

RESEARCH ARTICLE

# Characterization of polyclonal antibodies against nonstructural protein 9 from the porcine reproductive and respiratory syndrome virus

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**Abstract** Porcine reproductive and respiratory syndrome (PRRS) is considered to be one of the most important infectious diseases impacting the swine industry and is characterized by reproductive failure in late term gestation in sows and respiratory disease in pigs of all ages. The nonstructural protein 9 gene, *Nsp9*, encoding the RNA-dependent RNA polymerase, is generally regarded as fairly conserved when compared to other viral proteins. Antibodies against *Nsp9* will be of great importance for the diagnosis and treatment of the causal agent, PRRS virus. A study was undertaken to generate polyclonal antibodies against the immunodominant *Nsp9*. For this purpose, the *Nsp9* was expressed in *Escherichia coli* and subsequently used as an antigen to immunize New Zealand rabbits. Antiserum was identified via an indirect ELISA, and then verified based on the ability to react with both naturally and artificially expressed *Nsp9*. Results of virus neutralization test showed that this antiserum could not neutralize the PRRSV. Nevertheless, this antiserum as a diagnostic core reagent should prove invaluable for further investigations into the mechanism of PRRS pathogenesis.

**Keywords** PRRSV, *Nsp9*, expression, antibody, neutralize

## 1 Introduction

Porcine reproductive and respiratory syndrome (PRRS) is one of the most important threats to the swine industry. Since it was first identified in the USA in 1987, then in Europe in 1990<sup>[1]</sup> and later in China in 1995, the clinical manifestations of PRRS are severe reproductive failure in sows, which includes early farrowing with stillborn piglets and late term abortion, respiratory distress in piglets and growing pigs. Since 2006, a highly pathological PRRS virus (PRRSV), which is characterized by high fever and a high proportion of deaths in pigs of all ages, has emerged in some swine farms in China<sup>[2,3]</sup>.

The porcine reproductive and respiratory syndrome virus (PRRSV), the causal agent of PRRS, is a spherical, enveloped, single-stranded, positive-sense RNA virus. PRRSV belongs to the family Arteriviridae, which also includes the equine arteritis virus, the simian hemorrhagic fever virus and the lactate dehydrogenase virus. The PRRSV genome is approximately 15 kb in length and contains ten open reading frames (ORF). ORF1a and ORF1b comprise 80% of the genome in terms of size and encode the viral replicase polyproteins. In contrast, ORF2a, ORF2b and ORF 3–7 encode viral structural proteins GP2, E, GP3, GP4, GP5, GP5a, M and N.

RNA-dependent RNA polymerase (RdRp) functions as the catalytic subunit of the viral replication and transcription complex. RdRps are required for the replication and transcription of all positive stranded RNA viruses and work in concert with host proteins and sometimes other viral proteins. Indeed the RdRp domain is essential for RNA replication<sup>[4–6]</sup>. The nonstructural proteins, *Nsp9* and

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*Nsp10*, might contribute to the fatal virulence of highly pathogenic porcine reproductive and respiratory syndrome virus emerging in China<sup>[7]</sup>. The interaction of *Nsp9* with retinoblastoma protein benefits the replication of genotype 2 PRRSV *in vitro*<sup>[8]</sup>. The interaction between host Annexin A2 and viral *Nsp9* is beneficial for replication of PRRSV<sup>[9]</sup>. The DEAD-box RNA helicase 5 positively regulates the replication of PRRSV by interacting with *Nsp9 in vitro*<sup>[10]</sup>. Two residues of the SDD motif of RdRp are critical and essential for PRRSV transcription and represent a sequence variant of the GDD motif in PS RNA viruses<sup>[11]</sup>. There are 12 amino acid mutations between the common PRRSV and the highly pathogenic PRRSV<sup>[12,13]</sup>. An RT-qPCR based technique was developed which targets the viral RNA-dependent RNA polymerase gene (*Nsp9*), which is unique to genomic RNA, and has the ability to assess and compare PRRSV replication in a variety of tissues and between divergent strains, including highly pathogenic strains of considerable concern to the global swine industry<sup>[14]</sup>. A previous study<sup>[15]</sup> showed that *Cryptosporidium parvum* extract inhibits the PRRSV RNA and protein synthesis by directly inhibiting PRRSV RdRp activity, which indicates that RdRp can be used as a target protein for drug discovery and antiviral research.

*Nsp9* encodes the RdRp, and during the evolution of Arteriviridae it has been retained in PRRSV. RdRp is only produced when the virus is in the process of replication and does not reside in the host cell<sup>[16]</sup>, so the antigen targeting of the fusion protein of RdRp could discriminate the wild strain from the inactivated vaccine, which may be helpful for the diagnosis of PRRSV<sup>[17–19]</sup>.

The structure of the RdRp determined to date resembles a cupped right hand, with finger, palm and thumb domains, providing the correct geometrical arrangement of substrate molecules and metal ions at the active site for catalysis<sup>[20,21]</sup>. In contrast to other polymerases adopting this fold, RdRps are rather compact molecules, with finger and thumb domains making contact and enclosing the active site. The high-resolution structure of RdRp of positive and double strand RNA viruses revealed clear similarities despite sharing little sequence identity, suggesting an evolutionary link between the RdRp of these RNA viruses<sup>[11,20]</sup>.

PRRSV can be inhibited by a specific siRNA targeting *Nsp9*<sup>[22]</sup>, so we postulated that an antibody to *Nsp9* might inhibit PRRSV. Given that the *Nsp9* is involved in virus adsorption to host cells, an antiserum could conceivably neutralize PRRSV. To test this possibility, the relative infectivity of PRRSV preparations after incubation with antiserum was measured *in vitro* using a modified fluorescent focus neutralization assay. To explore the function and structure of RdRp, prokaryotic expression of *Nsp9* and antibody production to *Nsp9* were performed. Such detailed molecular knowledge of viral RdRps may contribute to the identification of selective inhibitors of this

important class of viral enzymes.

## 2 Materials and methods

### 2.1 Strains, vectors, virus and main reagents

In this study, *Escherichia coli* strains DH5a and BL21 (DE3), the expression vector pET-32a (+), and the plasmids pMD18-T-*Nsp9* were preserved in the laboratory. Platinum pfx DNA polymerase was purchased from Invitrogen (Invitrogen, Shanghai, China). Restriction enzymes, DNA markers, and isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) were purchased from TaKaRa (TaKaRa, Dalian, China). T4 DNA ligase and protein molecular weight markers were purchased from Thermo Scientific (Thermo Scientific, Shanghai, China). Plasmid Mini Kits and Gel Extraction Kits were purchased from OMEGA (OMEGA, Pinnacle way suit 450, Norcross, CA, USA). Ni Sepharose 6 Fast Flow was purchased from GE Healthcare (GE Healthcare, Beijing, China). The PRRSV GD-XH strain (GenBank accession No. EU624117) was isolated in our laboratory in 2007. The rabbit experiments were approved by the Institutional Animal Care and Use Committee of South China Agricultural University (Certification Number: CNAS BL0011).

### 2.2 Generation of expression plasmids pET-32a (+)-*Nsp9*

Based on the *Nsp9* sequence, the primers for the amplification of *Nsp9* were designed using the biological software Oligo 6.71 (Molecular Biology Insights, Inc., West Cascade, CO, and USA) and synthesized by Invitrogen. The forward primer was 5'-CGCAAGC TTATTAAACTGCTAGCCGCCAG-3', and the reverse primer was 5'-GCCTCGAGATCTCAGGCAATCATGAG-3' and these primers contained the *Hind* III and *Xho* I restriction sites respectively. Using the plasmids pMD18-T-*Nsp9* as the template, PCR reactions (100  $\mu$ L per tube) were performed using 10  $\mu$ L of 10  $\times$  pfx buffer, 8  $\mu$ L of dNTP mix (10 mmol  $\cdot$  L<sup>-1</sup>), 2  $\mu$ L of MgSO<sub>4</sub> (50 mmol  $\cdot$  L<sup>-1</sup>), 2  $\mu$ L of Platinum pfx DNA polymerase, 2  $\mu$ L of each primer (10  $\mu$ mol  $\cdot$  L<sup>-1</sup>), 1  $\mu$ L of DNA template, and 73  $\mu$ L of water. The conditions of the PCR amplification were initial denaturation at 94°C for 3 min, followed by 30 consecutive cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 68°C for 120 s, and then a final extension at 68°C for 10 min. The amplified products were analyzed by electrophoresis on a 1% agarose gel. The PCR products of *Nsp9* were digested by *Hind* III and *Xho* I and ligated into the digested expression vector, pET-32a (+). The ligation mixture was transformed into competent *E. coli* DH5a cells. The positive colony was identified by double restricted enzymatic digestion analysis and sequencing analysis.

The extracted positive plasmids were transformed into competent *E. coli* strain BL21 (DE3).

### 2.3 Protein expression, purification, and polyclonal antibody production

The pET-32a-*Nsp9* positive cloning strains were inoculated into 5 mL of LB/Amp liquid medium and cultivated overnight. The 50  $\mu$ L cultures were inoculated with 50 mL of LB/Amp for activation. When the bacterium reached the logarithmic phase (at OD<sub>600</sub> 0.5–0.6), IPTG was added in order to induce the expression of *Nsp9*. The level of protein expression was analyzed by SDS-PAGE. The non-induced and control cultures were analyzed in parallel. To increase the production of the recombinant proteins, the expression conditions, including the duration of induction, the concentrations of IPTG, and the composition of the binding buffer (with compounds 20 mmol·L<sup>-1</sup> Na<sub>3</sub>PO<sub>4</sub>, 0.5 mol·L<sup>-1</sup> NaCl, 20 mmol·L<sup>-1</sup> imidazole, 0.5%  $\beta$ -Mercaptoethanol, 1.3 mol·L<sup>-1</sup> urea, 0.5% Tween 20, 3% glycerol, 1% SDS, pH 7.4; without compounds 20 mmol·L<sup>-1</sup> Na<sub>3</sub>PO<sub>4</sub>, 0.5 mol·L<sup>-1</sup> NaCl, 20 mmol·L<sup>-1</sup> imidazole, pH 7.4) were optimized. *Nsp9* was purified by Ni Sepharose 6 Fast Flow. The samples from the Ni-column were assessed by SDS-PAGE. The purified protein was used to immunize the New Zealand rabbits to raise antibody. New Zealand rabbits were immunized intraperitoneally (i.p.) with 100  $\mu$ g *Nsp9* plus Freund's complete adjuvant. Four weeks after priming, rabbits were boosted four times in a 2 weeks interval by i.p. injection of 100  $\mu$ g *Nsp9* per rabbit. After 9 weeks, antiserum was collected from the rabbits that had been placed under terminal halothane anesthesia.

### 2.4 Enzyme-linked immunosorbent assay

Wells of 96-well plates were each coated with 100  $\mu$ L of purified *Nsp9* (1  $\mu$ g·mL<sup>-1</sup>) at 4°C overnight, blocked for 2 h at 4°C with 5% skimmed milk in TBST (20 mmol·L<sup>-1</sup> Tris-HCl, 150 mmol·L<sup>-1</sup> NaCl, 0.05% Tween20), and incubated with antiserum for 1 h at 37°C. then the wells were incubated for 30 min with goat anti-rabbit antibodies labeled with horseradish peroxidase, washed four times with PBS(137 mmol·L<sup>-1</sup> NaCl, 2.7 mmol·L<sup>-1</sup> KCl, 10 mmol·L<sup>-1</sup> Na<sub>2</sub>HPO<sub>4</sub>, 2 mmol·L<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>), incubated with TMB (3,3',5,5'-Tetramethylbenzidine) substrate for 10 min and the OD<sub>450</sub> of the solutions measured.

### 2.5 Western blot analysis

Western blot was used to evaluate the expression of *Nsp9*. The purified samples were subjected to SDS-PAGE with a 12% gel and transferred to a nitrocellulose membrane. No/*Nsp9* specific antibody binding sites were blocked with 5% skimmed milk in TBST overnight at 4°C. The membranes were incubated with a 1:1000 dilution of rabbit antiserum to *Nsp9* at 37°C for 1 h and then washed four times with TBST

(5 min each). The blot was probed with a 1:15000 dilution of IRDye 800CW Goat anti-rabbit IgG (H + L) secondary antibody (LI-COR) (LI-COR, Lincoln, Nebraska, USA) for 1 h in the dark at 37°C. Then the membrane was washed five times with TBST and then twice with TBS (20 mmol·L<sup>-1</sup> Tris-HCl, 150 mmol·L<sup>-1</sup> NaCl). The blot was analyzed using the Odyssey Infrared Imaging System (LI-COR) (LI-COR, Lincoln, Nebraska, USA).

### 2.6 Indirect fluorescent antibody assay

An indirect fluorescent antibody assay was performed for the detection of viral antigens. Marc-145 cells were infected with PRRSV GD-XH strain at a MOI (multiplicity of infection) of 0.1. At 48 h post inoculation (h.p.i.), infected cells were washed five times with PBS, followed by fixation in 4% formaldehyde. The cells were incubated with the antiserum (1:100 dilutions) at 37°C for 2 h. After being extensively washed with PBS, the cells were incubated for 1 h with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (1:1000 dilution). The cells were washed five times with PBS. Finally, the fluorescence was observed under an Olympus (Olympus, Tokyo, Japan) inverted fluorescence microscope.

### 2.7 Neutralization test

The ability of polyclonal antibody to neutralize PRRSV was examined using a modified fluorescent focus neutralization assay. Briefly, 50  $\mu$ L of four twofold dilutions of purified polyclonal antibody, normal rabbit serum in MEM (minimum essential medium) supplemented with 5% fetal bovine serum or just MEM supplemented with 5% fetal bovine serum were added to individual wells of a 96-well plate. An equal volume of virus, at a concentration of 100 TCID<sub>50</sub> (Tissue culture infective dose 50) per milliliter, was added to each well and after 1 h at 37°C, the total mix was transferred to separate wells of a 96-well plate containing a confluent monolayer of Marc-145 cells. After 24 h, the monolayers were fixed with 4% formaldehyde at ambient temperature for 1 h and the infected cells were detected based on reactivity with antiserum. The fluorescent foci were observed and counted using an inverted fluorescence microscope. The percentage of PRRSV neutralization was defined according to the following formula: ((PNV-PNS)/PNV)  $\times$  100, where PNV was the average number of plaques made by the untreated PRRSV and PNS was the average number of plaques made by the PRRSV after exposure to antiserum.

### 2.8 Statistical analysis

Generally, experiments were performed with at least three independent experiments, with three technical replicates for each experiment. Results were analyzed by GraphPad Prism (GraphPad Software, San Diego, CA, USA) using

Student's *t*-test. The threshold for statistically significant differences between measurements was  $P = 0.05$ .

### 3 Results

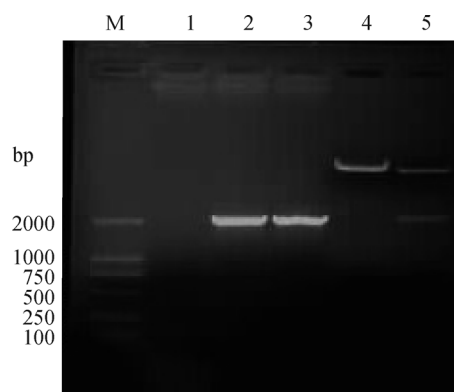
#### 3.1 Gene amplification and construction of expression plasmids

*Nsp9* was amplified using pMD-18T-*Nsp9* plasmids as template. The electrophoretic analysis of the amplified products showed that specific bands of about 1929 bp had been amplified (Fig. 1). The PCR product of *Nsp9* was digested by *Hind* III and *Xho* I, and inserted into the pET-32a (+) plasmid in order to construct the expression plasmids. The restriction digestion analysis showed that the pET-32a (+)-*Nsp9* expression plasmid had been successfully constructed.

#### 3.2 Optimized time of inducing expression by SDS-PAGE analysis

After being transformed into BL21 competent cells, the pET-32a-*Nsp9* plasmids were induced with IPTG, and SDS-PAGE electrophoresis was performed to analyze products. In contrast to the empty expression vector pET-32a (+), the bacteria containing the recombinant pET-32a (+)-*Nsp9* gave an 82 kDa band that appeared at the expected size for the recombinant protein (Fig. 2).

Judging from the effect of different induction times, the quantity of protein reached its peak with a 6 h induction. As induction time increased, the expression quantity

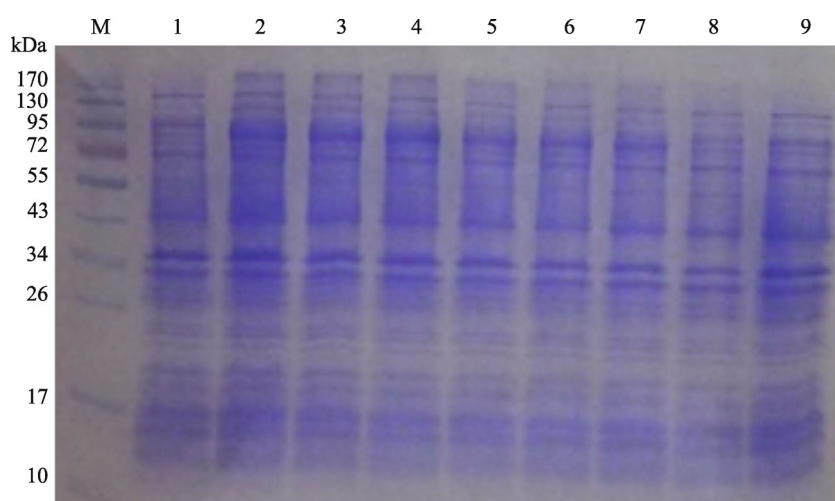


**Fig. 1** *Nsp9* amplification results and the double restricted enzymatic digestion of pET-32a (+)-*Nsp9*. M, Markers; 1, Negative control; 2, *Nsp9* PCR reactions; 3, Restriction enzyme digestion of the *Nsp9* PCR reactions; 4, Restriction enzyme digestion of the pET-32a (+); 5, Restriction enzyme digestion of pET-32a-*Nsp9*.

remained almost constant; so a 6 h induction time for the expression of the recombinant protein was selected.

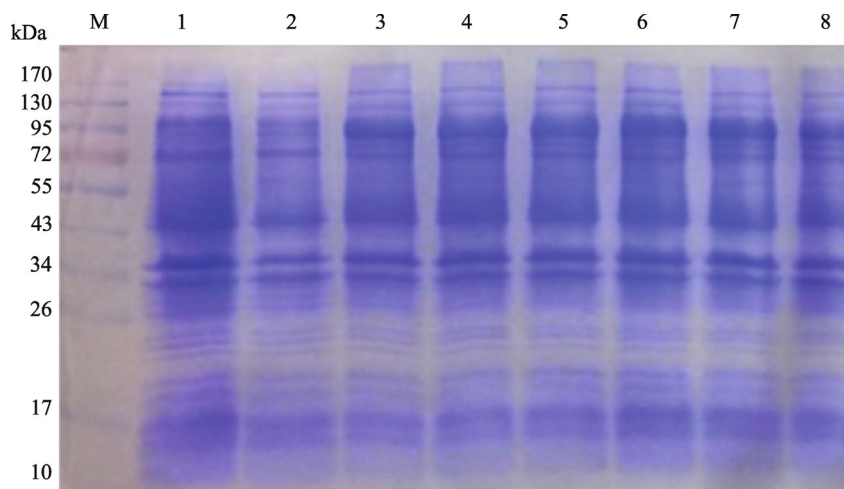
#### 3.3 SDS-PAGE analysis of the different concentration of IPTG induction

Positive recombinant bacteria were cultured at 37°C until the OD<sub>600</sub> reached 0.4–0.6, and then final concentrations of 0.2, 0.4, 0.6, 0.8, 1.0 and 2.0 mmol·L<sup>-1</sup> IPTG were added to the bacteria for induction at 37°C for 4 h. The results of SDS-PAGE analysis are shown in Fig. 3, and 0.8 mmol·L<sup>-1</sup> IPTG was selected as the final induction concentration.



**Fig. 2** SDS-PAGE results of the different times of IPTG induction of fusion protein expression. M, Protein markers; 1, Recombinant plasmid pET-32a (+)-*Nsp9* before induction; 2, Recombinant plasmid pET-32a (+)-*Nsp9* induced after 6 h; 3, Recombinant plasmid pET-32a (+)-*Nsp9* induced after 5 h; 4, Recombinant plasmid pET-32a (+)-*Nsp9* induced after 4 h; 5, Recombinant plasmid pET-32a (+)-*Nsp9* induced after 3 h; 6, Recombinant plasmid pET-32a (+)-*Nsp9* induced after 2 h; 7, Recombinant plasmid pET-32a (+)-*Nsp9* induced after 1 h; 8, pET-32a (+) before induction; 9, pET-32a (+) induced after 6 h.





**Fig. 3** SDS-PAGE results of different concentrations of IPTG induction of fusion protein expression. M, Protein markers; 1, pET-32a(+) before being induced; 2, pET-32a(+) IPTG induction concentration of  $0.2 \text{ mol} \cdot \text{L}^{-1}$ ; 3, pET-32a(+)-*Nsp9* IPTG induction concentration of  $0.2 \text{ mol} \cdot \text{L}^{-1}$ ; 4, pET-32a(+)-*Nsp9* IPTG induction concentration of  $0.4 \text{ mol} \cdot \text{L}^{-1}$ ; 5, pET-32a(+)-*Nsp9* IPTG induction concentration of  $0.6 \text{ mol} \cdot \text{L}^{-1}$ ; 6, pET-32a(+)-*Nsp9* IPTG induction concentration of  $0.8 \text{ mol} \cdot \text{L}^{-1}$ ; 7, pET-32a(+)-*Nsp9* IPTG induction concentration of  $1.0 \text{ mol} \cdot \text{L}^{-1}$ ; 8, pET-32a(+)-*Nsp9* IPTG induction concentration of  $2.0 \text{ mol} \cdot \text{L}^{-1}$ .

### 3.4 Solubility analysis of the protein

Equal volumes of sonicated bacteria in the supernatant and pellet were tested by SDS-PAGE electrophoresis (Fig. 4). In the solubility analysis, soluble proteins were found in the supernatant, but also in the pellet. However, the amount of pellet was greater, so the target protein was presumed to be insoluble.

The proteins were highly expressed in the supernatant from the *Nsp9*-producing cells that were incubated with a binding buffer containing the following compounds: 0.5%  $\beta$ -mercaptoethanol,  $1.3 \text{ mol} \cdot \text{L}^{-1}$  urea, 0.5% Tween 20, 3% glycerol, 1% SDS, while proteins were rarely expressed in the supernatant from the *Nsp9*-producing cells that were incubated with binding buffer without these compounds (Fig. 4).

### 3.5 Purification of expressed protein

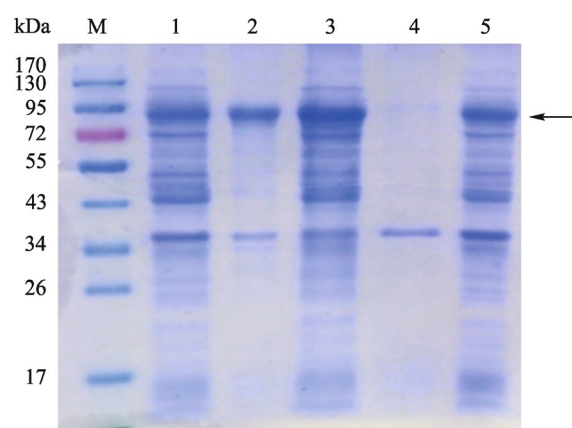
The recombinant *Nsp9* from the induced bacterial cells were purified using Ni Sepharose 6 Fast Flow gravity-flow columns and the purified protein is shown in Fig. 5.

### 3.6 ELISA titer of antiserum

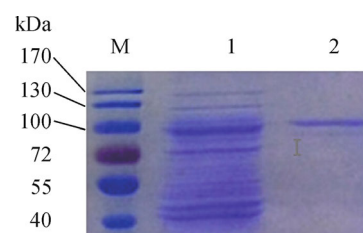
The *Nsp9* specific antibody responses were determined using an indirect ELISA, with purified recombinant *Nsp9* used as the antigen. The results indicated that antibody titer was 1:1024000 (Table 1).

### 3.7 Western blot identification of expressed protein

The purified proteins were used to immunize New Zealand



**Fig. 4** Results of the solubility analysis of the fusion protein. M, Protein markers; 1, Bacterial supernatant after bacterial sonication; 2, Precipitate after bacterial sonication; 3, Supernatant after bacterial sonication with added compounds; 4, Precipitate after bacterial sonication with added compounds; 5, Whole bacteria after bacterial sonication.

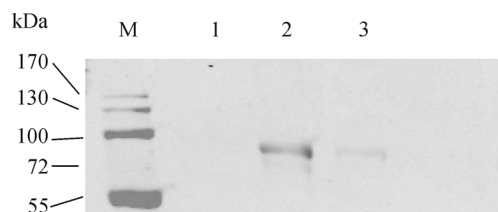


**Fig. 5** SDS-PAGE analysis of the purification of *Nsp9* proteins. M, Markers; 1, Flow through; 2, Elution with imidazole of  $200 \text{ mmol} \cdot \text{L}^{-1}$ .

**Table 1** ELISA detection of antiserum

| Antibody  | Antigen/( $\mu\text{g} \cdot \text{mL}^{-1}$ ) |       |       |       |                |       |       |       |
|-----------|--|-------|-------|-------|----------------|-------|-------|-------|
|           | Test serum                                     |       |       |       | Negative serum |       |       |       |
|           | 10   | 5     | 2.5   | 1.25  | 10             | 5     | 2.5   | 1.25  |
| 1:500     | 2.970  | 2.943 | 2.93  | 2.884 | 0.494          | 0.383 | 0.175 | 0.087 |
| 1:1000    | 2.842  | 2.831 | 2.822 | 2.816 | 0.265          | 0.205 | 0.104 | 0.071 |
| 1:2000    | 2.793  | 2.785 | 2.783 | 2.773 | 0.143          | 0.123 | 0.087 | 0.062 |
| 1:4000    | 2.779  | 2.783 | 2.681 | 2.744 | 0.137          | 0.113 | 0.073 | 0.063 |
| 1:8000    | 2.789  | 2.720 | 2.710 | 2.778 | 0.124          | 0.104 | 0.060 | 0.058 |
| 1:16000   | 2.727  | 2.704 | 2.703 | 2.703 | 0.080          | 0.075 | 0.065 | 0.050 |
| 1:32000   | 2.712  | 2.703 | 2.706 | 2.613 | 0.070          | 0.061 | 0.058 | 0.054 |
| 1:64000   | 2.700  | 2.700 | 2.652 | 2.392 | 0.062          | 0.056 | 0.057 | 0.045 |
| 1:128000  | 2.681  | 2.670 | 2.629 | 2.099 | 0.062          | 0.055 | 0.054 | 0.045 |
| 1:256000  | 2.298  | 2.276 | 2.014 | 1.371 | 0.061          | 0.054 | 0.055 | 0.047 |
| 1:512000  | 1.757  | 1.723 | 1.490 | 0.769 | 0.057          | 0.057 | 0.054 | 0.047 |
| 1:1024000 | 1.165  | 1.097 | 0.904 | 0.470 | 0.055          | 0.054 | 0.041 | 0.048 |

rabbits. After 4 injections, the antiserum was collected by arterial sampling and stored at  $-70^{\circ}\text{C}$ . The results showed that the recombinant *Nsp9* that was expressed in the *E. coli* exhibits a polyclonal antibody specificity binding reaction (Fig. 6).



**Fig. 6** Western-blot identification of fusion protein. M, Protein markers; 1, Blank control; 2, Precipitate after bacterial sonication; 3, Supernatant after bacterial sonication.

### 3.8 Immunofluorescent detection of *Nsp9* with antiserum

To determine whether antiserum could be used for the immunofluorescent detection of the *Nsp9* in virus-infected Marc-145 cells, monolayers of Marc-145 cells were infected with PRRSV-XH-GD at a MOI of 0.1 at 48 h.p.i. As shown in Fig. 7, the intracellular *Nsp9* could be detected by using antiserum; the green fluorescence was in the cell nucleus.

### 3.9 Neutralization assay

As the RdRp protein is involved in virus duplication, if the antiserum binds to this protein, antiserum could conceivably neutralize PRRSV. To test this possibility, the relative infectivity of a PRRSV XH-GD preparation after incubation with antiserum was measured *in vitro* using a modified fluorescent focus neutralization assay. When

compared to normal rabbit serum used as a negative control, there was no statistically significant reduction in virus infectivity detected with pre-exposure of the virus to the any dilution of anti-*Nsp9* antiserum. Thus, anti-*Nsp9* antiserum was not able to neutralize PRRSV.

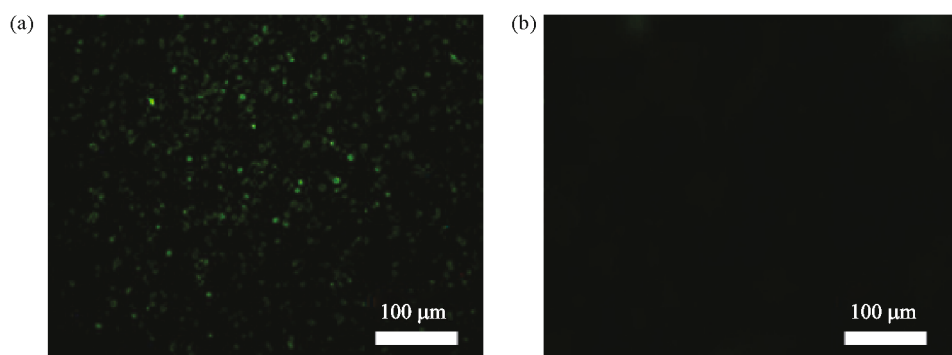
## 4 Discussion

Like all plus-strand RNA viruses, PRRSV carries an RdRp enzyme that functions as the catalytic subunit of the viral replication and transcription complex, directing viral RNA synthesis in concert with other viral or cellular proteins<sup>[23]</sup>.

ORF1b is the most conserved sequence found in the arterivirus genomes and encodes key enzymes involved in RNA templated RNA synthesis, i.e., RdRp (*Nsp9*) and RNA helicase (*Nsp10*), which were originally identified by comparative sequence analysis. RdRp functions as the catalytic subunit of the viral replication and transcription complex. RdRps are required for the replication and transcription of all positive-stranded RNA viruses and work in concert with host proteins and sometimes other viral proteins. Indeed, the RdRp domain is essential for RNA replication.

The *Nsps* are synthesized when the virus is replicating, so healthy animals that are vaccinated with inactivated virus would only be expected to elicit antibodies to structural proteins, so the anti-*Nsp* antibodies in animal serum is a clear indication of virus activity<sup>[24]</sup>.

Among the nonstructural genes of PRRSV, *Nsp9* contained within ORF1b is the most conserved region of Arteriviridae genomes and encodes RdRp. However, there are no data on the structure, function or *in vitro* activity of the RdRps of arteriviruses. In this study, we report the expression of *Nsp9* in *E. coli* and the purification of PRRSV *Nsp9*/RdRp. A gene encoding the full-length



**Fig. 7** Assay of polyclonal antiserum with indirect fluorescence antibody, The green fluorescence was analyzed by fluorescence microscopy using a 100 × objective. absorption wavelength was 490–495 nm, emission wavelength was 520–530 nm. (a) PRRSV infected Marc-145 cells; (b) normal Marc-145 cells.

PRRSV *Nsp9*/RdRp, was cloned in the pET-32a(+) expression vector for expression in *E. coli*.

PRRSV is usually diagnosed by pathological examination and clinical signs, but in early stages of the disease, it is difficult to recognize. Specific antibodies have a wide range of valuable applications in diagnostics and research. The polyclonal antibodies produced in this study can be used for diagnostic and epidemiological investigation using ELISA, Western blot and IFA detection assays, for studying the mechanism of pathogenesis by detecting the virus entry and location using the immunoprecipitation, immunofluorescence, immunogold assays and immunohistochemistry, and also for developing a possible subunit vaccine and therapeutic medicine.

The results presented here demonstrate that the anti-*Nsp9* polyclonal antibodies are specific, and can be used as valid tools for further research on the mechanism of PRRSV pathogenesis and development of an immunodiagnostic assay.

In the expression of *Nsp9*, different vectors and competent cells were also compared, but the expression level did not increase greatly. It is possible that the hydrophobic amino acid content influenced its accumulation. Here the whole *Nsp9* sequence was cloned and expressed and these anti-*Nsp9* polyclonal antibodies will be a useful tool to control PRRSV.

The *Nsp9*/RdRp-His protein was purified to near homogeneity using a Ni-NTA chromatography column. PRRSV *Nsp9*/RdRp-His eluted at its expected molecular size, demonstrating its monomeric state.

RdRp obtained by prokaryotic expression needs further research. Such detailed molecular knowledge of viral *Nsp9* may also contribute to the identification of selective inhibitors of this important class of viral protein.

A recent study showed that structural proteins, GP5 and GP6<sup>[25]</sup>, have a neutralizing ability, however, the antiserum obtained did not inhibit PRRSV. The antiserum could react with the Marc-145 cells infected with PRRSV, but could

not neutralize the PRRSV. It is possible that there is no neutralizing epitope does in *Nsp9*.

The ability to purify an enzymatically active PRRSV RdRp will facilitate its further functional and structural characterization and will provide insight into the molecular basis of RNA polymerization<sup>[26]</sup>. Such detailed molecular knowledge of viral RdRps may also contribute to the identification of selective inhibitors of this important class of viral enzymes.

## 5 Conclusions

Polyclonal antibodies against nonstructural protein 9 from the porcine reproductive and respiratory syndrome virus were generated, both naturally and artificially expressed *Nsp9* were able to react with it.

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

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