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Article

Greater Protection against Virulent Infectious Bronchitis Virus Challenge Conferred by Recombinant Baculovirus Co-expressing S1 and N Proteins

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Abstract: Avian infectious bronchitis virus (IBV) is the causative agent of infectious bronchitis, which causes considerable economic losses to the poultry industry worldwide. It is imperative to develop safe and efficient candidate vaccines to control IBV infection. In the current study, recombinant baculoviruses co-expressing S1 and N proteins, mono-expressing S1 or N proteins alone of IBV were constructed and prepared into subunit vaccines rHBM-S1-N, rHBM-S1 and rHBM-N. The levels of immune protection of these subunit vaccines were evaluated by inoculating specific pathogen-free (SPF) chickens at 14 days of age, boosting with the same dose 14 days later, and following challenge with a virulent GX-YL5 strain of IBV 14 days post-booster (dpb). The commercial vaccine strain H120 was used as a control. The IBV-specific antibody levels as well as the percentages of CD4+ and CD8+ T lymphocytes were detected within 28 days post-vaccination (dpv). The morbidity, mortality, and re-isolation of virus from the tracheas and kidneys of challenged birds were evaluated at 5 days post-challenge (dpc). The results showed that the IBV-specific antibody levels and the percentages of CD4+ and CD8+ T lymphocyte in rHBM-S1-N group were higher than those of rHBM-S1 and rHBM-N groups, especially the cellular immunity response. At 5 dpc, the mortality, morbidity and virus re-isolation rate of rHBM-S1-N were slightly higher than those of H120 group, but were lower than those of rHBM-S1 group and rHBM-N group. The present study demonstrated that the protection of recombinant baculovirus co-expressing S1 and N proteins was better than that of recombinant baculoviruses mono-expressing S1 or N protein alone. Thus, the recombinant baculovirus co-expressing S1 and N proteins could serve as a potential IBV vaccine and this demonstrates that the bivalent subunit vaccine including the S1 and N proteins might be a strategy for the development of an IBV subunit vaccine.

Keywords: infectious bronchitis virus; protection; co-expressing; subunit vaccine; challenge

1. Introduction

Avian infectious bronchitis (IB) is a highly contagious disease of chickens and caused by the infectious bronchitis virus (IBV) belonging to the Gammacoronavirus genus within the Coronaviridae [1]. IBV affects chickens of all ages and types and primarily infects the respiratory and urogenital systems of chickens, causing massive economic losses to the poultry industry worldwide [2-4]. The IBV genome is variable and there are dozens of IBV serotypes. There is little or

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no cross-protection between different serotypes of IBVs or circulating variant viruses, frequently leading to immune failures and making it extremely difficult to control the disease [5-7].

Control of the disease currently relies on conventional live-attenuated and inactivated vaccines [8-10]. However, live-attenuated vaccines can result in the occurring of vaccine-like viruses with increased virulence and persistence due to point mutation as well as provide genetic material for recombination with other vaccine or field strains [11-13]. Inactivated vaccines have disadvantages of high manufacturing costs and lack of long-term immunity. Application of inactivated vaccines alone frequently failed to induce strong cellular immunity, resulting in providing little or no protection [14, 15]. Despite the widespread application of these conventional vaccines, IB continues to cause severe economic losses in many countries, underscoring the high demand to develop safer and more effective new candidate vaccines for the practical control of IBV.

Among new vaccines, subunit vaccines have been showed to elicit strong humoral and cellular immune responses [16-18]. Subunit vaccines have significant advantages of efficient antigenic presentation, high stability, and flexibility in proteins or epitopes selection compared with live-attenuated and inactivated vaccines [16]. In addition, no infectious viral particles are involved in the course of vaccine production, so they are very safe. Subunit vaccines are genetically engineered and thus easy to mass produce [19]. In addition, subunit vaccines allow the creation of multivalent vaccines. Thus, subunit vaccines may have the potential to act as safe and effective vaccine candidates.

IBV contains four structural proteins, the spike (S) glycoprotein, the envelope (E) protein, the membrane (M) glycoprotein and the nucleocapsid (N) protein [20]. The S glycoprotein is post-translationally cleaved into S1 and S2 subunits [21]. S1 protein determines the antigenicity and immunogenicity of the virus and plays an important role in the induction of specific neutralizing antibody and hemagglutination inhibition antibody, tissue affinity and cell adsorption, which can induce the body to produce humoral immune response [22-24]. N protein plays an extremely important role in the replication and assembly of IBV with its good immunogenicity, which can induce the body to produce cellular immune response [25-27]. Hence, the S1 and N proteins are the most promising subunit vaccine candidates against IBV. Therefore, the development of subunit vaccines including both S1 and N proteins has promising prospects.

The baculovirus expression system (BES) is an efficient system for gene expression because of its good security, high expression, and complete protein processing system compared with other expression systems. A major advantage of the BES is the ease of scale-up from the laboratory to a large-scale production system [28, 29]. In addition, baculoviruses are insect pathogens and thus non-pathogenic for vertebrates. Therefore, the BES is currently used widely for protein expression and vaccine production. There were a few studies on the expressing IBV structure proteins by BES [18, 30-33], but no previous study has focused on developing of subunit vaccine from baculovirus co-expressing S1 and N proteins of IBV and evaluating the immune response of this type of subunit vaccine. Hence, it is necessary to co-express the S1 and N proteins by BES and develop the subunit vaccine including these two proteins.

In this study, the recombinant baculovirus co-expressing the S1 and N proteins of IBV by BES was constructed by introducing the honeybee melittin (HBM) signal peptide recognized by insect cells. Moreover, immune protection of recombinant subunit vaccine including both the S1and N proteins was evaluated. We also compared the immune protection with those induced by recombinant subunit vaccines including S1 protein or N protein alone and H120 inactivated vaccine. It is first study on co-expressing the S1 and N proteins by introduction of HBM aiming to obtain safer and more effective subunit vaccines, thus providing a novel subunit vaccine candidate for IBV.

2. Materials and Methods

2.1. Cells, virus and expression vector

Sf9 insect cells were purchased from Qiyin Biological Technology Co., Ltd (Jiangsu, China) and cultured in serum-free SF900II medium (GIBCO, Grand Island, NY, USA) at 27℃. The virulent

GX-YL5 strain of IBV was isolated from broilers with nephritis disease and is the representative dominant serotype isolate in southern China and shared heterologous serotype with H120 strain [34]. The 50% tracheal organ culture infection dose (TOC-ID_{50}) of IBV GX-YL5 strain was determined as previously description [34]. Euokaryotic expression vector pFastBac™ Dual was the product of Invitrogen (USA).

2.2. *Gene cloning and the recombinant baculoviruses' construction*

In order to increase expression and yield of heterologous secreted proteins in insect cells, the HBM signal peptide recognized by insect cells was introduced into the BES. The coding mature S1 and N protein genes of IBV GX-YL5 strain were amplified by RT-PCR. The insect signal peptide HBM gene was amplified by PCR using a pair of primers with partial overlapping sequence. Then the fusion genes HBM-S1 and HBM-N were obtained by fusion PCR, cloned into pEasy-T1 vector and then subcloned into the transfer vector pFastBacTM Dual at the BamH I/Pst I sites as well as Xho I/Kpn I sites under the control of PH and P10 promotors, respectively (Figure 1A and 1B) to obtain recombinant transposon vectors pFast-HBM-S1 and pFast-HBM-N. A 6×His tag and a TEV protease cleavage site were added before the stop codon to facilitate the identification and purification of the expressed proteins. The HBM-N gene was then directly subcloned into pFast-HBM-S1 to yield recombinant transposon vector pFast-HBM-S1-N with the S1 gene being inserted under the control of promotor PH and the N gene under the control of promoter P10 (Figure 1C). All the recombinant transposon vectors were verified by PCR, restriction endonuclease digestion and sequencing. The verified recombinant transposon vectors were then transformed into DH10BacTM E. coli cells to generate recombinant bacmids rHBM-S1, rHBM-N and rHBM-S1-N, which were identified by PCR with M13 primers. The purified recombinant bacmids rHBM-S1, rHBM-N and rHBM-S1-N were obtained after several times of screening and transfected into Sf9 insect cells to obtain recombinant baculovirus through lipofectin-mediated transfection following the manufacturer's instructions (Invitrogen, USA). The recombinant baculovirus rHBM-S1, rHBM-N and rHBM-S1-N were identified by PCR with IBV specific primers and M13 primers, respectively. The recombinant baculovirus titers were determined using end-point dilution analysis. All the primers used in this study are showed in table S1.

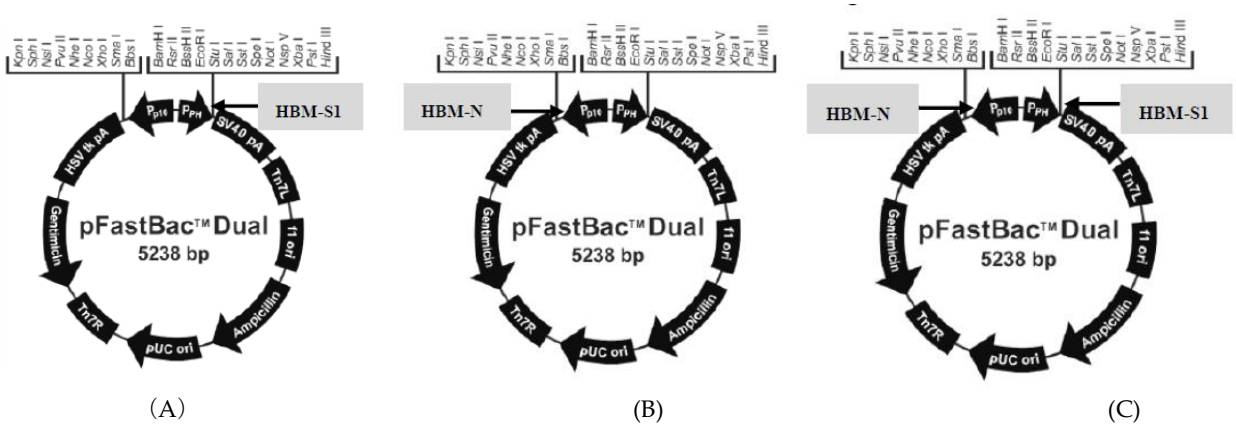

Figure 1. Schematic diagram of S1, N and S1-N genes baculovirus expression system. (**A**) The HBM-S1 gene was subcloned into the vector pFastBac™ Dual under the control of PH promotor. (**B**) The HBM-N gene was subcloned into the vector pFastBac™ Dual under the control of P10 promotor. (**C**) HBM-S1 and HBM-N genes were subcloned into the vector pFastBacTM Dual under the control of PH and P10 promotors, respectively. The resulting recombinant transposon vectors were named as pFast-HBM-S1, pFast-HBM-N and pFast-HBM-S1-N, respectively.

2.3. *Analysis of recombinants' protein expression*

Expression of recombinant proteins S1, N and S1-N in Sf9 cells were detected by indirect immunofluorescence assay (IFA) and Western blot. For IFA, Sf9 cells were infected with the recombinant baculoviruses rHBM-S1, rHBM-N and rHBM-S1-N at a multiplicity of infection (MOI) of 5, respectively, and then fixed with 4% paraformaldehyde at 72 h post-infection. Mouse anti-His

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IgG (1:2000 dilution, CWBio, Beijing, China) was used as primary antibody, while fluorescein isothiocyanate (FITC)-labeled goat anti-mouse antibody IgG (1:2000 dilution, CWBio, Beijing, China) used as the secondary antibody. Specific fluorescent signals in Sf9 cells were observed by fluorescent microscopy.

At 72 h post-infection, cell culture supernatants and cell lysates infected with the recombinant baculoviruses rHBM-S1, rHBM-N and rHBM-S1-N were collected for analysis of recombinant proteins by Western blot. Briefly, protein samples were separated by 12% SDS-PAGE, transferred onto PVDF membranes, blocked in 5% skim milk with PBST buffer. Mouse anti-His IgG (1:2000 dilution, CWBio, Beijing, China) was used as primary antibody, and horseradish peroxidase (HRP)-labeled goat anti-mouse antibody IgG (1:2000 dilution, CWBio, Beijing, China) used as the secondary antibody. Normal Sf9 cells and Sf9 cells infected with the same amount of wild-type baculovirus were set as negative controls. The proteins were visualized using DAB substrate kit as recommended by the supplier.

2.4. *Immunization of the birds and the virus challenge*

The specific-pathogen-free (SPF) chickens used in this study were hatched from fertilized white leghorn SPF eggs (Beijing Merial Vital Laboratory Animal Technology Co., Ltd, China) in our facilities and the chickens were housed in separate isolation units until reaching 14 days of age. 14-day-old SPF chickens were assigned randomly into six groups (n=10 chickens/group). Each bird in rHBM-S1-N, rHBM-S1and rHBM-N groups was injected subcutaneously with 0.5ml (50μg) S1-N, S1 and N recombinant proteins, respectively, emulsified in Freund's complete adjuvant on day of 14, and boosted with the same dose of proteins emulsified in Freund's incomplete adjuvant on day of 28. Birds in the H120 group, set as a positive control, were injected with H120 inactivated vaccine. The WT-B group and cell group, serving as negative controls, were set up by replacing the recombinant proteins with wild-type baculovirus and normal Sf9 cell lysates, respectively. At 14 days post-booster (dpb), all the birds from each group were challenged with 10^5 TOC-ID_{50} of IBV GX-YL5 strain in 0.2 mL by the nasal-ocular route and were recorded daily for clinical signs, morbidity and mortality, and euthanized at 5 days post-challenge (dpc). Any birds that died during the observation period were immediately necropsied. Any remaining birds were euthanized at the end of the observation period. The trachea and kidney samples were collected from each bird aseptically for re-isolation of IB virus. Animal experiments were approved by the Animal Care & Welfare Committee of Guangxi University (approval number GXU2016-011) and were performed in accordance with animal ethics guidelines and approved protocols.

2.5. *Detection of IBV-specific antibody*

Blood samples were collected from the wing vein of 10 birds in each group prior to prime immunization (0 day) and at 7, 14, 21, and 28 days post-vaccination (dpv) for the detection of IBV-specific antibody by commercial enzyme-linked immunosorbent assay (ELISA) kits (IDEXX Laboratory, Inc., Westbrook, ME, USA) according to the manufacturer's recommendations.

2.6. *Quantification of CD4+ and CD8+ T lymphocytes*

Peripheral blood samples were collected from the wing vein of 10 birds in each group at 0, 7, 14, 21, and 28 dpv. Peripheral blood lymphocytes were isolated and adjusted to 1 × 106 cells/mL, stained with 1μL Mouse Anti-Chicken CD4-PE and CD8a-FITC antibodies (Wu Han AmyJet Scientific Inc., China) respectively and analyzed for the percentages of CD4+ and CD8+ T lymphocytes by a BD AccuriTM C6 Flow Cytometer (Becton, Dickinson and Company, USA).

2.7. *Virus re-isolation*

All birds were euthanized at 5 dpc. Tracheas and kidneys mixture were collected for virus re-isolation by inoculation into 10-day-old SPF chicken embryos via the allantoic cavity route. Allantoic fluid was harvested at 72 h post-inoculation. Three passages in embryonated eggs were conducted. Viral RNA in allantoic fluid was extracted using EasyPure RNA Purification Kit

(TransGen Biotech, Beijing, China) and subjected to quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) according to our previous description [35]. The absence of detectable virus in the trachea and kidney was considered to be protected by the subunit vaccines against the challenge.

2.8. Statistical analysis

All data were shown as mean values ± standard deviation (mean ± SD) and were compared by One-way analysis of variance (ANOVA) and Student's t-test using SPSS 18.0 (SPSS Inc., Chicago, IL) biostatistics software. P values less than 0.05 were regarded as significant and those less than 0.01 were regarded as highly significant.

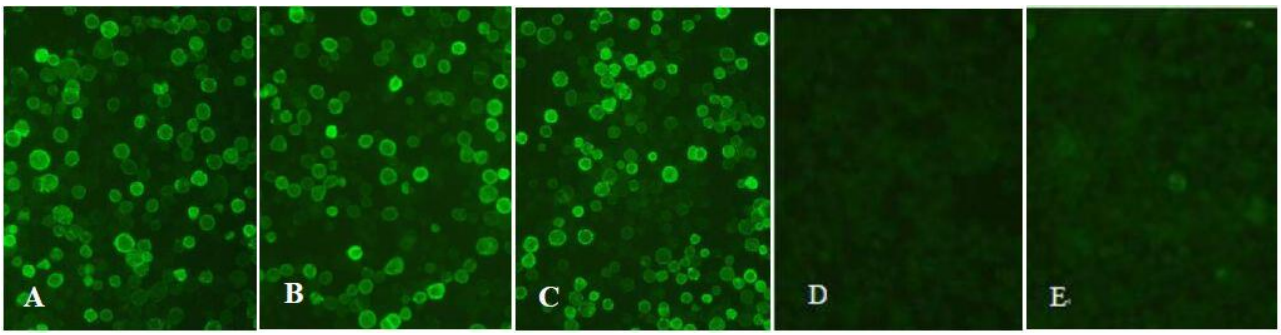
3. Results

3.1. Confirmation of the recombinant baculoviruses

The results of PCR, restriction enzyme digestion analysis (Figure S1) and sequencing showed that recombinant transposon vectors pFast-HBM-S1, pFast-HBM-N and pFast-HBM-S1-N were successfully constructed. The recombinant bacmids rHBM-S1, rHBM-N and rHBM-S1-N were obtained by transforming the verified recombinant transposon vectors to DH10Bac TM E. coli cells and identified by PCR. The results of PCR identification showed that the recombinant bacmids rHBM-S1, rHBM-N and rHBM-S1-N were generated (Figure S2). The purified recombinant bacmids rHBM-S1, rHBM-N and rHBM-S1-N were transfected into Sf9 insect cells to obtain recombinant baculovirus. The recombinant baculovirus rHBM-S1, rHBM-N and rHBM-S1-N were identified by PCR with IBV specific primers and M13 primers, respectively. The sizes of amplified bands matched those of the predicted products (Figure S3).

3.2. Identification of the recombinant proteins

Strong specific immunofluorescence signals were observed in the Sf9 cells infected with the recombinant baculovirus rHBM-S1 (Figure 2A), rHBM-N (Figure 2B) and rHBM-S1-N (Figure 2C) at 72 h post-infection, but no fluorescence was detected in the Sf9 cells infected with wild-type baculovirus (Figure 2D) and normal Sf9 cells (Figure 2E). The expected sizes of 93 kDa (Figure 3A) and 51 kDa (Figure 3B) proteins were detected in the culture supernatant and cell lysate infected with rHBM-S1 and rHBM-N, respectively. Two specific bands of 93 kDa and 51 kDa (Figure 3C) proteins were detected in both the culture supernatant and cell lysate infected with rHBM-S1-N. No bands were observed in the culture supernatant or cell lysate infected with wild-type baculovirus and normal Sf9 cells. The results showed that the sizes of expressed recombinant proteins are consistent with the sizes of native S1 or N proteins and these recombinant proteins retained their antigenicity.



（A） (B) (C) (D) (E)

Figure 2. Detection of the recombinant proteins' expression by IFA (200×). Sf9 cells were infected with recombinant baculoviruses at a MOI of 5 and detected by fluorescence microscopy 72 h post-infection. (**A**) Sf9 cells infected with baculovirus rHBM-S1; (**B**) Sf9 cells infected with baculovirus rHBM-N; (**C**) Sf9 cells infected with baculovirus rHBM-S1-N; (**D**) Sf9 cells infected with wild-type baculovirus ; (**E**) Normal Sf9 cells.

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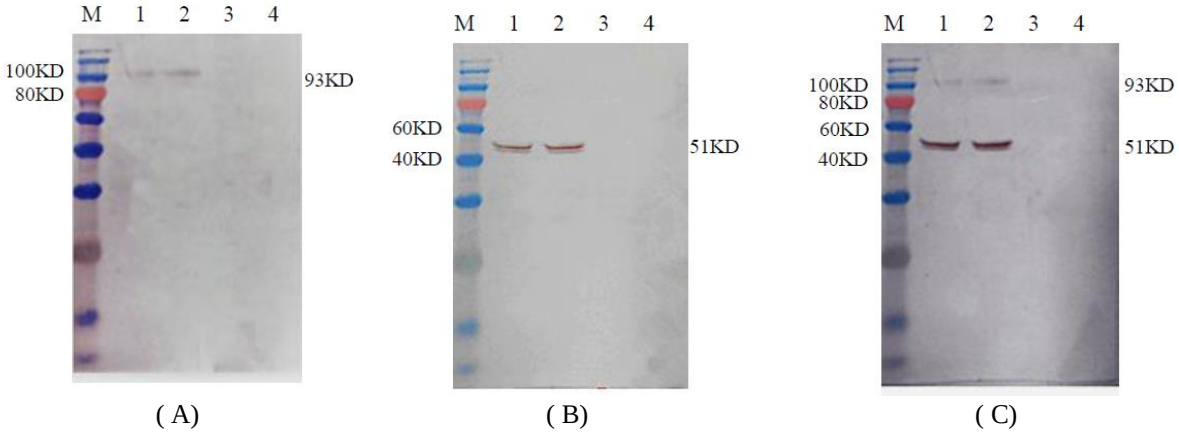

Figure 3. Detection of the recombinant proteins' expression by Western blot. Sf9 cells were infected with recombinant baculovirus at a MOI of 5. The recombinant proteins were detected 72 h post-infection. (**A**) The recombinant proteins expressed by baculovirus rHBM-S1; (**B**) The recombinant proteins expressed by baculovirus rHBM-N; (**C**) The recombinant proteins expressed by baculovirus rHBM-S1-N. Lane M. Blue Plus IV Protein Marker ; Lane 1. Culture supernatant; Lane 2. Cell lysate; Lane 3. Wild-type baculovirus; Lane 4. Normal Sf9 cells.

3.3. *Antibody responses following the immunizations*

At 14 dpv, IBV-specific antibody levels in birds immunized with subunit vaccines rHBM-S1-N, rHBM-S1, rHBM-N and inactivated vaccine strain H120 started to rise ($p>0.05$). The antibody levels of the vaccinated groups increased notably at 21 and 28 dpv (7 and 14 dpb) and were very significantly higher than those of the negative control groups ($p<0.01$) (Figure 4). The highest IBV antibodies in each vaccinated group were observed at 7 dpb. The antibody levels in the rHBM-S1-N group were always higher than those in rHBM-S1 and rHBM-N groups, but the difference was not significant ($P>0.05$). Similarly, the antibody titers in rHBM-S1 groups were always slightly higher than those in rHBM-N group ($P>0.05$). Chickens in the H120 group developed the highest antibody titers throughout the experimental period, but the difference was not significant ($P>0.05$) compared with those in the rHBM-S1-N, rHBM-S1 and rHBM-N groups.

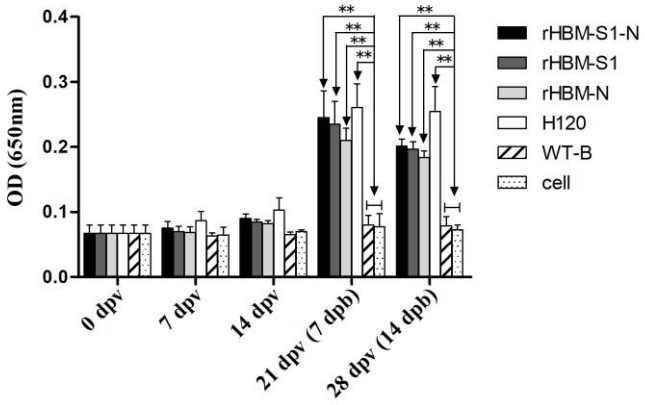

Figure 4. Antibody responses to IBV after vaccination. Serum samples were taken from the six groups of chickens at 0, 7, 14, 21 and 28 dpv and measured by indirect ELISA. Optical densities were read at 650 nm. Values are expressed as mean values ± standard deviation (mean ± SD) in each group. Statistically significant differences are indicated by * ($p<0.05$) or ** ($p<0.01$). (n = 10 chickens/group).

3.4. *Dynamic changes of CD4+ and CD8+ T lymphocytes in peripheral blood*

The results showed that the percentages of CD4+ and CD8+ T lymphocyte in vaccinated birds remained on increasing trend at 7 dpv (Figure 5). At 21 and 28 dpv (7 and 14 dpb), the percentages of the CD4+ and CD8+ T lymphocytes in the rHBM-S1-N and rHBM-N groups were very significantly higher than those of the negative groups ($p<0.01$). The percentages of CD4+ T lymphocyte in rHBM-S1 group were significantly higher than those of the negative controls ($p<0.05$), and the percentages of CD8+ T lymphocyte were very significantly higher ($p<0.01$) compared with the negative controls. The percentages of CD4+ and CD8+ T lymphocyte from high to low in the vaccinated groups at 21 and 28 dpv (7 and 14 dpb) were always rHBM-S1-N group, rHBM-N group, H120 group, rHBM-S1 group, with a significant difference between the rHBM-S1-N and rHBM-S1 groups ($P<0.05$), while no significant difference was noted between other groups ($p>0.05$).

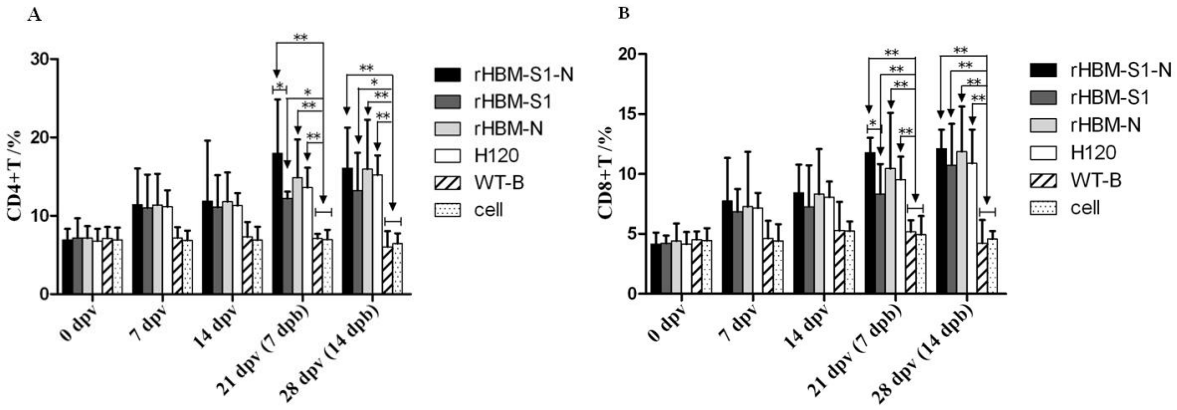

Figure 5. Detection of the percentages of CD4+ and CD8+ T lymphocytes in peripheral blood of vaccinated groups. T lymphocytes were isolated from the six groups of chickens at 0, 7, 14, 21 and 28 dpv. The percentages of (**A**) CD4+ and (**B**) CD8+ T lymphocytes were analyzed by flow cytometry. The data are shown as mean values ± standard deviation (mean ± SD) in each group. Statistically significant differences are indicated by * ($p<0.05$) or ** ($p<0.01$). (n = 10 chickens/group).

3.5. Protections against the IBV challenge

At 4 dpc, chickens began to show clinical signs or died. Morbidity, mortality, virus re-isolation rate and protection rate at 5 dpc were summarized in Table 1. Most of chickens in the two negative control groups (WT-B and cell groups) showed typical signs of IBV infection, such as coughing, sneezing, gasping, tracheal rale and wet droppings, with 60%-70% morbidity rate and 30%-40% mortality. In contrast, 20%, 30%, 40% and 10% morbidity as well as 0%, 0%, 20% and 0% mortality was observed in the rHBM-S1-N, rHBM-S1, rHBM-N and H120 groups, respectively. Birds in the four vaccinated groups exhibited milder clinical signs and gross lesions compared with those in the two negative control groups. In the necropsy at 5 dpc, the kidneys of infected birds were swollen and pale, and showed a white sludge of urate deposition, and some birds showed trachitis with exudates.

In order to further evaluate the level of protection, the collected trachea and kidney from the challenged birds at 5 dpc were inoculated into 10-day-old SPF chicken embryos for three passages for virus re-isolation and viral RNA was detected from the infected allantoic fluid of the embryonated eggs by qRT-PCR. The virus re-isolation rates for the birds in the rHBM-S1-N, rHBM-S1, rHBM-N and H120 groups were 40%, 50%, 60% and 20% respectively, while those in the WT-B and cell groups were 80% and 100% respectively (Table1). Birds in the rHBM-S1-N, rHBM-S1and rHBM-N groups developed higher levels of protection (60%, 50%, 40%) than those in the WT-B and cell groups (20%, 0%), but lower than that in the H120 group (80%). No protection was observed in the cell group, and low level of protection (20%) in the WT-B group.

Table 1 Protective efficacy of recombinant subunit vaccines against the challenge of the virulent GX-YL5 strain of IBV

Groups	Morbidity(%)[a]	Mortality(%)[b]	Virus re-isolation rate (%)[c]	Protection rate (%)[d]
rHBM-S1-N	20(2/10)	0(0/10)	40(4/10)	60(6/10)
rHBM-S1	30(3/10)	0(0/10)	50(5/10)	50(5/10)
rHBM-N	40(4/10)	20(2/10)	60(6/10)	40(4/10)
H120	10(1/10)	0(0/10)	20(2/10)	80(8/10)
WT-B	60(6/10)	30(3/10)	80(8/10)	20(2/10)
Cell	70(7/10)	40(4/10)	100(10/10)	0(0/10)

[a] Morbidity was determined by the number of affected chickens/total number of chickens in each group.

[b] Mortality was determined by the number of dead chickens/total number of chickens in each group.

[c] Virus re-isolation rate was determined by the number of chickens with virus re-isolation from tracheas and kidneys of challenged chickens/total number of chickens in each group.

[d] Protection rate was determined by the number of unaffected chickens (without detection in trachea and kidney) / total number of chickens in each group.

4. Discussion

IB causes massive economic losses to the poultry industry worldwide. Live-attenuated and inactivated vaccines are commonly used to control this disease. However, live-attenuated vaccines may become a source of mutation and recombination, resulting in the occurrence of new serotypes or variants of IBV [11-13]. Also, inactivated vaccines often fail to induce strong cellular immunity and have the disadvantage of high manufacturing costs [14-15]. Hence, there is an urgent need to develop new, safe and effective vaccines to control the disease. Recombinant subunit vaccines have been shown to elicit strong humoral and cellular immune responses and be very safe [16-18]. Therefore subunit vaccines may serve as potential vaccine candidates in the future.

In the current study, significantly higher antibody levels, as well as higher percentages of CD4+, CD8+ T lymphocytes as well as higher protection rates in the subunit vaccines rHBM-S1-N，rHBM-S1 and rHBM-N vaccinated groups compared to the WT-B and cell groups at 7 and 14 dpb were demonstrated. Therefore, the recombinant subunit vaccines rHBM-S1-N，rHBM-S1 and rHBM-N could induce the immunized chickens to produce humoral and cellular immunity to resist and eliminate the IBV infection, indicating the generation of recombinant baculovirus in the current study laid a feasible foundation for the development of effective vaccines against infection of IBV in the future.

The immune efficacy of subunit vaccine rHBM-S1 containing S1 protein was stronger than that of subunit vaccine rHBM-N containing N protein in humoral immune responses, while the immune efficacy of subunit vaccine rHBM-N was stronger than that of rHBM-S1 in cellular immune responses. The results confirmed that S1 protein and N protein play a major role in humoral and cellular immune responses, respectively, which were in accord with the previous investigations [23, 26, 36]. The immune efficacy of subunit vaccine rHBM-S1-N was better than that of subunit vaccines rHBM-S1 or rHBM-S1-N in humoral and cellular immune responses. The reason was that rHBM-S1-N expressed both the S1 and N proteins of IBV and stimulated both the humoral and cellular immune responses simultaneously, therefore the subunit vaccine rHBM-S1-N performed better in the activation of virus-specific immune responses. Although the S1 subunit is the major inducer of neutralizing antibodies, vaccination with S1 protein couldn't confer adequate protection against challenge [17]. A previous study even found that the antigenicity of N protein is better than that of the spike protein [37]. In another study, the cell-mediated immune response induced by N protein was higher than that induced by either S1 protein or H120 [38]. These results and ours indicated that it's imperative to generate the subunit vaccine including S1 and N proteins.

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A previous study showed that the BacMam virus Ac-CMV-S1 expressing the S1 glycoprotein of IBV-M41 were deficient in the induction of IBV-specific antibody compared with that induced by the inactivated vaccine [18]. In our study, the antibody levels in the groups rHBM-S1-N, rHBM-S1 and rHBM-N were slightly lower than that of the H120 group at 7 and 14 dpb, which agreed with the previous study results mentioned above [18]. The possible reason was that the H120 vaccine was derived from the whole virus and was comprised of almost all the epitopes of IBV. Another reason may be related to the fact that the coated antigen of ELISA kit used in this study is the Mass-type virus strain. On the other hand, the percentages of CD4+ and CD8+ T lymphocyte from high to low were always rHBM-S1-N group, rHBM-N group, H120 group, rHBM-S1 group. It is surprise to find that cellular immune response in rHBM-N group was higher than that in the H120 group although the difference was not found to be statistically significant. A previous research showed that Ac-CMV-S1 induced a significantly better cellular immune response in SPF chickens compared with that induced by the inactivated vaccine [18], which was consistent with our results. The cell-mediated immune response induced by rHBM-N was higher than that induced by either H120 or rHBM-S1. One possible explanation is a more efficient presentation of N peptides by antigen-presenting cells following effective uptake, processing, or presentation on MHC receptors of these cells [38]. Therefore, the necessity of co-expressing the S1 and N proteins is again stressed.

In the present study, chickens in the H120 group didn't obtain 100% protection against IBV GX-YL5 strain challenge and possibly it was because the vaccine H120 strain shared the heterologous serotype with the GX-YL5 strain [34], which was consistent with previous studies [39-41]. Another reason is the absence of live priming for the inactivated H120 vaccine [42, 43]. In addition, the protection rates of subunit vaccines rHBM-S1-N (60%), rHBM-S1 (50%), and rHBM-N (40%) were lower than that of traditional vaccine H120 (80%). The protection rates of subunit vaccine rHBM-S1 and rHBM-N were lower than that of the traditional vaccine strain H120. A previous description reported 55% protection against the virulent M41 challenge from the constructed baculovirus expressing the S1 protein of IBV M41 strain, which was lower than that for the inactivated vaccine (72%) [44]. Only 50% protection was obtained against the homologous KM91 strain challenge after three immunizations with IBV S1 glycoprotein expressed by a recombinant baculovirus [32]. A similar result was shown in another previous research [45]. It was surprising that the protection rate of rHBM-S1-N (60%) against the homologous GX-YL5 strain challenge was lower than that of H120 (80%) against the heterologous GX-YL5 strain challenge. There are some possible explanations for that. First of all, the constructed subunit vaccines contained only part of the whole immunogenicity of the whole virus, which couldn't lead to an immune effect that was as good as the whole-virus vaccine H120. Secondly, the cell lysates were used to prepare the oil emulsion vaccines for birds' immunizations and it is unknown whether the complicated composition of the cell lysate had an effect on the immunized birds. Thirdly, the immune dose of 50μg/bird may be not enough. Doses of 150μg/bird were used to immunize chickens in other studies [38, 46]. Finally, two immunizations may be not enough to elicit an effective immune response. A previous investigation pointed out that at least four immunizations with purified S1 glycoprotein were needed to induce protection against the homologues N1/62 strain challenge [47]. Similarly, three immunizations were performed and only 50% protection was obtained against the homologous IBV in the previous study mentioned above [32]. In this study, we only immunized twice with the recombinant proteins, which may be a reason for the low protection conferred by the subunit vaccines.

Live attenuated vaccines are applied from day-old to achieve early protection and boosted with the inactivated vaccines in the case of future layers and breeders. Although subunit vaccine rHBM-S1-N couldn't provide complete protection against IBV infection, it conferred higher protection than rHBM-S1 and rHBM-N. So it still can be used as alternative vaccine for boosting the primary vaccination with the traditional vaccine/vaccines against this disease. As we know, IBV continuously evolves and there are scores of serotypes. Little or no cross-protection confers between different serotypes of IBVs [6, 7]. Therefore, the universal IBV vaccines that protect against varying serotypes of IBV are imperative. Our results should enable the generation of multivalent vaccines to prevent more serotypes of IBV, especially the newly emerging virus strains. The vaccine rHBM-S1-N can also be applied by combining it with the traditional vaccines to reduce the occurrence of variants caused by virus mutations and recombination. Therefore, the subunit vaccine rHBM-S1-N is an

alternative to the traditional IBV vaccine. Our study also provides reference and ideas for the development of subsequent new vaccines. Further study should be needed to determine the effectiveness of combining the subunit vaccines with traditional vaccines.

In this study, a 20% protection rate was conferred following IBV challenge in the wild-type baculoviruses control group, and the protection rate of wild-type baculoviruses control group was slightly higher than that of cell control group, which was consistent with previous reports [18, 42]. The reason is that the baculovirus genome consists of double-stranded DNA, which provokes innate immunity through Toll-like receptor 9 and MyD88-dependent signaling pathway and stimulates the production of various inflammatory cytokines [48]. Therefore, vaccination of baculovirus alone may result in non-specific immunity, which could provide only a small amount of protection against an IBV challenge.

It is critical to obtain high-level secreted expression of recombinant proteins with native activities. HBM is an insect-derived signal peptide. It has been reported that introduction of HBM signal peptide could increase the expression of foreign proteins, enhance the activity of expressed protein and achieve the secretion of foreign proteins in the baculovirus system [49-51]. There are currently a few studies on expressing IBV proteins by BES [18, 30-33], but no previous study has focused on introduction of the HBM signal peptide into BES to express IBV proteins. Therefore, the HBM signal peptide was introduced in the present study. The results showed that the recombinant proteins S1-N, S1 and N could be expressed both in culture supernatant and cell lysate. So introduction of HBM signal peptide into BES can be applied in the expressing of other proteins from other pathogens.

In summary, the current study showed that the constructed recombinant baculoviruses could elicit both the cellular and humoral immune responses to a certain degree, and the protection of the recombinant baculovirus co-expressing S1 and N proteins was better than that of recombinant baculoviruses containing S1 or N protein alone. To our knowledge, this is the first report that proved that bivalent subunit vaccines, including S1 and N proteins of IBV, could induce higher immune response and provide greater protection against the infection. The recombinant baculovirus co-expressing S1 and N proteins could serves as a potential IBV vaccine and a bivalent including the S1 and N proteins might be promising for better subunit vaccines and/or viral vectored strategies.

SupplementaryMaterials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: Identification of recombinant transposon vectors pFast-HBM-S1, pFast-HBM-N and pFast-HBM-S1-N by PCR and restriction endonuclease digestion. Figure S2: Identification of recombinant bacmids rHBM-S1, rHBM-N and rHBM-S1-N by PCR with M13 primers. Figure S3: Identification of recombinant baculovirus by PCR with IBV specific primers and M13 primers. Table S1: All the primers used in this study.

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Conflicts of Interest: The authors have no conflict of interest to declare.

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