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Preparation and characterisation of monoclonal antibodies against the N protein of the SHpd/2012 strain of porcine epidemic diarrhoea virus

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ABSTRACT: Porcine epidemic diarrhoea is caused by the porcine epidemic diarrhoea virus, and is a highly contagious disease which affects the intestines of new-born piglets resulting in intense diarrhoea. Historically, the virus has caused enormous economic losses in the pig industry. In particular, the emergence of new epidemic strains means there is a pressing need for prevention and control of the disease. Owing to the specificity of the monoclonal antibodies now available, study of the pathogenesis, immune mechanisms and new diagnostic methods can be performed. In this study, 13 strains of positive hybridoma cells were prepared by immunising mice with purified whole porcine epidemic diarrhoea virus, and analysis was performed using ELISA and Western blotting. Three cell strains specifically recognised the porcine epidemic diarrhoea virus nucleocapsid protein (N protein). In this study, we report the characterisation of effective tools for the establishment of porcine epidemic diarrhoea virus diagnostic methods and we have specifically generated primary antibodies for ELISA, IFA, test strip and Western blotting.

Keywords: porcine epidemic diarrhoea virus; PEDV; nucleocapsid protein; N protein; monoclonal antibodies; MAbs

Porcine epidemic diarrhoea virus (PEDV), which is classified as a member of the alpha coronaviruses, causes porcine epidemic diarrhoea (PED) (Song and Park 2012). The performance of the infected pigs is affected by clinical symptoms including reduced food intake, weight loss, acute watery diarrhoea and frequent vomiting (Song and Park 2012). The virus is transmitted by the faecal-oral route and infects pigs of all ages. The rate of infection is particularly high for new-born piglets, and due to watery diarrhoea, the rate of mortality is as high as 90–100% (Li et al. 2012a). PED was first observed in Europe in 1971 (Oldham 1972), and many countries and regions with developed pig industries have also reported this disease, including South Korea, China, Japan, the Philippines and Thailand (Lei et al. 2015). In China, PED was reported in 1976 for the first time (Cheng et al. 1992). In the second half of 2010, there was again a large-scale outbreak of PED in China, and subsequently, the pig industry suffered significant economic losses (Li et al. 2012a; Li et al. 2012b; Sun et al. 2012; Gao et al. 2013; Wang et al. 2013; Zhang et al. 2013; Li et al. 2014; Chen et al. 2015; Li et al. 2016; Sun et al. 2016). The PEDV genome is 28 033 nt in size. The genome encodes four major structural proteins, including the spike

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(S) protein; the small envelope (E) protein; membrane glycoprotein (M); and the nucleocapsid (N) protein, as well as four non-structural proteins (1a, 1b and 3a, 3b) (Wu et al. 2012). Among them, the N protein of PEDV accounts for the largest proportion of the protein structure of the virion, which can reach a high level of expression in infected cells.

Pigs are able to produce high levels of PEDV N protein antibodies in the early stage of PEDV infection. Given the high conservation of the N protein, it seems rational to use the N protein as a basis on which to establish molecular diagnostic tools for PEDV. We predicted that if we use whole PEDV virion to infect mice, we would probably obtain monoclonal antibodies against the N protein. Such antibodies could then be used for the establishment of detection kits, e.g., ELISA, IFA and test strip. Thus, in this study, we used the purified whole PEDV virus as an immunogen in the lymphocyte hybridoma technique to prepare mAbs against the PEDV N protein. Our findings provide a foundation for further characterisation of PEDV N protein function in basic research.

MATERIAL AND METHODS

Animals. Seven-week-old female BALB/c mice (Shanghai Silaike experimental animal Co. Ltd) were raised in the laboratory with natural light, sterile water and special feed. The experiments on animals were conducted in accordance with local ethical committee laws and regulations as regards care and use of laboratory animals. All studies were performed with the approval of the guidelines of Institutional Animal Care and Use Committee of Shanghai (A2018056).

Virus strain, cells, plasmids and antisera. The currently circulating PEDV strain SHpd/2012 was isolated and passaged in Vero cell lines supplemented with 5 μg/ml trypsin (Sigma-Aldrich, St Louis, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Carlsbad, USA) supplemented with 10% foetal bovine serum (Gibco, Carlsbad, USA). The PEDV antisera were previously produced from pigs immunised with strain SHpd/2012 and were stored in our laboratory. The antisera used in this study were collected one day before and 14 and 21 days after immunisation. SP 2/0 myeloma cells, pGEX 6P-1 plasmids and pGEX 6P-N recombinant plasmids were all acquired from the Shanghai Jiao Tong University Laboratory of Zoonotic Diseases.

Purification of PEDV. PEDV was inoculated onto healthy Vero cells at a multiplicity of infection (MOI) of 0.01 and cultured at 37 °C and 5% CO_2. After 24 h, when the cell lesions reached 80%, cells were transferred to the –80 °C freezer (FORMA 900 Series, Thermo, USA), and were only freeze-thawed a maximum of three times at 8000 *g* for 15 min at 4 °C. The supernatant was left to precipitate and was centrifuged at 4 °C at 10 000 *g* for 15 min to allow collection. A total of 60 ml of the supernatant containing the virus were centrifuged. In gradient ultracentrifugation (Beckman FC 500 MPL, Beckman coulter, USA), the sample was first subjected to 100 000 *g* for 2 h at 4 °C. Following centrifugation, the supernatant was discarded carefully, and phosphate-buffered saline (PBS) was used to suspend the sediment by centrifugation at 200 000 *g* for 1 h at 4 °C. The supernatant was discarded carefully. The sediment was resuspended and packaged and finally optical density (OD) was measured at 280 nm with a spectrophotometer (Eppendorf, Hauppauge, USA). Next, the amount of viral protein was calculated, and the suspended liquid was stored at –80 °C for later use for both animal experiments and virus-specific ELISA.

Production of mAbs. (1) Immunisation procedure. The purified PEDV suspension was mixed 1 : 1 with Freund's complete adjuvant. Next, five BALB/c mice were intraperitoneally (*i.p.*) injected with the emulsified PEDV particles at a concentration of 100 μg per mouse. The interval between each injection was two weeks. After the third injection, spleen cells were removed and fused with SP2/0 cells to form hybridomas under aseptic conditions. (2) Cell fusion and cloning. The immunised mice sacrificed on the third day after booster immunisation and were immersed in 75% alcohol for 5 min. The spleens of the mice were aseptically removed and placed in a dish containing 5 to 10 ml of DMEM medium. After gently rinsing, the spleens were transferred to another dish containing about 20 ml of DMEM culture medium. The DMEM culture medium was then transferred into the spleen with a sterile syringe, and cells were rinsed inside the spleen several times until the spleen became white. Then, the spleen cells were transferred into a 50 ml centrifuge tube and centrifuged at 941 g for 10 min. The

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supernatant was discarded and the pellet was resuspended as back-up. SP 2/0 myeloma cells were fused with the spleen cells of the immunised mice with PEG1450 (Dow Chemical, USA) according to a ratio of 1 : 6~8. The fused cell suspension was added to a 96-well culture plate which had been seeded with feeder cells, and cultured at 37 °C and 5% CO_2 in an incubator. An indirect enzyme-linked immunosorbent assay (ELISA) and indirect immunofluorescence assay (IFA) were used to screen when 1/4~1/3 of the bottom of the plate was confluent. When the results of three tests were positive, cells were used for cloning using the limited dilution method (Wang et al. 2016). (3) Expansion of the tumour cell strains. Positive hybridoma cell strains were expanded from 96-well plates into T25 flasks, and were then seeded into 24-well plates, six-well plates and T25 flasks intermediated with half of liquid. The medium was changed as follows: from 20% HAT (Sigma-Aldrich, St Louis, USA) selective medium to 20% HT (Sigma-Aldrich, St Louis, USA) selective medium, to 20% foetal bovine serum (FBS, Gibco, Carlsbad, USA) culture media and finally with 10% FBS medium and then passaged.

Detection of IFA in the supernatant of hybrid tumour cells. Vero cells were inoculated with PEDV 24 h after infection, and the cell lines were fixed with 80% ice-cold ethanol. The serum from unimmunised mice, and Vero cells without PEDV infection were used as negative controls. The supernatant from the hybridoma cell lines was used as the primary antibody and incubated for 1 h at 37 °C, then washed three times with PBS for 5 min/wash. Then, an FITC-labelled goat anti-mouse IgG was added as secondary antibody (1 : 2000 dilution) and incubated at 37 °C for 45 min. The samples were washed three times with PBS (5 min/wash), and were then viewed under an inverted fluorescence microscope and photographed.

Induced expression of the recombinant N protein. PGEX 6P-N plasmids were induced to express the recombinant N protein of PEDV. A volume of 50 µl *E. coli* BL21 was transformed with 1 µl pGEX 6P-N plasmids and put on ice for 30 min. The EP tube was heat-shocked for 90 s in a 42 °C water bath, then slowly put on ice for 2 min after removal from the water bath. The tube was then mixed with 1 ml LB medium without antibiotics, with shaking at 37 °C for 1 h at 200 *g* in a sterile clean Taichung. Afterwards, the tube was centrifuged at 3000 *g* for 2 min, and the supernatant was discarded leaving approximately 100 µl of suspended sediment as supernatant. This sediment was then used to coat a LB plate containing ampicillin, placed in a bacteria incubator set to 37 °C, inverted and observed 12 h later. When the individual colonies flattened out, single colonies were chosen and placed into 4-ml tubes containing LB culture medium with ampicillin. When the concentration of the bacteria reached an OD about 0.6~0.8, 100 µl of the bacterial liquid were used to perform a PCR identification and to confirm the identity of the strains. After PCR verification PCR, isopropyl β-D-thiogalactoside (IPTG) (0.1 mM) was used to induce gene expression, and the strain identity was finally confirmed by sequencing.

Western blot analysis of recombinant N protein expression. To verify induced recombinant N protein expression, the recombinant N protein was purified by gel recovery as an antigen, and positive serum containing anti-PEDV antibodies were used as the primary antibodies for a Western blot analysis. Specific operating procedures were as follows: the N protein and marker were separated by electrophoresis and transferred onto a nitrocellulose (NC) membrane. After 2~3 h of blocking in skimmed milk at room temperature, the membranes were exposed to the supernatant of hybrid tumour cells for 1~2 h at room temperature and were washed five times with PBS (10 mM, pH 7.4) containing 0.1% Tween-20 for 3 min/wash. Then, the membranes were immersed in secondary horseradish peroxidase (HRP, Sigma-Aldrich, St Louis, USA) conjugated anti-mouse antibodies (1 : 6000). After washing, the membranes were finally detected using 3,3'-diaminobenzidine (DAB).

After the activation of the pGEX-6p-N recombinant plasmid, a single positive colony was selected to for induction with 1 mM of IPTG. The expressed products were used for subsequent ELISAs and Western blots.

Indirect ELISA detection of anti-PEDV antibodies in the hybrid tumour cell supernatant. The following procedures were used for the ELISA detection of anti-PEDV antibodies: (1) coating: polystyrene microtiter plates were coated with 100 µl of the viral N protein (5 µg/ml) in 50 mM carbonate buffer (pH 9.6) overnight at 4 °C and washed five times with PBS (50 mM, pH 7.4) with 0.05% Tween-20 for 3 min/wash; (2) blocking: 5% skimmed milk at 37 °C for 1~2 h and washing as

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described above; (3) primary antibodies: unmeasured hybridoma supernatants were used. Each plate contained the empty vector pGEX-6P-1 induced products and GST as the negative control and were incubated at 37 °C for 1 h with washes as described above; (4) secondary antibodies: HRP-

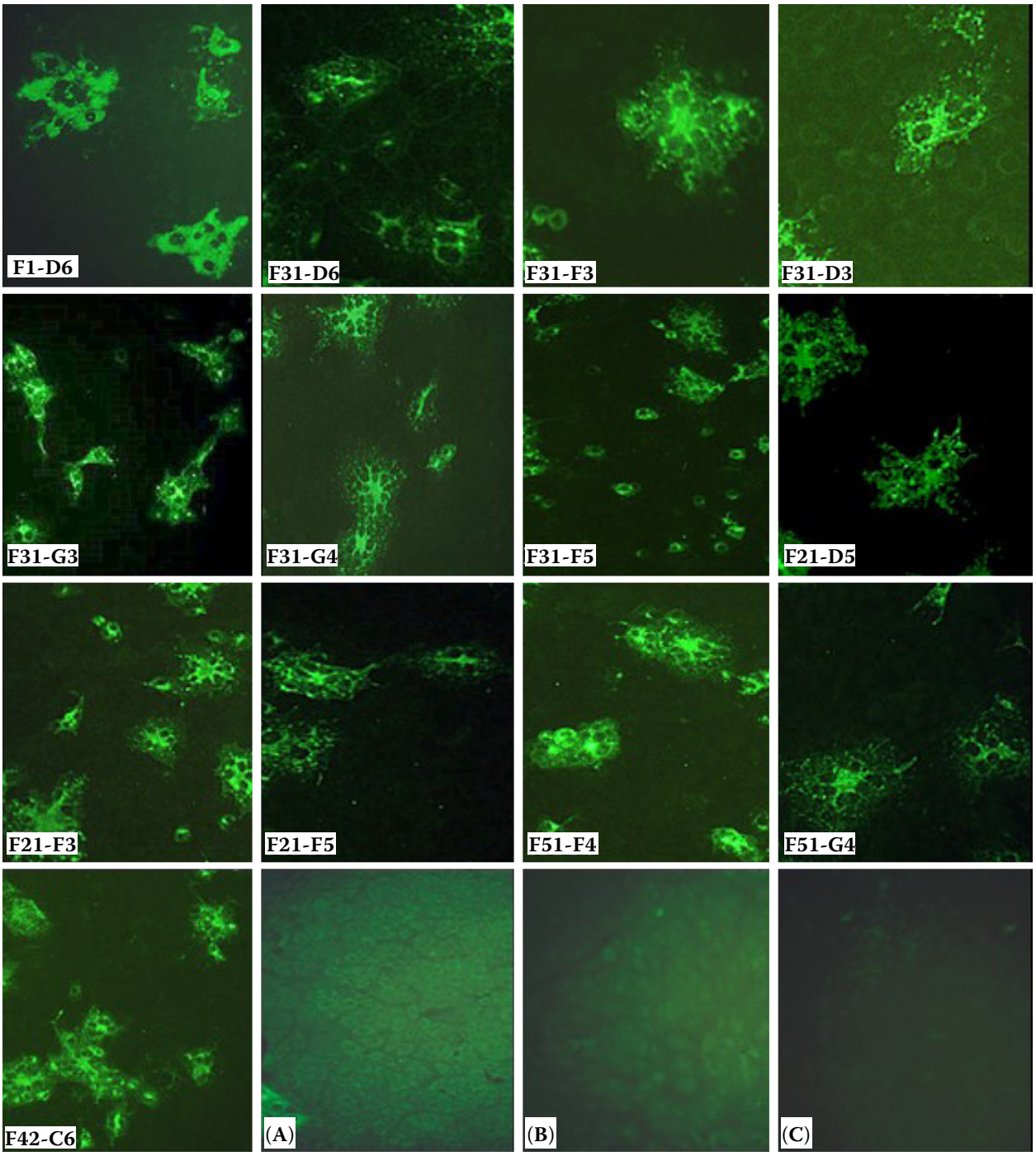

Figure 1. Immunofluorescence assay analysis of porcine epidemic diarrhoea virus-infected Vero cells. (**A**) Positive result , using mouse serum as primary antibodies; (**B**) negative result, using hybridoma supernatants from Vero cells that were not infected with porcine epidemic diarrhoea virus as primary antibodies; (**C**) the negative result, using negative mouse serum as primary antibodies; **F1-D6**, **F31-D6**, **F31-F3**, **F31-D3**, **F31-G3**, **F31-G4**, **F31-F5**, **F21-D5**, **F21-F3**, **F21-F5**, **F51-F4**, **F51-G4** and **F42-C6** panels correspond to hybridoma supernatants containing the respective primary antibodies

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conjugated anti-mouse antibodies (1 : 10 000 dilution) were added, and the plates were incubated for 0.5 h at 37 °C and washed as described above; (5) colour solution: to develop the colour, 100 µl of TMB were added to each well, and the plates were incubated for 10~15 min at room temperature out of direct light; and (6) stopping solution: the addition of 50 µl H_2SO_4 (2 M) was used to stop the colour reaction, and OD was determined at 450 nm using a microplate reader.

Western blot of the hybrid tumour cell supernatant for PEDV N protein identification. The recombinant PEDV N protein was used as an antigen to determine which supernatants from the hybrid tumour cell lines were able to bind to the N protein in Western blot analysis. Similarly, using the purified virus as an antigen, the same ultracentrifugation processed Vero cell supernatant was used as a negative control. Western blotting was used to analyse the supernatants of the hybrid tumor cell lines which contained antiboies against the PEDV N protein, and the characteristics of the monoclonal antibodies were further verified. The procedures were as follows: the N protein and marker were separated by electrophoresis and transferred onto nitrocellulose (NC) membranes. After 2~3 h blocking with skimmed milk at room temperature, the membranes were exposed to the supernatants of the three hybrid tumour strains for 1~2 h at room temperature and were washed five times with PBS (10 mM, pH 7.4) containing 0.1% Tween-20 for 3 min per wash. Then, the membranes were immersed in the HRP-conjugated anti-mouse secondary antibodies (1 : 6000 diluted). After washing, the membranes were finally detected using DAB.

RESULTS

IFA for identification of mAbs against the PEDV N protein

For cell fusion, a limited dilution method was used for several subclones to select positive hybridoma cell lines. To confirm whether the strains were producing antibodies against the N protein, the hybridoma supernatants were subjected to IFA (Figure 1). Wells containing the negative mouse serum with the primary antibodies and the wells of hybridoma supernatant containing primary antibodies from Vero cells which were not infected with PEDV did not exhibit specific fluorescence. In contrast, wells containing the positive mouse serum exhibited extremely specific fluorescence. As shown in Figure 1, 13 positive hybrid tumour cell lines were obtained and denoted as: F1-D6, F21-D5, F21-F3, F21-F5, F31-D3, F31-D6, F31-F3, F31-F5, F31-G3, F31-G4, F42-C6, F51-F4 and F51-G4.

Expression and identification of the recombinant N protein

The prokaryotic expression of the PEDV N protein is shown in Figure 2A. Following the IPTG induction, different bands of approximately 82 kDa in size corresponding to the N protein were observed. Importantly, the GST tag and empty vector GST did not give obvious bands at the size of 82 kDa. The results using recombinant N protein as an antigen and the anti-PEDV anti-pig immune serum as the primary antibodies in the Western blot analysis are shown in Figure 2B. The lane containing the recombinant gel-purified N protein appeared revealed a band at about 82 kDa, and the vector used as a negative control did not result in any bands. This finding indicated that the expression of the viral N protein can be recognised by

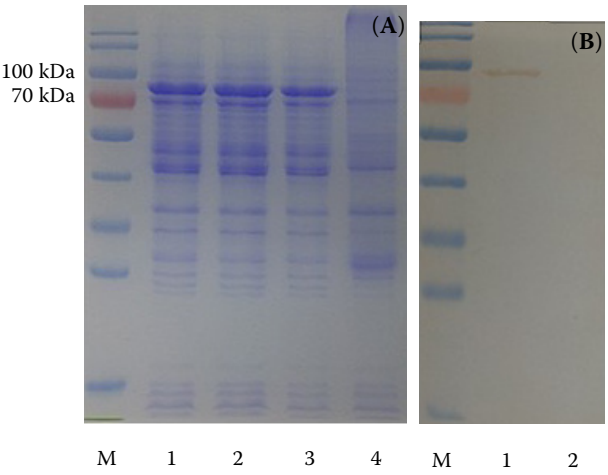

Figure 2. SDS-PAGE and Western blot analysis of recombinant protein N expression

(**A**) M = marker; 1, 2, 3 = different strains expressing the N protein; 4 = negative control pGEX 6P-1

(**B**) M = marker; 1 = recombinant N protein antigen; 2 = negative control pGEX 6P-1

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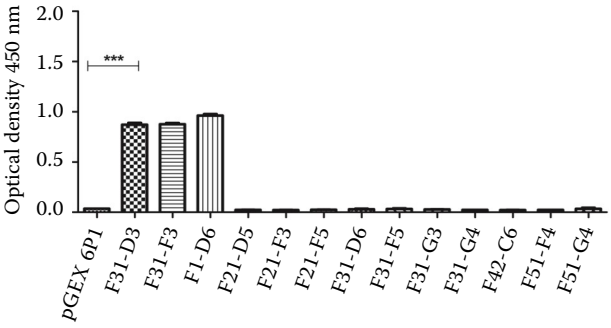

Figure 3. Porcine epidemic diarrhoea virus N protein-specific ELISA responses exhibited by the different hybridoma cells

positive serum containing antibodies specific to PEDV.

ELISA-mediated detection of the N protein using hybrid tumour cell line supernatants

The PEDV N fusion protein that was used as the antigen, and the protein which was successfully induced by empty vector pGEX 6P-1 that was used as the negative antigen were coated on an ELISA plate. A total of 13 supernatants from hybridoma cell line strains were used as the primary antibodies, and HRP goat anti-mouse IgG was used as the secondary antibody. TMB was used for the colour reaction, and the absorbance was measured. The results are presented in Figure 3, which shows that the absorbance of the three strains of the hybridoma cell line supernatants (i.e., F31-D3, F31-F3 and F1-D6) used as the primary antibody were above 0.7. Moreover, the absorbance of the empty vector control exhibited significant differences regarding the capacity to specifically recognise the N protein.

Western blot analysis of the hybrid tumour supernatants for identification specific antibodies against N protein

The recombinant N protein and the whole virus, which was subjected to ultracentrifugation were used as the antigen, while pGEX 6P-1 and the ultracentrifuged Vero supernatant were used as negative control. The monoclonal antibodies F31-D3, F31-F3 and F1-D6, were used as primary antibodies for Western blot analysis, and the results are shown in Figure 4. As can be seen from the diagram, in all of the lanes from experiments in which recombinant N protein was used as antigen bands at about 82 kDa were observed, while the vector only negative control gave no bands. All of the lanes of the whole virus as antigen exhibited bands at about 57 kDa, which corresponds to the size of PEDV N protein reported in the literature (Zheng 2014). At the same time, the negative control from Vero cell supernatants did not give bands. It was thus proven that monoclonal antibodies from three specific strains could bind to recombinant N protein specifically.

DISCUSSION

Porcine epidemic diarrhoea disease caused by PEDV is associated with heavy economic losses to the pig industry. Moreover, the clinical symptoms are very similar to those of other diarrhoea viruses, and thus, the development of a more efficient, sensitive and rapid detection method for PEDV is particularly important (Wang et al. 2015a). Monoclonal antibodies have high purity, specificity and good reproducibility. Moreover, they can be used as ho-

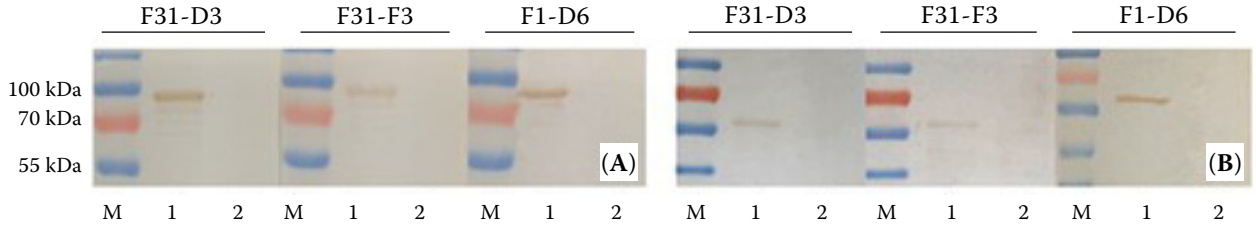

Figure 4. Western blot analysis of the supernatants of hybridoma cells expressing porcine epidemic diarrhoea virus protein N

(**A**) M = marker; 1 = recombinant N protein was used as primary antibody; 2 = negative control: pGEX 6P-1

(**B**) M = marker; 1 = whole virus was used as primary antibody; 2 = negative control: Vero cell supernatant; horizontal lines on the top of the picture indicate the supernatants of the respective hybridoma cell lines which were used as the primary antibodies

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mogeneous antibodies both *in vitro* and *in vivo*. In addition, they can also be used as diagnostic antibodies to avoid the many shortcomings of diagnosis made using polyclonal antisera (Rodak et al. 2005). The N protein of the PEDV virus is one of its largest structural proteins, and is highly expressed in infected cells. Therefore, pigs will produce high levels of PEDV N protein antibodies during the early stages of PEDV infection. Furthermore, the coronavirus N protein is highly conserved, and molecular PEDV diagnostic techniques based on the N protein have good application prospects (Lee and Yeo 2003). Therefore, many researchers have studied PEDV monoclonal antibodies. Wang et al. (2017) used recombinant expression of the PEDV S protein as an antigen to prepare monoclonal antibodies and then established a double antibody sandwich ELISA method. Lei et al. (2016) purified PEDV Chinese mutant S protein N-terminal hypervariable region (22~380 aa) recombinant protein as an antigen for immunised mice and obtained a stable anti-S protein specific monoclonal antibody cell line, which had good specificity. Using the prokaryotic expression of the fusion protein GST-N as the immunogen, Liu et al. (2015) prepared three stable and specific mAbs. Wang et al. (2015b) used the truncated PEDV S1 protein to immunise BALB/c mice and obtained three specific mAbs. Shi et al. (2014) prepared four hybridoma cell lines against PEDV with purified recombinant N protein, which were stable and specific. Zhang et al. (2011) used truncated His-M recombinant protein to immunise BALB/c mice and prepared a stable anti-M protein mAb which could recognise PEDV but did not cross-react with TGEV and IBV. Pei et al. (2008) immunised mice with recombinant N protein, and prepared two strains of mAbs. These strains of mAbs did not cross-react with TGEV, PRV and PRV, but showed a good specific reaction with PEDV. Purified recombinant PEDV N protein was used as an antigen to immunise BALB/c mice by Feng et al. (2008). Seven hybridoma cell strains which could stably secret anti-recombinant N protein-specific monoclonal antibodies were obtained using the lymphocyte hybridoma technique. These mAbs had good reactivity. Sun et al. (2007) made PEDV S protein neutralizing epitope (SID) recombinant protein as an antigen to immunise BALB/c mice and obtained six stable anti-SID region-specific monoclonal antibody cell lines, which could specifically recognise the natural PEDV S protein.

The mAbs prepared for the different proteins of PEDV had been proven to have good antigenicity. However, the preparation of these mAbs was based on the *in vitro* expression of recombinant protein as the immunogen, and most of them are classical PEDV strains. Compared with the above mAbs, natural mAbs maintain the natural space conformation and the good antigenicity of various proteins of the virus. Therefore, using natural virus as an immunogen can simulate the reaction of animals infected with PEDV in natural conditions, and monoclonal antibodies will have better biological activity. In addition, the genome structure and pathogenicity of the epidemic strains have changed greatly. The mAbs expressed by the classic strains are not necessarily suitable. In this study, we used the purified PEDV epidemic strain SHpd/2012 whole viral protein as an immune antigen. Thirteen hybridoma cell lines were screened using the hybridoma technique, indirect ELISA and IFA. A western blot test and ELISA proved that three of the tested monoclonal antibodies (i.e., F31-D3, F31-F3 and F1-D6) recognised the PEDV N protein with strong specificity. This study thus reports the characterisation of effective tools for PEDV diagnostics and related basic research.

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