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RESEARCH ARTICLE

Research and development of a novel subunit vaccine for the currently circulating pseudorabies virus variant in China

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Abstract Pseudorabies (PR) is a devastating viral disease which leads to fatal encephalitis and respiratory disorders in pigs. Commercial gE-deleted live pseudorabies virus (PRV) vaccine has been widely used to control this disease in China. However, the new-emerging variants of PRV compromises the protection provided by current vaccines and lead to the outbreak of PR in vaccinated pig herds. Several killed and live vaccine candidates based on current PRV variants have been reported to be effective to control the disease. A subunit vaccine based on gB protein, one major PRV glycoprotein which elicits strong humoral and cellular immune responses, however, was never evaluated for protection against the current circulating PRV variants. In this study, full-length PRV gB protein was successfully expressed in baculovirus/insect cells in the soluble format and was tested on 3-week-old piglets as a subunit vaccine. Compared with unvaccinated pigs, the gB-vaccinated pigs developed specific antibody-mediated responses and were protected from the virulent PRV HN1201 challenge. All vaccinated pigs survived without showing any PRV-specific respiratory and neurological signs, but all unvaccinated pigs died within 7 days after HN1201 challenge. Hence, this novel gB-based vaccine could be applied as an effective subunit vaccine to control PRV variant in China.

Keywords pseudorabies virus, glycoprotein B protein, subunit vaccine

1 Introduction

Pseudorabies virus (PRV), also known as Aujeszky's disease virus, is an economically important swine viral disease worldwide[1]. In China, the disease has been well controlled in recent decades by application of gene-deleted modified vaccines accompanied by the use of ELISA serological screening[2]. However, since 2011, the PRV variants were reported to be circulating in vaccinated pig herds and caused PR-specific clinical symptoms with a high mortality rate[3–5]. A range of killed and live vaccines based on current PRV variant strains were developed and tested on pigs, and the results showed these vaccines can provide useful protection against the PRV infection[6–8]. By contrast, the development and efficacy of subunit vaccines based on current PRV variants has not been reported. Compared to the other types of vaccines, the gB-based subunit vaccine is safer and less expensive but provides good protection to PRV infection.

PRV has a double-stranded linear DNA genome 150 kb in length and contains a unique long region, a unique short region, a terminal repeat sequence, and internal repeat sequences[9]. There are at least 11 different glycoproteins of PRV that have been identified and some of them have been implicated as important antigens in protective immunity against infection[10]. Among these, gB protein was the major glycoprotein that induced both humoral and cellular immunity and its role in the induction of a protective immune response against virus infection has been reported in vaccination experiments with mouse and pig models[11]. Many gB-based subunit or DNA vaccines have been tested in mice and pigs, and found to provide useful substitutes for the current commercial killed/live PRV vaccines[12–14]. However, gB protein of the currently circulating variants differ considerably as a consequence of multiple mutations, insertions and deletions compared to the common PRV vaccine strains such as Barth-K61 and

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other PRV isolates[3]. The alterations of some important amino acids may compromise the efficacy of gB-based subunit vaccine against the PRV infection. Therefore, in this study, a novel gB subunit vaccine based on PRV variant HN1201 was developed and its efficacy tested on 3-week-old piglets.

2 Materials and methods

2.1 Expression of gB protein in the baculovirus/insect cells expression system

The full-length PRV HN1201 *gB* gene was amplified by PCR and was inserted into pFastBac I plasmid (Invitrogen, Carlsbad, CA, USA). After transformation into DH10Bac cells, the white recombinant bacmid colonies were selected and their DNA extracted. The recombinant bacmid DNA was used to transfect Sf9 insect cells for subsequent gB protein expression and purification. The recombinant gB protein was purified from the culture supernatant by immunoaffinity chromatography using Ni-NTA His·Bind Resin (Novagen, Madison, WI, USA) according to the manufacturer's instructions. The recombinant gB protein was subjected to SDS-PAGE and western blot. The specificity of recombinant protein was verified by anti-His and gB-specific monoclonal antibodies.

2.2 Preparation of gB subunit vaccine and animal experiments

Two hundred microgram of purified gB protein was homogenized with the Montanide™ ISA 206 adjuvant (SEPPIC, Puteaux, France) at a ratio of 46∶54 (v/v) to make a final one dose vaccine (2 mL).

Ten 3-week-old pigs free of PRV, porcine reproductive and respiratory syndrome virus, classical swine fever virus, and porcine circovirus 2 were randomly divided into vaccinated group and unvaccinated groups. Pigs in the vaccinated group were inoculated intramuscularly with one dose of 2 mL (200 μg) gB subunit vaccine, and pigs in the unvaccinated group received 2 mL Dulbecco minimum essential medium (DMEM) medium. After vaccination, rectal temperature and clinical signs were recorded on a daily basis. The serum samples were collected to monitor PRV gB and gE antibodies at designated days. Four weeks after vaccination, pigs were challenged intranasally with 2 mL HN1201 ($10^{7.0}$ $TCID_{50}\cdot mL^{-1}$) to assess protection. After challenge, pigs were observed and scored daily for 7 days for clinical signs of disease as previously described[15,16]. At 14 days post challenge (dpc), all surviving pigs were euthanized, and different tissue samples were collected for histopathology and immunohistochemistry examination. The animal trial in this study was approved by the Animal Care and Ethics Committee of China National Research Center for Veterinary Medicine and conventional animal welfare regulations and standards were followed.

2.3 Enzyme-linked immunosorbent assay

PRV-specific gB and gE (BioChek, Holland) antibodies were detected by using commercially available enzyme-linked immunosorbent assay kits according to the manufacturer's instructions.

2.4 Histopathology and immunohistochemistry staining

The tonsil, lung, brain, cerebellum and trigeminal samples were collected for hematoxylin and eosin staining with a Leica fully automatic dyeing machine according to standard procedures, and immunohistochemistry staining using previously described methods[17]. The slides were photographed at 400× magnification.

2.5 Statistical analysis

The differences in body temperature, bodyweight gain and clinical score of pigs between two groups were determined by *t*-test (GraphPad Prism 5.0 Software, San Diego, CA, USA). Differences at $P < 0.05$ were considered statistically significant.

3 Results

3.1 Expression and purification of gB protein

The *gB* gene was amplified and inserted into pFastBac I vector. The sequencing confirmed the *gB* gene was in the correct orientation and reading code frame in the expression plasmid (data now shown). The plasmid was transformed into DH10Bac cells and the positive recombinant bacmid was selected by white/blue colony screening. The recombinant bacmid was then transfected into Sf9 insect cells. After 3 days, the supernatant containing the recombinant gB protein was collected when apparent CPE was observed. SDS-PAGE analysis showed that the recombinant gB protein was successfully expressed in the culture supernatant (Fig. 1a). The recombinant protein was next purified by immunoaffinity chromatography and was recognized by His tag and gB-specific monoclonal antibodies, respectively (Fig. 1b).

3.2 Clinical symptoms after vaccination and PRV HN1201 challenge

The purified gB protein was combined with mineral Montanide™ ISA 206 adjuvant for pig vaccination. All pigs in both vaccinated group and control group remained clinically healthy and showed no adverse reactions after vaccination (data not shown). After 28 days, the pigs in

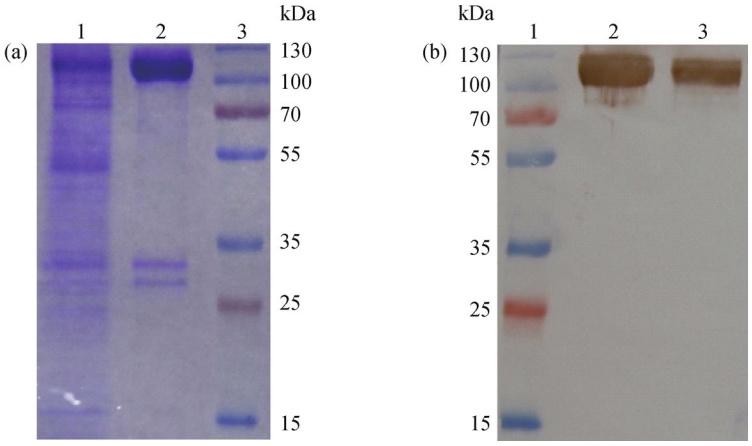

Fig. 1 Expression and purification of gB protein analysis by SDS-PAGE (a) and western blot (b). (a) Lane 1, the supernatant of sf9 insect cell culture; lane 2, purified S1 protein using immunoaffinity chromatography; lane 3, protein marker; (b) lane 1, protein marker; lane 2, the recombinant protein was recognized by HIS-tag monoclonal antibody; lane 3, the recombinant protein was recognized by PRV-gB specific monoclonal antibody.

both groups were challenged via intranasal inoculation with $10^{7.0}$ $TCID_{50}$ virulent PRV variant HN1201. Pigs in both groups showed fever (⩾40°C) after challenge as shown in Fig. 2a. However, the body temperature of vaccinated pigs returned normal at 5 days. By contrast, the body temperature of unvaccinated pigs kept increasing to 41°C until death at 5 to 7 dpc.

Besides fever, the vaccinated pigs did not develop any other clinical symptoms. In contrast, the unvaccinated pigs showed typical PR-clinical symptoms such as respiratory distress, excessive salvation and neurological signs including convulsion, muscle tremor, posterior paralysis, and ataxia. The average clinical scores in unvaccinated group were significantly higher than those in the vaccinated group (Fig. 2b).

The unvaccinated pigs lost body weight for the first 5 days after PRV HN1201 challenge (Fig. 3a). In contrast, the vaccinated pigs gained weight and the body weight of the vaccinated group was significantly higher than that of the unvaccinated group (Fig. 3a). One pig in the unvaccinated group was found dead at 5 dpc, three pigs were found dead at 6 dpc, and one pig was found dead at 7 dpi (Fig. 3b). All pigs in the vaccinated group were euthanized at 14 dpc.

3.3 Antibody response after vaccination and PRV HN1201 challenge

Three out of five pigs in the vaccinated group developed gB-specific antibodies at 7 days post-immunization (dpi), and all pigs seroconverted positively at 14 dpi (Fig. 4a). Antibody titers kept increasing steadily with an average 3.0 of OD_{405} before challenge. The unvaccinated pigs did not develop any gB antibodies after inoculating with DMEM medium as expected. After PRV HN1201 challenge, gB-antibody titer in vaccinated group dropped to an average of 2.0 at OD_{405} at 5 dpc and returned to an average of 2.2 at OD_{405} at the end of the study (Fig. 4a). By contrast, gB-antibody titer in the DMEM group kept increasing and reached an average of 0.5 OD_{405} at 5 dpc.

As for the gE antibody, as expected, both vaccinated pigs and unvaccinated pigs did not develop any gE antibodies before PRV HN1201 challenge (28 dpi), and only unvaccinated pigs showed positive gE antibodies at 5

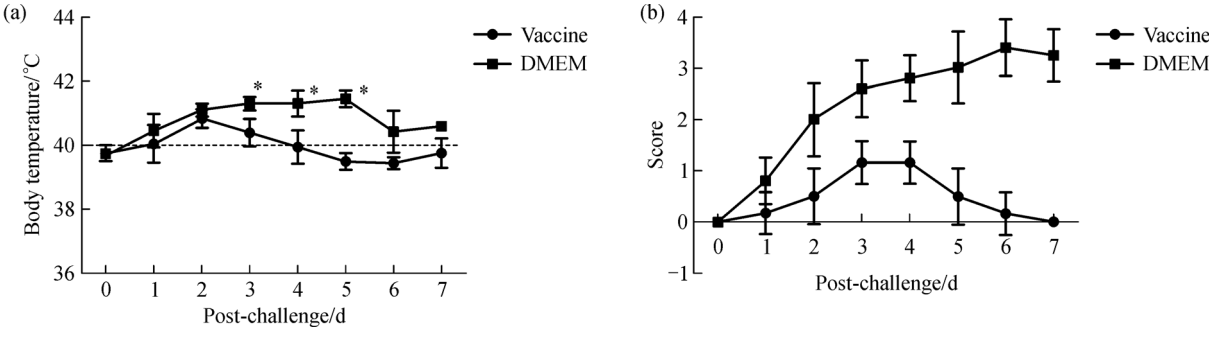

Fig. 2 Body temperature (a) and clinical symptom score (b) of pigs within first 7 days after PRV HN1201 challenge. *$P < 0.05$.

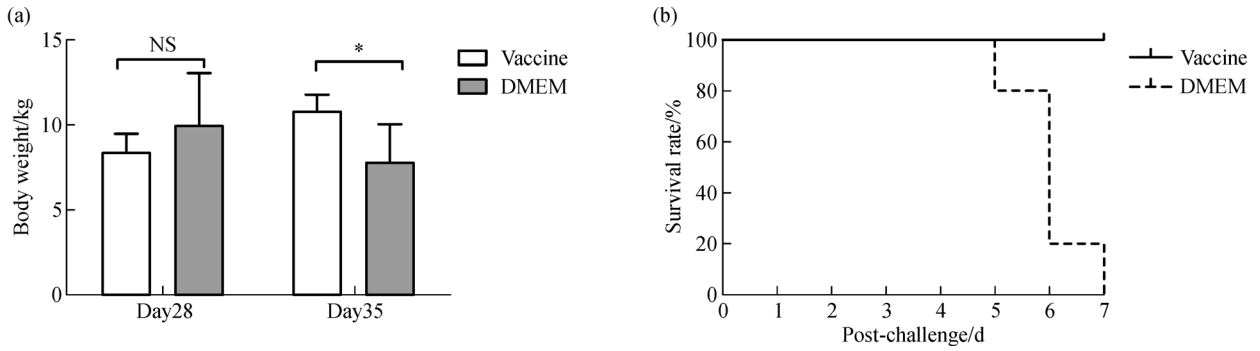

Fig. 3 Body weight gain (a) and survival rate of pigs (b) after PRV HN1201 challenge. $*P < 0.05$.

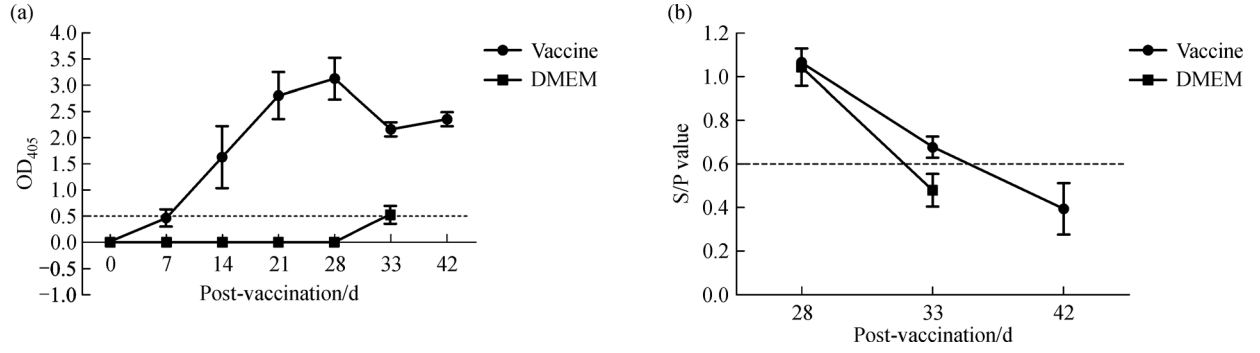

Fig. 4 Profile of PRV gB-specific (a) and gE-specific (b) antibody responses after vaccination and challenge

dpc (Fig. 4b). All vaccinated pigs showed gE antibody positive at the end of study (14 dpc).

3.4 Pathological examination

All pigs in the unvaccinated group were found dead and were subjected to necropsy before the end of study. The unvaccinated pigs showed hemorrhage and necrosis in the tonsil, severe pulmonary consolidation and necrosis in the lung, encephalic hemorrhage in the brain and cerebellum. All pigs in the vaccinated group were euthanized at 14 dpc. There were no visible gross pathological changes for the pigs in the vaccinated group.

Histopathological examination results showed that none of vaccinated piglets displayed any histopathological changes (Fig. 5a–5e). The unvaccinated piglets had hemorrhages and necrosis in tonsil and lung samples (Fig. 5f–5g). The infected pigs also showed nonsuppurative meningoencephalitis (Fig. 5h), Purkinje cell degeneration and necrosis in the cerebellum (Fig. 5i), and infiltration of lymphocytes around the trigeminal ganglia (Fig. 5j). Consistent with the pathological results, immu-

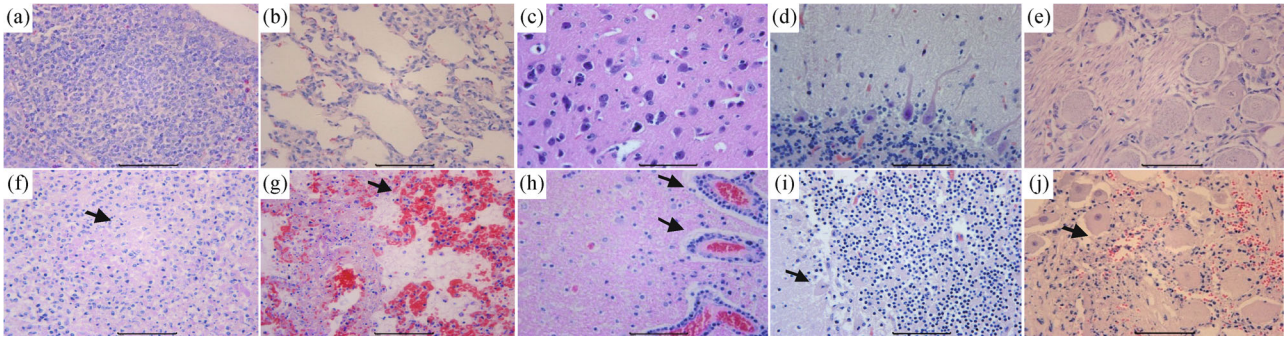

Fig. 5 Histopathological results of vaccinated (a–e) and unvaccinated pigs (f–j). (a–e) Tonsil, lung, brain, cerebellum and trigeminal ganglia samples from vaccinated pigs, respectively. No obvious histopathological changes were observed; (f) tonsil. Tonsillar lymphoid tissue necrosis and formation of large necrotic foci; (g) lung. Massive vascular congestion and hemorrhage; (h) brain. Nonsuppurative meningoencephalitis; (i) cerebellum. Purkinje cell degeneration and necrosis; (j) trigeminal ganglia. Infiltration of lymphocytes around the neuron of trigeminal ganglia. Original magnification 400×.

nochemistry results also showed no positive staining was detected in the above tissues of vaccinated pigs (Fig. 6a–6e). Positive staining in the tonsil, lung, brain, cerebellum and trigeminal ganglia samples of unvaccinated pigs (Fig. 6f–6j).

4 Discussion

The new-emerging PRV variants have recently been reported to be widespread in China and caused significant economic losses in vaccinated pig herds[3,5].These PRV variants are antigenically different from previously common PRV and current commercially-available PRV vaccines cannot provide complete protection to the circulating variants[2,3]. To address this problem, several killed and modified live vaccine based on current circulating PRV variants were developed and showed useful protection against infection[6–8]. Although the origin of current circulating PRV variants remains unknown, the massive use of modified live vaccines could have provided sufficient selection pressure for these variant to emerge. Therefore development of a more effective and safe PRV vaccines, such as novel subunit vaccine, is urgently needed to control the disease.

The PRV vaccines based on glycoprotein gB, gC and gD of vaccine strains were expressed in different systems including baculovirus, adenovirus and Sindbis virus[11,13,18]. These vaccines showed useful protection to the viral challenge by previously common PRV strains in mice and/or pig models because they elicit good humoral and cellular immunity. However, with the emergence of new PRV variants, the *gB* gene changes with multiple sites of insertions, deletions and mutations when compared with previously common RPV, which may alter the important epitopes which induce protective immunity. Therefore, in this study, the recombinant PRV variant HN1201 gB protein was produced in a baculovirus/insect cell expression system and tested on 3-week-old piglets as a subunit vaccine.

The gB subunit vaccine appears to be safe as no pigs developed clinical symptoms including fever after vaccination. At 28 dpi, the pigs were challenged with virulent PRV HN1201 to test the efficacy of vaccine. The unvaccinated pigs started to show PR-specific clinical symptoms and died between 5 and 7 days, demonstrating the high virulence of PRV HN1201 variant as previously reported[5]. In contrast, no vaccinated pigs showed any clinical symptoms except a transient fever. As found with the killed PRV vaccine[17], this fever in vaccinated groups lasted for 4 days when body temperature returned to normal. In contrast, the unvaccinated pigs had lasting high fever until they died.

The glycoprotein gB of PRV is immunogenic and related to viral defense, and the level of gB antibodies may work as an indicator that correlates with the degree of protection[19]. The vaccinated pigs developed high levels of gB antibodies after vaccination (Fig. 4a). However, unlike the PRV killed vaccine previously reported[17], the gB antibody titers of vaccinated pigs dropped with average OD_{405} of 2.0 after PRV HN1201 challenge. Although this subunit vaccine may not induce gB-specific antibodies lasting as long as the killed vaccine did, the high titer of gB antibodies were still enough to provide useful protection to PRV HN1201 challenge. Two vaccination regimes may work better than the current one vaccination protocol, and may need to be explored in the near future.

The gE antibodies work as an indicator of field PRV infection since gB subunit vaccine did not elicit the generation of gE antibodies. As shown in Fig. 4b, all vaccinated pigs were gE antibody negative. In contrast, three out of five unvaccinated pigs were gE antibody positive, which indicated the infection of PRV HN1201. These results indicate the vaccination of gB subunit vaccine may provide protection at the early stage of virus infection. Consistent with other experimental PRV vaccines including killed and live modified vaccines, all vaccinated pigs were gE antibody positive at 14 dpc. To explore the mechanisms behind the phenomena, besides humoral antibody responses, the cellular immunity could also be important in elimination of PRV in the tissues and needs to be further studied.

The immunohistochemistry results showed that the PRV antigen could not be detected in tonsil, lung, brain and cerebellum samples of the vaccinated pigs. However, the PRV antigen was present in three out of five vaccinated pigs in trigeminal ganglia (Table 1). In pigs, neurons in the trigeminal ganglia are the primary site for PRV latency[20].

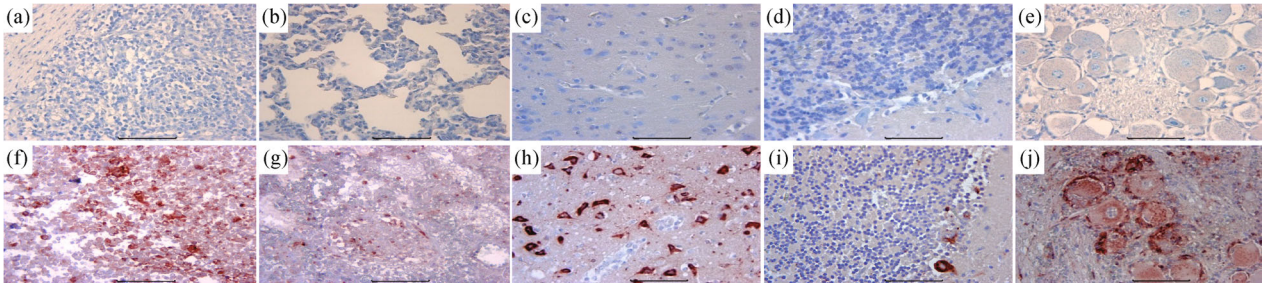

Fig. 6 Immunochemistry staining of tonsil (a, f), lung (b, g), brain (c, h), cerebellum (d, i), and trigeminal ganglia (e, j) samples of vaccinated (a–e) and unvaccinated pigs (f–j). Original magnification 400×.

Table 1 Virus antigen distribution and intensity in different organs detected by immunohistochemistry staining

Groups	Tonsil	Lung	Brain	Cerebellum	Trigeminal ganglia
DMEM	++	–	++	-	+
	+++	+++	+++	+	+
	+++	++	+++	+	+
	–	+ +	–	+	+
	+++	++	–	–	+
Vaccine	–	–	–	–	–
	–	–	–	–	+
	–	–	–	–	+
	–	–	–	–	+
	–	–	–	–	–

Note: The positive staining signals were interpreted as negative (–), low (+), moderate (++), intense (+++) according to the intensity of staining. Each row represents one pig from the corresponding group.

No previous PRV vaccine studies included antigen detection in the trigeminal ganglia because it is technically demanding. This is the first reported the PRV antigen distribution in the neurons of trigeminal ganglia after vaccination, which may indicate no current PRV vaccines can prevent the transmission of virus to the neurons in the trigeminal ganglia and virus latency and partially explained the generation of gE-specific antibodies.

5 Conclusions

To conclude, PRV gB was successfully produced in baculovirus/insect cell expression system and was tested for efficacy as a novel subunit vaccine combined with commercial adjuvant on 3-week-old pigs. The results showed that vaccination can provide complete protection to virulent PRV HN1201 challenge by the evidence of high level of gB and absence of viral antigen in multiple tissues. Therefore, this subunit vaccine is likely to provide a useful substitute to other types of PRV vaccines.

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Compliance with ethics guidelines Yuzhou Wang, Tongyan Wang, He Yan, Fanli Yang, Linghua Guo, Qingyuan Yang, Xule Hu, Feifei Tan, Yan Xiao, Xiangdong Li, and Kegong Tian declared that they have no conflict of interest or financial conflicts to disclose.

All applicable institutional and national guidelines for the care and use of animals were followed.

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