FULL PAPER

Virology

Efficacy of a novel *in ovo*-attenuated live vaccine and recombinant vaccine against a very virulent infectious bursal disease virus in chickens

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ABSTRACT. Infectious bursal disease (IBD) causes severe economic damage to the poultry industry worldwide. To prevent IBD virus (IBDV) infection, live virus vaccines have been widely used in chickens having wide-ranging levels of maternally derived antibodies. But, the risks of infection with other pathogens because of lesions related to atrophy of the bursa of Fabricius in vaccinated chickens are a concern. To resolve the problems, a recombinant turkey herpesvirus (HVT) vaccine expressing IBDV-VP2 protein (rHVT-IBD) has been developed. However, the induction of neutralizing antibodies by rHVT-IBD against a virulent IBDV might be delayed compared with that by the live IBD vaccine, leading to the high risks of IBDV infection for young chickens. To find the best selection of IBDV vaccine for the onset of immunity, we examine the protective efficacy of a novel *in ovo*-attenuated live IBDV (IBD-CA) vaccine and the rHVT-IBD vaccine in young chickens challenged with a very virulent IBDV (vvIBDV) strain. We show that the protective efficacy of IBD-CA vaccine was higher than that of the rHVT-IBD vaccine in 14-day-old chickens challenged with the vvIBDV strain, leading to the risk of IBDV infection for young chickens when vaccinated with rHVT-IBD. Our results suggest that farmers should select the best vaccines to maximize vaccine efficacy in consideration of the vaccine characteristics, prevalence levels of IBDV in the areas, and initial MDA levels of the chickens since the attenuated live and recombinant vaccines play a role in the different vaccine efficacies.

KEY WORDS: attenuated live infectious bursal disease virus vaccine, infectious bursal disease virus viral protein 2 expression, infectious bursal disease virus, *in ovo* inoculation, recombinant turkey herpesvirus

Infectious bursal disease (IBD) is one of the most important poultry diseases with high mortality and morbidity worldwide, and is caused by the infectious bursal disease virus (IBDV) [10]. IBDV is a highly contagious pathogenic virus that especially infects young chickens, and has a major economic impact on the poultry industry worldwide. IBDV infection mainly induces inflammatory responses and subsequent atrophy of the bursa of Fabricius due to the depletion of B cells due to apoptosis, which leads to immunosuppression [6].

IBDV, a member of the genus *Avibirnavirus* in the family Birnaviridae, is a non-enveloped virus with a bi-segmented (segments A and B) double-stranded negative-sense RNA genome [11]. Segment A in the IBDV genome encodes large open reading frames that are translated into precursor polyproteins, including precursors VP2, VP3, and VP4. VP2 is further processed by VP4 protease into mature capsid protein VP2, which mainly constitutes the icosahedral capsid of the virus particles [16]. Therefore, the VP2 protein contains major antigenic determinants that are primarily responsible for eliciting neutralizing antibody responses against IBDV [9, 18, 20].

Attenuated IBDV live vaccines are applied to prevent IBDV infection and successfully control the disease when used at an appropriate timing of vaccination. However, most of these live vaccines might cause atrophy of the bursa of Fabricius when they replicate, and subsequently, cause mild or severe lesions due to temporal depletion of lymphocytes. One of the issues associated with the use of IBDV live vaccines is the high susceptibility of the vaccine viruses to maternally derived antibodies (MDAs), which can neutralize such viruses [1, 19]. Therefore, if MDAs interfere with the replication of vaccine viruses, the vaccinated chickens might not be fully protected from virulent or very virulent IBDV (vvIBDV) strains in the field. To overcome this issue,

vaccines consisting of strains at various levels of attenuation have been developed and extensively used according to wide-ranging MDA levels in different poultry farms. Although IBDV infection is controlled by these vaccinations, another concern is the potential risk of infection by other pathogens because of mild or severe lesions related to bursa atrophy in vaccinated chickens.

An alternative to attenuated live IBDV vaccines is the recombinant turkey herpesvirus (rHVT) vaccine, which has been successfully developed by insertion of the IBDV-VP2 gene into the HVT genome (rHVT-IBD) [7]. The expression of the VP2 protein from rHVT elicits a neutralizing antibody response against IBDV [8]. rHVT-IBD does not cause any damage to the bursa; hence, it is considered a safer vaccine than attenuated IBDV live vaccines, and this vaccine virus is likely to be unsusceptible to IBDV MDAs. Hence, rHVT-IBD has the potential to solve the safety and efficacy issues of IBDV vaccines. However, a previous report showed that an rHVT-IBD vaccine, Vaxxitek, only partially protected 30- and 60-week-old specific-pathogen-free (SPF) chickens from several IBDV viruses upon challenge at 10 or 14 days post-vaccination (dpv) because of inefficient induction of IBDV antibodies in the serum [3]. This report suggests that the onset of immunity of the rHVT-IBD vaccine is slower than that of live attenuated IBDV vaccines, and longer periods are required to elicit protective immunity after vaccination. This would lead to the risk of creating a window of susceptibility to IBDV infection. Therefore, the comparison information of vaccine efficacy for the attenuated live and recombinant vaccines against the vv IBDV is valuable to understand the onset of immunity by the *in ovo* vaccines.

In this study, we focused on the protective efficacy of a novel *in ovo*-attenuated live IBDV vaccine (IBD-CA) consisting of IBDV-infected cells, and an rHVT-IBD vaccine, against virulent IBDV in 7, 9, 11, and 18 days old SPF chicks after *in ovo* vaccination. The IBD-CA vaccine showed higher protective efficacy than rHVT-IBD at earlier ages in chickens. Our data also show that the IBDV live vaccine induced higher levels of neutralizing antibodies against IBDV than rHVT-IBD. Our results provide important information for determining the optimal choice of IBDV vaccines with different characteristics for *in ovo* vaccination.

MATERIALS AND METHODS

Cells, vaccines, and virus

SPF embryonated eggs of White Leghorn (VALO Biomedia, Osterholz-Scharmbeck, Germany) at 10 days of age were used to prepare chicken embryonated fibroblast (CEF) cells for titration of antibodies against IBDV and HVT. The CEF cells were maintained in Eagle's minimum essential medium (EMEM) supplemented with 5% fetal bovine serum and antibiotics. The CEF cells were then seeded in 96-well microtiter plates (1×10^5 cells/well). A commercial recombinant vector vaccine, HVT-IBD (Vaxxitek®) (Boehringer Ingelheim, Ingelheim, Germany), expressing VP2 protein of IBDV classic strain Faragher 52/70; Vaxxon IBD-CA vaccine, which consists of IBDV Lukert strain-infected CEF cells; bivalent live MD vaccine (HVT+SB-1); and monovalent MD vaccine (HVT) were used in this study. The vvIBDV 68–20 strain was provided by AGRI-BIO CORP. (Ithaca, NY, USA) and was grown in embryonated chicken eggs for the challenge test. The IBDV Lukert vaccine strain was used for the virus neutralization test. The original HVT FC126 strain was provided by Maine Biological Laboratories (Waterville, ME, USA), and the HVT strain was established by propagation among CEF cells for the assays.

Animal experimental designs and sampling

SPF eggs from White Leghorn chickens (certified IBDV serotype 1 and 2-free) were purchased from VALO Biomedia and used for experiments. After hatching, the chickens were housed in isolation units in our laboratory. All animal experimental procedures were conducted in accordance with the relevant national and international guidelines defined in our laboratory for the humane use and care of chickens and approved by a committee of our institutes (The approval numbers: AU400-C-EX-20-001).

To compare vaccine efficacy against vvIBDV, 5 groups (*n*=15/flock) were used: Unimmunized+no challenge, Challenge control, rHVT-IBD, HVT+SB-1+IBD-CA, and HVT+IBD-CA. Eighteen-day-old embryonated eggs were vaccinated with one dose of each vaccine *in ovo* and further incubated until they hatched. At 3, 5, 7, and 14 days after hatching, the bursa of 5 chickens from each flock were collected to observe the effect of vaccination and to calculate the bursa/body weight (B/B) ratio. At various periods after the vaccination, the vvIBDV 68-20 strain was orally administered at 3.5×10^3 50% egg infectious dose/0.1 ml to 10 chickens in each flock. At 4 days post-challenge (dpc), the chickens were euthanized and necropsied to collect the bursa for the evaluation of gross lesions such as yellowish peribursal edema and hemorrhage (Fig. 1A). For the histopathological test, the bursa from each flock at 7 days of age (at 4 dpc after challenge to 3-day-old chickens) were fixed with 10% phosphate-buffered formalin, and the fixed tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E).

To quantify the HVT DNA in the temporal study, the thymus, bursa, spleen, and feather follicle epithelium (FFE) were collected at various time points (1, 2, 3, 4, 5, 6, 7, and 14 days). The five lymphoid organs from each flock were minced with scissors to release lymphocytes into the supernatant, and the lymphocytes were suspended in Eagle's Minimum Essential Medium supplemented with 5% fetal bovine serum and 10% dimethyl sulfoxide, and stored at −70°C until use. Feathers from 5 chickens were removed, and the FFE was obtained from the feather sheath. The FFE was also minced with scissors and briefly sonicated for 1 min in sucrose-phosphate-glutamine-albumin buffer to release the virus from the FFE into the supernatant; this yielded FFE extract containing the virus. Both lymphocytes and FFE extracts were subjected to qPCR for the quantification of HVT DNA.

To examine the immunosuppression of chickens by *in ovo*-inoculation with IBD-CA, 18-day-old embryonated eggs were vaccinated with five doses of IBD-CA. After hatching, one dose of our vaccine for Newcastle disease virus (NDV) B1 strain through the intraocular route was vaccinated with 7-day-old chickens. At 3 weeks post-vaccination of the NDV vaccine, the blood from each vaccinated chicken was collected and the isolated serum was subjected to hemagglutinin inhibition (HI) assay
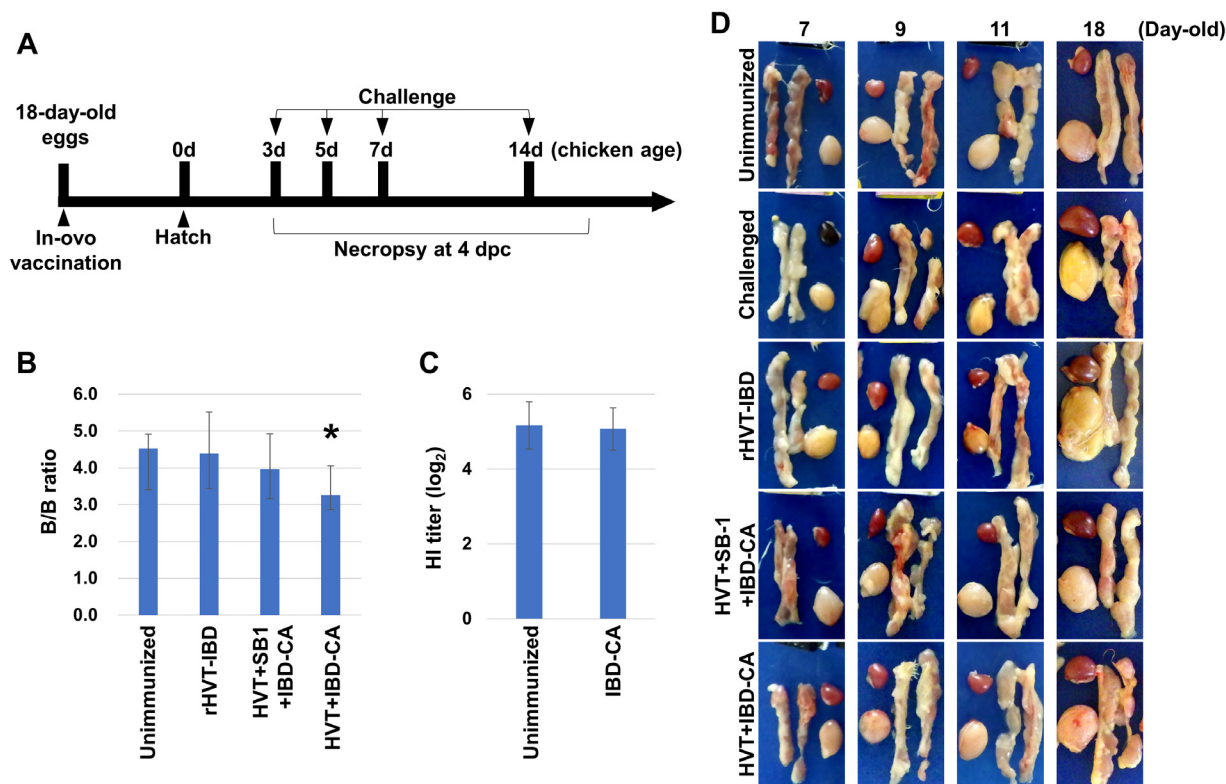
Fig. 1. Lesions in lymphoid organs after vaccination and challenge. Eighteen-day-old eggs were vaccinated with different vaccines *in ovo*. After hatching, 3-, 5-, 7-, and 14-day-old chickens were challenged with a very virulent infectious bursal disease virus (IBDV) strain, and bursa and blood were collected at the indicated time points. At 4 days post-challenge (dpc), challenged chickens were necropsied **(A)**. *In ovo* vaccination of 18-day-old embryonated eggs was carried out and the bursa was collected from 5 chickens at the age of 14 days in each flock. The average bursa/body weight (B/B) ratio was calculated and the B/B ratio of the Unimmunized group was compared with that obtained after each vaccination. An asterisk represents a statistically significant difference ($P<0.05$) by Student's *t*-test **(B)**. Eighteen-day-old embryonated eggs were vaccinated with five doses of IBD-CA vaccine followed by the further vaccination of Newcastle disease virus B1 strain vaccine to 7-day-old chickens after hatching. At 21 dpv, the serum was collected from each chicken and subjected to HI assay to measure the HI antibody titers of NDV. No statistically significant difference was observed between unimmunized and IBD-CA groups **(C)**. At 4 dpc, the thymus, spleen, and bursa were collected from chickens at ages of 7, 9, 11, and 18 days in each flock (indicated), and lesions in each organ were observed **(D)**.

to measure the antibody titer against NDV. The NDV virus was incubated with serial dilutions of the serum obtained from each chicken for 30 min at 37°C and mixed with 0.5% chicken erythrocytes for 1 hr at room temperature. The HI antibody titers were shown as the minimum concentrations of antibodies that completely inhibited 4 hemagglutinin units of virus.

To examine the vaccine take of IBD-CA after *in ovo* inoculation, the 19-day-old embryonated broiler eggs with various MDA levels were vaccinated with one dose of IBD-CA vaccine. Following hatching, the blood was randomly collected from 8–10 chickens at 0, 7, 14, 21, 27, 34, 41 days of age and isolated serum was subjected to a virus neutralization test to measure the neutralizing antibody levels.

Titration of antibodies in serum specific for IBDV and HVT

For IBDV, 200 median tissue culture infectious dose ($TCID_{50}$) of IBDV Lukert strain was mixed with serial dilutions of the serum in EMEM at 37°C for 1 hr. The virus-serum mixtures were inoculated into CEF cells in a 96-well microtiter plate, and the cells were incubated at 37°C for 5 days. Neutralizing titers were defined as the minimum concentration of serum required to neutralize a $TCID_{50}$ of 200 of IBDV in CEF cells. For HVT, 50 plaque formation units (PFUs) of cells infected with HVT FC126 strain were infected to CEF cells, which were then incubated for 3 days. Following fixation and blocking, the cells were incubated with serial dilutions of the primary serum and subsequently with a secondary antibody (Ab) conjugated with horseradish peroxidase. The antibody titer in serum specific for HVT was defined as the highest dilution factor at more than 1.5-fold of the absorbance value of the negative control (uninfected cells) as a cut-off value.

Quantification of amount of HVT DNA using duplex qPCR

Viral DNA of the lymphocytes or viruses isolated from the FFE was extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The purified viral DNA was subjected to duplex qPCR using specific primers

for HVT SORF1 and chicken α2 (VI) collagen genes. qPCR was carried out using a StepOne real-time system (Applied Biosystems, Foster City, CA, USA) with TaqMan probes according to the manufacturer's protocols. The ΔCt values of the viral DNA from lymphocytes in each organ or the FFE extract were calculated with each Ct value of the chicken α2 (VI) collagen gene as an internal control, and the relative expression levels of the viral DNA were determined using the comparative Ct ($2^{-\Delta\Delta Ct}$) method.

Statistical analysis

For the B/B ratio and HI antibody titer of NDV, the statistical significance was determined by Student's *t*-test between the unimmunized and vaccinated groups. For the gross and histopathological lesions, the statistical significance was determined by χ^2-test between the challenge group and other vaccinated groups. *P* values of <0.05 or 0.01 were considered statistically significant.

RESULTS

IBD-CA induced atrophy of bursa of Fabricius but protected vaccinated chickens from vvIBDV

To examine the effect of *in ovo* vaccination on the bursa of Fabricius, vaccines were inoculated into 18-day-old eggs. Then, the bursa were collected from the chickens at various ages, and the bursa and body weights were measured to determine the B/B ratios. For 3-, 5-, and 7-day olds, significant atrophy of the bursa was not observed in all flocks, but the B/B ratios of the HVT+IBD-CA flock for 14-day olds were significantly lower than those of the unimmunized flock. In contrast, the B/B ratios of the rHVT-IBD flock showed no significant reduction compared with those of the unimmunized flock (Fig. 1B), suggesting that the IBD-CA vaccine, but not rHVT-IBD, induced slight bursa atrophy. Although the NDV vaccine was also vaccinated to 7 day-old chickens following the *in ovo* vaccination of the IBD-CA, no significant decrease in HI antibody titer against NDV was observed, indicating no immunosuppression occurred by the vaccination of IBD-CA even slight bursa atrophy (Fig. 1C). To confirm whether these vaccines showed protective efficacy against challenge of vvIBDV, the vvIBDV 68-20 strain was administered to the vaccinated chickens at 3 days of age. At 4 dpc, the bursa, thymus, and spleen were collected from the chickens, and their gross lesions, especially in the bursa, were observed (Fig. 1D). The bursa of the chickens vaccinated with rHVT-IBD showed yellowish edema, similar to the gross lesions in the bursa of the chickens in the challenge control group. Interestingly, there were no gross lesions in the bursa of chickens vaccinated with HVT+SB-1+IBD-CA and HVT+IBD-CA although the bursa were slightly atrophied due to IBD-CA vaccination (Table 1), indicating that the IBD-CA vaccine showed higher protective efficacy than rHVT-IBD within 14 days of *in ovo* vaccination.

Infection of vvIBDV strain caused lymphocyte depletion in lymphoid follicles of bursa from chickens vaccinated with rHVT-IBD

The bursa collected from 7-day-old chickens (at 4 dpc of 3-day-old chickens) were subjected to histopathological assays to observe tissue lesions. For the challenge and rHVT-IBD administered groups, the follicles filled with lymphocytes were lost and the tissues were predominantly stained with red-pink color with eosin since lymphocytes stained with purplish-blue were severely depleted from follicles and replaced with eosinophilic reticular cells and connective tissues (Table 2 and Fig. 2C and 2E). Furthermore, the capsular was thickened, compared with those of unimmunized and HVT-SB-+IBD-CA administered groups, due to the infiltration of excess lymphocytes and fluid from blood vessels into the capsular by the inflammation, leading to capsular edema (Table 2 and Fig. 2D and 2F). In contrast, the bursa of chickens vaccinated with the HVT+SB-1+IBD-CA vaccine showed mild depletion of lymphocytes and slight infiltration of lymphocytes into capsular, but no follicle disappearance, capsular inflammation, and edema, consistent with the results of gross lesion observation. These results indicated that the protective immunity conferred by rHVT-IBDV against vvIBDV was delayed in chickens under 14 days of age.

Table 1. Gross lesions of the bursa in vaccinated chickens at different ages after challenge

Age (days)	Flock			
	Challenged	rHVT-IBD	HVT+SB-1 +IBD-CA	HVT +IBD-CA
7	10/10	8/10	0/10*	0/10*
9	7/10	3/10	0/10*	0/10*
11	10/10	5/10*	0/10*	0/10*
18	10/10	4/10*	0/10*	0/10*

The numbers of chickens showing gross lesions with yellowish peribursal edema in bursa / total numbers of tested chickens are shown. rHVT-IBD, commercial recombinant vector vaccine; HVT+SB-1+IBD-CA, bivalent live MD vaccine; HVT+IBD-CA, monovalent live MD vaccine. The gross lesion rate differences between the Challenge group and other groups were significant according to the χ^2 test (*P*<0.05) at each age marked with asterisks (*).

Table 2. Histopathological analysis of bursa in chickens at 7-day-old vaccinated chickens vaccinated with each vaccine after challenge

Tissue lesions	Flock				
	Unimmunized	Challenged	rHVT-IBD	HVT+SB-1 +IBD-CA	HVT +IBD-CA
Lymphocyte deletion	0/10	10/10	10/10	10/10	10/10
Capsular inflammation	0/10	10/10	8/10	0/10*	0/10*
Capsular edema	0/10	7/10	7/10	0/10*	0/10*

The numbers of chickens showing indicated tissue lesions in bursa / total numbers of tested chickens are shown. rHVT-IBD, commercial recombinant vector vaccine; HVT+SB-1+IBD-CA, bivalent live MD vaccine; HVT+IBD-CA, monovalent live MD vaccine. The histopathological lesion rate differences between the Challenge group and other groups except for the Unimmunized group were significant according to the χ^2 test (*P*<0.05) at each age marked with asterisks (*).
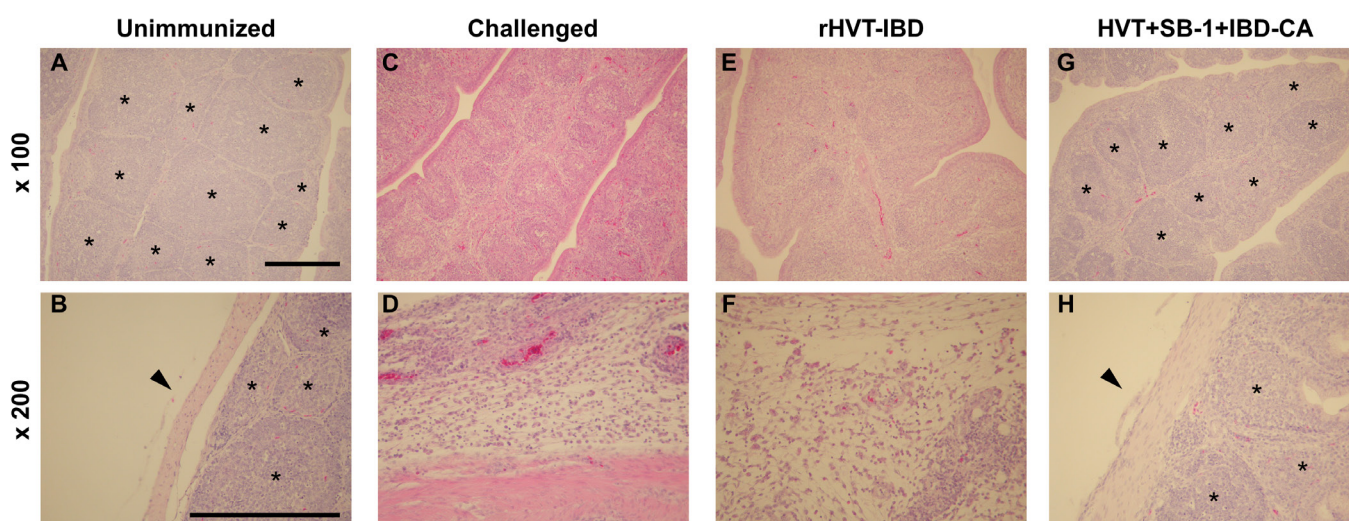

Fig. 2. Bursa sections from challenged chickens after hematoxylin and eosin (H&E) staining. The bursa collected from 7-day-old chickens from different flocks were subjected to histopathological analysis. Images taken at high magnifications of ×100 (A, C, E, and G) and ×200 (B, D, F, and H) are shown. Follicles filled with lymphocytes and the capsular are shown by asterisks and arrowheads, respectively (A, G, B, and H). Bar: 250 µm.

Recombinant HVT was abundant in chickens but induced low levels of neutralizing antibodies against IBDV

The amount of HVT DNA temporally extracted from lymphoid organs such as the thymus, bursa, spleen, and FFE was quantified by qPCR. The qPCR experiments showed that the relative amount of HVT DNA in the thymus and spleen obtained from the chickens vaccinated with rHVT-IBD was slightly higher than that of the DNA from chickens vaccinated with conventional HVT, and almost similar kinetics were observed in the bursa (Fig. 3), indicating that the recombinant HVT grew well in lymphoid organs. We next examined whether the virus-neutralizing antibody in the serum against IBDV was sufficiently induced by the vaccinations. The virus neutralization test revealed that a very low level of neutralizing antibody was induced by the vaccination of rHVT-IBD in 14-day-old chickens, whereas high levels of neutralizing antibody titer were shown by the serum obtained from chickens vaccinated with attenuated live IBD-CA vaccine (Fig. 4A). These results indicated that the rHVT-IBD vaccine, but not attenuated live IBDV vaccine, only induced much lower levels of the neutralizing antibody in chickens under 14 days of age via the expression of IBDV-VP2 from recombinant HVT. We also determined the antibody titer against HVT for the serum obtained from chickens aged 3, 5, 7, and 14 days. The antibody titers in HVT+SB-1+IBD-CA and HVT+IBD-CA flocks gradually increased for 14 days, whereas those in the rHVT-IBD flock dramatically increased (Fig. 4B), presumably due to the higher amount of HVT in the thymus and spleen (Fig. 3A and 3C), leading to the strong induction of antibodies against HVT.

DISCUSSION

Attenuated live IBDV vaccines are categorized as mild, intermediate, intermediate-plus, and hot vaccines based on the various attenuation and break-through levels of MDAs [13]. Intermediate, intermediate-plus, and hot vaccines can be used to better induce neutralizing antibodies against virulent IBDV strains in the field. However, live vaccines with low attenuation levels generally induce moderate or severe atrophy of the bursa of Fabricius, which is associated with depletion of lymphocytes, resulting in immunosuppression [6]. A previous report showing the pathogenicities of rHVT expressing IBDV-VP2 protein, hot (Winterfield 2512), and intermediate
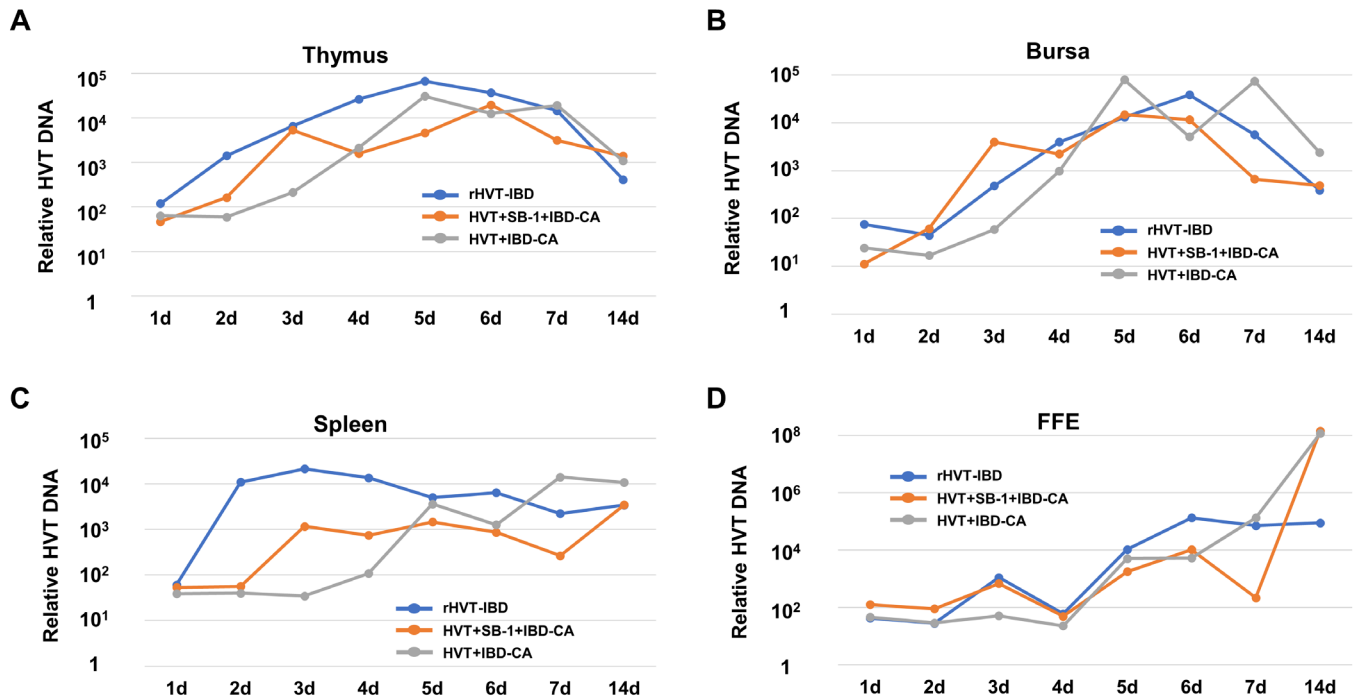
Fig. 3. Time course study of turkey herpesvirus (HVT) growth in vaccinated chickens. After *in ovo* vaccination, the thymus **(A)**, bursa **(B)**, spleen **(C)**, and feather follicle epithelium (FFE) **(D)** from vaccinated chickens were temporally harvested at various time points (indicated). Viral DNA was extracted from lymphocytes of lymphoid organs and the FFE, and subjected to a duplex quantitative PCR using gene-specific primers for the SORF1 and chicken α2 (VI) collagen genes as the internal controls. The relative amounts of HVT DNA were calculated using the comparative Ct (ΔΔCt) method by normalizing the expression level of viral DNA to that of the chicken α2 (VI) collagen gene.
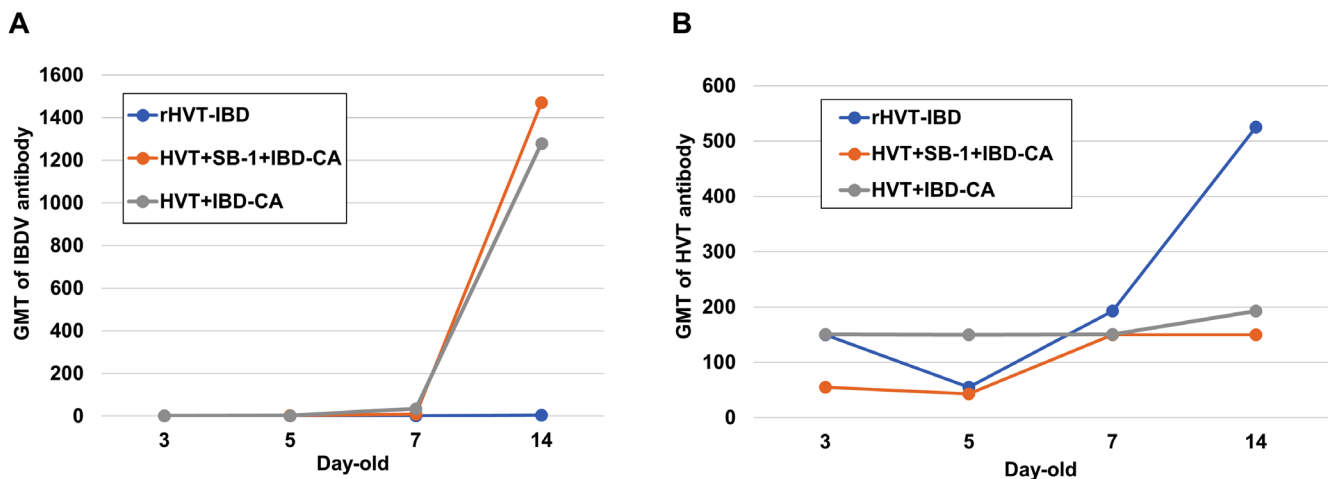
Fig. 4. Antibody titration against infectious bursal disease virus (IBDV) and turkey herpesvirus (HVT) upon vaccination. Increases in antibody titers against IBDV and HVT were measured using the virus neutralization and immunoperoxidase monolayer assays, respectively, with serum obtained from 5 chickens of different flocks at ages of 3, 5, 7, and 14 days. Geometric mean titers (GMT) of IBDV- **(A)** and HVT-specific **(B)** antibodies in the serum obtained from chickens vaccinated with a commercial recombinant vector vaccine (rHVT-IBD), bivalent live MD vaccine (HVT+SB-1+IBD-CA), and monovalent live MD vaccine (HVT+IBD-CA) are shown. The data shown are the average values of the two experiments.
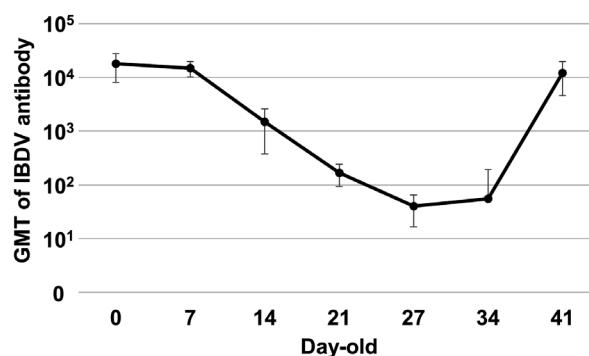
(Lukert) vaccine strains after administration to one-day-old chicks revealed that the bursa was severely atrophied by the administration of hot strain. In contrast, the bursa from the chickens administered with the rHVT expressing IBDV-VP2 protein and intermediate strains showed no gross lesions [2]. Our results also showed slight atrophy and mild depletion of lymphocytes in the bursa of chickens vaccinated with the IBD-CA vaccine (Fig. 1B). However, in our test on whether vaccination induces immunosuppression, no significant difference in the induction levels of HI antibody against NDV was observed for the IBD-CA-vaccinated and unimmunized groups (Fig.

1C). This finding indicated that the bursa atrophy induced by the IBD-CA vaccine did not cause immunosuppression. Our data from the challenge experiment in this study suggested that the rHVT-IBD vaccine insufficiently protected chickens against the IBDV challenge strain (Figs. 1 and 2). The IBD-CA vaccine showed strong induction of the neutralizing antibody against IBDV at 14 dpv (Fig. 4A), consistent with the results of a previous study [5]. In contrast, the antibody titer against IBDV induced by vaccination with rHVT-IBD was very low, even at 14 dpv (Fig. 4A). We examined whether the ability of recombinant HVT to replicate in chickens is low, leading to a low expression level of IBDV-VP2 by the genome of rHVT. However, when HVT DNA from each lymphoid organ and FFE was quantified, the expression level of HVT DNA from the chickens vaccinated with rHVT-vv IBDV was higher than that from chickens in other groups (Fig. 3), indicating the high replication ability of rHVT in each lymphoid organ. These results suggest that the onset of immunity against IBDV in chickens can be critically delayed only by the expression of VP2 from rHVT-IBD. Previous reports also showed that low ELISA antibody titers against IBDV after rHVT-IBD vaccination in SPF chickens showed poor protection against IBDV challenge strains at 10 and 14 dpv [3], indicating a correlation between increasing antibody titers and protection rates against IBDV. *In ovo* vaccination can induce immune reactions in

Fig. 5. Vaccine take of IBD-CA after *in ovo* vaccination to the chickens with maternally derived antibody. The 19-day-old embryonated broiler eggs with various MDA levels were vaccinated with IBD-CA. After hatching, the blood was collected from the 8–10 chickens randomly selected at 0, 7, 14, 21, 27, 34, and 41 days of age. The serum was subjected to the virus neutralization test to measure the neutralizing antibody levels against IBDV.

embryos, and it has been suggested that 18-day-old embryos have the ability to activate cellular immunity to regulate the stimulation of cytokines such as interferon (IFN)-γ, interleukin (IL)-1β, and transforming growth factor-β [12, 15]. A previous study on *in ovo* booster showed that a DNA vaccine encoding chicken IL-2 and IFN-γ as adjuvants enhanced the protection of chickens against vvIBDV challenge [14]. The cell-mediated immune responses of IFN-γ induction against IBDV with a peak at 3 weeks post-vaccination were observed in chickens vaccinated with both attenuated live vaccines and rHVT-IBD via the intraocular and subcutaneous route, respectively [4, 5]. Although it remains to be elucidated how *in ovo* vaccination with live IBDV vaccines contributes to the induction of cell-mediated immunity in young chickens, it is conceivable that other cell-mediated immune responses specific for IBDV might be elicited by the IBD-CA vaccine but not rHVT-IBD. Therefore, it might be possible that the IBD-CA vaccine can protect chickens better than rHVT-IBD against the challenge strain.

MDA levels against IBDV in chickens must be considered to avoid inadequate vaccination programs for IBDV in the field. To overcome the interference of high levels of MDAs in chickens, intermediate-plus and hot IBDV vaccines have been developed. However, these commercial vaccines induce bursa lesions owing to the residual virulence of the vaccine viruses, leading to immunosuppression. Our preliminary data indicated that IBDV-infected cells in the IBD-CA vaccine produced virus particles continuously, and the virus particles were partially neutralized by MDAs. However, when the MDA levels in the chickens declined, protective immunity such as neutralizing antibodies against IBDV could be induced after *in ovo* inoculation (Fig. 5). In addition, the rHVT-IBD vaccine potentially has strong advantages in that the vaccine virus is unlikely to suffer interference from high MDA levels, and protects chickens from IBDV challenge strains [3, 17] without bursa atrophy (Fig. 1B), creating less of a window of susceptibility to early IBDV infection in chickens. However, as demonstrated in this study, the increase in neutralizing antibody titer against IBDV was greatly delayed (Fig. 4A), resulting in insufficient protection against the challenge strain (Tables 1 and 2). These results suggest that there could be risks of IBDV infection for the *in ovo* rHVT-IBD vaccinated young chickens with low initial MDA levels and they did not acquire the high levels of neutralizing antibody at an early time to prevent the IBDV infection. It is possible that the rHVT-IBD vaccine could not show high vaccine efficacies at a particularly high risk of very virulent IBDV infection in the high-incidence areas. Therefore, farmers should select the best vaccines to maximize vaccine efficacy in consideration of the vaccine characteristics, prevalence levels in the areas, and initial MDA levels of the chicks.

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