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Full Length Research Paper

Immunogenicity of the glycoprotein 5 truncated trans-membrane regions of porcine reproductive and respiratory syndrome virus

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A fusion gene encoding glycoprotein 5 (GP5) of porcine reproductive and respiratory syndrome virus (PRRSV) truncated trans-membrane regions was constructed successfully through a linker-based reconstruction strategy and overlap polymerase chain reaction (PCR). High-level expression of the truncated GP5 protein with a molecular weight of 16 kDa was obtained in *Escherichia coli* BL21 (DE3) through subcloning of the gene into a prokaryotic expression vector pET-28a. Western blot indicated that the truncated GP5 protein could react specifically with pig positive serum against PRRSV and anti-His_6 mAb, respectively. After recovering from inclusion bodies through nickel affinity purification and refolding by gradient dialysis, the truncated GP5 protein showed high specific reaction to the PRRSV positive serum in enzyme-linked immunosorbent assay (ELISA) and was used to immunize BALB/c mice. Immunoperoxidase monolayer assay (IPMA) indicated that mouse polyclonal antibody could react apparently with MARC-145 cells infected with PRRSV as pig positive serum against PRRSV did. Thus, the truncated GP5 was demonstrated to have good immunogenicity and would be very useful for PRRSV antibody detection as well as for vaccine development.

Key words: Overlap polymerase chain reaction (PCR), truncated GP5 protein, heterologous expression, immunogenicity.

INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS) is considered to be one of the most economically important infectious diseases of swine, causing late-term reproductive failure in pregnant sows and severe pneumonia in neonatal pigs (Snijder and Meulenberg, 2001). The disease was first reported in the United States in 1987, and the causative agent (PRRSV) was isolated and characterized for the first time in Europe in 1991 and 1 year later in the United States (Keffaber, 1989; Collins et al., 1992).

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The genome of PRRSV is a single-stranded, non-segmented, positive-sense and polyadenylated RNA of approximately 15 kb in length that contains nine open reading frames (ORF1a, ORF1b, ORF2a, ORF2b and ORFs 3 through 7) (Conzelmann et al., 1993; VanNieuwstadt et al., 1996; Meulenberg et al., 1997). ORF1a and ORF1b comprise of 80% of the genome size and encode viral replicase polyprotein which is predicted to be autoproteolytically cleaved at 12 sites, producing 13 non-structural proteins ultimately, while ORF2a, ORF2b and ORFs 3-7 encode the viral structural proteins GP2, E, GP3, GP4, GP5, M and N, respectively (Bautista et al., 2002; Stadejek et al., 2002). Of these, neutralizing antibodies to GP5 appear to be most relevant for protection. PRRSV neutralization is correlated with antibodies directed against the GP5 protein, both *in vivo* (Yoon et al., 1995; Pirzadeh and Dea, 1998; Kwang et al.,

Table 1. Names, sequences and features of the primers.

Name	Sequences	Introduced domain
P1	5'-CGC*GGATCC*AGCAACAACAGCAGCTCT-3'	*Bam*HI
P2	5'-***AGAACCACCACCACCAGAACCACCACCACC***TGCCCAGTCAAATTTTTGTGTC-3'	*Linker*
P3	5'-***GGTGGTGGTGGTTCTGGTGGTGGTGGTTCT***AACTGCATGTCCTGGCGC-3'	*Linker*
P4	5'- CCC*AAGCTT*TTACTATAGACGACCCCATTG-3'	*Hin*dIII

Restriction enzyme cutting sites of *Bam*HI and *Hind*III were placed at the 5 prime end of P1 and P4, respectively (underlined parts). A linker sequence encoding two repeated amino acid sequences (GGGGS) was introduced into the 5 prime end of P3, with its reverse complement sequences in the 5 prime end of P2 (the linker sequence is in black bold).

1999; Gonin et al., 1999) and *in vitro* (Pirzadeh and Dea, 1997; Zhang et al., 1998; Weiland et al., 1999; Yang et al., 2000). Antibody to GP3 is in a very low level in pigs and is involved in viral neutralization together with antibodies to the GP5 and M proteins (Cancel-Tirado et al., 2004).

Although, monoclonal antibodies (mAbs) against GP4 protein have also been found to be neutralizing (Meulenberg et al., 1997), mAbs against the GP5 protein appeared to be much more effective (Weiland et al., 1999).

At present, both modified live and killed vaccines used have inherent drawbacks. Killed vaccines cannot always provide protective immunity at the herd level, and the intrinsic risk of reversion to virulence under farm conditions has restricted the use of live vaccines (Meng et al., 2000). Since GP5 is associated with the development of neutralizing antibodies and viral protection, it becomes a leading target for vaccine design and antibody development (Ostrowski et al., 2002; Plagemann, 2004; Ansari et al., 2006). GP5 has been expressed in mammalian cells, recombinant viruses, tobacco plant and host animals (Pirzadeh and Dea, 1998; Lee et al., 2004; Chia et al., 2009; Yun et al., 2009). Nevertheless, as far as the high-level expression is concerned, the *Escherichia coli* (*E. coli*)-based prokaryotic expression system is a powerful host cell system for expressing heterologous genes (Yin et al., 2007).

In this study, a fusion GP5 gene of PRRSV lacking trans-membrane regions was cloned using a linker-based reconstruction strategy and overlap polymerase chain reaction (PCR), and the truncated GP5 protein (designated as ΔGP5) was over-expressed in *E. coli* system. After recovering from inclusion bodies through nickel affinity purification and refolding by gradient dialysis, the truncated GP5 protein (ΔGP5) proved to have good immunogenicity.

MATERIALS AND METHODS

The full-length GP5 gene in pMDT-GP5 was cloned from the PRRSV Chinese isolate HN07-1 (GenBank No. FJ147205.1), and the PRRSV negative and positive sera were obtained from the healthy and HN07-1 immunized pigs, respectively (Qiao et al., 2010).

Primer design and overlap PCR

After analyzing the molecular characteristics of GP5 by DNAStar software (DNASTAR, Inc., Wisconsin, USA), two pairs of primers were designed for overlap PCR amplification based on the gene sequence of PRRSV HN07-1 using Primer Premier 5.0 (PREMIER Biosoft International, Silicon Valley, USA). The nucleotide sequences of the primers are listed in Table 1. In overlap PCR, Primers P1 and P2 were used for amplification of GP5a gene fragment encoding the N-terminal part of GP5 (residues 32-63), while the GP5b gene fragment encoding the C-terminal part (residues 130-200) was amplified by primers P3 and P4. To maintain the conformational structure of GP5, a flexible linker sequence encoding two repeated amino acid sequences (GGGGS) was incorporated into the fusion gene by primers P2 and P3, and used for ligation of GP5a and GP5b gene fragments. *Bam*HI and *Hind*III restriction enzyme cutting sites were introduced at the ends of the fusion gene by primers P1 and P4, respectively, to insert it into the prokaryotic expression vector pET-28a (Figure 1). In each step, PCR products were detected by electrophoresis in 1.0% agarose gel stained with ethidium bromide, and purified by Gel Extraction Kit (Takara).

Construction of the prokaryotic expression plasmid

The overlap PCR products were digested with *Bam*HI and *Hind*III, and subcloned into the prokaryotic expression vector pET-28a using standard molecular cloning techniques. Designated as pET-GP5, the recombinant expression plasmid was sequenced by Sangon Biotech (Shanghai) Co., Ltd.

Expression and purification of ΔGP5

E. coli BL21(DE3) cells transformed by pET-GP5 were cultured in LB liquid medium to a mid-log growth phase, and induced by 1 mM isopropy-β-D-thiogalactoside (IPTG) at 37℃ for 6 h with the control of *E. coli* BL21(DE3) cells harboring pET-28a. The induced cells were harvested and resuspended in phosphate buffered saline (PBS), and disrupted by incubation with 100 μg/ml lysozyme for 30 min at 30℃ followed by sonication on ice. After centrifugation at 12000 rpm for 20 min at 4℃, the pellet was collected and dissolved in 8 M urea for purification through Ni-chelating affinity column and refolding by gradient dialysis. The recombinant protein was designated as ΔGP5.

Immunization of BALB/c mice with ΔGP5

After refolding, ΔGP5 was used to immunize BALB/c mice. For the first immunization, each mouse was injected subcutaneously at two sites with 50 μg of ΔGP5 emulsified with Freund's complete adjuvant. Three weeks later, the same amount of ΔGP5 emulsified

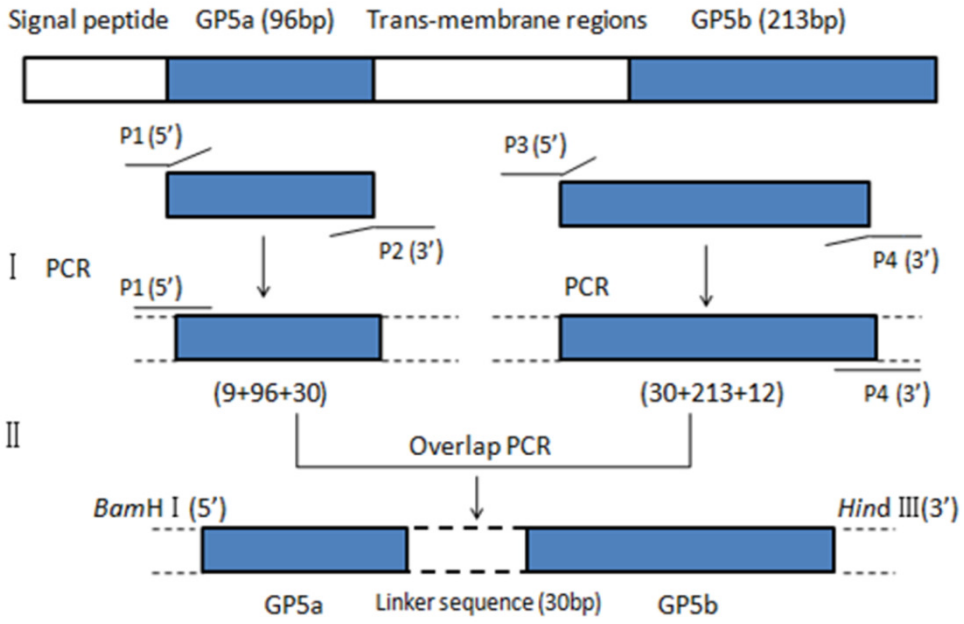

Figure 1. Schematic diagram of the truncated GP5 gene construction. The gene fragments of GP5a and GP5b were amplified from the full-length GP5 gene in pMDT-GP5 using two pairs of primers P1/P2 and P3/P4, respectively. The earlier mentioned gene fragments were then ligated with a flexible linker sequence encoding two repeated amino acid sequences (GGGGS) by overlap PCR to generate a fusion gene of GP5 lacking trans-membrane regions. The restriction enzyme cutting sites of *Bam*HI and *Hind*III were introduced at the ends of the truncated gene to insert it into the prokaryotic expression vector of pET-28a.

with Freund's incomplete adjuvant was injected into each mouse the same way as the first injection of ΔGP5. The third and fourth immunization was carried out the same way as the second immunization at intervals of two weeks. After the fourth immunization, mouse sera against ΔGP5 were collected and then used for detection in ELISA and IPMA.

SDS-PAGE and Western blot

The expressed and refolded ΔGP5 were analyzed by 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and confirmed by Western blot. The recombinant proteins resuspended in SDS-PAGE reduced buffer were subjected to 12% SDS-PAGE, and stained with 0.25% coomassie brilliant blue R250 or transferred onto the polyvinylidene difluoride (PVDF) membrane (Roche) by semi-dry electrophoretic transfer cell (Bio-Rad, Nazareth, Belgium). Blocked with 5% skimmed milk at 4℃ overnight, the transferred membrane was incubated with positive sera against PRRSV, and anti-His_6 mAb (1:1000 diluted in PBST) at 37℃ for 1 h. Horseradish peroxidase (HRP)-conjugated goat anti pig antibody and HRP-conjugated goat anti mouse antibody were used as secondary antibodies, respectively (1:1000 diluted in blocking buffer), and 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma-Aldrich) was used as a staining substrate.

ELISA

The immunogenicity of the refolded ΔGP5 was detected and identified by indirect ELISA routinely. ΔGP5 were diluted to 10, 7.5, 5, 2.5, 2, 1, 0.5, 0.25, 0.125, 0.0625 and 0.03125 μg/ml in carbonate buffered saline (CBS) and coated onto ninety-six well plates, respectively. PRRSV positive and negative sera were used as primary antibodies, and HRP-conjugated goat anti pig antibody was employed as secondary antibody. 3,3',5,5'-Tetramethylbenzidine (TMB) was used as the chromogen for color development, and absorbance at 450 nm was measured with an automatic plate reader. The well with a P/N value of the PRRSV positive and negative sera ($OD_{positive}/OD_{negative}$) over 2.5 was considered to be positive.

Immuno-peroxidase monolayer assay (IPMA)

Monolayers of MARC-145 cells were grown in 96-well microtiter plates. After washing once with Hanks balanced salt solution containing 0.01% TPCK-treated trypsin, the monolayers were incubated with PRRSV. Then, IPMA was carried out routinely. Pig positive sera against PRRSV and mouse polyclonal antibodies were respectively employed as primary antibodies, and rabbit anti-swine IgG-HRP and goat anti-mouse IgG-HRP were applied as secondary antibodies. AEC buffer (pH 5.0) (18.8 ml 0.2 M acetic acid, 35.2 ml 0.2 M sodium acetate and 50 ml distilled water) was used for color development. The color reaction was allowed to develop for 5 min, and the plates was washed once with PBS and then examined under a light microscope.

RESULTS

Molecular characteristic of the full-length GP5 protein

The molecular characteristics of the full-length GP5 were analyzed by using DNAStar software (Figure 2). The first

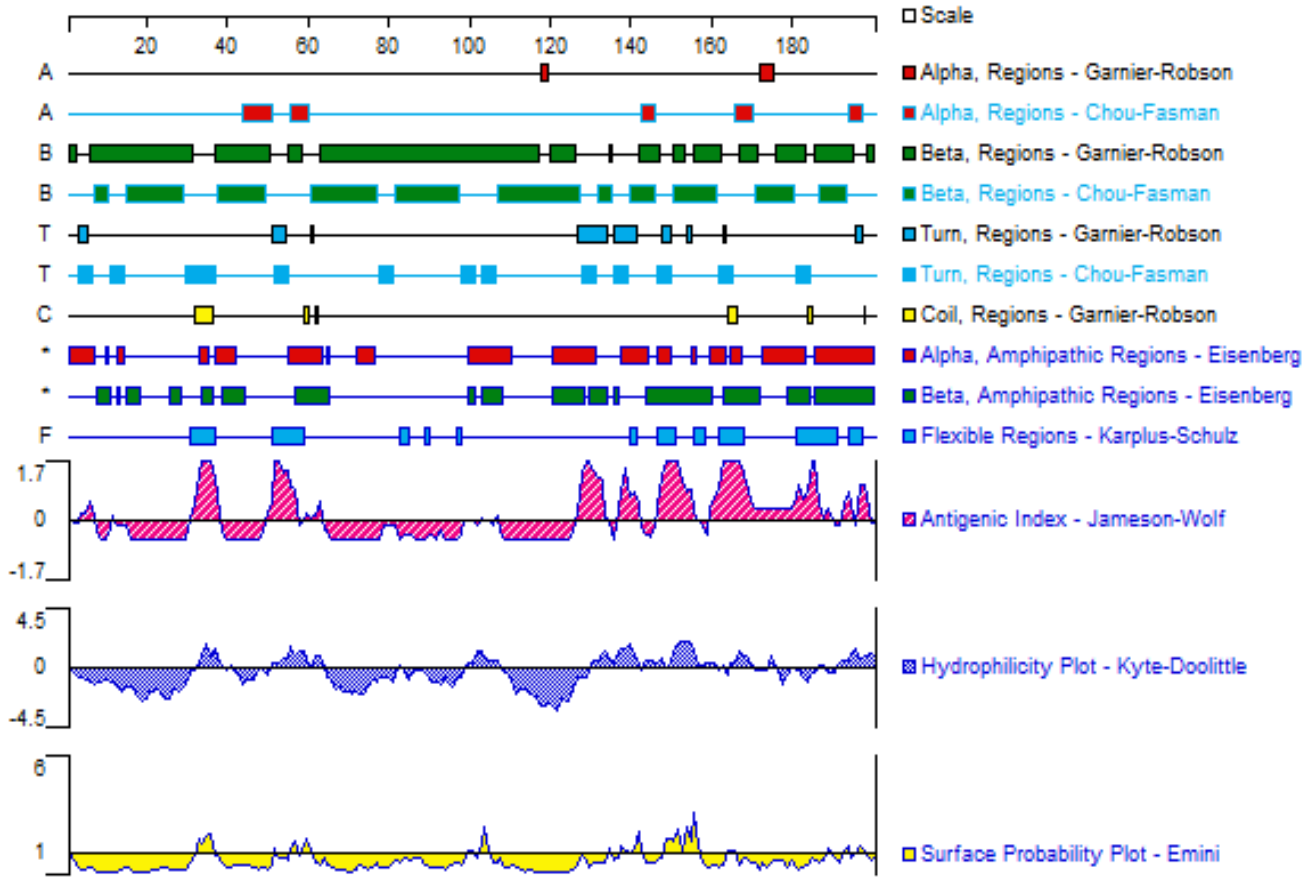

Figure 2. Molecular characteristics of the full-length GP5. The molecular characteristics of the full-length GP5 were analyzed using DNAStar software. The signal peptide (residues 1-31) and three trans-membrane regions (residues 64-82, 87-99 and 107-129) show strong hydrophobicity. And these regions also have poor antigenic index and surface probability inferred from the predicted profile.

31 amino acids and three regions in the middle of GP5 (residues 64-82, 87-99 and 107-129) show poor antigenic index and surface probability and are highly hydrophobic. This is consistent with the report that the GP5 gene contains a signal peptide sequence in its N terminal and three centrally located trans-membrane regions (Kimman et al., 2009). Amino acids 32-63 and 130-200 are highly hydrophilic and possess strong antigenic index and surface probability. So these two parts were selected to be ligated by a flexible linker in overlap PCR and expressed in *E. coli* system.

Construction of the truncated GP5 gene and its expression plasmid

At the beginning of this study, GP5 gene lacking only the signal peptide sequence was introduced into pET-28a and pET-32a using restriction enzyme cutting sites of *Bam*HI and *Hind*III, but the positive recombinant clones of both pET28a-GP5 and pET32a-GP5 failed to be expressed in two host cells: *E. coli* BL21(DE3) and *E. coli. Rossetta* (data not shown). So the truncated GP5 gene lacking the trans-membrane regions was constructed by overlap PCR. As shown in Figure 3, the primary PCR products of GP5a and GP5b were 135 and 255 bp, respectively, and the overlap PCR product of the fusion gene was 390 bp. The gene of interest was then inserted into the prokaryotic expression vector of pET-28a under the control of T7 promoter, which was expected to express a fusion protein of 146 amino acids with a molecular weight of 16 kDa.

Expression of ΔGP5

Analyzed by SDS-PAGE, a protein band with a molecular weight of about 16 kDa was found in the induced bacteria containing pET-GP5 when compared with the control cells with pET-28a, indicating the successful expression of ΔGP5. In the solubility analysis, most of the expressed protein was found in the insoluble fractions of the induced *E. coli* cells extract, suggesting that the recombinant ΔGP5 protein mainly exists as inclusion bodies. ΔGP5

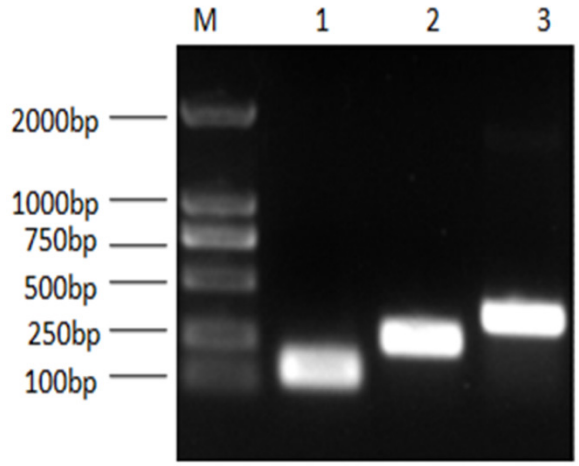

Figure 3. Overlap PCR amplification. The gene fragments of GP5a and GP5b were amplified from the full-length GP5 gene in pMDT-GP5 using two pairs of primers P1/P2 and P3/P4, respectively. The earlier mentioned gene fragments were then ligated with a flexible linker sequence encoding two repeated amino acid sequences (GGGGS) by overlap PCR to generate a fusion gene of GP5 lacking trans-membrane regions. The primary PCR products of GP5a and GP5b were 135 and 255 bp, respectively, and the overlap PCR product of the fusion gene was 390 bp. Lane 1: DNA marker (DS 2000); lane 2: GP5a; lane 3: GP5b; lane 4: recombinant GP5.

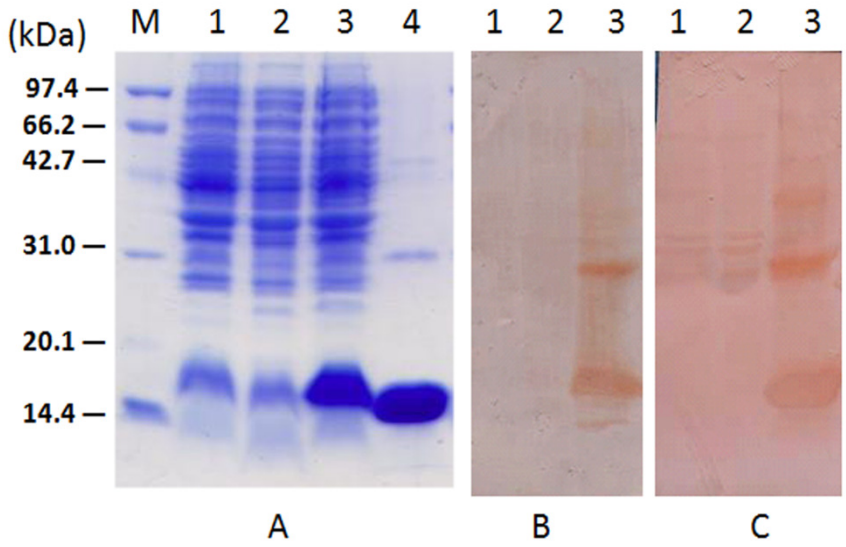

Figure 4. Expression analysis of the truncated GP5 protein by SDS-PAGE and Western blot. The expressed protein was separated by 12% SDS-PAGE (A), and analyzed by western blot using PRRSV positive serum (B) and anti-His_6 mAb (C). Lane M, Mid-range protein molecular weight markers (kDa), lane 1, BL21 (DE3) harboring pET-28a induced; lane 2, BL21 (DE3) harboring pET-GP5 uninduced; lane 3, BL21 (DE3) harboring pET-GP5 induced; lane 4, the purified recombinant protein.

was shown to be recognized specifically by the positive serum against PRRSV and anti-His_6 mAb, respectively in Western blot analysis (Figure 4).

Immunogenicity of ΔGP5

The immunogenicity of ΔGP5 was evaluated by indirect

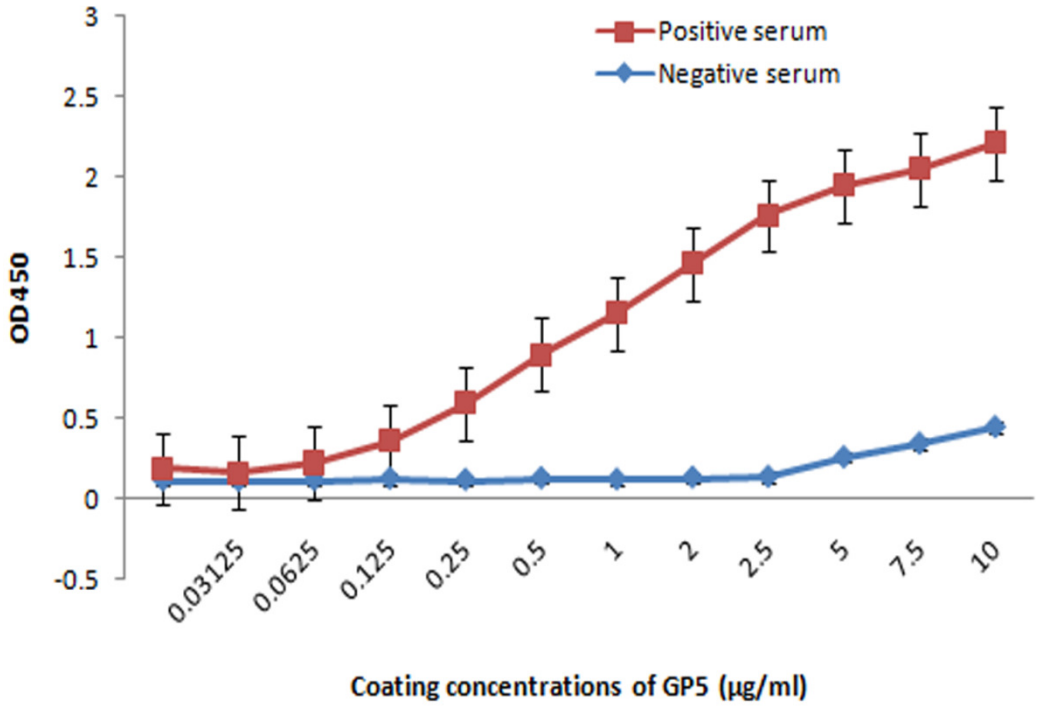

Figure 5. The immunogenicity of ΔGP5 was analyzed by indirect ELISA. ΔGP5 were diluted to 10, 7.5, 5, 2.5, 2, 1, 0.5, 0.25, 0.125, 0.0625 and 0.03125 μg/ml and coated onto ninety-six well plates, respectively. 400-fold diluted PRRSV positive and negative reference sera were used as primary antibodies in ELISA. The result showed that the wells coated with as low as 0.125 μg/ml of the interested protein could still be detected by the PRRSV positive serum specifically with a P/N value of 2.86.

ELISA and IPMA. The result shows that the wells of the ELISA plate coated with as low as 0.125 μg/ml of ΔGP5 could still be detected by the PRRSV positive serum specifically with a P/N value of 2.86, indicating that ΔGP5 maintained well bioactivity (Figure 5). IPMA indicated that mouse polyclonal antibodies produced by exposure to ΔGP5 could react apparently with MARC-145 cells infected with PRRSV as positive serum against PRRSV, suggesting that mouse polyclonal antibodies against ΔGP5 possess good immunoreactivity with natural PRRSV (Figure 6).

DISCUSSION

PRRS has devastated the swine industry by causing tremendous economic losses throughout the world and is now considered to be one of the most important diseases in countries with intensive swine industries (Jiang et al., 2009). GP5 (encoded by ORF5, a 25 kDa envelope protein) is essential in the virus particle and responsible for the development of neutralizing antibodies, antigenic variability, apoptosis and possibly antibody-dependent enhancement phenomena (Meulenberg et al., 1995; Mardassi et al. 1996; Pirzadeh and Dea, 1997; Pirzadeh and Dea, 1998; Weiland et al., 1999; Yang et al., 2000).

In this study, truncated GP5 gene lacking both the signal peptide sequence and the sequences encoding the trans-membrane regions was successfully expressed at high level in the *E. coli*-based prokaryotic system. After predicting the amino acid sequence of GP5 by DNAStar software, it has been recognized that there is one N-terminal signal peptide (31 amino acids in length) and three trans-membrane regions (residues 64-82, 87-99, and 107-129) in the gene sequence of GP5. Since the existence of the trans-membrane regions affects the expression of GP5, truncated GP5 gene lacking both the signal peptide sequence and the sequences encoding the trans-membrane regions was selected to be expressed. Through overlap PCR, GP5a and GP5b were successfully ligated with each other by a flexible linker encoding two repeated amino acid sequences (GGGGS). Western blot showed that ΔGP5 could react with PRRSV positive sera and anti-His_6 mAb. Following nickel affinity purification and refolding, ΔGP5 showed high specificity to PRRSV positive sera in ELISA. In IPMA, mouse polyclonal antibodies against ΔGP5 could react apparently with PRRSV-infected MARC-145 cells as pig positive serum to PRRSV did.

In conclusion, the genes encoding GP5a and GP5b were successfully ligated with each other through a flexible linker gene sequence by overlap PCR which

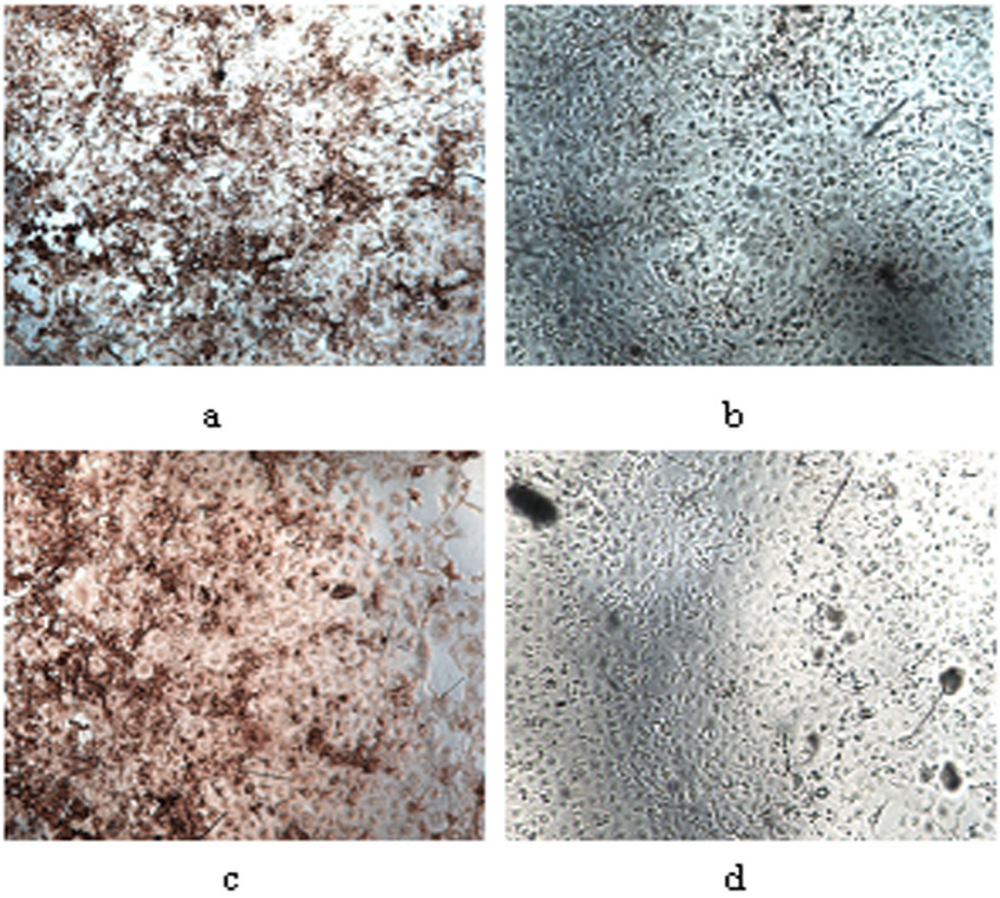

Figure 6. IPMA of the mouse polyclonal antibodies with PRRSV-infected cells. Monolayers of MARC-145 cells were grown in 96-well microtiter plates and infected by PRRSV for 48 h. Polyclonal antibodies from the vaccinated mouse were employed as the primary antibody for incubation with PRRSV-infected cells, and pig positive sera against PRRSV were used as positive control. a: Positive pig sera against PRRSV were used as primary antibody. b: Negative pig sera against PRRSV were used as primary antibody. c: Mouse polyclonal antibodies were used as primary antibody. d: Negative mouse serum was used as primary antibody.

proved to be an efficient way for the expression of certain genes. High-level expression of biologically active ΔGP5 was achieved in *E. coli*-based prokaryotic system, which provided important material for the study of PRRSV. Moreover, ΔGP5 could be used as diagnostic agent for PRRSV antibody detection in clinical field, and monoclonal antibodies against PRRSV could also be produced through immunization of mice with this protein.

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