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Goose parvovirus structural proteins expressed by recombinant baculoviruses self-assemble into virus-like particles with strong immunogenicity in goose

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ABSTRACT

Goose parvovirus (GPV), a small non-enveloped ssDNA virus, can cause Derzsy's disease, and three capsid proteins of VP1, VP2, and VP3 are encoded by an overlapping nucleotide sequence. However, little is known on whether recombinant viral proteins (VPs) could spontaneously assemble into virus-like particles (VLPs) in insect cells and whether these VLPs could retain their immunoreactivity and immunogenicity in susceptible geese. To address these issues, genes for these GPV VPs were amplified by PCR, and the recombinant VPs proteins were expressed in insect cells using a baculovirus expression system for the characterization of their structures, immunoreactivity, and immunogenicity. The rVP1, rVP2, and rVP3 expressed in Sf9 cells were detected by anti-GPV sera, anti-VP3 sera, and anti-His antibodies, respectively. Electron microscopy revealed that these rVPs spontaneously assembled into VLPs in insect cells, similar to that of the purified wild-type GPV virions. In addition, vaccination with individual types of VLPs, particularly with the rVP2-VLPs, induced higher titers of antibodies and neutralized different strains of GPVs in primary goose and duck embryo fibroblast cells *in vitro*. These data indicated that these VLPs retained immunoreactivity and had strong immunogenicity in susceptible geese. Therefore, our findings may provide a framework for development of new vaccines for the prevention of Derzsy's disease and vehicles for the delivery of drugs.

1. Introduction

Goose parvovirus (GPV) infection can cause a highly contagious and lethal Derzsy's disease in goslings and muscovy ducklings. Derzsy's disease is widespread in all major goose farming countries of Europe and Asia [1,2], leading to a huge economic loss. GPV is a small icosahedral virus with a diameter of 20–22 nm and a self-replicating parvovirus of the genus Dependovirus of the Parvoviridae family [3]. The GPV genome is comprised of two open reading frames, encoding non-structural and structural proteins, including three capsid proteins of VP1, VP2, and VP3. These three proteins are generated from the same mRNA by alternative splicing for VP1 and VP2 or post-translational cleavage for the VP3, respectively. The wild-type of GPV predominately contains VP3 and varying amounts of VP1 and VP2 [4,5].

Baculovirus expression system (BES) has demonstrated to be a valuable tool for producing a variety of complex eukaryotic proteins. This system has been used for investigating capsid formation of many viruses [6], including rotavirus [7], piconavirus [8], orbivirus [9], calicivirus [10], papillomavirus [11], herpesvirus [12], and parvoviruses. The structural proteins of many parvoviruses can self-assemble into virus-like particles (VLPs), including adeno-associated virus [13], Aleutian mink disease parvovirus [14], human parvovirus B19 [15,16], porcine parvovirus [17], canine parvovirus [18], AAV Type 2 [19], and Muscovy duck parvovirus [20]. The generated VLPs usually have characteristics, similar to natural viral particles, in size, shape, cellular uptake, and intracellular trafficking, but lack infectivity [17–20]. Therefore, VLPs may be used as subunit vaccines, and diagnostic and therapeutic reagents. It is well known that GPV-based vaccines can induce potent immune protection against GPV-induced Derzsy's disease. Currently, the available GPV vaccines are produced by growing the native virus in goose or duck embryos, which is expensive and laborious, and has a potential risk for spreading GPV in farms. Therefore, the development of new strategies for cheaply producing effective vaccines will be of great significance. However, there is no information on whether different recombinant VPs of GPV generated in the BES could self-assemble into VLPs and what the stabilities and immunogenicities of these recombinant VLPs are.

In present study, we found that recombinant VP proteins were able to self-assemble into VLPs with characteristics similar to that of GPV virions. Immunization with individual types of VLPs

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induced high titers of antibodies, which effectively neutralized wild-type of GPV *in vitro*. Apparently, the GPV-VLPs may be used as subunit vaccines and diagnostic reagents for the prevention and diagnosis of GPV infection.

2. Materials and methods

2.1. Cloning and construction of recombinant baculoviruses

The sequence encoding VP1, VP2, and VP3 of GPV were amplified by PCR using the complete genomic sequence of GPV as template and the specific primers were designed (Table 1). The PCR products were digested with *Bam*HI and *Xho*I, and cloned into the corresponding restriction sites of plasmid pFastBacHTB with a His-tag at the N-terminus (Invitrogen, Germany). The cloned different VP regions were transferred into the bacmid using the Bac-to-Bac Baculovirus expression system, according to the manufacturers' instruction (Invitrogen).

2.2. Cells culture and expression of recombinant proteins

Spodoptera frugiperda (Sf9, Invitrogen) insect cells were maintained in Grace's insect cell culture medium supplemented with 10% fetal calf serum (FCS, Gibco, USA) in 25 cm^2 plastic flakes at 27 °C. The cells were transfected with the resulting recombinant bacmids to generate recombinant baculoviruses using Cellfectin® Reagent (Invitrogen). The recombinant baculoviruses were propagated and amplified according to standard procedures.

2.3. SDS–PAGE and Western blot analysis

Samples were boiled for 5 min in reducing Laemmli sample buffer [21]. Prepared samples were separated on the 10% slab gels and stained with Coomassie blue or transferred to polyvinylidene fluoride (PVDF) membrane for Western blot analysis. Probing of proteins was conducted with anti-His monoclonal antibody (1:10,000, Sigma, USA), GPV immune sera generated (1:2000), or rabbit anti-VP3 hyperimmune sera (1:2000, maintained in our lab). The bound antibodies were detected with horseradish peroxidase (HRP)-conjugated goat anti-mouse (1:10,000), HRP-rabbit anti-goose (1:10,000), or HRP-goat anti-rabbit (1:10,000, Sigma, USA). The immune complex was visualized with EasySee Western Blot Kit (Transgen, China), according to the manufacturers' instruction.

2.4. Immunofluorescence analysis

Sf9 cells were infected in triplicate with 1 MOI of individual types of recombinant baculoviruses in 12-well tissue-culture plates for 72 h. The cells were washed with PBS (pH 7.4) and fixed with 4% paraformaldehyde (PFA–PBS, 20 min, RT). Probing of proteins was conducted with anti-His monoclonal antibody (1:10,000, Sigma, USA) or rabbit anti-VP3 hyperimmune sera (1:2000). Detection of antibody was performed using FITC-goat anti-mouse (1:10,000, Sigma) or FITC-goat anti-rabbit (1:10,000, Sigma), and examined under a fluorescence microscope (TE2000U, Nikon, Japan).

Table 1
The sequences of primers.

Genes	The sequences	The site
VP1	Forward: 5′-GGCGCGGATCCATGTCTACTTTTTTAGAT-3′	*Bam*HI
	Reverse: 5′-GGGGCTCGAGTTACAGATTTTGAGTTAG-3′,	*Xho*I
VP2	Forward: 5′-CTGGATCCATGGCACCTGCAAA-3′[a]	*Bam*HI
	Reverse: 5′-GGGGCTCGAGTTACAGATTTTGAGTTAG-3′	*Xho*I
VP3	Forward: 5′-GGGGATCCATGGCAGAGGGAG-3′	*Bam*HI
	Reverse: 5′-GGGGCTCGAGTTACAGATTTTGAGTTAG-3′,	*Xho*I

[a] The original code of ACG was changed to ATG for efficient initiation of translation.

2.5. Production and purification of VLPs

The cells infected with recombinant baculoviruses were washed with cold PBS and re-suspended in buffer (1% deoxycholate, 10 mM Tris–HC1 pH 8.0) supplemented with protease inhibitors on ice for 30 min, followed by centrifugation at 12,000*g* for 10 min at 4 °C, loaded on sucrose gradients (10–40%) and ultracentrifuged at 30,000*g* for 2 h at 4 °C. The pellets were re-suspended in PBS, and protein concentrations of the VLP preparations were determined using a Bradford assay kit.

2.6. Electron microscopy

Specimens for electron microscopy (EM) were prepared from the samples obtained by sucrose gradient purification, as described previously with minor modifications [22]. Briefly, the tested virus and VLP samples were mounted on a carbon-coated paper grid at RT for 10 min, and carefully drained with a filter paper. After being negatively stained with 2% phosphotungstic acid (pH 7.4) for 1 min, the specimens were examined under a transmission electron microscope (TEM) (H-7650, HITACHI). For immunoelectron microscopy (IEM), samples were first incubated with 1:50 diluted of anti-GPV sera or 50 μg/mL of anti-His monoclonal antibody at 37 °C for 1 h, and then centrifuged at 12,000*g* for 30 min at 4 °C. The precipitated immune complexes were mounted and stained as described above, followed by examination under a TEM.

2.7. Stability and composition of the VLPs

To determine the stability, individual types of VLPs were incubated with 40 mM EDTA or 40 mM DTT at 4 °C for 30 min, respectively, and then examined under a TEM as described above. To evaluate the composition of the VLPs, individual types of VLPs were treated with, or without, 10% β-mercaptoethanol (β-ME) and subjected to SDS–PAGE analysis under reducing or non-reducing conditions.

2.8. Animals and immunization

Geese at four days and Goose/Duck embryo at 10 days were from the Datong Farm and Harbin Veterinary Research Institute, respectively, and housed in a specific pathogen-free facility at the Experimental Animal Center of NEAU with free access to water and food *ad libitum*. The geese were randomized into six groups (n = 15 per group), and individual geese were vaccinated subcutaneously with 20 μg of individual types of VLPs in 50% mineral oil (Sigma), similar protein amount of 200 ELD_{50}/0.2 ml inactivated GPV vaccine (Binzhou Huahong Biological Products, Shandong, China) in 50% mineral oil, and 105 ELD_{50}/0.1 ml attenuated Gosling Plague vaccine (SYG41-50 Strain, Yang Zhou Vacbio Bio-Engineering, China), respectively. An additional group of geese was injected with the same amount of mineral oil and used as negative controls. Their blood samples were obtained from their leg veins weekly up to 8 weeks post immunization, and their sera were prepared and stored at −20 °C.

2.9. Neutralizing activity of antibodies against native GPV

The neutralizing activities of individual goose serum samples against native GPV were evaluated by virus neutralization assay (VN) *in vitro*. GPV H (kindly provided by the First Bio-product Manufactory of Heilongjiang Province, China), GPV 98E, and duck

embryo-adapted 98E strains (both have been maintained in our laboratory) were used and the primary goose/duck embryo fibroblast cells (1.2×10^6 cells/well) were infected in triplicate with 100 $TCID_{50}$ of individual types of viruses as positive controls, with the mixture of 100 $TCID_{50}$ ($TCID_{50}$ values were $10^{5.26}$/0.1 ml for GPV H, $10^{3.62}$/0.1 ml for GPV 98E strain, and $10^{5.68}$/0.1 ml for 98E Duck embryo-adapted strain) of individual types of virus (100 μl) and serially diluted of serum samples that had been cultured at 37 °C for 1 h, or with diluted sera alone as negative controls, respectively. The cells were cultured at 37 °C, 5% CO_2 for 144 h.

2.10. Statistical analyses

Data are expressed as mean ± SD. The difference among groups was analyzed by ANOVA and Student's *t*-test using SPSS version 13 software. A p value of <0.05 was considered statistically significant.

3. Results

3.1. Expression and characterization of individual GPV capsid proteins in insect cells

Sf9 cells were infected with recombinant baculoviruses (AcVP1, AcVP2, and AcVP3), respectively, and expression of VP1, VP2, and VP3 in Sf9 cells was characterized by SDS–PAGE (Fig. 1A). Sf9 cells infected with AcVP1, AcVP2, or AcVP3 for 72 h exhibited a dark band with molecular weight of 90, 75, or 65 kDa, which were similar to native 87, 70, and 60 kDa of VP1, VP2, and VP3 [20]. Further analysis by Western blot and Immunofluorescence assays using anti-His, anti-GPV or anti-VP3 antibodies revealed infection with recombinant baculovirus induced high levels of rVP1, rVP2, and rVP3 expression in Sf9 cells. (Fig. 1B–D).

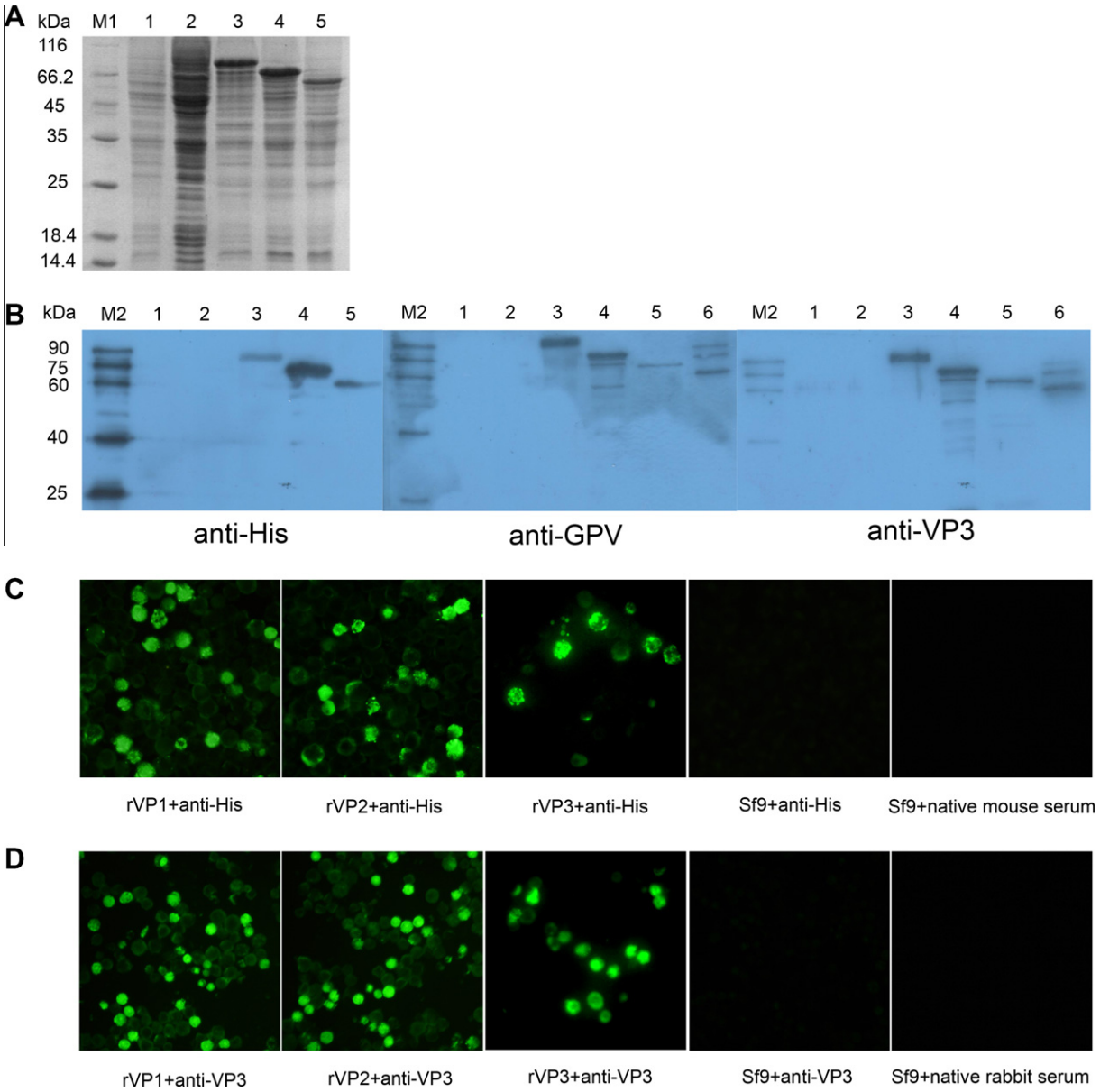

Fig. 1. Characterization analysis of recombinant rVP proteins. Identification of the recombinant proteins from cell lysates, recombinant proteins were characterized by SDS–PAGE (A), Western blot (B), and immunofluorescent assays (C and D). Lane M: protein molecular weight markers; Lane 1: Sf9 cells infected by wild-type baculovirus; Lane 2: Sf9 cells; Lane 3: Sf9 cells infected with AcVP1 recombinant baculovirus; Lane 4: Sf9 cells infected with AcVP2 recombinant baculovirus; Lane 5: Sf9 cells infected with AcVP3 recombinant baculovirus; Lane 6: The native GPV viruses.

3.2. The rVPs self-assemble VLPs

To study assembly of recombinant proteins, Sf9 cells infected with the recombinant baculoviruses AcVP1, AcVP2, or AcVP3 were collected at 72 h, solubilized, and exposed to sucrose gradient centrifugation, respectively. The potential virion particles were collected in PBS and subjected to electron microscopy analysis. Following infection with GPV, the typical shape of parvovirus particles with a diameter of approximately 22 nm was displayed (Fig. 2A). Immunoelectron microscopy analysis revealed that these VLPs were recognized by anti-GPV and anti-His antibodies (Fig. 2B and C), suggesting that these VLPs retained GPV immune epitopes and that the His-tag at the N-terminus likely existed on the surface of VLPs. These data indicated that the expressing rVP1, rVP2, and rVP3 had spontaneously assembled into VLPs in Sf9 cells.

3.3. Stability and composition of GPV-VLPs

To test stability of GPV-VLPs, individual types of VLPs were treated with EDTA or DTT, respectively, and then assessed by TEM. Treatment with 40 mM EDTA did not affect the structure of VLPs because the shape and size of VLPs were similar to that of untreated VLPs, suggesting that there was no metal irons in these VLPs (data not shown). However, treatment with 40 mM DTT that can destroy the disulfide bonds resulted in the disappearance of VLPs, suggesting that disulfide bonds are required to maintain the structure of these VLPs (data not shown). To elucidate the composition of VLPs, individual types of VLPs were characterized by SDS–PAGE under reducing and non-reducing conditions. The purified VP1-VLPs displayed a single protein band with a molecular weight of approximately 95 kDa, similar to that of recombinant VP1 from *Escherchia coli* under a reducing condition (Fig. 3).

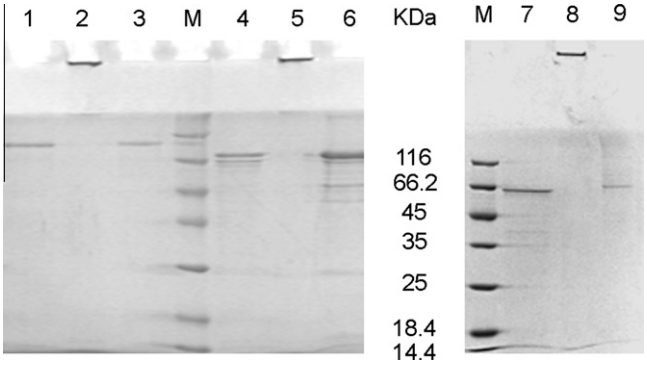

Fig. 3. SDS–PAGE analysis of GPV-VLPs under reducing and non-reducing conditions. The purified rVP-VLPs were characterized by SDS–PAGE under reducing and non-reducing conditions. Lane M: Protein molecular weight markers; Lane 1, 4, and 7: the purified VP1-VLPs, VP2-VLPs, and VP3-VLPs dissolved under a reducing condition; Lane 2, 5, and 8: the purified VP1-VLPs, VP2-VLPs, and VP3-VLPs dissolved under a non-reducing condition; Lane 3, 6, and 9: The purified rVP1, rVP2, and rVP3 proteins from *E.coli* dissolved under a non-reducing condition.

However the purified VP1-VLPs exhibited a protein band with a huge molecular weight under a non-reducing condition. Similar patterns of VP2-VLPs and VP3-VLPs were observed under reducing and non-reducing conditions.

3.4. Immunization with single type of VLP induces virus-neutralizing antibodies in geese

Neutralizing antibodies are critical for the control of GPV infection. The titers of virus neutralizing antibodies were tested by

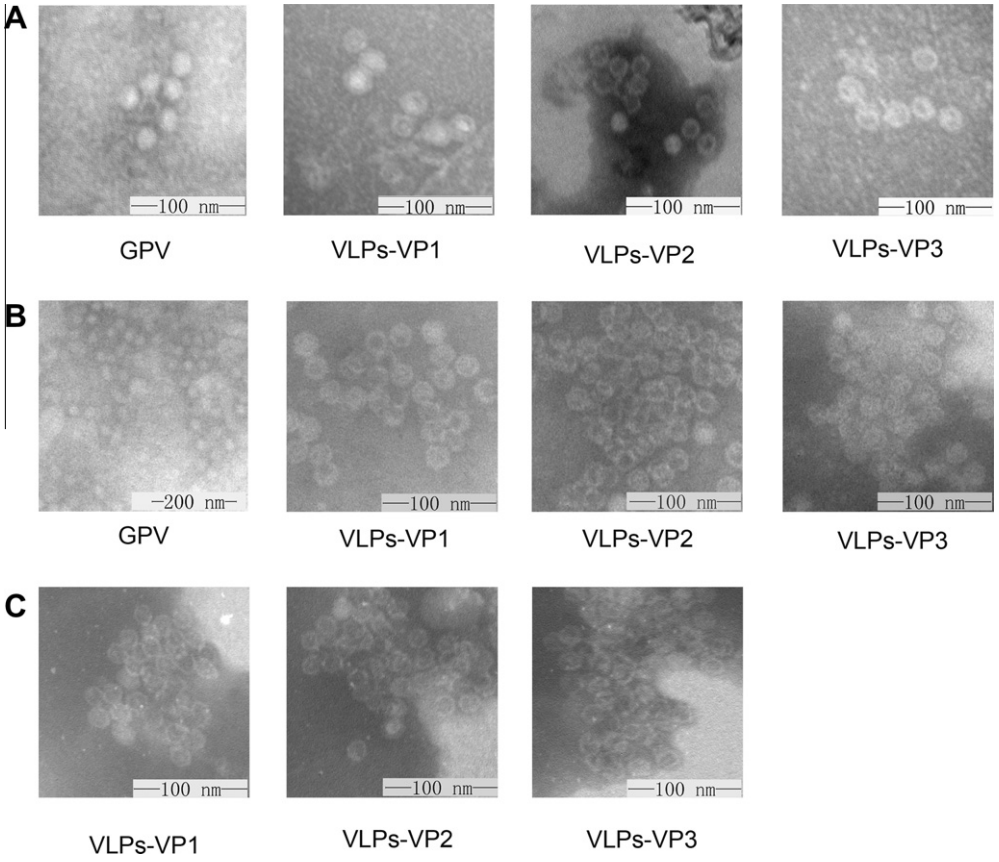

Fig. 2. Electron micrographs of GPV-VLPs. Identification of VLPs from sucrose gradient centrifuging, the VLPs, together with the purified GPV virions, were examined under a TEM (A). The purified VLPs and native GPV virions were reacted with anti-GPV sera (B) or anti-His monoclonal antibody (C), respectively, and examined under a TEM.

in vitro virus neutralization assay (VN). The primary goose and duck embryo fibroblast cells were infected with, or without, 100 $TCID_{50}$ of GPV H, 98E, or duck embryo-adapted 98E or a mixture of 100 $TCID_{50}$ of each type of virus and different diluted of individual sera ($1:2^1$–$1:2^{16}$), respectively for 144 h. The titers of neutralizing antibodies in individual sera against each strain of GPV were analyzed quantitatively in Fig. 4. These data indicated that rVP-VLP-induced antibodies had strong GPV-neutralizing activity in our experimental system.

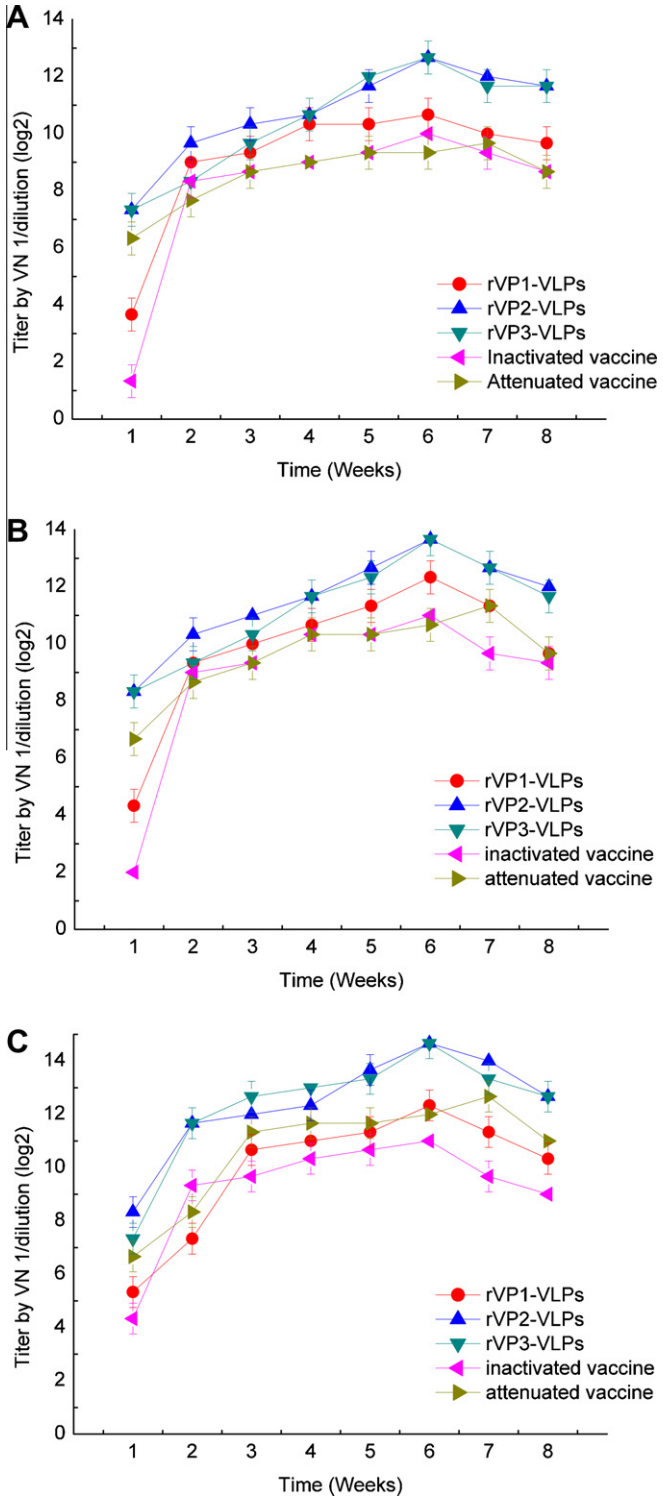

Fig. 4. The titers of neutralizing antibodies against different strains of GPV. The titers of neutralizing antibodies against GPV H (A), GPV 98E (B), and the duck embryo-adapted GPV 98E (C) were determined, according the cell path effect and GPV antigen detection by immunofluorescent assays. Data are expressed as mean ± SD of each groups of sera ($n = 15$ per group) at indicated time points from three separate experiments. There was no cell path effect observed in the negative control cells, while majority of the positive cells displayed cell path (data not shown).

4. Discussion

GPV is a virulent pathogen causing goose plaque, a devastating disease in goslings with a high rate of mortality. A previous study expressed the rVP1 in insect cells, but did not report the VLP formation [23]. However, whether the VP2 and VP3 could form VLPs has not been tested. In this study, these indicate that recombinant proteins have immunoreactivity with the specific antibodies. The rVP1, rVP2, and rVP3 spontaneously formed VLPs with a size and appearance similar to that of wild-type of GPV virions, individual VPs were able to self-assemble into GPV-like empty capsids. These data extended previous findings regarding the formation of VLPs of AAV2 [19] and some autonomous parvoviruses [16,24–27]. The spontaneous formation of VLPs by rVP3 was different from other autonomous parvoviruses [19,28]. Given that the VP3 is a major component of GPV capsids and has high immunogenicity, it is possible that the VP3 is a scaffolding protein for the formation and stability of GPV virions.

Immunoelectron microscopy analysis indicated that individual VLPs were recognized by anti-His. These data suggest thatHis-tag at the N-terminus of the rVPs does not hinder the self-assembling process and that the His-tag at the N-terminus of fusion protein may be located on the outer surface of the VLPs. This phenomenon is consistent with some results of previous studies that an enhanced green fluorescent protein (EGFP) fused to the N-terminus of the human parvovirus B19 VLPs [29] and adeno-associated virus type 2 VLPs [30], canine parvovirus (CPV) VLPs [29], and porcine parvovirus (PPV) VLPs are displayed on the surface of VLPs.

A previous study has shown that the assembly of HPV-16 L1 protein into VLPs requires the formation of disulfide bonds [31]. We found that treatment with DTT resulted in the disappearance of VLPs, as examined under a TEM, suggesting that the formation of disulfide bonds is critical for the maintenance of VLP structure. Furthermore, calcium ions are required in the assembling process of capsids of different viruses, including simian virus 40 [32] and mouse polymavirus [15,33,34]. We found that treatment with EDTA did not change the size and shape of VLPs, indicating that the formation and stability of VLPs are metal ion-independent. In addition, analysis of VLP components revealed that individual VLPs were assembled by the single type of multimeric rVP because a single protein band was resolved for individual types of VLPs by SDS–PAGE under a reducing condition. Moreover, individual types of VLPs displayed mobilization behaviors, similar to that of the purified corresponding rVP under a reducing condition, but presented huge proteins under a non-reducing condition. Therefore, our data indicated that individual VLPs were assembled by single types of multimeric rVP and that their structure stability is dependent on the sulfide bonds. We are interested in further investigating the process of different VLPs.

Recombinant subunit vaccines are safe and effective in inducing immune responses. There are numerous types of rVLPs generated from at least 30 different viruses that infect humans and animals [6]. VLPs, particularly for those from DNA viruses, usually have high immunogenicity because they contain important antigen epitopes and determinants, which are presented to immune cells in a native conformation. Vaccination with rVLPs has been demonstrated to protect from virus infection and to reduce the virulence of virus infection [17,35]. The small VLPs of the surface antigen of hepatitis B virus have been used as a vaccine in human [31,36]. Furthermore, the rVLPs assembled from recombinant capsid

proteins of the human papilloma virus have been used as a vaccine against cervical cancer [37–39], and other rVLPs from influenza [40,41], Rotavirus [32,42], and Filovirus infections [35,43] have been tested at clinic. We tested the immunogenicity of individual types of VLPs and found that immunization of geese with individual types of VLPs induced humoral responses. The rVLPs-stimulated antibodies effectively neutralized infection of wild-type strains of GPV H, 98E, and the embryo duck-adapted 98E *in vitro*, and neutralizing activities of the antibodies, particularly for antibodies from the rVP2-VLPs and rVP3-VLPs-immunized geese, were significantly higher than that of antibodies from the inactivated and attenuated GPV vaccines. Given that high titers of neutralizing antibodies have life-long protection against GPV-related diseases, vaccination with the rVP-VLPs may induce strong immunity against GPV infection in susceptible animals.

Currently, the rVLPs, as antigens, have more advantages in the detection of antibodies because they usually retain conformation-dependent epitopes and can be produced in large quantities. In addition, the generated rVP-VLPs may be used as diagnostic reagents for the detection of GPV-specific antibodies and as vehicles for the development of vaccines and therapeutic reagents for other diseases.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.04.129.

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