VP2 Virus-Like Particles elicit protective immunity against duckling short beak and dwarfism syndrome in duck and chicks

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Abstract

Duckling short beak and dwarfism syndrome virus (SBDSV), an emerging goose parvovirus, that has caused serious outbreaks of short beaks and dwarfism syndrome (SBDS) in Chinese duck flocks since 2015. Presently, there is no commercial vaccine against SBDS. In the present study, a virus-like particle (VLP) based candidate vaccine was developed against this disease. A baculovirus expression system was used to efficiently express the SBDSV VP2 protein in Sf9 cells. Immunofluorescence assay, SDS-PAGE, and Western blot were used to confirm protein expression. Furthermore, transmission electron microscopy was used to observe the formation of VLPs. Finally, VLPs were formulated in an oil-adjuvanted vaccine to evaluate humoral responses in ducks and chicks via latex particle agglutination inhibition assay (LAI) and microneutralization assay. A single injection with this oil-adjuvanted VLP vaccine induced high levels of LAI and neutralization antibodies in ducks and chickens, with LAI peak titer as 4.9 ± 1.20, 4.4 ± 1.07, and neutralization peak titers as 7.1 ± 1.20 and 6.2 ± 1.03 (log2) in ducks and chickens, respectively. The average LAI titer of yolk antibodies in duck eggs receiving 2 doses was 5.3 ± 1.09 (log2). These results

indicate that SBDSV VLPs could be a promising vaccine candidate for SBDS control.

1. Introduction

Short beak and dwarfism syndrome virus (SBDSV) is a distinct goose parvovirus (GPV) that causes atrophic beak development (and the characteristic result of protruding tongues), growth retardation, diarrhea, fragile bones, and feather dysplasia in mule and Cherry valley ducklings [1-4]. Other domesticated waterfowl such as Muscovy ducks, Sheldrake ducks, and geese challenged with SBDSV present with the characteristic clinical signs including growth retardation, anorexia, and diarrhea, but not the protruding tongue [5]. Since 2015, SBDS has spread widely in China, resulting in 10-100 % morbidity and 0-10 % mortality, in addition to significant economic losses [6-11]. However, SBDS differs from classical goose parvovirus (GPV) and Muscovy duck parvovirus (MDPV) with respect to the clinical signs and susceptible animals. Specifically, GPV can cause lethargy, anorexia, prostration and weight loss mainly in young goslings and sometimes in Muscovy ducklings with high morbidity and mortality [12]. Conversely, MDPV can induce watery diarrhea, wheezing, and locomotor dysfunction only in young Muscovy ducklings under 4 weeks old [13].

SBDSV belongs to the *Anseriform dependoparvovirus 1* [14] and contains a single-strand DNA genome of approximately 5.1 kb. There are two large open reading frames (ORFs) in the middle of the genome which encode for the regulator (Rep) and viral capsid (VP1-3) proteins. Both ends of the genome contain inverted terminal repeat sequences (ITRs). The viral structural proteins VP1, VP2, and VP3 are derived from the same gene by differential splicing, which is associated with viral infectivity, receptor recognition, and nuclear translocation, capsid assembly, and virion stability, respectively [15]. Importantly, VP2 is the most abundantly expressed viral protein, and as a result, induces the strongest host immune response. Expression of VP2 alone can result in spontaneous assembly, forming virus-like particles (VLPs) [16]. Therefore, this protein makes an ideal candidate for development as a VLP-based vaccine against SBDS.

Previously, we isolated and identified a strain of SBDSV that was named SBDSV M15 (GenBank accession no. KU844283) [2]. A preliminary study on a potential vaccine against the virus produced an inactivated, oil-adjuvanted vaccine, and characterized the host immunological response to the vaccine [17]. However, there are no commercially available vaccines against SBDS. In this study, VLPs of the SBDSV M15 VP2 were produced using a baculovirus expression system. Immunogenicity of SBDSV VLPs in ducks and chicks were investigated, along with yolk antibody determination of vaccinated ducks.

2. Materials and Methods

2.1. Antibodies, reagents, Cell lines, and viruses

A monoclonal antibody targeting the VP2 of GPV E16 and GPV MAb E16 based latex agglutination reagent for detection of GPV antigens were prepared in our lab.

Spodoptera frugiperda (Sf9) insect cells (Invitrogen, USA) were cultured in SIM SF Expression Medium (Sino Biological, Beijing, China) at 27 ℃ with shaking at 110 rpm. Duck embryo fibroblast (DEF) cells were prepared from 16-day-old specific pathogen-free (SPF) embryonated duck eggs (Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences) and were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum. The M15 strain of SBDSV was cultured in DEF cells, and the 50% tissue culture infective dose ($TCID_{50}$) was determined.

2.3. Plasmid construction and virus rescue

The M15 strain DNA was extracted using the OMEGA Viral DNA kit (Lot. D3892-01). The VP2 gene was obtained by PCR amplification from the viral genome. The specific primers designed and used here are as follows: forward, 5'-ACGCGTCGACATGGCTCCTGCAAAAAAAAATACAG-3' (containing a SalI restriction site), reverse, 5'- ATAAGAATGCGGCCGCTTACAGATTCTGAGT -3' (containing a NotI restriction site). Following enzymatic digestion with SalI and NotI, the PCR product was cloned into the pFastBac Dual vector (Invitrogen). The recombinant pFastBac Dual plasmid was transformed into Trans10 Chemically Competent *E. coli* cells (Transgen Biotech, Beijing, China) for expansion of the

plasmid. The recombinant pFastBac Dual plasmid DNA was extracted and purified using the EasyPure Plasmid MiniPrep Kit (Transgen Biotech, Beijing, China). The resulting plasmid was then transformed into DH10Bac *E. coli* to generate the recombinant bacmid. Recombinant baculoviruses (rBVs) were generated using the X-tremeGENE HP DNA Transfection Reagent (Roche, Basel, Switzerland) in Sf9 insect cells according to the manufacturer's instructions. The GPV VP2 protein was expressed by infecting Sf9 cells with SBDSV-rBVs, and the VP2 spontaneously assembled into SBDSV VLPs [16,18]. The multiplicity of infection (MOI) of rBVs was determined by viral plaque assay. To generate sufficient quantities of SBDSV VLPs, 2 × 10^6 cells/mL Sf9 insect cells were infected with SBDSV-rBVs at an MOI of 5. At 5 dpi, VLPs were harvested by 3 freeze-thaw cycles, followed by centrifugation at 8000 rpm for 10 min to remove cellular debris.

The pFastBac-Gus plasmid (Invitrogen) was used to generate Gus-rBVs as a control.

2.4. Immunofluorescence assay (IFA)

A total of 8 × 10^5 Sf9 cells were seeded in each well of 6 well culture plates (Corning Costar), and cultured at 27℃ in SIM SF Expression Medium. The next day, cells were inoculated with SBDSV-rBVs and Gus-rBVs at an MOI of 5. At 3 dpi, the culture supernatant was carefully removed, and the cell monolayers were fixed with cold acetone at -20℃ for 30 min. The cells were then washed with PBST, and then incubated with GPV MAb E16 (diluted 1:100 in PBS) at 37℃ for 2 h. After washing 5 times, the plates were stained with FITC Conjugated AffiniPure Goat Anti-mouse IgG (H+L) (BOSTER Wuhan, China) diluted 1:100 dilution in PBST, and incubated at 37℃ for 30 min. Following three more washes, the plates were examined using a fluorescent microscope.

2.5. Western blotting

Characterization of SBDSV VLPs was conducted by SDS-PAGE and Western blotting, using standard protocols, to determine molecular weight and purity of the protein preparation. The primary and secondary antibodies for the Western blot analysis were GPV MAb E16 and HRP-AffiniPure Goat Anti-Mouse IgG (H+L)

(BOSTER Wuhan, China), respectively.

2.6. Transmission electron microscopy (TEM)

Assembly of SBDSV VLPs in Sf9 cells was characterized by TEM. The SBDSV-rBV infected Sf9 cells were harvested at 3 dpi by centrifugation at 1000 rpm for 10 min, fixed in 2.5% glutaraldehyde, post-fixed in 1% osmiumtetroxide (OsO_4), and embedded in EMbed 812 (Electron Microscopy Sciences, Hatfield, PA, USA). Ultrathin sections were cut to an approximate thickness of 70 nm, and stained with 2% uranyl acetate saturated alcohol solution for ultrastructural analysis under a Hitachi HT-7800 transmission electron microscope (Hitachi High-Technologies America, Inc., Pleasanton, CA, USA).

For characterization of the shape and size of SBDSV VLPs, the SBDSV-rBV infected Sf9 cells were harvested at 5 dpi by centrifugation at 8000 rpm for 10 min after 3 freeze-thaw cycles. The supernatant was then centrifuged at 50,000 rpm for 120 min to pellet the SBDSV VLPs. The pellet was resuspended in PBS, and VLPs were visualized by negative stain electron microscopy.

2.7. Immunization studies in ducks and chicks

One volume SBDSV VLPs (LPA titer is 5 log2) and 1.5 volume of oil adjuvant were mixed together. The Gus-rBVs and oil adjuvant were prepared in the same manner as a negative control vaccine. Thirty 130-day-old Cherry Valley ducks (negative for waterfowl-parvovirus antibodies as determined by LAI) were divided into 3 groups. For the immunization and control groups, each duck was injected with 1 mL vaccine or empty vector vaccine, respectively, intramuscularly in the leg, while PBS was administered to the mock group. Thirty 10-day-old Hyline brown chicks, all negative for WPV antibodies, were treated in the same way, but the immunizing dose was 0.5 mL per chick. The condition of the birds in each group was monitored and recorded daily. Sera samples were collected at 2, 3, 4, 5, 6, and 7 wpi for the detection of GPV specific antibodies.

Twenty 130-day-old breeding female Cherry Valley ducks, which were free from WPV antibodies, were divided into 2 groups for laying eggs. Each duck in the immunization group was injected with 1 mL SBDSV VLP oil-adjuvanted vaccine.

Animals received a booster vaccination of the same dose 3 weeks later. For the mock group, ducks were injected with sterile PBS. At 4 weeks post boost, 50 eggs from each group were harvested for yolk antibody determination.

2.8. Serologic tests by LAI and microneutralization assay

The LAI protocol for detection GPV specific antibodies was described previously [2,19]. Briefly, 20 μL 2-fold serial dilutions of inactivated serum samples were mixed with 20 μL (4 agglutination units) and incubated at room temperature for 60 min. Then, 10 μL of incubation mix was added to an equal volume of GPV MAb-based latex agglutination reagent in plates and incubated at room temperature for 20 min, and LAI titers were observed. For the microneutralization assay, 2-fold serial dilutions of inactivated serum samples were mixed with 100 $TCID_{50}$ SBDSV M15 virus and incubated at 37℃ for 60 min. The serum/virus mixture was overlaid on DEF monolayers, and the plates were incubated at 37℃ in a 5% CO_2 cell culture incubator. The inverse of the highest serum dilution in which no cytopathic effect was observed was recorded as the neutralizing antibody titer.

2.9 Determination of yolk antibody titer

The yolk antibody against GPV was determined by LAI as described in 2.8.

2.9. Statistical analysis

Data were presented as the mean ± SD. Correlation analysis was carried out using Pearson's statistics. Pearson correlation coefficient was represented by the letter r. When $r > 0$, a positive relationship was observed, and an inverse relationship[was observed when $r < 0$. As the value of r approaches zero, the correlation becomes weaker. Significant was determined using a two-tailed test.

2.10. Ethics statement

The animal protocol used in this study was approved by the Animal Care and Use Committee of the Institute of Animal Husbandry and Veterinary Medicine, Fujian Academy of Agriculture Sciences. The procedures were conducted in full accordance with the approved guidelines.

3. Results

3.1. Expression of SBDSV VP2 in insect cells

After infection with SBDSV-rBV, the diameters of both the Sf9 cells and the nuclei increased. In addition, the GPV VP2 antigen was successfully detected in SBDSV-rBV infected Sf9 cells, but not in Gus-rBV infected or control Sf9 cells by IFA assay at 3 dpi (Fig. 1). Next, SBDSV-rBV infected Sf9 cells were assayed by Western blot at 5 dpi for the presence of the VP2 protein. As was expected, the VP2 protein, with molecular weight of 65 kDa, was apparently detected by GPV MAb E16 (Fig. 2). These results showed that SBDSV VP2 protein was correctly expressed in the insect cells.

3.2. The formation of parvovirus-like particles

To confirm whether parvovirus-like particles could be assembled correctly, the SBDSV-rBV infected Sf9 cells were either processed for TEM observation, or collected by ultracentrifugation. The nuclei size of SBDSV-rBV infected Sf9 cells increased, and both baculoviruses and SBDSV VLPs were success assembled within the cell nuclei (Fig. 3 A, B). The baculovirus vectors used here were derived from *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV), which consists of helical virions, approximately 50×300 nm in size. The SBDSV particles were icosahedral in the shape, with diameters of about 20-22 nm. The micrographs showed that both baculoviruses and SBDSV VLPs of the correct size and shapes were contained within viroplasts (Fig. 3 C and D).

3.3. Ducks and chicks vaccinated with SBDSV VLPs produced strong neutralizing antibody responses against SBDSV

No obvious adverse reactions were observed in vaccinated birds during the experiment. Evaluation of antibody responses in vaccinated birds was assessed by LAI and microneutralization assays. Both ducks and chicks receiving the SBDSV VLP oil-adjuvanted vaccine produced antibodies against SBDSV (Fig. 4). No SBDSV antibodies were detected in birds in the Gus-rBV or mock groups. The LAI antibody levels were similar to neutralizing antibody levels observed in both ducks and chicks. Antibody titers increased gradually from 2 to 4 wpv, and decreased mildly from 5 to 7 wpv. The highest LAI titers observed in ducks and chickens were 4.9 ± 1.20 (log2) at 4 wpv, and 4.4 ± 1.07 (log2) at 5 wpv, respectively (Fig. 4 A and B). The peak

neutralization titers in ducks and chickens were 7.1 ± 1.20 (log2) at 4 wpv and 6.2 ± 1.03 (log2) at 5 wpv, respectively. The LAI titers were significantly and positively correlated with neutralization titers according to Pearson's statistics. The r value for ducks and chickens were 0.513 and 0.482, respectively, with $P < 0.01$. The antibody levels of ducks were positively correlated with chickens as well ($r = 0.598$, $P < 0.01$ for LAI titers; $r=0.424$, $P < 0.01$ for neutralization titers).

The yolk antibodies of 50 eggs from 130-day-old Cherry Valley ducks, which were vaccinated twice, were determined by LAI during the 4th weeks after the second immunization. The LAI titers of the immunization group ranged from 4 to 8 log2 (average 5.3 ± 1.09 (log2)), while no anti-GPV antibody was detected in the control group (Table 1).

4. Discussion

When considering synthetic or recombinant antigens, VLPs have the most similar structure to the natural virus, but are formed without viral nucleic acids present. They can be produced using *E.coli* [20], baculovirus [21,22], yeast [23,24], pseudorabies virus [25], plant [26], and cell-free expression systems [27]. There are many advantages for VLP based vaccines over inactivated virus or other subunit vaccines, such as better immunogenicity, easier to modify, and increased safety [14]. If a mutant virus were to rapidly emerge and cause an outbreak, sequencing the mutant capsid genes to produce VLPs might be a faster way to develop a vaccine. Among *Anseriform dependoparvovirus 1* species, GPV [16,18,28] and MDPV [29,30] VLPs have been successfully made. But the formation and immunological characteristics of the mutant virus SBDSV VLPs has yet to be studied.

In this study, SBDSV VLPs were produced by cloning VP2 into a baculovirus expression system. The 65 Kd VP2 protein was successfully expressed in the Sf9 cells, as determined by IFA, SDS-PAGE, and Western blotting. Furthermore, the GPV mAb E16, which can distinguish GPV associated antigen from MDPV, was used to identify the SBDSV VP2 protein [2,31]. Transmission electron microscopy observation clearly visualized baculovirus vector virions and SBDSV VLPs, both exhibiting the correct size and morphology accumulating within the nuclei of the cells.

Ducks (130-day-old) and chicks (10-day-old) immunized with an oil adjuvanted SBDSV VLP vaccine induced strong neutralizing antibodies against SBDSV. After a single dose immunization, peak neutralizing antibody titers were observed at 4 wpi for ducks, and 5 wpi for chicks (Fig. 4). The peak antibody titer in ducks came earlier than in chicks, with LAI and neutralization titers was 4.9 ± 1.20, 4.4 ± 1.07, 7.1 ± 1.20 and 6.2 ± 1.03 (log2) respectively. A previous study showed that 4-day-old geese immunized with an oil-adjuvanted GPV VLP vaccine developed peak neutralizing antibody titers at 6 wpi [16,18]. The difference in time for ducks and chicks to reach peak antibody titers could be the result of an immature immune system of 10-day-old chicks, or species specific differences. Considering the susceptible age of ducks to SBDSV was under 21 days, the rate at which ducklings immunized with our SBDSV VLP vaccine was not mounted quickly enough to defend against SBDSV. Therefore, it is likely to be more appropriately utilized in breeding ducks.

The kinetics of antibody titer development followed similar patterns between ducks and chickens (Fig. 4). As such, it is possible to use chicks for vaccine and immunologic studies in the context of GPV and extrapolate the data, as it can be difficult to source ducklings seronegative for waterfowl parvoviruses. Moreover, antibody titers measured by LAI were positively correlated with that of the microneutralization assay. This correlation has been repeatedly corroborated with abundant unpublished data from the author's laboratory.

The yolk antibody titers in Cherry Valley ducks receiving 2 vaccine doses was examined by LAI, which ranged from 4 -8 log2 with an average 5.3 ± 1.09 (log2). For SBDSV, the observed yolk antibody titers are indirectly reflective of the maternal antibody titers. The maternal antibody can protect ducklings from SBDSV at 8-day-old with the initial titer above 2 (log2) [17]. It can be inferred that breeding Cherry Valley ducks vaccinated with oil-adjuvanted SBDSV VLPs can protect its offspring from SBDSV infection. In conclusion, SBDSV VLPs show great promise as a vaccine platform for the prevention of SBDS.

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Figure legends

Figure 1. IFA detection of VP2 protein in Sf9 cells infected. Sf9 cells were infected with SBDSV-rBV and Gus-rBV at an MOI of 5 for 3 days. (A-C) mock infected cells; (D-F) Gus-rBV control; (G-I) SBDSV-rBV infected. Sf9 cells infected with rBVs

showed an increased cytoplasmic and nuclear size (E, H) compared to normal cells (B). GPV antigen was detected in SBDSV-rBV infected cells (G, I) while the control (D, F) and mock infected groups (A, C) did not exhibit any fluorescent labeling. Magnification, 200×.

Figure 2. SDS-PAGE and Western blot analysis of VP2 protein expression in Sf9 cells. (A) The whole-cell protein of SBDSV-rBV infected Sf9 cells resolved on an SDS-PAGE. Lane M, protein marker; lane 1, Sf9 whole-cell protein; lane 2, whole-cell protein from SBDSV-rBV infected Sf9 cells. (B) Western blot detection of VP2. Lane 1, Sf9 whole-cell protein; lane 2, the whole-cell protein of SBDSV-rBV infected Sf9 cells; lane 3, ultracentrifugation pellets of SBDSV-rBV infected Sf9 cells; lane 4, ultracentrifugation pelleted SBDSV M15 strain.

Figure 3. Transmission electron microscopy of SBDSV VLPs. Ultrathin sections of SBDSV-rBV infected Sf9 cell pellets (A, B, C) and ultracentrifuged SBDSV VLPs (D). SBDSV VLPs and baculoviruses are indicated by black and red arrows, respectively. SBDSV VLPs are 20-22 nm diameter icosahedrons. Baculoviruses are about 50×300 nm and cylindrical. (B) Higher magnification view of the red box in (A). The magnification is 2,500× for A, 7,000× for B and C, 20,000× for D.

Figure 4. Anti-SBDSV VLP antibody titers in ducks and chickens. 130-day-old Cherry Valley ducks and 10-day-old Hyline brown chicks were immunized with oil-adjuvanted SBDSV VLPs, blood samples were collected for latex agglutination inhibition assay and microneutralization assay.

Table 1. Yolk antibody titers of eggs from ducks receiving 2 doses of SBDSV VLP vaccine