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# Protective humoral immune response induced by an inactivated porcine reproductive and respiratory syndrome virus expressing the hypo-glycosylated glycoprotein 5

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ABSTRACT

Porcine reproductive and respiratory syndrome (PRRS) causes significant economic losses to the swine industry worldwide. Although inactivated and live vaccines are commercially available for the control of PRRS, both types of vaccine have not always proven successful in terms of generating a protective immune response, particularly in the case of inactivated vaccines. In this study, we tested whether an inactivated vaccine could induce a humoral immune response to PRRS during a homologous challenge. Amino acid substitutions were introduced into glycoprotein (GP) 5 of the FL12 strain of the PRRS virus (PRRSV) using site-directed mutagenesis with a pFL12 infectious clone. The substitutions led to double deglycosylation in the putative glycosylation moieties on GP5. The mutant virus was subsequently inactivated with binary ethylenimine. The efficacy of the inactivated mutant virus was compared with that of the inactivated wild-type PRRSV. Only the inactivated mutant PRRSV induced serum neutralizing antibodies at six weeks post-vaccination. The group that was administered the inactivated mutant virus twice exhibited a significantly increased neutralizing antibody titer after a challenge with the virulent homologous strain and exhibited more rapid clearing of viremia compared to other groups, including the groups that were administered either the inactivated mutant or wild-type virus only once and the group that was administered the inactivated wild-type virus twice. Histopathological examination of lung tissue sections revealed that the group that was administered the inactivated mutant virus twice exhibited significantly thinner alveolar septa, whereas the thickness of the alveolar septa of the other groups were markedly increased due to lymphocyte infiltration. These results indicated that the deglycosylation of GP5 enhanced the immunogenicity of the inactivated mutant PRRSV and that twice administrations of the inactivated mutant virus conferred better protection against the homologous challenge. These findings suggest that the inactivated PRRSV that expresses a hypo-glycosylated GP5 is a potential inactivated vaccine candidate and a valuable tool for controlling PRRS for the swine industry.



## 1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is one of the most important infectious diseases in pigs and is responsible for substantial economic losses in the swine industry worldwide. The PRRS virus (PRRSV) causes severe reproductive failure in pregnant sows and is associated with porcine respiratory disease complex (PRDC) in combination with other viral and bacterial infections in young piglets [1–3]. To help control outbreaks of PRRS, strategies, such as management, biosecurity and vaccination, have been applied with various levels of success [4–6]. The control PRRS is complicated due to its pattern of persistent, subclinical infections with occasional epidemic outbreaks, the great heterogeneity of the virus and the poor antibody response that is insufficient to completely block viral re-infection [7–10]. Although the current vaccines need to be improved, and new vaccine technologies are required, vaccination is the most cost-effective and reliable strategy that is currently available.

There are two types of commercially available PRRS vaccines. The first is a modified-live virus (MLV) vaccine, and the second is

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an inactivated vaccine. The PRRS MLV vaccine is well-recognized for its protective efficacy against PRRSV infection in the field, but this vaccine has a limited efficacy against challenges with heterologous viruses. Additionally, the PRRS MLV vaccine has an intrinsic risk for reversion to a virulent strain [4]. The PRRS inactivated vaccine is much safer than the PRRS MLV vaccine. However, this advantage of the inactivated vaccine is diminished by its insufficient immunogenicity. The commercially available PRRS inactivated vaccine does not induce a sufficient immune response and does not adequately protect pigs from viremia when challenged with PRRSVs [11–13]. Although previous studies have shown that PRRS inactivated vaccines are able to inhibit viral shedding and induce neutralizing antibodies, these results vary depending on the virus strain and the type of tissue culture used to produce the vaccines [11,14]. Numerous efforts have been made to develop an ideal PRRS inactivated vaccine that would offer broad protection and high immunogenicity [15,16], but these efforts have been unsuccessful.

Previous studies have determined that the neutralizing epitopes are located in the structural proteins, including glycoprotein (GP) 3, GP4, GP5 and the non-glycosylated membrane protein (M) [17–19]. Among these, the neutralizing epitopes in GP5 induce the primary neutralizing antibodies [20–23]. GP5 is encoded by open reading frame (ORF) 5 of the PRRSV viral genome and is the major envelope glycoprotein of PRRSV. GP5 has been suggested to be involved in the viral entry and assembly of PRRSV [24]. A small ectodomain located on the N-terminus of GP5 plays an important role in the attachment of PRRSV to the macrophage-specific receptor [24,25]. Two epitopes located in this ectodomain have previously been identified and characterized, based on their neutralizing capabilities, as a decoy epitope and a major neutralizing epitope [22]. The delayed production of neutralizing antibodies to GP5 is a characteristic of the immune response to PRRSV and is caused by the rapid induction of non-neutralizing antibodies against the decoy epitope [24,26]. PRRSV-specific non-neutralizing antibodies have been detected at one week post-inoculation (PI), while neutralizing antibodies have been observed to appear from three weeks PI [27–29].

The GP5 of PRRSV has two to five potential N-linked glycosylation sites located in the ectodomain, and the presence of glycans around the major neutralizing epitopes has been suggested to reduce the immunogenicity of PRRSV [30]. It has been reported that glycan shielding on PRRSV GPs interferes with the neutralizing antibody response [31–34]. Previous studies have also demonstrated that weak neutralizing antibody responses are obtained in the presence of glycans on the GPs of other viruses, such as lactate dehydrogenase-elevating virus, hepatitis B virus, human immunodeficiency virus and influenza virus [35–38].

In previous studies, multiple mutants of PRRSV containing various combinations of deglycosylations on GP5 have been produced using reverse genetics [31,33,39,40]. Among these mutants, the mutant virus containing a double deglycosylation of N34 and N51 of GP5 shows decreased viral replication but induces a higher level of neutralizing antibodies in in vivo experiments compared to its parental strain of PRRSV [31]. In this study, we attempted to develop an inactivated PRRSV vaccine using this mutant strain of PRRSV, which contains a double deglycosylation of GP5 and evaluated the efficacy of this vaccine using a challenge infection with a homologous strain of PRRSV.

## 2. Materials and methods

### 2.1. cDNA clone, cells and viruses

A full-length PRRSV infectious cDNA clone (pFL12) generated from the North American type II PRRSV isolate was used to construct a mutant plasmid carrying mutations in the putative *N*-glycosylation sites by site-directed mutagenesis as previously described [41]. To produce wild-type (wt) and mutant viruses, the pFL12 and mutant plasmids were linearized by digestion with AclI and transcribed in vitro using the mMESSAGE mMACHINE Ultra T7 kit (Ambion, Austin, TX). The produced RNAs were electroporated into the MARC-145 cell lines, cultured in low-glucose Dulbecco's Modified Eagle's Medium (DMEM) and supplemented with 10% fetal bovine serum (FBS) [42] All of the rescued wt and mutant viruses were subjected to RT-PCR amplification using appropriate primers, and the PCR fragments were subjected to DNA sequencing to confirm the presence of the desired mutations and the absence of unwanted mutations. The *N*-glycosylation profiles of the GP5 for the wt and deglycosylated mutant viruses were confirmed by western blotting. The viruses were treated for 16 h at 37 °C with the 10 U peptide *N*-glycosidase F (PNGaseF) according to the manufacturer's instructions (NEB, Beverly, MA). Subsequently, the proteins were separated by 12% SDS-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane (GE Healthcare, Milwaukee, WI). The membrane was blocked with 5% skim milk in phosphate-buffered saline-Tween 20 and incubated with porcine serum containing a high level of PRRSV-specific primary antibody and horseradish peroxidase-conjugated goat anti-porcine IgG (Southern Biotech, Birmingham, AL) secondary antibody. The blots were imaged using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare).

### 2.2. Production of inactivated PRRSV vaccines

Both wt FL12 and the mutant virus FL12/GP5DM containing double-amino acid substitutions at the 34 and 51 potential *N*-glycosylation residues in GP5 [31] were used to produce the inactivated vaccines used in this study.

Both the wt and mutant viruses were concentrated at $10^8$ $TCID_{50}$/ml and inactivated with binary ethylenimine (BEI) as described previously [43]. Briefly, BEI was prepared as a 0.1 M stock solution by stirring 0.1 M 2-bromoethylamine hydrobromide (Sigma-Aldrich, St. Louis, MO) in 0.175 M NaOH at 37 °C for 1 h. The BEI stock solution was added to the virus suspension, and the mixture was incubated at 37 °C for 24 h. The remaining BEI was subsequently neutralized with 0.1 mM sodium thiosulfate (Sigma-Aldrich) for 2 h. To confirm the inactivation of the virus, MARC-145 cells were inoculated with BEI-treated virus suspension and cultured at 37 °C for five days. A total of 1 ml of inactivated viral antigen containing $10^8$ $TCID_{50}$/ml of the BEI-inactivated PRRSV was mixed with an equal volume of Montanide™ IMS1313 VG adjuvant (SEPPIC, Paris, France). The aqueous adjuvant was suitable for inactivated and live vaccines in pigs [44,45].

### 2.3. Evaluation of the efficacy of the inactivated PRRSV vaccine

The experimental design involving the inactivated PRRSV vaccination and the challenge is summarized in Table 1. Fifteen three-week old piglets were purchased from a PRRS-free farm and confirmed to be serologically PRRSV-negative. The piglets were randomly allocated into five groups (G1 to G5). The piglets in G1 and G2 were intramuscularly administered inactivated wt virus vaccine, and the piglets in G3 and G4 were intramuscularly administered inactivated mutant virus vaccine. The G5 piglets were injected with DMEM alone as a control. Three weeks after the primary vaccination, the piglets in G2 and G4 were boost-vaccinated using the same vaccines they had received earlier. Serum samples were collected at 14, 28, 42, 49 and 56 days post-vaccination and stored in −20 °C. Five weeks after the booster vaccination, all of the groups were intramuscularly challenged with $10^5$ $TCID_{50}$/ml of wt virus. Serum samples were collected at 2, 3, 4, 7, 8, 9 and 13 days after the challenge. The collected sera were examined for viremia via

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