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Immunogenicity of the envelope GP3 protein of porcine reproductive and respiratory syndrome virus displayed on baculovirus

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Summary. – Porcine reproductive and respiratory syndrome virus (PRRSV) has been recognized as one of the most important pathogens of pigs throughout the world. The minor envelope protein GP3 of PRRSV plays an important role in clearing of the virus infection and protecting the animals. In this study, a recombinant baculovirus (BacSC-GP3) expressing His6-tagged GP3 with the transmembrane (TM) and cytoplasmic (CT) domains of envelope protein gp64 was constructed and its immunogenicity was evaluated in mouse and piglet models. The His6-tagged GP3 was successfully displayed on the surface of virions as well as virus-infected Sf-9 cells. The animals immunized with BacSC-GP3 gave a slightly higher (piglets) up to a markedly higher (mice) humoral and lymphocyte proliferation responses than those that received a commercial killed vaccine. This is the first study on the immunogenicity of recombinant GP3-baculovirus, which indicates that the latter can represent an alternative strategy for developing a more effective PRRSV vaccine.

Keywords: baculovirus; GP3 protein; porcine reproductive and respiratory syndrome virus

Introduction

Porcine reproductive and respiratory syndrome is characterized by a severe reproductive failure in sows and respiratory disease in pigs of all ages and is considered one of the most economically important diseases affecting the swine industry worldwide (Meulenberg, 2000). PRRSV, the causative agent, is an enveloped, positive-stranded RNA virus belonging to the family *Arteriviridae* (Dea *et al.*, 2000). The viral genome is approximately 15 kbp in length and contains 9 ORFs (ORF1a/1b, ORF2a/2b, ORF3-7). ORF1a and ORF1b are located at the 5′ end of the genome and code for the proteins with replicase and polymerase activities, whereas ORF2-ORF7 are located at the 3′ end of the genome and code for the major (GP5, M, and N) and minor structural proteins (GP2, GP3, and GP4) (Meulenberg *et al.*, 1995; Dea *et al.*, 2000). Several experimental vaccines expressing GP5 and M, respectively, or simultaneously, have been developed recently and tested against PRRSV (Qiu *et al.*, 2005; Jiang *et al.*, 2006b; Zheng *et al.*, 2007). However, only a few studies have been reported on the role of GP3 being a part of vaccine. The GP3 protein encoded by ORF3 is one of the important minor envelope glycoproteins of PRRSV. It is comprised of 254 aa and is highly glycosylated potentially by seven N-linked oligosaccharides (Gonin *et al.*, 1998). The first 57 aa of the protein constitutes N-terminal signal sequence (SS) and hydrophobic domain according to the protein structure analysis. Some reports indicated that the GP3 was highly antigenic (Katz *et al.*, 1995) and could provide a protection of piglets against PRRSV infection (Plana Duran *et al.*, 1997). However, the immunogenicity of GP3 was not fully characterized in their study.

Baculovirus naturally infects the insect cells and the virus entry into cell is mediated by an envelope glycoprotein gp64,

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Abbreviations: CT = cytoplasmic; MCS = multiple cloning sites; PBMCs = peripheral blood mononuclear cells; PRRSV = porcine reproductive and respiratory syndrome virus; SI = stimulation index; SS = signal sequence; TM = transmembrane

which comprises N-terminal SS and a mature part that includes TM and CT domains (Monsma *et al.*, 1996; Kitagawa *et al.*, 2005). The significance of gp64 in virus budding has been exploited for the surface display of exogenous peptides by inserting a heterologous gene between SS and mature domain of gp64 (Yang *et al.*, 2007). The fusion protein is transported to the plasma membrane and incorporated into baculoviral envelope. This method has been extended to develop various recombinant baculoviruses as a potential vaccine delivery platform used for expressing avian influenza virus hemagglutinin protein (Yang *et al.*, 2007), classic swine fever virus E2 and NS3 proteins (Xu and Liu, 2008; Xu *et al.*, 2009), and avian reovirus σC and σB proteins (Lin *et al.*, 2008).

The aim of this study was to construct a recombinant baculovirus displaying on its surface the envelope GP3 protein of PRRSV and evaluate its immunogenicity in mouse and piglet models. The results showed that (i) GP3 was displayed on the surface of recombinant virions as well as virus-infected cells, and (ii) the animals immunized with the recombinant baculovirus induced satisfactory cellular and humoral immune responses.

Materials and Methods

Virus and cells. PRRSV Shaanxi strain, a highly pathogenic North American type isolate was used in this study (Wang *et al.*, 2010). PRRSV inactivated vaccine NVDC-JXA1 strain was purchased from Qilu Animal Health Products, China. PRRSV was propagated and titrated in MARC-145 cells. Recombinant baculoviruses were propagated and titrated in Sf-9 cells. MARC-145 cells were grown and maintained in DMEM (Invitrogen) supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C with 5% CO_2. Insect Sf-9 cells were propagated in Grace′s insect supplemented media (Invitrogen) at 27°C.

Recombinant baculovirus construction and preparation. The baculovirus surface display system pBacSC plasmid was described previously (Lin *et al.*, 2008). To generate recombinant baculovirus BacSC-GP3, the DNA fragment with deleted ORF3 N-terminal SS and hydrophobic region of 57 aa was amplified from the plasmid pGEM-GP3 (containing the complete cDNA of ORF3 gene of the PRRSV Shaanxi strain) by PCR with forward primer (PRRSV-*Xho*I), 5′-GCTCTCGAGCCAACCCGG-3′, and reverse primer (PRRSV-*Pst*I), 5′-GATCTGCAGTCGCCGTGC-3′. The amplified product was inserted into the corresponding sites in pBacSC plasmid resulting in the recombinant transfer plasmid pBacSC-GP3 (Fig. 1). The recombinant baculovirus was subsequently generated by using the Bac-to-Bac® system (Invitrogen) following the manufacturer's instructions. Recombinant baculovirus was further amplified by propagation in Sf-9 cells. Virus purification was performed as described (Abe *et al.*, 2003). The virus titer was determined by a plaque assay according to the manufacturer's manual (Invitrogen).

SDS-PAGE and Western blot analysis. The infected cell lysates were subjected to 12% SDS-PAGE and blotted to PVDF membranes (Amersham). Two primary antibodies were used to detect GP3 in the blots – mouse monoclonal His-tag antibody (1:3,000 dilution, Invitrogen) and porcine polyclonal PRRSV antibody (1:1,000 dilution, Center of Animal Health and Epidemiology, China). The secondary antibodies were goat anti-mouse and rabbit anti-porcine IgG conjugated to HRP (1:3,000 dilution, Invitrogen). The protein bands were visualized by the ECL chemiluminescence kit (Pierce). The purified virus particles were also detected by SDS-PAGE and Western blot analysis following the same protocol.

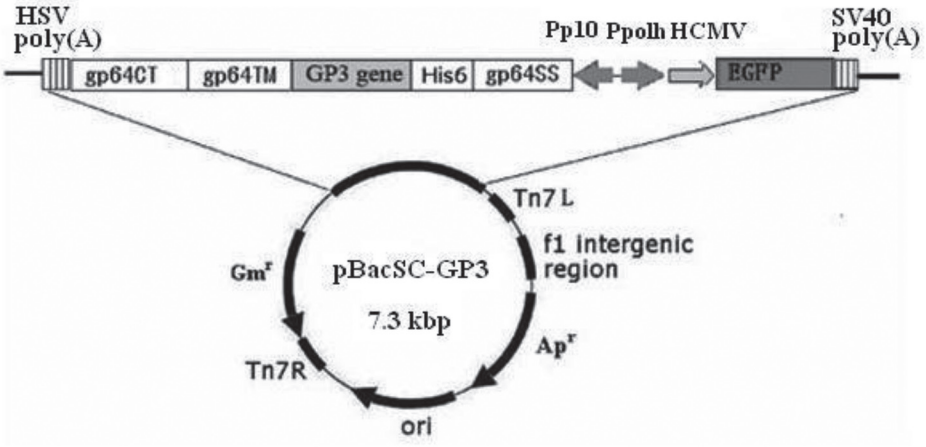


Fig. 1

Structure of recombinant baculovirus pBacSC-GP3

HSV – herpes simplex virus; HCMV – human cytomegalovirus; SV40 – Simian virus 40; Pp10 – p10 promoter; Ppolh – polyhedron promoter.

Confocal immunofluorescence microscopy. The confocal microscopy was carried out as described previously (Yang *et al.*, 2007). The cells were incubated with the porcine polyclonal PRRSV antibody (1:300 dilution) following by FITC-conjugated rabbit anti-porcine IgG (1:100 dilution, Invitrogen). The negative control cells were treated in the same way. Protein localization was visualized using a confocal microscope LSM 510 META, Zeiss.

Electron microscopy. The immunogold electron microscopy was performed as described (Hu *et al.*, 2003). The grids were incubated with the porcine polyclonal PRRSV antibody (1:300 dilution) following by rabbit anti-porcine IgG conjugated with 10-nm gold particles (1:50 dilution, Sigma). The grids were then examined under the transmission electron microscope H-7500, Hitachi.

Immunization. To evaluate whether the GP3 protein can elicit immune responses, four groups of animals were set for immunization by BacSC-GP3, PRRSV vaccine, BacSC, and PBS. There were five mice or five piglets in each group. Six-week-old female BALB/c mice (Animal Center, The Fourth Military Medical University, Xi′an, China) were inoculated intraperitoneally twice at 2-week internal with purified BacSC-GP3 (1.0×10^9 PFU), PRRSV vaccine (1.0×10^6 PFU), purified BacSC (1.0×10^9 PFU), and PBS, respectively. Four-week-old piglets (PRRS-free farm, Xi′an, China, seronegative for PRRSV) were vaccinated intramuscularly following the protocol of the mice. Serum samples for assay of antibodies were collected from the mouse retro-orbital plexus and swine *V. jugularis* external 6 weeks after the initial shot. At the same time, the lymphocyte proliferation assay was performed using the splenocytes of immunized mice or peripheral blood mononuclear cells (PBMCs) of piglets.

Assay of antibodies. GP3-specific antibody titers were determined using indirect ELISA with the purified PRRSV as coating antigen (Jiang *et al.*, 2006a). The results were expressed as the ratio of A_{490} produced by the serum samples compared with the negative control serum. Serum with a ratio value higher than 2.1 was considered as a positive serum. The titer was expressed as the reciprocal of the highest dilution of serum producing ratio value of 2.1 (Fan *et al.*, 2008). Serum neutralization assay was performed as described previously (Jiang *et al.*, 2006b). The neutralization titer was expressed as the reciprocal of highest serum dilution showing no CPE.

Lymphocyte proliferation assay. Mouse splenocytes or piglet PBMCs were isolated from the spleen of immunized mice or the whole blood of immunized piglets with lymphocyte separation medium (TBD). The lymphocyte proliferation assay was performed as described previously (Jiang *et al.*, 2006b). Proliferation response was calculated from the mean of triplicate wells and expressed as a stimulation index (SI) that was defined as the average A_{490} of PRRSV stimulated cells/average A_{490} of non-stimulated cells.

Statistical analysis. The Student′s test was used to evaluate a significance of differences and the value of $P \leq 0.05$ indicated the significance.

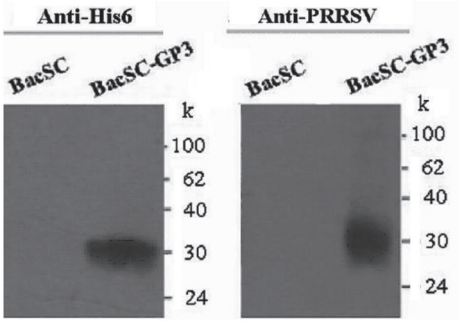


Fig. 2

Western blot analysis of GP3 expression in insect cells

Proteins were detected with His6 and PRRSV antibodies. Size markers on the right.

Results

GP3 expression from recombinant baculovirus in insect cells

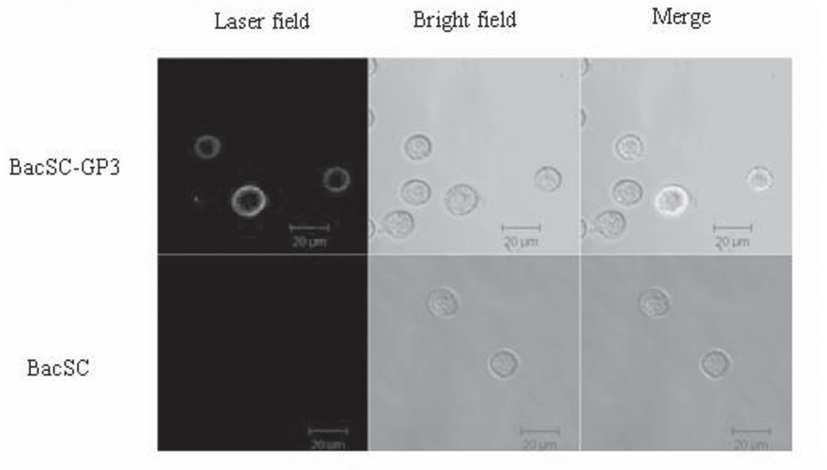
To find whether the His6-tagged GP3 was expressed in Sf-9 cells, the cells were infected separately by BacSC-GP3 or BacSC at MOI 10 and harvested 3 days later. The Western blot analysis showed that there was no protein detected by both His6-tag and PRRSV antibodies in the cells infected BacSC. In contrast, the cells infected BacSC-GP3 expressed approx. 30 K protein (Fig. 2).

To determine whether the His6-tagged GP3 was translocated properly to the cell surface, the cells were cultured on cover slips, infected separately with BacSC-GP3 or BacSC at MOI 10 and subjected to the immunofluorescence assay by confocal microscopy 2 days after the infection. The result showed that the GP3 protein could be detected by the PRRSV antibody in BacSC-GP3-infected cells, but not in BacSC-infected cells (Fig. 3). This protein was localized in the plasma membrane, thus demonstrating the anchoring of GP3 on the surface of Sf-9 cells.

Display of GP3 on recombinant baculovirus

To investigate whether the His6-tagged GP3 was incorporated into the baculovirus, the purified virus particles were analyzed by SDS-PAGE and Western blot. The result showed that there was no target protein expression in the negative control (BacSC). In contrast, the protein expressed by BacSC-GP3 could be detected by both His6-tag and PRRSV antibodies (Fig. 4).

To examine whether the GP3 protein was displayed successfully on the baculoviral envelope, BacSC-GP3 and BacSC recombinant viruses were purified by sucrose gradient ultracentrifugation and visualized by immunogold electron microscopy using porcine PRRSV primary antibody and



Confocal immunofluorescence microscopy of GP3 expression in insect cells

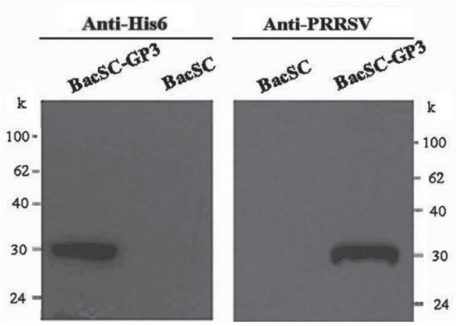


Fig. 4

Western blot analysis of purified recombinant baculovirus

Proteins were detected with His6 and PRRSV antibodies. Size markers on the left and right, respectively.

secondary anti-porcine IgG antibody conjugated with 10 nm gold particles. The results showed that gold particles were displayed on the surface of baculovirus BacSC-GP3, but not on the surface of baculovirus BacSC indicating the incorporation of His6-tagged GP3 and its display on the baculoviral envelope. Nevertheless, the incorporation of GP3 did not alter the virus morphology.

GP3-specific humoral immune response in mice

To explore whether the recombinant baculovirus BacSC-GP3 could serve as an immunogen *in vivo*, the BALB/c mice were immunized intraperitoneally with BacSC-GP3, PRRSV vaccine, BacSC or PBS, respectively. GP3-specific antibodies were examined by ELISA 6 weeks after the initial immunization. The average GP3-specific antibody titers reached up to 1:4,100 and 1:3,000 in mice immunized with BacSC-GP3 or PRRSV vaccine, respectively ($P < 0.05$), whereas no antibody against GP3 could be detected in the groups immunized with BacSC or PBS after the control immunization (Table 1). Obtained data indicated that the recombinant baculovirus BacSC-GP3 successfully elicited formation of the specific antibodies.

Serum samples were further examined for their ability to neutralize PRRSV *in vitro* by serum neutralization assay. Six weeks after the initial immunization shot, the mice inoculated with BacSC-GP3 developed neutralizing antibody with the mean titer of 1:12.8 that was significantly higher than the titer 1:8 found in mice immunized with PRRSV vaccine ($P < 0.05$), whereas no neutralizing antibody against PRRSV could be detected in the control mice immunized with BacSC or PBS throughout the experiment (Table 1). The results suggested that GP3 protein of PRRSV was related to the induction of neutralizing antibodies.

GP3-specific lymphocyte proliferation response in mice

To investigate whether the recombinant baculovirus BacSC-GP3 could also induce cell-mediated immune response, the lymphocyte proliferative response was analyzed 6 weeks after the primary immunization. The SI value was significantly higher ($P < 0.05$) in mice immunized with the BacSC-GP3 than in those immunized with PRRSV vaccine (Table 1). These results indicated that recombinant BacSC-GP3 could also induce an obvious cellular immune response.

Table 1. Humoral and lymphocyte proliferative responses of animals to recombinant baculovirus

	Mice			Piglets		
	Serum antibodies (ELISA)	Neutralizing antibodies	Lymphocyte proliferative response (SI)	Serum antibodies (ELISA)	Neutralizing antibodies	Lymphocyte proliferative response (SI)
BacSC-GP3	4100.0 ± 223.6[a]	12.80 ± 4.38[a]	1.75 ± 0.07[a]	32.0 ± 19.6[a]	7.60 ± 5.36[a]	1.59 ± 0.09[a]
Vaccine	3000.0 ± 612.4[b]	8.00 ± 4.90[b]	1.44 ± 0.07[b]	48.0 ± 22.6[a]	6.40 ± 2.19[a]	1.42 ± 0.12[b]
BacSC	0	0	1.15 ± 0.12[c]	0	0	1.12 ± 0.09[b]
PBS	0	0	1.02 ± 0.03[c]	0	0	1.08 ± 0.13[b]

Data represent the mean ± S.D. The same letters represent no significant difference. The different letters represent significant difference.

GP3-specific immune responses in piglets

Encouraged by the efficient humoral and cellular immune responses demonstrated in the mouse model, we further investigated the immunogenicity of BacSC-GP3 in pigs, the natural host. Four groups of piglets were vaccinated intramuscularly twice at 2-week intervals with BacSC-GP3, PRRSV vaccine, BacSC or PBS, respectively. Sera from immunized piglets were tested 6 weeks after the primary immunization for the presence of GP3-specific antibodies. Low levels of ELISA and neutralizing antibodies were observed in piglets immunized with BacSC-GP3 or PRRSV vaccine 6 weeks after the primary immunization (Table 1). Piglets vaccinated with BacSC-GP3 produced sera with ELISA antibody titers of 1:32 and neutralizing antibody titers of 1:7.6, while those immunized with PRRSV vaccine produced sera with ELISA antibody titers of 1:48 and neutralizing antibody titers of 1:6.4. These results showed that the difference between groups immunized with BacSC-GP3 or PRRSV vaccine was not statistically significant ($P > 0.05$). No GP3-specific ELISA or neutralizing antibodies could be detected in the control piglets immunized with BacSC and PBS throughout this experiment.

Six weeks after the primary immunization, PBMCs were isolated and restimulated *in vitro* with the purified PRRSV (20 μg/ml) to analyze the cellular immune response. Piglets immunized with BacSC-GP3 showed a significantly higher lymphocyte proliferative response compared to the piglets immunized with PRRSV vaccine ($P < 0.05$) (Table 1). Taken together, the recombinant baculovirus BacSC-GP3 can induce a weak humoral and cellular immune response in the natural host.

Discussion

In recent years, the baculovirus surface display system utilizing the gp64 SS, TM, and CT domains has been used increasingly as an alternative to the traditional expression systems. The gp64 SS facilitates the translocation of protein to the insect cell plasma membrane, thus exposing the His6 tag to the outer surface, while the gp64 TM enable the protein to anchor into the plasma membrane. CT domain from baculovirus gp64 mediates the interaction of plasma membrane with the budding nucleocapsid and incorporation into the virus envelope (Oomens *et al.*, 1999). Furthermore, the virus appeared as to be able to display the fusion protein in native conformation presumably with all the necessary posttranslational modifications in the insect cells. This virus can augment the immunoreactivity of displayed antigens, but can not influence the replication of recombinant baculovirus in insect cells. In addition, the expression of protein through the baculovirus display system allows a rapid generation of effective antigens without the need for purification of recombinant proteins and high titers (>10^{10} PFU/ml) can be easily reached. The recombinant baculovirus is non-cytotoxic and non-replicative in mammalian cells even at high MOI. There are no pre-existing antibodies against baculovirus in animals that might interfere with the administration of recombinant genes (Hu, 2005). Thus, the baculovirus surface display system should be a promising vaccine candidate vector over the other forms of viral vectors.

The effective vaccination of piglets against PRRSV is still an unanswered problem, because of the broad variability of PRRSV and its immunomodulatory effect. Two types of commercial vaccine currently on the market (live attenuated and killed vaccine) have been wildly used, but they cannot provide an effective protection against PRRSV infection (Meng, 2000; Mengeling *et al.*, 2003; Nilubol *et al.*, 2004). Furthermore, the vaccines containing live PRRSV have the potential to revert virus to its original virulence (Opriessnig *et al.*, 2002). Thus, the development of a safer vaccine with higher efficacy has become a major research focus. To date, researchers have developed some genetically engineered PRRSV vaccines by expressing the major immunogenic proteins GP5 and/or M, such as DNA vaccine (Jiang *et al.*, 2006b), Mycobacterium bovis BCG vaccine (Bastos *et al.*, 2004), recombinant adenovirus vaccine (Kheyar *et al.*, 2005; Jiang *et al.*, 2006a), and recombinant pseudorabies virus vaccine (Qiu *et al.*, 2005; Jiang *et al.*, 2007b). However, only a limited protective immunity could be obtained with

these experimental vaccines. Hence, new strategies should be developed for the more efficacious vaccines against PRRSV infection. Up to now, a few vaccine studies have been reported based on the minor envelope protein GP3 of PRRSV. So, the identification of vaccine immunogenicity would provide an important basis for the development of efficient vaccine to control the porcine reproductive and respiratory syndrome.

GP3 is one of the important minor envelope proteins of PRRSV that has been reported as a part of the European and North American PRRSV strains (Meulenberg *et al.*, 1995; Lima *et al.,* 2009). The GP3 protein of European and North American strains displays a very low identity and the most important difference lies within the first 35 aa, where only 29% identity is found (Murtaugh *et al.*, 1995). On the other hand, the degree of aa identity of GP3 is very high among the different PRRSV isolates in China and varies from 91.4 to 98.8% (Zhang *et al.*, 2010). However, the potential N-linked glycosylation sites as well as the general hydropathy profiles of GP3 protein of different PRRSV strains are highly conserved. Moreover, GP3 of North American and European strains has been shown to be antigenic (Dea *et al.*, 2000).

Compared to the complete GP3 protein, the truncated GP3 protein without signal peptide and hydrophobic region could induce a higher immune response (Jiang *et al.*, 2007a). In this study, the recombinant baculovirus BacSC-GP3 containing GP3 protein with the N-terminal 57 aa deleted was constructed and its immunogenicity was evaluated in the mouse and piglet models. The results showed that the fused protein was displayed on the virion surface as well as on the surface of virus-infected cells. Mice and piglets immunized with BacSC-GP3 induced successfully GP3-specific ELISA antibodies, neutralizing antibodies, and lymphocyte proliferative responses 6 weeks after the primary immunization, though the antibody titers were low in piglets. Similar results were observed in the immunization experiments with recombinant adenovirus rAd-tGP3-infected cells (Jiang *et al.*, 2007a). However, GP3 played certain role in the clearing of viral infection in the absence of a noticeable neutralizing antibody response and might be involved in the viral neutralization along with GP5 and M proteins (Plana Duran *et al.*, 1997; Cancel-Tirado *et al.*, 2004).

To investigate whether the recombinant baculovirus BacSC-GP3 can protect against the PRRSV infection, the animal protection test is ongoing in our laboratory. Since GP3 of Shaanxi strain showed a high aa identity among the different PRRSV isolates in China, we only tested the homologous reactivity of GP3-specific antibodies induced with recombinant baculovirus BacSC-GP3. In addition, we have constructed a novel vector, which can co-display GP3 and GP5 proteins of PRRSV on the virion envelope. Currently, we are investigating whether the co-expression of GP3 and GP5 proteins can significantly enhance the vaccine immunogenicity and protect pigs against a virulent PRRSV challenge.

An effective vaccine should not only elicit strong humoral and cellular immunity, but also induce the effective cross-protection against PRRSV infection. It is known that a high degree of genetic and antigenic variability exists among different PRRSV isolates, what creates a problem in the development of an efficacious PRRSV vaccine. It has been demonstrated that the cross-neutralizing activity could be affected by the genetic and antigenic variability of PRRSV (Kim *et al.*, 2007). Thus, it would be an intriguing idea to induce the broadly-ranged neutralizing antibodies against a variety of PRRSV isolates. Nevertheless, this work provided the new experimental approach for development of a new vaccine to PRRSV. So far, our data have demonstrated only the effect of immunogenicity elicited with BacSC-GP3 immunization of mice and piglets. It is necessary to investigate further the protective efficacy of vaccine against PRRSV.

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