-linked immunosorbent assay (ELISA)

To detect chicken ELISA antibody to VP3 protein or VP4 protein, ELISA test was performed with some modifications as previously described (Shang et al., 2009). Briefly, 96-well plates were coated with 100 μl of a recombinant IBDV-VP3 protein or IBDV-VP4 protein at a concentration of 0.5 μg/ml, which were diluted with coating buffer (0.14 M NaCl, 1 M Na_2CO_3, 1 M $NaHCO_3$, pH 9.6). After coating at 4 °C overnight, blocking procedure was carried out by incubating the antigens with 200 μl of blocking reagent (5% skim milk in PBS) at 37 °C for 1 h. After washing with PBST three times, 100× diluted serum samples were added and the plates were incubated at 37 °C for 2 h, and then washed with PBST buffer three times, each for 10 min. The plates were incubated with horseradish peroxidase (HRP)-conjugated goat anti-chicken IgG (KPL, Gaithersburg, Maryland, USA) diluted to 1:10,000 in PBST buffer for 1 h at 37 °C. Following similar washes, 100 μl of TMB chromogenic substrate (Sigma) was added to each well for color development. Then 50 μl of 2 M H_2SO_4 was added to each well to stop the reaction and the values of absorbance were measured at 450 nm. The ELISA data are presented as the sample absorbance values, and each datum point represents the mean of at least nine separate chickens.

2.8. Virus neutralizing antibodies

After the chicken serum samples were inactivated at 56 °C for 30 min, the neutralizing test was performed as stated (Hu et al., 2007; Zhou et al., 2003). Briefly, the serum was diluted serially at 1:2 in MEM, and mixed equally with 100 $TCID_{50}$ of IBDV. After incubation for 1 h at 37 °C, the antibody–virus mixture was added to the CEF monolayer. Cell pathogenic effects were observed everyday until 5 days post inoculation during incubation at 37 °C. The level of protection was evaluated by visual screening of the infected monolayers. IBDV- and mock-infected CEF monolayers were used as positive and negative controls. The virus neutralization titer of antibodies in a serum sample was determined as the reciprocal value of the highest.

2.9. Histopathology and Immunohistochemistry (IHC)

Tissues were fixed by 10% neutral buffered formalin, routinely processed, and embedded in paraffin. Sections were stained with hematoxylin and eosin (HE). Duplicate sections were performed the immunohistochemical staining. Briefly, the sections were treated with 0.3% H_2O_2 in PBS to inactivate endogenous peroxidase and washed three times in PBS, and were digested for 10 min at 37 °C by 0.1% trypsin (pH 7.6, 0.1% $CaCl_2$) for antigen retrieval, then the mAb specific for VP4 or VP3 was applied and allowed to incubate for 2 h at 37 °C. The primary antibody was then detected by the application of horseradish peroxidase (HRP) labeled goat anti-mouse IgG secondary antibody (KPL, Gaithersburg, Maryland, USA). Finally, the sections were counterstained with hematoxylin.

3. Result

3.1. General characterization of mAbs to VP4 and VP3

After the recombinant *E. coli* BL21 (DE3) respectively containing the plasmids pET-28-VP3 and pET-28-VP4 were induced by IPTG at 37 °C for the expression of VP3 and Vp4 proteins, SDS-PAGE analysis showed that the VP4 and VP3 proteins were produced as insoluble inclusion bodies. Western blot analysis also indicated that two protein bands with the approximate molecular weight of 28 kDa or 32 kDa could be recognized specifically with anti-His mAb, indicating that VP4 and VP3 proteins were expressed in *E. coli*.

The mice were injected respectively using the purified VP3 or VP4 proteins as immunogen. After cell fusion and screening by ELISA test, the hybridomas secreting mAb were established (Table 2), designated as mAbs 6H8, 4C3, 1G7, 1H9, 4D11, 4E10 and 6A4 specific for VP4, and mAbs 1B1, 2B12, 2D6, 2D12, 3F12, 2A2, 2H12, 4C6, 4G11, 9B4and 9H1 specific for VP3. These mAbs reacted specifically with the homologous protein expressed in *E. coli* BL21 (DE3), while showed no reaction with cell lysate of *E. coli* BL21 (DE3) harbouring the blank pET-28a (+) vector. IgG subtype analysis showed that all these mAbs belonged to IgG1, and their light chain was κ chain.

3.2. Binding ability of anti-VP3 and VP4 mAbs for native VP3 and VP4 proteins and intracellular distribution of VP4 protein of IBDV

To further identify the reactivity of the mAbs, Vero cells were respectively transfected with the expression vectors pCI-VP3 and

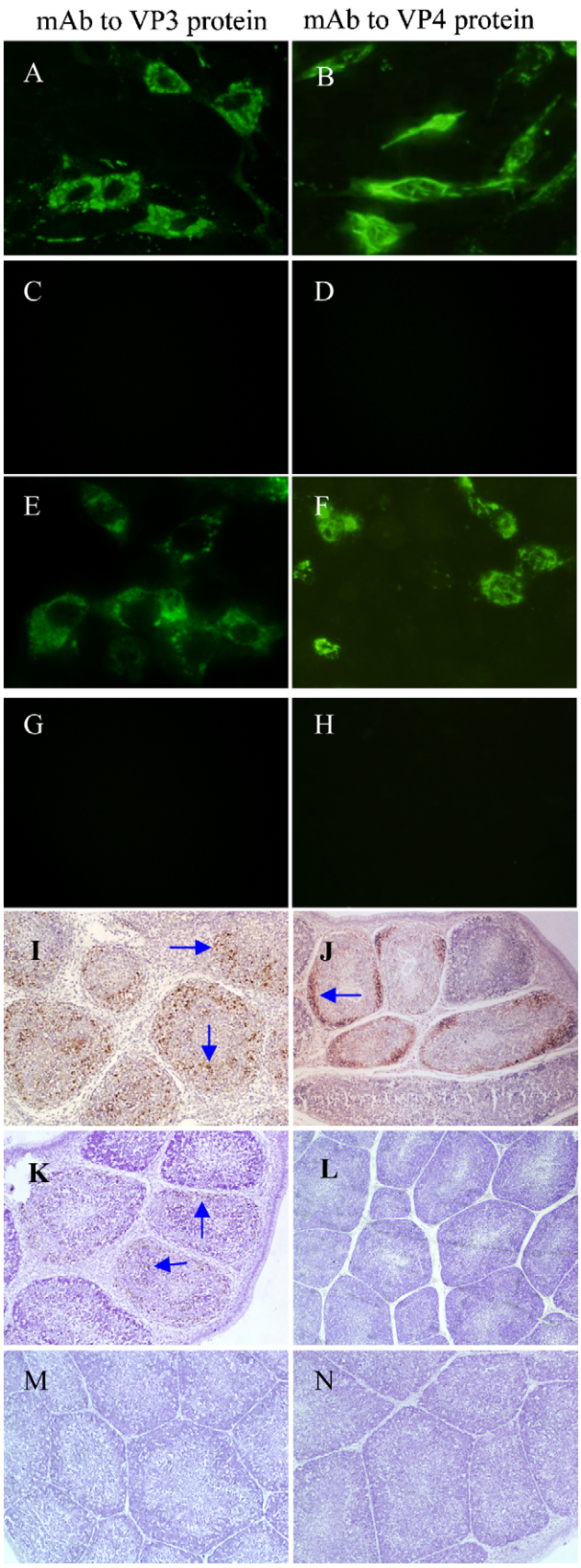

Fig. 1. Immunofluorescence assay and immunohistochemical staining of VP3 or VP4 gene-transfected/IBDV-infected cells. Each immunohistochemistry picture represents three treated chickens. A and E are an intracytoplasmic VP3 protein reacted with anti-VP3 mAb in IBDV-infected CEF monolayer and in Vero cell transfected with the vector pCI-VP3, respectively. B and F indicate the intracytoplasmic and intranuclear VP4 protein (needle-shaped fluorescence) recognized with anti-VP4 mAb in IBDV-infected CEF monolayer and in Vero cell transfected with the vector pCI-VP4, respectively. C and D reveal respectively mock-infected CEF monolayers reacted with anti-VP3 and anti-VP4 mAbs. G and H are mock-transfected Vero cell recognized by anti-VP3 and anti-VP4 mAbs respectively. I and J are the follicle lymphocytes recognized respectively with anti-VP3 and anti-VP4 mAbs in the cortex of bursa in pathogenic IBDV-infected chickens. K shows few follicle lymphocytes recognized with anti-VP3 mAb in the cortex of bursa in nonpathogenic IBDV-immunized chickens. L is the follicle lymphocytes not to be recognized with anti-VP4 mAb in the cortex of bursa in nonpathogenic IBDV-immunized chickens. M and N represent the follicle lymphocytes not to be recognized with anti-VP3 and anti-VP4 mAbs in the cortex of bursa in mock-infected chickens.

pCI-VP4, and CEF monolayer was inoculated with IBDV. In all produced mAbs to VP3 protein (Table 2), the mAbs 1B1, 2B12, 2D6, 2D12, 3F12, and 9H1 can recognize specifically with the VP3 protein expressed in Vero cells transfected with the plasmid pCI-VP3. But in all generated mAbs to VP4 protein (Table 2), three mAbs (6H8, 4C3 and 6A4) can react specifically with the VP4 protein expressed in Vero cells transfected with the plasmid pCI-VP4. Cross reaction of the mAbs was not found between VP3 and VP4 proteins, indicating that the generated mAbs to VP3 and VP4 proteins were specific. The mAbs against VP3 and VP4 proteins, reacted respectively with VP3 and VP4 proteins expressed in Vero cells and IBDV-infected CEF monolayer, shows that these mAbs have a strong binding capacity with native VP3 and VP4 proteins of IBDV. Furthermore, the intracellular distributions of VP3 and VP4 protein were located by these mAbs. The result shows that the VP3 protein only appears in the cytoplasm (Fig. 1A and E), and the VP4 protein reveals in both cytoplasm and nucleus as the line-shaped and needle-shaped structures (Fig. 1B and F).

3.3. Anti-VP4 and anti-VP3 antibodies in chickens infected with IBDV

By ELISA test, the antibodies to VP4 and VP3 proteins can be detected from sera in chickens infected with the pathogenic IBDV (Fig. 2A); in chickens immunized with the nonpathogenic IBDV (Fig. 2B), anti-VP3 antibody was only detectable when twice vaccinated and the anti-VP4 antibody is not seroconverted. Immunofluorescence assay further shows that anti-VP3 and anti-VP4 antibodies of the pathogenic IBDV-infected chickens can be detected respectively using the VP3 and VP4 proteins expressed in Vero cells, but anti-VP4 antibody of the nonpathogenic IBDV-vaccinated chickens is not still detectable, indicating that VP4 protein of the nonpathogenic IBDV cannot induce the humoral immune responses of the immunized chickens. In the pathogenic IBDV-infected chickens, compared to anti-VP4 antibody that begin to be detected at 23 day post inoculation (dpi), the virus neutralizing and anti-VP3 antibodies are detected at 20 dpi and have a high titer. In addition, anti-VP3 antibody of the pathogenic IBDV-infected chickens has a high titer and maintains a longer time than that of the nonpathogenic IBDV-immunized chickens does, revealing that the pathogenic IBDV antigenicity is stronger than the nonpathogenic IBDV.

3.4. Distribution of VP4 protein in Bursa of Fabriciusof chicken inoculated with IBDV

To identify VP4 protein in bursa of Fabricius, the chickens were respectively inoculated with the pathogenic and nonpathogenic IBDV. In the pathogenic IBDV-infected chickens, the clinical signs, and BF gross lesions were observed after 36 h post inoculation. However no clinical signs and BF gross lesions were revealed in the nonpathogenic IBDV-immunized chickens. Immunohistochemistry analysis reveals that the VP3 and VP4 proteins (Fig. 1I and J) have a stronger reactivity with mouse anti-VP3 and VP4 mAbs in BF of the

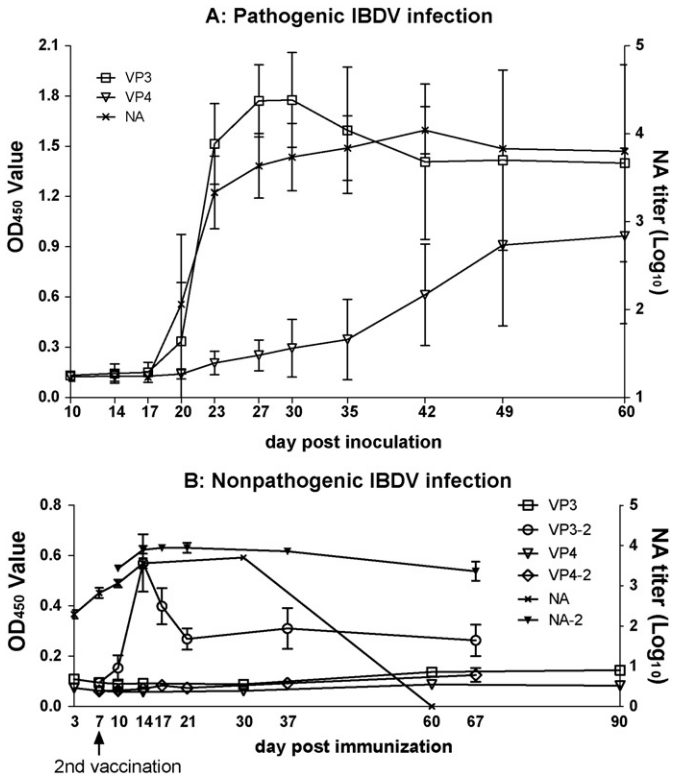

Fig. 2. Kinetic analysis of anti-VP3, anti-VP4 and viral neutralizing antibodies of chickens infected with pathogenic or nonpathogenic IBDV. Left *Y* axis represents the OD value (450 nm) tested by indirect ELISA; right *Y* axis represents the neutralizing antibody titer. In pathogenic IBDV infection, curves of VP3, VP4 and NA represents respectively the anti-VP3 antibodies, anti-VP4 antibodies and neutralizing antibody. In nonpathogenic IBDV infection, curves of VP3, VP3-2, VP4, VP4-2, NA, NA-2 represented respectively the anti-VP3 antibodies after the primary vaccination, the anti-VP3 antibodies after the boosted vaccination, the anti-VP4 antibodies after the primary immunization, the anti-VP4 antibodies after the boosted immunization, the neutralizing antibodies after the primary vaccination, the neutralizing antibodies after the boosted vaccination; the OD value of sera from control chickens were below 0.150 throughout the experiment. Geometric mean value was calculated for ten samples on each observation day.

pathogenic IBDV-infected chickens; but in the nonpathogenic IBDV-vaccinated chickens (Fig. 1K and L) the VP3 protein has a weaker reactivity with mouse anti-VP3 mAb, and the VP4 protein has not visible reactivity with mouse anti-VP4 mAb.

4. Discussion

IBDV genome encodes five viral proteins (Sanchez and Rodriguez, 1999). VP3 is considered to be a group-special antigen protein of IBDV (Birghan et al., 2000; Jagadish et al., 1988; Kibenge et al., 1997), and VP4 protein is described as a protease produced by the polyprotein VP243 during cleavage (Birghan et al., 2000; Feldman et al., 2006). The mAbs to various encoding proteins of IBDV are a critical tool studying the pathogenic mechanism. In this report, we generated the mAbs specific for VP3 and VP4 of IBDV using the recombinant proteins VP3 and VP4. Six mAbs to VP3 of IBDV and three mAbs to VP4 of IBDV with binding ability of IBDV, were identified by Vero cells respectively transfected with the plasmids pCI-VP3 and pCI-VP4, IBDV-infected CEF monolayer and BF tissue. These recombinant proteins VP3 and VP4 as well as mAbs against VP3 and VP4 proteins of IBDV provide an important tool for researching the pathogenic mechanism and developing a novel diagnostic method.

Of five viral proteins encoded by IBDV genome, the VP3 plays a key role in virus assembly as a scaffold protein (Maraver et al., 2003; Tacken et al., 2002). The interaction of the VP3 C-terminal with VP1 can remove the inherent structural blockade of the polymerase active site (Garriga et al., 2007). The VP3 protein acts as a transcriptional activator (Casanas et al., 2008). Currently, two linear epitopes of VP3 protein is mapped (Deng et al., 2007) and the VP3-ELISA was developed as a method detecting IBDV infection (Wang et al., 2008), considering that detecting VP3 antibody may be used as an efficient diagnostic method for IBDV infection in field chickens. In our study, anti-VP3-ELISA and neutralizing antibodies (Fig. 2A) appeared at 20 dpi in the pathogenic IBDV-infected chickens and at 10 dpi in the nonpathogenic IBDV-infected chickens, indicating that the time producing anti-VP3 antibody is identical to virus neutralizing antibody. Moreover, we also found that the relative sensitivity between virus neutralization and VP3-ELISA was identical, suggesting that the VP3-ELISA may be used to replace the virus neutralization assay for assessing the protective efficacy of IBDV vaccine. However, because anti-VP3 antibody can be detected in nonpathogenic IBDV-immunized chickens, hereby the antibody to VP3 protein of IBDV cannot use as a diagnostic marker of IBDV infection in the field chicken.

As known, VP4 is a non-structural protein of IBDV (Granzow et al., 1997) and a viral protease. Up to now, there is no report whether VP4 protein of IBDV can elicit antibody during IBDV infection. In our study, using VP4-ELISA and immunofluorescence assay, we firstly demonstrate that the anti-VP4 antibody is positive in all pathogenic IBDV-infected chickens, but no antibody specific for VP4 were detected in nonpathogenic IBDV-immunized chickens (Fig. 2B), and that the time eliciting anti-VP4 antibody is later than anti-VP3 and virus neutralizing antibodies in the pathogenic IBDV-infected chickens. This strongly suggests that the antibody to VP4 protein of IBDV is a biomarker discriminating the pathogenic and nonpathogenic IBDV infection in field chicken populations, and provides a method in IBDV epidemiology. However whether this evidence can be applied in all pathogenic and nonpathogenic IBDV strains needs further investigation.

Granzow et al. (1997) had found the intracytoplasmic and intranuclear needle-shaped structures recognized with anti-VP4 mAb in IBDV-infected cells. Here the intracytoplasmic and intranuclear needle-shaped fluorescence binding with anti-VP4 mAb not only reveals in IBDV-infected CEF monolayers, but is also observed in Vero cells transfected with VP4 gene of IBDV. In addition, we also found that VP4 protein appeared mainly in nucleus at 12 h post infection. It is not clear that the intracellular distribution of VP4 protein implies whatever biological role in replication and pathogenicity of IBDV. One previous report considered that sIgM-bearing B cells that is mainly sited in the medulla of lymphoid follicle (Withers et al., 2006), are the major target for IBDV infection (Hirai et al., 1981), but another evidence displayed that susceptibility of lymphoid bursa cells is not correlated with the expression of immunoglobulins on their surface (Muller, 1986). However, in IBDV-infected chickens, we further demonstrate that the cells recognized with anti-VP4 or anti-VP3 mAb is mainly located in the cortex of lymphoid follicle of BF (Fig. 1I and J). Thereby our results did not support that the target cell of IBDV is sIgM-bearing B cells distributing in the medulla of lymphoid follicle.

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