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Recombinant infectious bursal disease virus expressing Newcastle disease virus (NDV) neutralizing epitope confers partial protection against virulent NDV challenge in chickens

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ABSTRACT

In this study, the regions in the infectious bursal disease virus (IBDV) genome that are amenable to the introduction of a sequence encoding a virus-neutralizing epitope of Newcastle disease virus (NDV) hemagglutinin-neuraminidase (HN) protein were identified. By using the reverse genetics approach, insertions or substitutions of sequences encoding the NDV epitope were engineered in the exposed loops (P_{BC}, P_{HI} and $P_{AA'}$) of the VP2 capsid protein and the N terminus of the nonstructural VP5 protein as well as the pep7a and pep7b regions of the pVP2 precursor of a commonly used IBDV vaccine strain, Gt. Three recombinant IBDVs expressing the NDV epitopes were successfully rescued in the P_{BC}, pep7b and VP5 regions and the expressed epitope was recognized by anti-HN antibodies. Genetic analysis showed that the IBDV recombinants carrying the NDV epitopes were stable in cell cultures and in chickens. Animal studies demonstrated that the IBDV recombinants were innocuous in chickens. Vaccination with the recombinant viruses generated antibody responses against both IBDV and NDV, and provided 70–80% protection against IBDV and 50–60% protection against NDV. These results indicate that the recombinant IBDV has the potential to serve as a novel vaccine vector for other pathogens. In future studies, it is worth considering research to improve IBDV vector vaccine to get complete protection and safety of animals and humans.

1. Introduction

Infectious bursal disease virus (IBDV), a member of the genus *Avibirnavirus* in the family *Birnaviridae*, causes considerable economic losses in the poultry industry worldwide by inducing an acute and highly contagious disease in young chickens with high mortality (van den Berg, 2000). IBDV is a non-enveloped, icosahedral virus which has a polyploid genome containing two segments of double-stranded RNA. The smaller segment, B, encodes VP1, a 90-kDa viral RNA-dependent RNA polymerase (von Einem et al., 2004). The larger segment, A, contains two partially overlapping open reading frames (ORFs). The smaller ORF encodes VP5, a 17-kDa nonstructural protein. VP5 is dispensable for viral replication in vitro and in vivo (Yao et al., 1998), which makes it a prime candidate for the construction of marked vaccines carrying deletions. The larger ORF encodes a 110-kDa precursor polyprotein that is proteolytically cleaved by the viral protease VP4 (Birghan et al., 2000) to form the pVP2 precursor (48 kDa) as well as VP4 (28 kDa) and VP3 (32 kDa). Throughout virion maturation, pVP2 is further processed by several proteolytic cleavages at its C terminus for conversion into mature VP2 (41 kDa) and four structural peptides composed of 46, 7, 7 and 11 amino acids (pep46, pep7a, pep7b, and pep11), all associated with the virus particle (Chevalier et al., 2005; Da Costa et al., 2002). VP2 and VP3 are the major structural proteins, constituting 51% and 40% of the virion, respectively (van den Berg, 2000). The four peptides are located either at the external surface of the viral particle (pep7a, pep7b, and pep11) or underneath the surface at the fivefold or quasi sixfold axes (pep46) (Chevalier et al., 2005). It was showed that pep46 and pep11 are essential to viral assembly and play a key role in the viral cycle (Chevalier et al., 2005; Galloux et al., 2007). In contrast, pep7a and pep7b are not essential to viral assembly, and reverse genetics shows that they are dispensable for virus rescue (Chevalier et al., 2005; Da Costa et al., 2002).

The IBDV capsid consists of a single shell formed by 260 trimers of protein VP2 organized in a $T = 13$ icosahedral lattice (Coulibaly et al., 2005). The available structural data for VP2 (Coulibaly et al., 2005; Garriga et al., 2006; Lee et al., 2006) reveal that this protein is folded into three distinct domains named projection

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(P), shell (S), and base (B). Expression of VP2 by itself leads to dodecahedral subviral particles (SVP) containing 20 VP2 trimers (Castón et al., 2001) and exposing five loops of the P domain away from the surface of the SVP, in which loops P_{BC} (aa 219–224), P_{DE} (aa 250–254), P_{FG} (aa 283–287) and P_{HI} (aa 315–324) located at the head of domain P, while loop $P_{AA'}$ (aa 184–195) located near domain S (Coulibaly et al., 2005). Previous studies carried out on the virus cell tropism and virulence (Brandt et al., 2001; Qi et al., 2009) suggest that loops P_{DE} and P_{FG} containing residues 253 and 284 are involved in virus-cell receptor binding, and loops P_{BC} and P_{HI} are the most suitable sites for the insertion of foreign peptides (Rémond et al., 2009).

Recently, the potential of IBDV to serve as an antigen delivery system has been explored. It was shown that the immunodominant epitope of foot-and-mouth disease virus (FMDV) could be effectively inserted into the P_{BC} loop to produce IBDV SVPs with chimeric VP2 in insect cells (Rémond et al., 2009). The produced subviral particles reacted with FMDV monoclonal antibodies and elicited a neutralizing antibody response in immunized mice. More interestingly, Upadhyay et al. (2011) successfully recovered recombinant IBDVs expressing c-Myc and human hepatitis C virus (HCV) epitopes in the VP5 region of segment A. In spite of these promising results, the tolerable size and sites in the IBDV genome for the insertion have not been fully studied. Further exploration of the insertion sites used in previous studies and other sites with different antigenic epitopes would shed more light on the development of IBDV vectors.

Newcastle disease virus (NDV) is a member of the genus *Avulavirus* in the family *Paramyxoviridae*. NDV causes an economically important disease and has the potential to infect all species of birds worldwide, with the mortality and morbidity rates varying among species and strains of the virus (Alexander, 1997). The envelope of NDV contains two glycoproteins, the virus hemagglutinin-neuraminidase attachment protein, HN, and the fusion protein, F, which form spike-like protrusions on the outer surface of the virion. The HN and F proteins are important for virus infectivity and pathogenicity (Nagai et al., 1976). They produce virus neutralizing antibody responses and are the protective antigens (Boursnell et al., 1990; Cosset et al., 1991; Sun et al., 2008). Major antigenic determinants and epitopes that stimulate the production of virus-neutralizing antibodies have been determined for the HN (Chambers et al., 1988) and F proteins (Toyoda et al., 1988).

The present study explored the possibility of recovering recombinant IBDVs carrying NDV HN neutralizing epitopes with the aim of further studying the potential of IBDV vectors and developing a safe and efficient vaccine candidate against NDV. The insertion sites used in previous studies were further explored by inserting or substituting the NDV epitope in the P_{BC} and P_{HI} loops of the VP2 or the VP5 region. The previously unexplored regions, $P_{AA'}$ loop of VP2, pep7a and pep7b of pVP2 precursor, were also tested as potential insertion sites. Here, three recombinant IBDVs expressing the NDV epitopes were successfully recovered in the P_{BC}, pep7b and VP5 regions, and they elicited neutralizing antibody responses against both IBDV and NDV in immunized chickens. These results clearly suggest that it is possible to use IBDV as a novel vaccine vector for other pathogens and the recombinant IBDVs generated in this study have the potential for development into a bivalent vaccine candidate against virulent NDV as well as IBDV infection.

2. Materials and methods

2.1. Viruses, cells and plasmids

The very virulent IBDV (vvIBDV) strain Gx was isolated and identified as previously described (Wang et al., 2003). IBDV strain Gt was attenuated from vvIBDV Gx after blind passages in vivo (Wang et al., 2004). The NDV vaccine strain LaSota and the virulent strain F48E9 were originally received from the China Veterinary Culture Collection. The NDV live vaccine LaSota was obtained from a commercial manufacturer (Weike, Harbin, China). Primary chicken embryo fibroblast (CEF) cells were prepared from 10-day-old specific-pathogen-free (SPF) chicken embryos. DF1 (a CEF cell line) cells were kindly provided by Dr. Zhigao Bu, Harbin Veterinary Research Institute, the Chinese Academy of Agricultural Sciences, and cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA). The eukaryotic expression vector pCAGGS (Niwa et al., 1991) was kindly provided by Dr. J. Miyazaki, University of Tokyo, Japan.

2.2. Animals

SPF white leghorn chickens were obtained from the Experimental Animal Center of Harbin Veterinary Research Institute, the Chinese Academy of Agricultural Sciences and housed in negative-pressure-filtered air isolators. Animal experiments were approved by the Animal Ethics Committee of the Institute and performed in a biosafety level 2 facility in accordance with animal ethics guidelines and approved protocols.

2.3. Construction of plasmids

Construction of the plasmids pCAGGmGtAHRT and pCAGGmGtBHRT containing segments A and B of IBDV Gt flanked by the hammerhead ribozyme (HamRz) sequence at their 5′ ends and hepatitis delta ribozyme (HdvRz) sequence at their 3′ ends, respectively, has been described previously (Qi et al., 2007). Plasmid pCAGGmGtAHRT was used as template to construct different plasmids of IBDV segment A containing foreign sequences. Plasmid pCAGGmGtBHRT was used to co-transfect DF1 cells with plasmids of segment A for virus rescue. Ten different constructs of segment A were created by inserting or substituting a sequence encoding a conserved neutralizing epitope (PDEQDYQIR, residues 345–353) of NDV HN protein in the loops of VP2, the peptide regions of pVP2 or the N terminus of VP5, as shown in Fig. 1. First, plasmids pIBDV-1 and pIBDV-2 were constructed by inserting the NDV epitope sequence into the P_{BC} loop of VP2 (between nt 793 and 794 of segment A) or replacing the nucleotides encoding the P_{BC} loop (nt 785–802 of segment A) with the epitope sequence, respectively. The epitope was also inserted into the P_{HI} loop (between nt 1087 and 1088) for pIBDV-3 or replaced the P_{HI} loop (nt 1073–1102) for pIBDV-4. Additionally, the $P_{AA'}$ loop of VP2 was inserted (between nt 697 and 698) or replaced (nt 686–712) with this epitope for pIBDV-5 and pIBDV-6. Second, the NDV epitope was inserted into the pep7a region of pVP2 (between nt 1600 and 1601) for pIBDV-7 or the pep7b region (between nt 1621 and 1622) for pIBDV-8. Third, the NDV epitope was inserted into the N terminus of VP5 (between nt 99 and 100) for pIBDV-9 or replaced the N terminus of VP5 (nt 100–126) for pIBDV-10.

All the manipulations were done by performing overlapping PCR using the respective primers (Table 1). To construct pIBDV-1, two PCR fragments (878 bp and 2582 bp) were amplified with primer pairs HamCla1F/IBDV-1R and IBDV-1F/HdvKpn1R, respectively. These fragments were combined and amplified into one fragment using the flanking primer pair HamCla1F/HdvKpn1R. The amplified fragment was digested with ClaI/KpnI and ligated into pCAGGS vector under control of the hybrid CMV enhancer/chicken β-actin promoter to yield plasmid pIBDV-1. Similarly, plasmid pIBDV-2 was generated using the primer pairs HamCla1F/IBDV-2R, IBDV-2F/HdvKpn1R and HamCla1F/HdvKpn1R. The construction of plasmids pIBDV-3–pIBDV-10 was performed similarly using the respective primers. All the plasmids were sequenced to confirm the desired sequence changes in segment A.

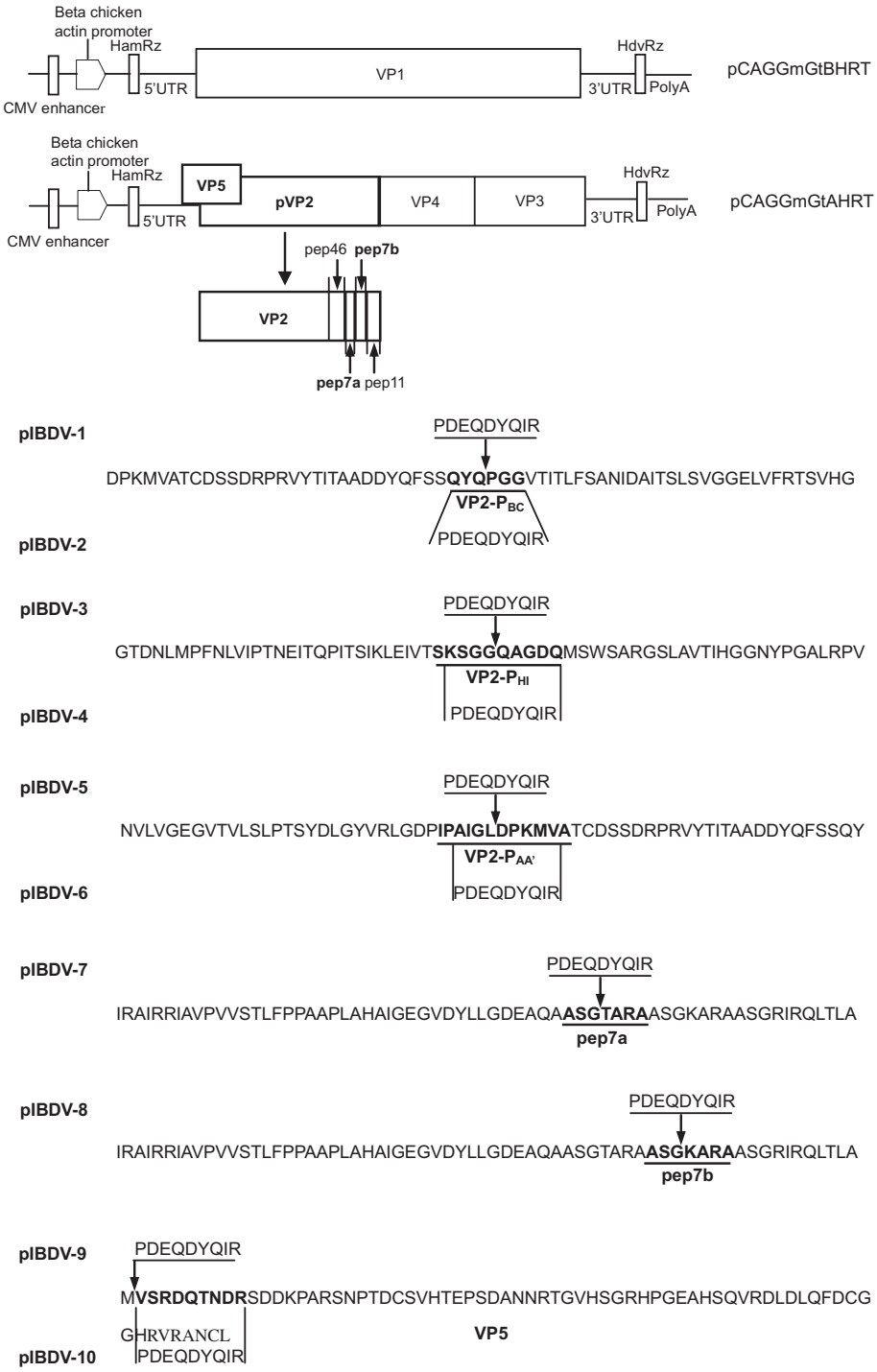

Fig. 1. Schematic diagrams of the recombinant plasmids containing the cDNAs of segment A (pCAGGmGtAHRT) and segment B (pCAGGmGtBHRT) of IBDV Gt strain. The cDNAs of both segments were preceded by a CMV enhancer and a beta chicken actin promoter and were flanked by the cDNAs of HamRz and HdvRz. Segment A was further modified to construct different plasmids with a NDV neutralizing epitope (PDEQDYQIR) as follows: pIBDV-1 and pIBDV-2, the NDV epitope was inserted in P_{BC} loop (between aa 221 and 222 of VP2) or replaced P_{BC} loop (aa 219–224 of VP2); pIBDV-3 and pIBDV-4, the NDV epitope was inserted in P_{HI} loop (between aa 319 and 320 of VP2) or replaced P_{HI} loop (corresponding to aa 316–324 of VP2); pIBDV-5 and pIBDV-6, the NDV epitope was inserted in $P_{AA'}$ loop (between aa 189 and 190 of VP2) or replaced $P_{AA'}$ loop (corresponding to aa 186–194 of VP2); pIBDV-7 and pIBDV-8, insertion of the NDV epitope in pep7a (between aa 490 and 491 of pVP2) or pep7b (between aa 497 and 498 of pVP2); pIBDV-9 and pIBDV-10, the NDV epitope was inserted at the N terminus of VP5 (between aa 1 and 2 of VP5) or replaced the N terminus of VP5 (aa 2–10 of VP5). DF1 cells were transfected with various IBDV segment A constructs plus segment B. The constructs pCAGGmGtAHRT, pIBDV-1, pIBDV-8, and pIBDV-9 were successful in generation of recombinant IBDVs while others were unsuccessful.

2.4. Virus rescue and identification

Highly purified plasmids carrying IBDV segment A or segment B were obtained by using Qiagen Plasmid Midi kits (Qiagen, Hilden, Germany) and transfected into DF1 cells to rescue viruses. Briefly, DF1 cells were co-transfected with 2 μg of each plasmid carrying segment A and 2 μg of pCAGGmGtBHRT using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA). The culture supernatant containing the virus stocks was harvested after 72 h of transfection and then blindly passaged into secondary CEF cells. The viruses were

Table 1
Oligonucleotide primers used for construction of various plasmids.

Primer	Sequence (5′–3′)[a]
HamCla1F	ATTA**ATCGAT**TGTTAAGCGTCTGATGAGTCC
HdvKpn1R	ATTA**GGTACC**CGCCCTCCCTTAGCCATCCGAGT
IBDV-1R	*CCGGATCTGGTAATCCTGCTCATCGGG*TTGGTACTGTGATGAGAATTG
IBDV-1F	*CCCGATGAGCAGGATTACCAGATCCGGC*CAGGTGGGGTAACAATCACA
IBDV-2R	*CCGGATCTGGTAATCCTGCTCATCGGG*TGATGAGAATTGGTAATCATC
IBDV-2F	*CCCGATGAGCAGGATTACCAGATCCGGG*TAACAATCACACTGTTCTCA
IBDV-3R	*CCGGATCTGGTAATCCTGCTCATCGGG*ACCACCACTTTTGGAGGTCAC
IBDV-3F	*CCCGATGAGCAGGATTACCAGATCCGGC*AGGCAGGGGATCAGATGTCA
IBDV-4R	*CCGGATCTGGTAATCCTGCTCATCGGGGGAGGT*CACTATCTCCAGTTTG
IBDV-4F	*CCCGATGAGCAGGATTACCAGATCCGGA*TGTCATGGTCGGCAAGAGGG
IBDV-5R	*CCGGATCTGGTAATCCTGCTCATCGGGAAGCCCT*ATTGCGGGAATGGG
IBDV-5F	*CCCGATGAGCAGGATTACCAGATCCGGGACCCAAAAAT*GGTAGCCACA
IBDV-6R	*CCGGATCTGGTAATCCTGCTCATCGGGGGGAAT*GGGGTCACCAAGCCT
IBDV-6F	*CCCGATGAGCAGGATTACCAGATCCGGGCCACATGT*GACAGCAGTGAC
IBDV-7R	*CCGGATCTGGTAATCCTGCTCATCGGG*TCCTGAAGCAGCCTGTGCCTC
IBDV-7F	*CCCGATGAGCAGGATTACCAGATCCGGA*CTGCTCGAGCCGCGTCAGGA
IBDV-8R	*CCGGATCTGGTAATCCTGCTCATCGGG*TCCTGACGCGGCTCGAGCAGT
IBDV-8F	*CCCGATGAGCAGGATTACCAGATCCGGAAAGCAAGAGCT*GCCTCAGGC
IBDV-9R	*CCGGATCTGGTAATCCTGCTCATCGGG*CATCAATGATAGCGTTGTAGA
IBDV-9F	*CCCGATGAGCAGGATTACCAGATCCGGG*TTAGTAGAGATCAGACAAAC
IBDV-10R	*CCGGATCTGGTAATCCTGCTCATCGGG*CATCAATGATAGCGTTGTAGA
IBDV-10F	*CCCGATGAGCAGGATTACCAGATCCGGA*GCGATGACAAACCTGCAAGA

[a] The restriction enzyme sites are shown in bold. NDV epitope sequences are marked in italics.

harvested from the infected cell cultures when visible cytopathic effect (CPE) appeared. To characterize the rescued virus, indirect immunofluorescence assay (IFA) with anti-VP2 monoclonal antibody (MAb, 1:100 dilution) and electron microscopy were performed as described previously (Qi et al., 2007). To verify whether the recombinant virus contained the introduced foreign sequence, genomic RNAs were isolated from the rescued virus and analyzed by RT-PCR (Qi et al., 2007). For each virus, the full-length genomes were amplified and sequenced.

2.5. Expression of HN epitope in cells infected with rescued viruses

The expression of the HN epitope by the recombinant virus was demonstrated in DF1 cells infected with virus stock by IFA. Briefly, confluent DF1 cells on six-well plates were infected with each of the recombinant viruses (IBDV-1, IBDV-8, IBDV-9) or IBDV-rGt at a multiplicity of infection (MOI) of 0.1. Uninfected DF1 cells were used as a negative control. After 24 h, the cells were washed with PBS and fixed with absolute ethanol for 20 min at room temperature. The fixed cells were incubated with a rabbit anti-HN polyclonal antiserum at 37 °C for 60 min, and reacted with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG antibody (Sigma, St. Louis, MO, USA) at 37 °C for 60 min. After being washed with PBS, the cells were examined by fluorescence microscopy. Other negative controls treated with negative rabbit serum were also included.

2.6. Growth kinetics and stability of the recombinant viruses in cell cultures

To compare the replication kinetics of the rescued viruses, the one-step growth was analyzed. Confluent monolayer of CEF cells in cell culture flasks (25 cm^2) was inoculated with 10^4 50% tissue culture infectious doses ($TCID_{50}$) of each virus based on the titer determined in CEF cells. Infected cell cultures were harvested at 12-h intervals and the titer of infectious progeny present in the culture was determined as $TCID_{50}$ per milliliter using the Reed–Muench formula. The mean values and standard deviations were calculated from three independent experiments.

To verify the genetic stability of the foreign epitope of the recombinant viruses, the viruses were serially passaged in CEF cells up to the 12th generation. For each passage, the viruses were inoculated to the secondary CEF cells at a MOI of 0.1. After 72 h of inoculation, the viruses were harvested and the viral RNAs were extracted. The full length genomes of the recombinant viruses were amplified by RT-PCR and sequenced as previously described (Qi et al., 2007). The expression of the NDV epitope was also confirmed by immunofluorescence assay.

2.7. Characterization of the recombinant viruses in vivo

To assess the pathogenicity of the recombinant viruses, seventy-five 2-week-old SPF chickens were randomly divided into five groups of 15 chickens each. Each group was maintained separately in a negative-pressure isolator. Chickens in groups 1–4 were inoculated intranasally and intraocularly with 10^5 $TCID_{50}$ of the rescued virus IBDV-1, IBDV-8, IBDV-9 and IBDV-rGt, respectively. The fifth group received DMEM medium without any virus as negative control. Chickens were monitored daily for clinical signs. At 3, 5, 7, 10 and 14 days post-inoculation (d p.i.), three chickens were randomly chosen from each group and euthanized for necropsy. Each chicken was examined for signs of pathological changes. The bursa and body weights of each chicken were determined, and the bursa:body-weight index (BBIX) was calculated according to the following formula: BBIX = (bursa:body-weight ratios)/(bursa:body-weight ratios in negative group). Bursae with a BBIX lower than 0.70 were considered atrophied (Lucio and Hitcher, 1979). The obtained bursa was divided into two parts. One part was used for extraction of viral RNA, and the other part was fixed in 10% neutral buffered formalin for histopathological analysis from which the severity of bursal follicular necrosis was recorded using the histopathologic bursal lesion score (HBLS) as described earlier (Schröder et al., 2000). In brief, the HBLS was scored on a scale of 0–5: 0, no lesion; 1, slight change; 2, scattered or partial bursal damage; 3, 50% or less follicle damage; 4, 51–75% follicle damage; 5, 76–100% bursal damage.

To evaluate the replication kinetics of the rescued viruses in vivo, viral RNA obtained from the bursa were quantified by real-time RT-PCR as described previously (Wang et al., 2009). The mean values and standard deviations of the data obtained from three independent experiments were calculated. To confirm the identity of the recombinant viruses and investigate whether changes occurred in the nucleotide acid sequence during inoculation, viral RNAs obtained from the bursa were amplified by RT-PCR and sequenced.

2.8. Immunization and challenge experiments in chickens

The immunogenicity and protective efficacy of the recombinant viruses were evaluated in SPF white leghorn chickens. A total of 110 chickens were randomly assigned to 11 treatment groups of 10 birds each. Each group of chickens was reared separately in a negative-pressure isolator. At 14 days of age, 8 groups of chickens were immunized by the eye and intranasal route with either the rescued virus IBDV-1, IBDV-8, IBDV-9, IBDV-rGt (10^5 $TCID_{50}$ per bird) or NDV commercial live vaccine LaSota ($10^{6.21}$ EID_{50} per bird as recommended) as shown in Table 2. Chickens in groups 9 and 10 were inoculated with DMEM medium by the same route as unvaccinated challenge control. In addition, group 11 was kept as a normal control which was unvaccinated and unchallenged during the experiment. Serum samples were collected from each group prior to immunization and at weekly intervals postimmunization for assessing IBDV and NDV antibodies.

Three weeks postimmunization, birds in groups 1–10 were challenged through the oculonasal route with 10^3 ELD_{50} of vvIBDV

Table 2
Animal experimental design.

Exptl. group	No. of chickens	Vaccination age (days)	Vaccine[a]	Challenge age (days)	Challenge virus[b]
1	10	14	IBDV-1	35	Gx
2	10	14	IBDV-1	35	F48E9
3	10	14	IBDV-8	35	Gx
4	10	14	IBDV-8	35	F48E9
5	10	14	IBDV-9	35	Gx
6	10	14	IBDV-9	35	F48E9
7	10	14	IBDV-rGt	35	Gx
8	10	14	NDV-LaSota	35	F48E9
9	10	14	DMEM	35	Gx
10	10	14	DMEM	35	F48E9
11	10	–[c]	None	–	None

[a] Vaccination was given by the eye and intranasal route with either the rescued virus IBDV-1, IBDV-8, IBDV-9, IBDV-rGt (10^5 $TCID_{50}$ per bird) or NDV commercial live vaccine LaSota ($10^{6.21}$ EID_{50} per bird as recommended). DMEM inoculated birds were kept for controls.
[b] Chickens were challenged through the oculonasal route with 10^3 ELD_{50} of vvIBDV Gx or 10^2 ELD_{50} of highly pathogenic NDV F48E9 three weeks after immunization.
[c] No vaccination or no challenge.

Gx or 10^2 ELD_{50} of highly pathogenic NDV F48E9, at 35 days of age, as indicated in Table 2. The birds challenged with vvIBDV Gx were monitored daily for clinical signs and mortality for 10 days. At 10 days postchallenge (d p.c.), the chickens were euthanized and weighed. Bursa samples from all chickens were weighed and then fixed in neutral buffered formalin for histopathological analysis. The BBIX and HBLS of each chicken were determined as described above. A chicken that survived with a BBIX higher than 0.70 and a HBLS of 1 was defined as protected against IBDV challenge. To evaluate whether the recombinant viruses could induce protective immunity against NDV, birds challenged with NDV strain F48E9 were observed daily for a period of 10 days for mortality and clinical signs of neurotrophic Newcastle disease. Birds were considered protected if they showed no central nervous system signs and survived. Postchallenge sera were also collected from the dead birds during the necropsy or the surviving birds before they were sacrificed at 10 d p.c. for further analysis.

2.9. Serological analysis

The antibody levels of serum samples collected from each group were evaluated by enzyme-linked immunosorbent assay (ELISA) and virus neutralization (VN) test. The ELISA antibody titers in sera against IBDV and NDV were tested using commercially available ELISA kits (IDEXX, Westbrook, Maine). The relative level of antibody titer in the unknown was determined by calculating the sample to positive (S/P) ratio. Endpoint titers were calculated with the equation: $\log_{10}\text{titer} = 1.09\ (\log_{10}S/P) + 3.36$ (IDEXX, Westbrook, Maine). The sera from each group were tested in triplicates and the sera with titers of higher than 396 were considered as positive for IBDV or NDV antibody according to the protocol.

For the virus neutralization test, 2-fold serial dilutions of 50 μL serum samples were heat-inactivated (56 °C for 30 min) and mixed with an equal volume containing 100 $TCID_{50}$ of IBDV Gt or 100 EID_{50} of NDV LaSota in DMEM. After incubation for 1 h, the IBDV- and NDV-serum mixtures were inoculated to DF1 cells or embryonated SPF chicken eggs, respectively, followed by further incubation at 37 °C for 72 h. The VN titers against IBDV and NDV were obtained by confirmation of the presence of virus in IBDV-infected DF1 cells by cytopathic effects or the allantoic fluid of NDV-inoculated eggs by hemagglutination assay. The test was read when virus was observed in the negative serum control and the virus positive control. The VN titer of the serum was determined as the $\log_2$ value of the reciprocal of the highest dilution that was capable of neutralizing 100 $TCID_{50}$ of Gt or 100 EID_{50} of LaSota. VN titer higher than 1.0 was considered positive and a geometric mean titer was calculated for each group.

2.10. Statistical analysis

All data were presented as the mean ± standard deviation (S.D.). One-way ANOVA was employed to evaluate the statistical differences among groups using SPSS 17.0 (SPSS Inc., Chicago, IL). Statistical significance was set at $P < 0.05$ for all tests.

3. Results

3.1. Generation of recombinant IBDV viruses expressing NDV HN epitope

To identify regions in the IBDV genome amenable to the introduction of a sequence encoding a NDV neutralizing epitope, ten different recombinant plasmids containing IBDV segment A with insertion or substitution of the epitope sequence in the P_{BC} (pIBDV-1 and pIBDV-2), P_{HI} (pIBDV-3 and pIBDV-4) or $P_{AA'}$ (pIBDV-5 and pIBDV-6) loops of VP2, the pep7a or pep7b of pVP2 (pIBDV-7 and pIBDV-8) or the N terminus of VP5 (pIBDV-9 and pIBDV-10) were constructed. The desired sequence changes in segment A were confirmed by sequencing for all the plasmids.

Successful virus rescue was achieved when cells were co-transfected with the plasmid of segment B and with the constructs pCAGGmGtAHRT, pIBDV-1, pIBDV-8, and pIBDV-9, yielding IBDV-rGt, IBDV-1, IBDV-8, and IBDV-9, respectively. Visible CPE appeared in infected cells from the second passage for IBDV-rGt and IBDV-8 and from the third passage for IBDV-1 and IBDV-9. IFA results demonstrated that cells infected with IBDV-rGt, IBDV-1, IBDV-8, and IBDV-9 had detectable fluorescent signals when incubated with VP2-specific MAb, and fluorescent signals were not observed in the mock-infected control (Fig. 2A–E). The rescue of the viruses was further confirmed by RT-PCR analysis of viral RNA template. Subsequent sequence analysis of the RT-PCR products confirmed the presence of the NDV epitope sequence in segment A of the recombinant viruses. Besides, the non-enveloped and icososahedral-shaped IBDV with a diameter of about 60 nm were observed by electron microscopy (data not shown).

Expression of the HN epitope by the rescued IBDVs was confirmed by immunostaining infected DF1 cells 24 h after inoculation. As expected, cells infected with IBDV-rGt were not stained by the rabbit antiserum against HN protein (Fig. 2F), but they were positive for immunostaining using VP2-specific MAb. In contrast, cells infected with IBDV-1, IBDV-8 or IBDV-9 were stained by the rabbit anti-HN antiserum (Fig. 2G–I) as well as the anti-VP2 MAb. The mock-infected control showed no fluorescence with either anti-HN antiserum (Fig. 2J) or anti-VP2 MAb. These results suggested that IBDV can sustain the insertion of the NDV epitope

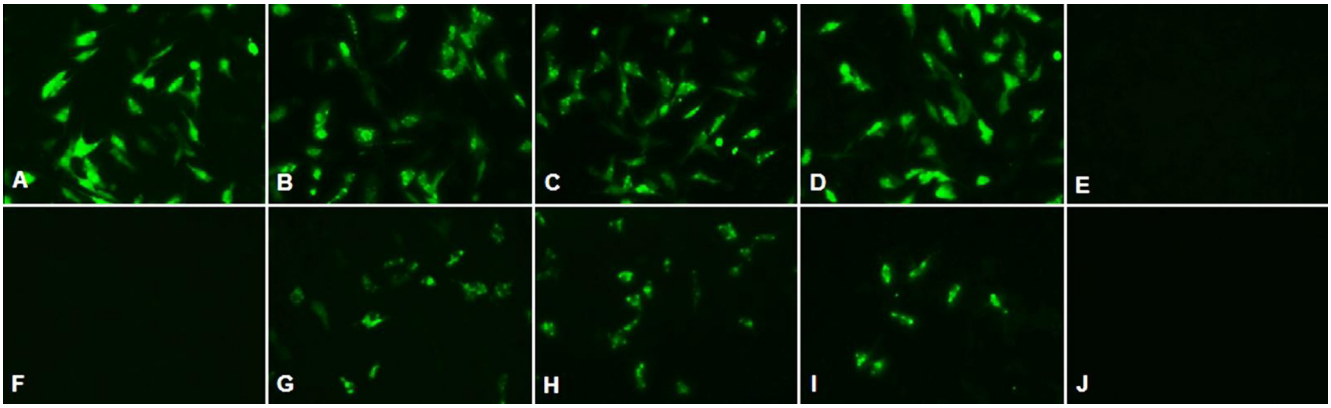

Fig. 2. Immunofluorescence analysis of NDV HN epitope expression. Confluent DF1 cells were infected with IBDV-rGt (A and F), IBDV-1 (B and G), IBDV-8 (C and H), or IBDV-9 (D and I) at a MOI of 0.1. Uninfected cells were used as negative controls (E and J). At 24 h post-infection, the cells were fixed and analyzed by immunofluorescence staining with mouse anti-VP2 monoclonal antibody (A, B, C, D, and E) or with rabbit anti-HN polyclonal antiserum (F, G, H, I, and J).

in the P_{BC} loop, pep7b and VP5 region, and the epitope was successfully expressed by the recombinant IBDVs.

3.2. Biological characterization and stability of the rescued viruses

To investigate whether the insertion of the foreign epitope affected the growth property of the IBDV vector virus, the replication kinetics of the recombinant IBDVs and the parental virus IBDV-rGt were compared both in CEF cells and in chickens. Triplicate monolayers of CEF cells were infected with 10^4 $TCID_{50}$ of each virus, and samples were collected at 12-h intervals to assess the virus titers. As shown in Fig. 3A, the kinetics and magnitude of replication for IBDV-8 in CEF cells were very similar to those for IBDV-rGt and the final virus yields were comparable. However, IBDV-1 showed a slight delay in growth and IBDV-9 replicated slowly after 36 h p.i., yielding titers approximately half a log lower than that of IBDV-rGt at 72 h after inoculation. The replication properties of the rescued viruses in bursa of chickens were determined by real-time RT-PCR. The results showed that viral loads of the virus strains reached the highest level at 5 d p.i., and kept the level to 7 d p.i. Then the viral loads decreased until the lowest point observed at 14 d p.i. Compared with IBDV-rGt, IBDV-8 exhibited a comparable viral load after inoculation, while IBDV-1 and IBDV-9 replicated slight slowly in bursa and had a load approximately 0.5–1.0 log lower than that of IBDV-rGt during the infection (Fig. 3B).

The pathogenicity of the recombinant IBDVs in vivo was investigated in 2-week-old SPF chickens. Throughout the 14-day experimental period, no death and no clinical symptom of IBD were observed for chickens receiving the recombinant IBDVs and the IBDV-rGt. The BBIX in different groups inoculated with IBDV-1, IBDV-8, IBDV-9 or IBDV-rGt were similar and kept above the critical value of 0.7 (Fig. 4), indicating that the bursal of chickens infected with the rescued viruses were not atrophic. Also, histopathological sections of bursae derived from all groups showed normal follicles and follicular connective tissues, and no microscopic lesion was observed (HBLS being 0–1). These results indicated that the insertion of the NDV epitopes did not increase the virulence of the IBDV vector virus.

To determine the stability of the NDV epitope in recombinant IBDV viruses, the rescued viruses were passaged 12 times in secondary CEF cells. Sequence analysis of the RT-PCR product confirmed the presence of the NDV epitope sequence in the viruses from each passage, and the sequence identity of the NDV epitope was preserved and stably maintained even after 12 passages in cell cultures (data not shown). The expression of the NDV epitope in the infected cells of each passage was also confirmed by immunofluorescence assays (data not shown). During the infection experiment in chickens, RT-PCR and sequencing confirmed the presence of the recombinant viruses in the bursae of chickens and there were no changes occurred in the epitope sequence. In addition, the recombinant viruses maintained similar BBIX and HBLS values after 12 passages in cell cultures (data not shown).

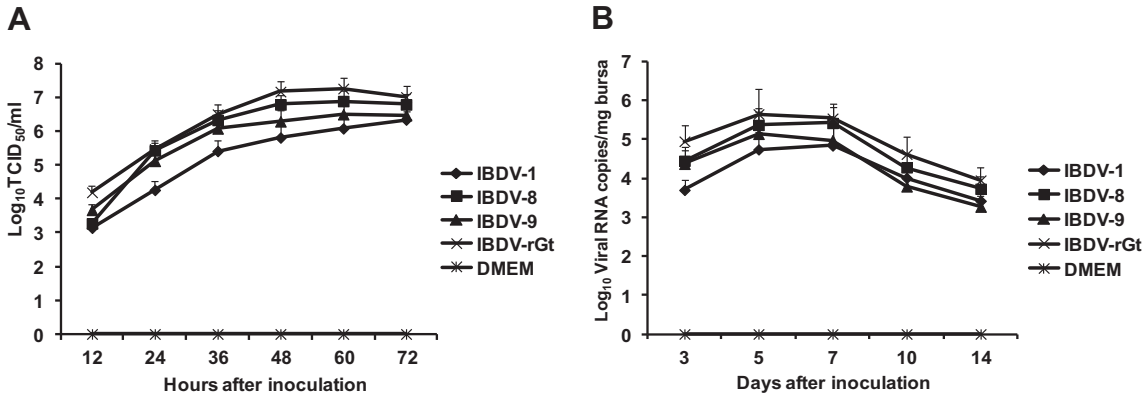

Fig. 3. Replication kinetics of the rescued viruses in cell cultures and in chickens. (A) Confluent secondary CEF cells were infected with 10^4 $TCID_{50}$ of each rescued virus. Infected cell cultures were harvested at 12-h intervals and the virus titers were determined as $TCID_{50}$ per milliliter. The mean values and standard deviations were calculated from three independent experiments. (B) Two-week-old SPF chickens were infected intranasally and intraocularly with 10^5 $TCID_{50}$ of the rescued virus. The bursa was collected and homogenized at indicated time points after infection. Subsequently, viral RNA loads were quantified using real-time RT-PCR. Data are the mean titer (log_{10} of viral RNA copies) per mg of tissue, and the error bars represent the standard deviations for each time point from three assays.

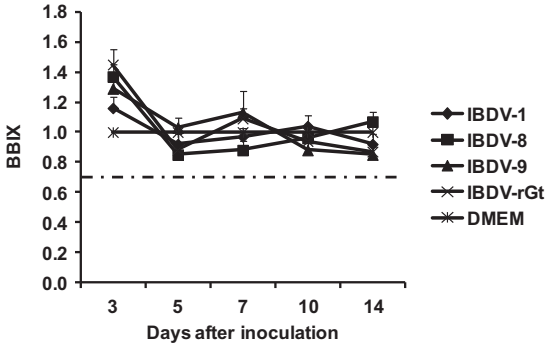

Fig. 4. The kinetic curves of bursa: body-weight index (BBIX) of chickens infected with the rescued viruses, or with DMEM alone without viruses (negative control). The BBIX was calculated as (bursa:body-weight ratios)/(bursa:body-weight ratios in negative group), and presented as the mean ± standard deviation from each group. Bursae with a BBIX lower than 0.70 were considered atrophied.

3.3. Protective efficacy in chickens against virulent IBDV and NDV challenges

The protective efficacy of the recombinant viruses against IBDV and NDV were examined in chickens. To determine the protective efficacy of the recombinant viruses against IBDV challenge, the immunized chickens and unvaccinated controls were challenged with vvIBDV Gx at 3 weeks postvaccination. Chickens of the DMEM control group were fully susceptible to the challenge, showing depression at 2 d p.c., subsequent to induction of typical signs of acute IBD including ruffled feathers, anorexia, diarrhoea, trembling, dehydration and prostration from 3 to 7 d p.c. with 5 chickens died at 4 d p.c. and 2 died at 5 d p.c. (Fig. 5A). In contrast, neither clinical signs nor mortality was observed in chickens vaccinated with IBDV-rGt or IBDV-9 after the lethal challenge with vvIBDV. Chickens vaccinated with IBDV-1 or IBDV-8 had 20% or 10% mortality, respectively, but the surviving chickens showed no clinical symptoms. In the DMEM group challenged with Gx, all the dead and surviving chickens had a BBIX value of below 0.7 and severe bursal gross lesions including depletion and necrosis of lymphocytes, fibroplasias, atrophy of follicles and follicular depletion (HBLS being 4–5). Of chickens vaccinated with IBDV-1, IBDV-8, IBDV-9 or IBDV-rGt, only 30%, 20%, 20% and 10% showed bursal atrophy after challenge, respectively, as indicated by BBIX. Histopathological examination of the infected bursae revealed that the surviving chickens vaccinated with the recombinant viruses or IBDV-rGt and challenged with Gx virus showed an HBLS value of 0–2 with no to mild bursal lesions (Table 3). Overall, based on these criteria, vaccination with IBDV-1, IBDV-8, IBDV-9 and IBDV-rGt conferred 70%, 80%, 80% and 90% protection, respectively, against challenge with vvIBDV.

To determine the protective efficacy of the recombinant viruses against NDV, chickens were challenged with the virulent NDV F48E9 at week 3 postvaccination. The control birds in the DMEM group exhibited severe clinical disease after challenge. Slight depression, anorexia and ruffled feathers were evident in some birds at 2 d p.c. While all the birds were depressed at 3 d p.c., and some had severe nervous signs such as strophocephalia, opisthotonos and incoordination as well as other clinical signs including oral discharge, diarrhoea and dyspnoea. Then, all infected birds in control group were dead within 5 d p.c. (Fig. 5B). Chickens immunized with the NDV LaSota live vaccine were completely protected from NDV challenge, showing no signs of disease or deaths for 10 days. For chickens vaccinated with IBDV-1, IBDV-8 or IBDV-9 virus, 4, 5, or 4 out of 10 birds developed clinical signs after challenge, respectively, and died between 5 and 7 d p.c. The symptoms of the vaccinated birds were less severe and developed later after challenge compared to the control birds. The other surviving birds were normal and healthy during the challenge experiment, suggesting that a partial protection of 50–60% was conferred by vaccination of the recombinant viruses.

3.4. Antibody responses in chickens induced by the rescued viruses

To assess the immunogenicity of the recombinant viruses in chickens, serum samples were collected and analyzed by ELISA and by VN test. The results showed that antibody titers from the ELISA and VN test were highly correlated. As shown in Figs. 6A and 7A, the ELISA titers and VN titers against IBDV were at high levels in chickens vaccinated with the recombinant viruses and IBDV-rGt. Both the ELISA and VN titers against IBDV were comparable in birds immunized with IBDV-8, IBDV-9 and IBDV-rGt during the experiment ($P > 0.05$). The IBDV antibody titers in group IBDV-1 were lower than other groups except DMEM at 14 d p.i. and 10 d p.c. ($P < 0.05$), but comparable at other time points ($P > 0.05$).

Chickens vaccinated with the recombinant viruses also developed NDV-specific antibodies as detected by ELISA and VN test, although the titers were lower than those in the group immunized with NDV LaSota vaccine ($P < 0.05$) (Figs. 6B and 7B). The ELISA and VN titers induced by the IBDV-1 were slightly higher than those elicited by IBDV-8 and IBDV-9 from 14 d p.i., but the differences among them were not statistically significant ($P > 0.05$). The DMEM group showed no detectable amounts of IBDV and NDV antibodies

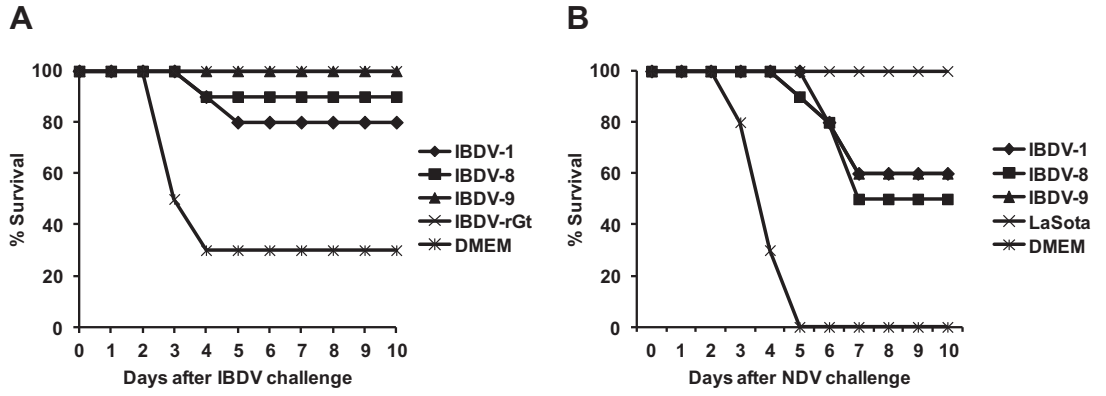

Fig. 5. Percent survival of chickens following IBDV and NDV challenge. (A) Chickens were vaccinated with the rescued viruses and challenged with 10^3 ELD_{50} of vvIBDV Gx three weeks after immunization. (B) Chickens were vaccinated with the recombinant viruses or NDV-LaSota vaccine, and challenged with 10^2 ELD_{50} of virulent NDV F48E9 three weeks after immunization. DMEM-immunized chickens were used as negative controls. After viral challenge, the birds were monitored daily for clinical signs and mortality for 10 days. Percentages of chickens surviving on different days postchallenge were recorded.

Table 3
Protection efficacies for vaccination against virulent IBDV and NDV challenge.

Groups[a]	Protection against IBDV								Protection against NDV	
	Mortality[b]	BBIX[c]	Histopathological bursal lesion scores[d]						Protection[e]	Mortality[b]	Protection (%)[f]
			0	1	2	3	4	5			
IBDV-1	2/10	0.81 ± 0.16 (B)	6	1	1	2	0	0	7/10 (70)	4/10	6/10 (60)
IBDV-8	1/10	0.84 ± 0.18 (AB)	6	2	1	1	0	0	8/10 (80)	5/10	5/10 (50)
IBDV-9	0/10	0.88 ± 0.12 (AB)	7	1	2	0	0	0	8/10 (80)	4/10	6/10 (60)
IBDV-rGt	0/10	0.89 ± 0.13 (AB)	7	2	1	0	0	0	9/10 (90)	–	–
LaSota	–	–	–	–	–	–	–	–	–	0/10	10/10 (100)
DMEM	7/10	0.18 ± 0.03 (C)	0	0	0	0	4	6	0/10 (0)	10/10	0/10 (0)
Normal control	0/10	1.00 ± 0.16 (A)	10	0	0	0	0	0	10/10 (100)	0/10	10/10 (100)

[a] Two-week-old SPF chickens were vaccinated by the eye and intranasal route with the recombinant viruses, IBDV-rGt, or commercial live vaccine NDV LaSota and challenged through the oculonasal route with 10^3 ELD_{50} of vvIBDV Gx or 10^2 ELD_{50} of highly pathogenic NDV F48E9 three weeks after vaccination following observation for 10 days. DMEM-inoculated chickens were used as unvaccinated challenge controls. The normal control chickens were unvaccinated yet unchallenged during the experiment.
[b] Mortality was presented as number of dead chickens/total number of chickens in each group.
[c] The bursa:body-weight index (BBIX) was calculated following the formula: BBIX = (bursa:body-weight ratios)/(bursa:body-weight ratios in negative group), and presented as the mean ± standard deviation from each group. Values followed by different letters within the column are significantly different ($P < 0.05$).
[d] Histopathologic bursal lesion score (HBLS) was determined on a scale of 0–5: 0, no lesion; 1, slight change; 2, scattered or partial bursal damage; 3, 50% or less follicle damage; 4, 51–75% follicle damage; 5, 76–100% bursal damage.
[e] A chicken that survived with a BBIX higher than 0.70 and a HBLS of 1 was defined as protected against IBDV challenge. Protection percent was determined as the number of protected chickens/total number of chickens in each group.
[f] Birds were considered protected from NDV challenge if they showed no central nervous system signs and survived. Protection percent was determined as the number of protected chickens/total number of chickens in each group.

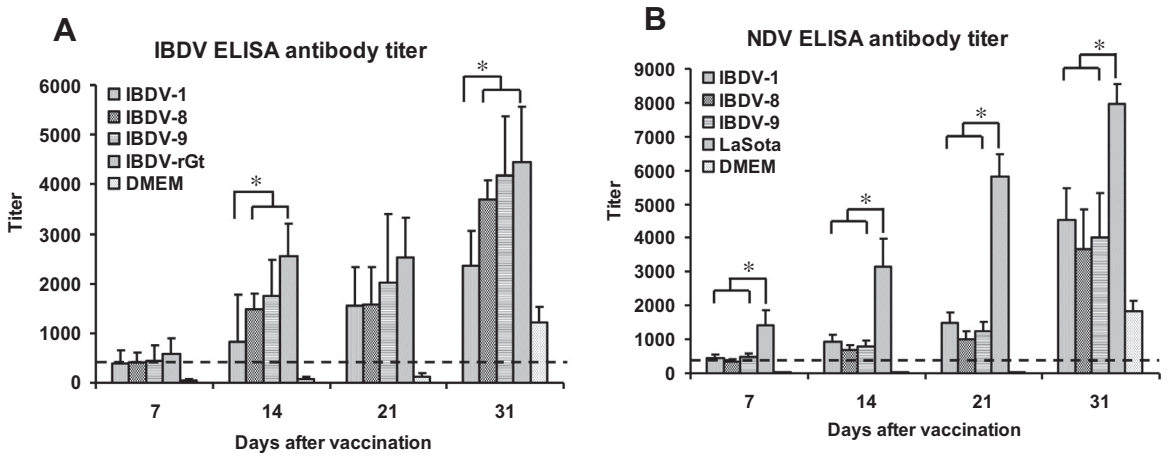

Fig. 6. IBDV- and NDV-specific antibody responses in chickens. Two-week-old chickens were immunized with the recombinant viruses, IBDV-rGt, or NDV-LaSota vaccine by the eye and intranasal route. DMEM-immunized chickens were used as negative controls. At 21 days after vaccination, chickens were challenged with vvIBDV Gx or NDV F48E9 and observed for 10 days. The ELISA antibody titers in sera were tested against IBDV (A) or NDV (B) using commercially available ELISA kits (IDEXX, Westbrook, Maine). The sera with titers of higher than 396 were considered as positive for IBDV or NDV antibody according to the protocol. Statistically significant differences were calculated by one-way ANOVA. *$P < 0.05$.

in the sera before challenge and significantly lower antibody titers after challenge compared to other groups ($P < 0.05$).

4. Discussion

The use of recombinant viral vaccines represents a novel and promising approach to preventing infectious diseases in humans as well as in veterinary medicine (Jackwood, 1999; Yokoyama et al., 1997). Recently, encouraging results have shown the potential of IBDV to serve as a novel vector system for the delivery of foreign epitopes of other pathogens, such as those of FMDV or HCV (Rémond et al., 2009; Upadhyay et al., 2011). There are several advantages to the use of avirulent IBDV strains as vaccine vectors. Due to its natural heat stability, ease of production, and widespread use, IBDV lends itself as a low-cost veterinary vaccine with proven track records of efficacy and safety. They can be administered through drinking water or spraying, making inoculation of large numbers of animals in the field feasible. Unlike other viral vectors that encode large number of proteins such as herpesviruses and poxviruses, IBDV has a simple genome encoding only a few proteins; therefore, there is less competition for immune responses between vector proteins and the expressed foreign antigen. Additionally, IBDV is an RNA virus that replicates in the cytoplasm, precluding concerns about integration into the host cell DNA. In this study, the potential utility of IBDV to carry NDV epitopes was explored. We rescued three recombinant IBDVs expressing NDV neutralizing epitopes and evaluated their potential use as a novel vectored vaccine against virulent NDV infection as well as against vvIBDV infection in chickens.

In this study, ten different constructs were created by inserting or substituting a sequence encoding a virus-neutralizing epitope of NDV HN protein in six different regions of IBDV segment A. Using a reverse genetics system, three recombinant IBDV viruses (IBDV-1, IBDV-8 and IBDV-9) expressing the epitope were generated by inserting the foreign sequence into the P_{BC} loop, pep7b or the N terminus of VP5, respectively, thereby increasing the length of genome segment A by 27 nucleotides. Upadhyay et al. (2011)

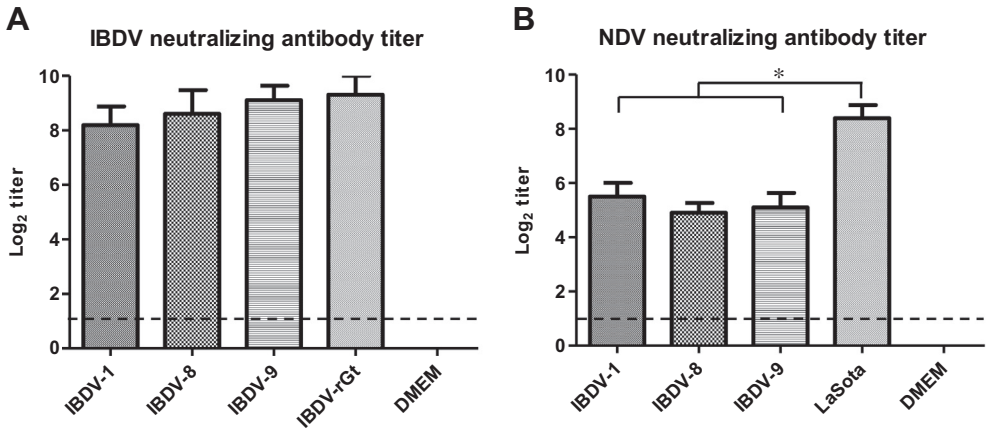

Fig. 7. IBDV and NDV neutralizing antibody titers in chickens. The serum neutralizing antibodies were determined against IBDV (A) or NDV (B) by virus neutralization test at 21 days after vaccination. The neutralizing antibody titers in sera were determined as mean reciprocal $\log_2$ titer. Statistically significant differences were calculated by one-way ANOVA. $^*P < 0.05$.

recently recovered a recombinant IBDV expressing a HCV epitope at the VP5 region, highlighting the potential value of IBDV to serve as a vaccine vector, even though the immunogenicity of this recombinant virus was not further evaluated in vivo. In this study, we successfully rescued recombinant IBDV-9 expressing a NDV epitope at the N terminus of VP5 protein, indicating that this region can endure the introduction of different foreign epitopes. It was reported previously that any insertion of sequences into VP2 region of IBDV may limit virus packaging and the function of VP2 (Upadhyay et al., 2011). In contrast, the recombinant IBDV-1 with inserted NDV epitope in VP2 P_{BC} loop was successfully rescued in our study, indicating that the loop regions of VP2 are amenable to the introduction and expression foreign peptides. The different finding may be due to the different size and structure of the NDV epitope compared to the HCV epitope they used. More importantly, the pep7b region was identified in this study for the first time as a novel site in IBDV segment A that can accept and express foreign epitopes. In this region, recombinant IBDV-8 was rescued with high efficiency. During virus recovery process, typical CPE appeared in infected CEF cells from the second passage for IBDV-8, the same as the parental virus IBDV-rGt, while from the third passage for IBDV-1 and IBDV-9. Furthermore, CPE caused by IBDV-8 started earlier and more severe than that of IBDV-1 and IBDV-9 after inoculated in CEF cells. The results indicated that the pep7b site was more efficient than P_{BC} loop and VP5 region in introducing foreign peptides into genome segment A.

Using reverse genetics system, it has been shown previously that the VP2 amino acids at positions 253 (loop P_{DE}) and 284 (loop P_{FG}) are involved in IBDV adaptation to cell culture and virulence (Brandt et al., 2001; Qi et al., 2009). Hence, loops P_{DE} and P_{FG} were not used in our study for inserting foreign peptides to prevent influencing the function of VP2 in virus attachment/entry. Attempts were made to introduce the NDV epitope in loops P_{BC}, P_{HI} and $P_{AA'}$ by insertions or substitutions and it was found that only loop P_{BC} could insert this epitope. The inability to rescue recombinant viruses in P_{HI} loop may be because P_{HI} is adjacent to P_{DE} and these two regions mutually stabilized by forming β-sheet structure (Lee et al., 2006), any insertion in P_{HI} may partially block the receptor binding region (P_{DE} and/or P_{FG}) to interfere the viral entry. While the lack of recovery of virus with foreign peptides inserted in $P_{AA'}$ loop might due to the interference of other functions of VP2 involved $P_{AA'}$ in virus packaging like the interactions of VP2 subunits and different trimers (Garriga et al., 2006).

Among the four structural peptides obtained from the cleavage of the C terminus of pVP2 precursor, pep46 and pep11 are crucial determinants of the viral assembly process (Chevalier et al., 2005). While pep7a and pep7b are not essential for viral assembly and virus rescue (Chevalier et al., 2005; Da Costa et al., 2002), indicating that these two regions might be used as inserting sites for foreign epitopes. Interestingly, we successfully rescued recombinant virus IBDV-8 with the NDV epitope inserted in pep7b region but not in pep7a. The inability to rescue recombinant IBDV-7 in pep7a is probably because this region is adjacent to pep46, and the insertion of foreign peptide in pep7a might block the cleavage of pep46 from pVP2 which is essential to viral assembly (Chevalier et al., 2005). In addition, two different strategies, insertion or substitution, were used to introduce foreign sequences into VP2 loops and VP5 region. The recombinant viruses were successfully rescued with the insertion approach but no virus was rescued by replacing the nucleotides encoding the loops or the VP5 region with foreign sequences, indicating that the original sequence is essential to the function of viral proteins in virus replication.

Although live attenuated NDV vaccines are routinely used in poultry industries, these vaccines do not completely prevent virulent NDV infection or shedding, nor do they possess genetic markers to allow differentiation between infected and vaccinated birds (Kumar et al., 2011; Miller et al., 2007). In this study, the recombinant IBDVs expressing NDV epitopes induced ELISA and neutralizing antibodies against both IBDV and NDV in chickens and conferred protection against virulent IBDV and NDV challenge. Results of the present study have evaluated, for the first time, the potential of the recombinant IBDV viruses to serve as bivalent vaccines. Although vaccination with the recombinant IBDVs provided partial protection against virulent NDV challenge in the study, we can devise strategies to improve the recombinant vaccines. Previous studies showed that other recombinant vaccines for ND also showed incomplete protection preliminarily prior to being optimized (Swayne et al., 2003) and the replication ability of the vector virus is an important determinant of the efficacy of recombinant vaccines. The recombinant virus expressing the HA gene of H7 avian influenza virus (AIV) using the highly attenuated NDV B1 strain only provided 40% protection against highly pathogenic H7 AIV or NDV challenge (Swayne et al., 2003). While the less attenuated NDV LaSota-based recombinant vaccine provides much better protection against both NDV and the targeted pathogen challenge than does the B1 strain (Ge et al., 2007; Veits et al., 2006). Correspondingly, it is reasonable to assume that the vaccine efficacy of the recombinant IBDVs could be enhanced by screening and using a vector virus with better replication efficiency and immunogenicity. Since three insertion sites, P_{BC} loop, pep7b and

VP5 region, were identified and shown to be efficacious in carrying the HN epitope, we can attempt to express three copies of the HN epitope or three different NDV neutralizing epitopes simultaneously from the same IBDV genome, which may further improve the immunogenicity of the recombinant vaccines. Additionally, the vaccine efficacy of the recombinant IBDVs can also be enhanced by boosting immunization with optimized dose.

IBDV is known to propagate in primate cells (Jackwood et al., 1987). Previous studies further described dogs and mice as potential carriers for IBDV and it was shown that the virus excreted from mice induced clinical disease in chickens, suggesting that IBDV maintained its viability and pathogenicity in mice (Pagès-Manté et al., 2004; Park et al., 2010). More importantly, IBDV was recently shown to be capable of replication in HEK293T cells, a specific cell line originally derived from human embryonic kidney cells (Upadhyay et al., 2011). The ability of IBDV to propagate in mice and in human cells suggests that IBDV might be able to replicate in humans, ascertaining the potential of IBDV to be used as vectors for prophylactic purposes. As an avian virus, IBDV is not known to be a hazard for transmission to other species, and IBDV has been used as a therapeutic agent without any toxicity in clinical trials with patients suffering from acute and chronic hepatitis infections (Bakács and Mehrishi, 2002; Csatary et al., 1999). Additionally, in vivo expression of a heterologous immunogen from an IBDV-based vaccine vector would not be limited by prior immunity since most human and animal populations are not exposed to IBDV. Taken together, these results indicate that IBDV is a promising vaccine vector not only for birds but also for humans and other mammals.

In this study, for the first time we demonstrated the insertion of a NDV neutralizing epitope in three different sites of IBDV segment A and showed that IBDV can be used as a vaccine vector for other avian pathogens. Further exploration of the insertion sites used in the present study with other antigenic epitopes will be highly beneficial to the development of novel IBDV vectors which could be used not only in veterinary research but potentially as a promising delivery system for human pathogens.

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