Rapid Transient Production of a Monoclonal Antibody Neutralizing the Porcine Epidemic Diarrhea Virus (PEDV) in Nicotiana benthamiana and Lactuca sativa

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ABSTRACT
Porcine epidemic diarrhea virus (PEDV) causes acute diarrhea, vomiting, dehydration, weight loss, and high mortality rate in neonatal piglets. Porcine epidemic diarrhea (PED) has been reported in Europe, America, and Asia including Thailand. The disease causes substantial losses to the swine industry in many countries. Presently, there is no effective PEDV vaccine available. In this study, we developed a plant-produced monoclonal antibody (mAb) 2C10 as a prophylactic candidate to prevent the PEDV infection. Recently, plant expression systems have gained interest as an alternative for the production of antibodies because of many advantages, such as low production cost, lack of human and animal pathogen, large scalability, etc. The 2C10 mAb was transiently expressed in Nicotiana benthamiana and lettuce using geminiviral vector. After purification by protein A affinity chromatography, the antibody was tested for the binding and neutralizing activity against PEDV. Our result showed that the plant produced 2C10 mAb can bind to the virus and also inhibit PEDV infection in vitro. These results show excellent potential for a plant-expressed 2C10 as a PEDV prophylaxis and a diagnostic for PEDV infection.

Introduction
Porcine epidemic diarrhea (PED) is an enteric disease that causes the significant loss for the swine industry. The disease first occurred in Europe in the early 1970s and the virus was first isolated in Belgium in 1978 [1]. Subsequently, the disease was also reported in the United States [2] and several countries in Asia, including China [3], Korea [4], Taiwan [5], Japan [6], and Thailand [7]. PED is characterized by intense diarrhea, vomiting, weight loss, and dehydration. These symptoms result in the death of newborn piglets and weight loss in all pig ages, which adversely affects growth performance of growing pigs [1, 8]. PED diagnosis cannot be made purely on the basis of clinical signs and histopathological lesions. Therefore, the diagnosis to confirm the presence of porcine epidemic diarrhea virus (PEDV) or its antigens must be conducted in the laboratory.

PEDV is an enveloped virus with a single-stranded positive-sense RNA and belongs to the Alphacoronavirus genus in the Coronavirus family (ICTV, 2012). Its genome is approximately 28 kb encoding seven viral proteins: ORF1A, ORF1B, spike (S),
OFR3, small envelope protein (E), membrane (M), and nucleocapsid (N) [9]. Among these viral proteins, S-protein is the key protein responsible for the viral entry into the host cell. It is the envelope type I glycoprotein that can bind to the receptor on the host cell, subsequently fuse the viral membrane to the host membrane, and then release the nucleocapsid protein into the host cell [10]. Vaccination with the recombinant S-protein was shown to protect the piglets against PEDV infection [11]. Thus, S-protein is one of the targets for PEDV vaccine development.

There are several neutralizing epitopes on the S-protein [12–14]. Among these epitopes, 2C10 is one of the peptides that are recognized by neutralizing monoclonal antibody (mAb) against PEDV [15]. The recombinant 2C10 single-chain antibody, consisting of variable regions of heavy (VH) and light chains (VL), was previously expressed in *Escherichia coli* and also showed the PEDV neutralization [16]. This can be developed to use as PEDV diagnostic and prophylaxis treatment.

The goal of this study was to develop 2C10 mAb in plants. Recently, plants have been used as factories to effectively produce several recombinant proteins because of several advantages over other protein expression systems, including low manufacturing cost, high protein expression level, scalability, and lack of human and animal pathogen [17, 18]. Moreover, plants contain the post-translational modification, which are often critical to the proper protein functions [19, 20]. The two main systems for producing proteins in plants are stable transgenic expression and transient expression. The establishment of transgenic plants is a time-consuming process with limitation of relatively low protein expression level. For transient expression, plant viral vectors were developed to increase the protein expression level [21]. Among different viral vectors, geminiviral vector was developed to produce several proteins in plants such as antigens and antibodies [22–24].

In this study, we used geminiviral vectors to produce 2C10 mAb in *Nicotiana benthamiana* Domin. (Solanaceae) and *Lactuca sativa* L. var. *longifolia* (Asteraceae) by transient expression. The seeds of *N. benthamiana* were provided from Julian Ma’s Lab (London, UK). *N. benthamiana* is the most common host plant for transient expression because of its high biomass yield. Lettuce is another rapid-growing plant that also produces high biomass and contains lower amount of phenolics and alkaloids, compared to tobacco plants. This is the advantage for the protein purification downstream process [24]. Lettuce was obtained commercially from a local grocery store with romaine lettuce labeled on the package. We tested the 2C10 mAb expressed in *N. benthamiana* and lettuce for PEDV binding and neutralizing activity. This plant-produced mAb can be applied to use as diagnostic kit or prophylactic treatment for PEDV infection, which will be very useful for the swine industry.

**Results**

The genes optimized for plant codon usage encoding the VL and VH chains of 2C10 (▶Fig. 1) were amplified and fused with constant regions of IgG1 mAb [25]. The fused genes were inserted into geminiviral replicon vector (pBY030.2R) (▶Fig. 2) and introduced to *Agrobacterium tumefaciens* strain GV3101. The 2C10 mAb was expressed in *N. benthamiana* by co-infiltration with the mixture of *A. tumefaciens* containing pBY2C10-kappa, pBY2C10-kamma, and p19. The p19 used in this study was the gene silencing inhibitor p19 from tomato bushy stunt virus using the non-replicating expression vector. At day 5 post-infiltration, the 2C10 mAb in plant extract was detected by western blot analysis using mouse anti-human IgG antiserum, Fc specific and mouse anti-kappa light chain antiserum. The result showed that 2C10 IgG was produced in *N. benthamiana*, containing both heavy chain and light chain at approximately 50 and 25 kDa in reducing condition, respectively (▶Fig. 3A). The assembly of 2C10 IgG was determined after protein A affinity purification by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The 2C10 mAb purified from plant proteins were analyzed under reducing and non-reducing conditions. Under reducing condition, two bands of heavy and light chains were detected at 50 kDa and 25 kDa, respectively (▶Fig. 3B), while the purified protein had the band at 150 kDa (▶Fig. 3B), which confirms the assembly of the whole IgG molecule.

To test the effectiveness of geminiviral vector in producing the 2C10 mAb in lettuce, we investigated the transient expression of 2C10 mAb in romaine lettuce from the local store. *Agrobacterium* cultures using for lettuce were similar to the experiment above. At day 4 post-infiltration, western blot analysis of infiltrated lettuce leaves confirmed the expression of both heavy chain and light chain of 2C10 IgG at the expected size (▶Fig. 4A), similar to the 2C10 expressed in *N. benthamiana* (▶Fig. 3A). The 2C10 mAb was purified from the lettuce proteins with protein A affinity chromatography (▶Fig. 4B).

The PEDV binding ability of plant produced 2C10 mAb was tested by indirect ELISA (enzyme-link immunosorbent assay). Plant-produced 2C10 IgG from tobacco and lettuce was diluted 1:1000 before incubated in wells containing immobilized PEDV. Detection with HRP (horseradish peroxidase) labeled anti-human IgG antiserum yielded OD450 measurement. The results showed that 2C10 mAb produced in tobacco and lettuce could bind to PEDV approximately 1.7 and 1.6 times more than the negative control (▶Fig. 5). However, glutathione S-transferase (GST) fused with truncated S-protein of PEDV showed no binding to PEDV; the binding ratio to PEDV is approximately 1 compared to negative control. These results suggested that the plant-produced 2C10 mAb might be developed for use as a diagnostic for PEDV infection.

The 2C10 mAb showed some binding activity to PEDV (▶Fig. 5). To evaluate the neutralization potential of 2C10 mAb, the PEDV neutralization assay was performed using 2C10 produced from *N. benthamiana*. PEDV virus (100–300 TCID50/50 µL) was incubated with the 2C10 mAb for 1 h at 37°C. The antibody-PEDV mixture was added to Vero cell and incubated for 1 h at 37°C. The result showed that 1:32 dilution ratio of 2C10 mAb was able to neutralize PEDV infection in Vero cell (▶Table 1) because there is no cytopathic effect. However, after the antibody was diluted to 1:64, the cytopathic effect was shown. Thus, plant-produced 2C10 mAb retained neutralizing activity against infectious PEDV, which suggested the potential to use as PEDV prophylaxis treatment.
Discussion

PED is a major reason for the economic loss in swine industry [26, 27]. PEDV infection induces death in neonatal pigs due to watery diarrhea and dehydration. Previous study reported that there are over one million piglet deaths from PEDV infection during the outbreak in China in 2010, which created large economic losses [28]. For PEDV prevention, there are different types of PEDV vaccine commercially, including live viral vaccine and inactivated virus [29, 30]. However, these vaccines did not significantly reduce the...
morbidity rate due to diarrhea, because several factors including route of administration and strains of vaccine virus affected the poor lactogenic immunogenicity of these commercial vaccines [31]. Another potential approach to protection of neonatal piglets is passive immunity induced by effective quantities of protective antibodies obtained from sow colostrum and milk. Artificial passive immunization by oral administration of specific antibodies represents an attractive approach against gastrointestinal pathogens such as PEDV [26]. Several PEDV neutralizing antibodies were developed previously [15,16,32,33], and they might be good candidates for use as PEDV prophylactic agents. However, an effective antibody production system with low production cost is required for the animal industry.

Plant-based expression is becoming attractive for recombinant protein production. Several conventional systems, such as E. coli, yeast, mammalian cells, insect cells, etc., are available commercially. Among these production platforms, plants have several advantages over other systems, including low production cost, large scalability, lack of human and animal pathogen, and post-translational modification, which affect the function of many recombinant glycoproteins. In the present study, tobacco (N. benthamiana) and lettuce (L. sativa) were used to produce 2C10 mAb, which previously showed the PEDV neutralization [16].

Among all PEDV neutralizing mAb, 2C10 is one of a good candidate for using to inhibit PEDV infection [15,32,34–38]. The 2C10 mAb recognizes GPRLQPY motif found on carboxy-terminal region of PEDV S-protein [12]. Moreover, this mAb also showed strong binding to the peptides SHRLP(Y/Q)(P/V) or GPRLQPY or g3p minor coat protein and strong neutralizing activity against KPED-9 strain [15].

The nucleotide sequences of 2C10 VH and VL from previous work [24] were codon usage optimized for N. benthamiana and synthesized. After fusing both VH and VL to the constant region of heavy chain (CH) and light chain (CL), the 2C10 gamma and kappa gene were cloned into geminiviral replicon vector, separately. To test whether 2C10 mAb produced from plants can function, we used the human IgG constant region which is available in the lab. If we get the promising result, the porcine IgG constant region will be used for further study. Geminiviral replicon systems have been used for production of several recombinant proteins in plants, such as vaccine antigens [22–24,39,40] and antibodies [22,24,25]. The transient expression in plants using geminiviral vectors can rapidly produce high level of recombinant protein expression.

In this study, 2C10 mAb was transiently expressed in tobacco and lettuce on day 5 and day 4 after agroinfiltration, respectively (Fig. 3 A and Fig. 4 A). This expression process generated 2C10 mAb in a short period of time. After purification process, the assembly of full-size 2C10 IgG (150 kDa) produced from both plants was detected in SDS-PAGE under non-reducing condition (Fig. 3 B and Fig. 4 B). The quantity of corrected IgG was found in tobacco more than lettuce. However, the antibodies produced from both plants clearly showed binding to PEDV in vitro (Fig. 5). Our result showed that the binding of plant-produced 2C10 mAb to PEDV increased approximately 1.7 times. However, the binding of another protein, GST-truncated S, is similar to negative control (the binding ratio is approximately 1). The binding activity of plant-produced 2C10 mAb strongly suggests that it can be developed to use as a reagent in the assays for detection of PEDV.

To test the neutralization of PEDV by 2C10, PEDV strain ST was used in this study. The 2C10 IgG produced from N. benthamiana showed the neutralizing activity at dilution 1:32 (Table 1). In this study, the neutralizing activity of plant-produced 2C10 was not strong compared to the neutralizing activity of 2C10 mAb against KPED-9 strain in previous study [15]. The level of neutral-
izing activity might be different depending on the strain of the virus. This data confirmed that the plant-produced 2C10 mAb neutralized PEDV infection in vitro, suggesting the potential to use as PEDV prophylactic agent. Previous data reported that protecting suckling piglets from PEDV infection also depends on the secretory IgA (sIgA) antibodies in milk [41]. The sIgA is more resistant to protease than IgG, which is suitable to use as the oral vaccine [42]. Therefore sIgA is a good approach to use as a prophylactic and therapeutic treatment. Plants were shown to be an effective platform for sIgA production [43–45]. Thus, we can develop this 2C10 mAb to produce sIgA from plants in the future, which will be more effective PEDV prophylactic.

The results of the present study provided a proof of concept in which the PEDV specific antibody can be produced through plant expression system and the produced antibody has an in vitro efficacy against PEDV. Further in vivo investigation involving oral application of plant produce antibody in piglets against oral PEDV challenge is suggested. The in vivo study, although can address the efficacy of the produced antibody, is needed to control several factors including methods to prevent enzymatic activities in gastrointestinal tracts, pH factors, and delivery system in which is beyond the scope of the study. The level of antibody needed to protect young pigs from the PEDV infection is another study that is needed for further investigation. In this study, the neutralization showed at titer 1:32 and at higher concentration of the antibody. However, at which certain level is beyond the scope of the study as the level of antibody induced by either natural infection or vaccination that provide a complete protection is presently yet to be known.

In conclusion, we developed a novel plant-produced PEDV 2C10 mAb, which showed binding and neutralizing activity against PEDV. This technology provides an effective and low-cost platform to produce PEDV mAb, which could be developed for PEDV diagnostic and prophylactic agents. The platform developed here will be beneficial, especially to the swine industry.

Materials and Methods

Design of the constructs for producing 2C10 mAb in plants

We designed plant-optimized DNA sequences encoding 2C10 VH and VL based upon previous study [16], using codons that are preferred in tobacco. The VH was fused to the N-terminus of a gene encoding constant region of the humanized 62-71-3 mAb [46]. The synthetic VH gene was amplified using primers Ncol-VH-F and Pmll-VH-R whereas the 62-71-3 CH was amplified using Pmll-CH-F and Sacl-CH-R. All primer sequences are in ▶ Table 2. The overlapped extension PCR between VH and CH was performed using primers Ncol-VH-F and Sacl-CH-R. For the cloning of 2C10 light chain, the synthetic VL gene was amplified using primers Ncol-VL-F and Xhol-VL-R whereas the 62-71-3 CL was amplified using primers Xhol-CL-F and Sacl-CL-R. The overlapped PCR between VL and CL was performed using primers Ncol-VL-F and Sacl-CL-R. The genes of 2C10 heavy and light chain were inserted into the geminiviral vector via Ncol and SacI sites. Two geminiviral vectors, 2C10 heavy chain and light chain genes, was co-infiltrating with p19 vector.

Plant inoculation and protein expression

The seeds of N. benthamiana were provided from Julian Ma’s Lab (London, UK). N. benthamiana plants were co-infiltrated with Agrobacterium GV3101 strains containing pBY2C10-gamma, pBY2C10-kappa, and p19 that protects gene silencing of transient protein production in plant (▶ Fig. 2). The final OD600 of mixed Agrobacterium in equal amount of the three strains for vacuum infiltration is 0.25. Plants were maintained in the growth chamber for 5 d before protein extraction. Romaine lettuce (L. sativa) was obtained commercially from a local grocery store. The agroinfiltration of lettuce was performed as previously described [24]. After vacuum infiltration with Agrobacterium, lettuce heads were covered with Saran wrap and kept in the growth chamber for 4 d before protein extraction.

Protein purification

Protein purification Infiltrated tobacco and lettuce leaves were homogenized by using a blender with 1× phosphate-buffered saline (1× PBS: 137 mM NaCl; 2.7 mM KCl; 4.3 mM Na2 HPO4; 1.47 mM KH2PO4 at pH 7.4), pH 7.5. Crude extract was filtered through Miracloth and centrifuged at 26,000 g for 20 min. The solution was filtered with 0.2 µm filter before loading into the protein A bead column. After washing the column with PBS, pH 7.5, for 10 bed volumes, the protein was eluted with 100 mM glycine, pH 2.5. After the protein was eluted from the column, 1 M Tris base was added to neutralize to a final pH of 7.5.
The purified plant 2C10 mAb protein was mixed with SDS loading sample buffer (250 mM Tris-HCl pH 6.8, 10 % [w/v] SDS, 30 % [v/v] glycerol, 5 % [w/v] β-mercaptoethanol, and 0.02 % [w/v] bromophenol blue) for reducing SDS-PAGE or SDS loading sample buffer without β-mercaptoethanol for non-reducing SDS-PAGE and boiled for 5 min. Proteins were separated using 10 % SDS-PAGE. Proteins were stained with Coomassie Brilliant Blue G-250. For western blot, proteins were transferred to a nitrocellulose membrane (Bio-Rad) and probed with HRP-conjugated anti-human IgG antiserum, Fc specific, (diluted 1:1000 plant 2C10 mAb diluted in 1% skim milk at 37 °C for 2 h). The plate was washed again and developed using 3, 3′, 5′, 5′-tetramethy benzidine liquid substrate for 15 min at 23 °C in dark condition. The reactions were stopped with 1 N H2SO4 and determined with micro-plate reader at OD450.

Neutralization assay

Vero cells were seeded into the 96-well plates and grown until 80 % coverage of the plate. PEDV virus was diluted to TCID50 (50 % tissue culture infectious dose) in MM medium and incubated with 100 µl of different dilutions of plant-made 2C10 mAb (1:4, 1:8, 1:16, 1:32, 1: 64, and 1:256) at 37 °C for 1 h. The Vero cells were washed with PBS one time and MM containing 10 µg/ml trypsin one time. The viral mixed solutions were transferred to the Vero cells plate and incubated at 37 °C for 1 h. Following the incubation, the solution was removed and MM plus 2 % FBS was added. The Vero cells plate was incubated at 37 °C in a humidified 5 % CO2 for 5–7 d. Colostrum samples with and without neutralizing activity were used as positive and negative control, respectively. The assays were performed in duplicate. The cytopathic effect (CPE) were determined and compared with positive and negative control.

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**Conflict of Interest**

On behalf of my co-authors, I declare that there are no financial or other conflicts of interest in the present manuscript. This work was original research, and the data presented in this article have not been published in elsewhere.

**References**

[1] Pensaert MB, de Bouck P. A new coronavirus-like particle associated with diarrhea in swine. Arch Virol 1978; 58: 243–247

[2] Chen Q, Li G, Stasko J, Thomas JT, Stensland WR, Pillatzki AE, Gaucer PG, Schwartz KJ, Madson D, Yoon KJ, Stevenson GW, Burrough ER, Harmon KM, Main RG, Zhang J. Isolation and characterization of porcine epidemic diarrhoea viruses associated with the 2013 disease outbreak among swine in the United States. J Clin Microbiol 2014; 52: 234–243

[3] Li W, Li H, Liu Y, Pan Y, Deng F, Song Y, Tang X, He Q. New variants of porcine epidemic diarrhea virus, China. 2011. Emerg Infect Dis 2012; 18: 1350–1353

[4] Chung HC, Lee JC, Nguyen VG, Huynh TM, Lee GE, Moon HJ, Park SJ, Kim HK, Park BK. New emergence pattern with variant porcine epidemic diarrhoea viruses, South Korea, 2012–2015. Virus Res 2016; 226: 14–19

[5] Lin CN, Chung WB, Chang SW, Wen CC, Liu H, Chien CH, Chiuo MT. US-like strain of porcine epidemic diarrhoea virus outbreaks in Taiwan, 2013–2014. J Vet Med Sci 2016; 76: 1297–1299

[6] Islam MT, Kubota T, Ujike M, Yahara Y, Taguchi F. Phylogenetic and antigenic characterization of newly isolated porcine epidemic diarrhoea viruses in Japan. Virus Res 2016; 222: 113–119

[7] Cheun-Arom T, Temeeyasen G, Tripipat T, Kaewprommal P, Piriyapongsa J, Sukrong S, Chongcharoen W, Tantituvanont A, Nilubol D. Full-length genome analysis of two genetically distinct variants of porcine epidemic diarrhoea virus. Res Vet Sci 1978; 25: 255–256

[8] Kocherhans R, Bridgen A, Ackermann M, Tobler K. Completion of the genome of recombinant S1 domain of the porcine epidemic diarrhoea virus spike protein. Arch Virol 2014; 159: 2977–2987

[9] Cruz DJ, Kim CJ, Shin HJ. The GPRLQPY motif located at the carboxy-terminal of the spike protein induces antibodies that neutralize Porcine epidemic diarrhoea virus. Virus Res 2008; 132: 192–196

[10] Sun D, Feng J, Shi H, Chen J, Cui X, Chen H, Liu S, Tong Y, Wang Y, Tong G. Identification of two novel B cell epitopes on porcine epidemic diarrhoea virus spike protein. Vet Microbiol 2008; 131: 73–81

[11] Chang SH, Bae JL, Kang Tj, Kim J, Chung GH, Lim CW, Laude H, Yang MS, Jang YS. Identification of the epitope region capable of inducing neutralizing antibodies against the porcine epidemic diarrhoea virus. Mol Cells 2002; 14: 295–299

[12] Cruz DJ, Kim CJ, Shin HJ. Phage-displayed peptides having antigenic similarities with porcine epidemic diarrhoea virus (PEDV) neutralizing epitopes. Virology 2006; 354: 28–34

[13] Pyo HM, Kim Ij, Kim SH, Cho SD, Cho IS, Hyun BH. Escherichia coli expressing single-chain Fv on the cell surface as a potential prophylactic of porcine epidemic diarrhoea virus. Vaccine 2009; 27: 2030–2036

[14] Tschofen M, Knopp D, Hood E, Stoger E. Plant molecular farming: much more than medicines. Annu Rev Anal Chem (Palo Alto Calif) 2016; 9: 271–294

[15] Topp E, Irwin R, Mcallister T, Lessard M, Joensuu J, Kolotilin I, Conrad U, Stoger E, Mor T, Warzecha H, Hall JC, McLean MD, Cox E, Devriendt B, Potter A, Depicker A, Virdi V, Holbrook L, Doshi K, Dussault M, Friendship R, Yarosh O, Yao HS. Understanding the process of virus-made antibody production. Biotechnol Adv 2016; 34: 597–604

[16] Schneider JD, Castillo A, Neumann L, Altmann F, Loos A, Kannan L, Mor TS, Steinkellner H. Expression of human butyrylcholinesterase with an engineered glycosylation profile resembling the plasma-derived orthologue. Biotechnol J 2014; 9: 501–510

[17] Loos A, Gach JS, Hacket T, Maresch D, Henkel T, Porodko A, Bui-Minh D, Sommeregger W, Wozniak-Knopp G, Forthal DN, Altmann F, Steinkellner H, Mach L. Glycan modulation and subfoeengineering of anti-HIV-1 monoclonal antibody PC9 in plants. Proc Natl Acad Sci U S A 2015: 112: 12675–12680

[18] Fischer R, Emans N, Schuster F, Hellwig S, Drossard J. Towards molecular farming in the future: using plant-cell-suspension cultures as bioreactors. Biotechnol Appl Biochem 1999; 30: 109–112

[19] Chen Q, He J, Phoolchooaren W, Mason HS. Geminoviral vectors based on bean yellow dwarf virus for production of vaccine antigens and monoclonal antibodies in plants. Hum Vaccin 2011; 7: 331–338

[20] Phoolchooaren W, Bho S, Hsi L, Ma J, Amstzen Cj, Chen Q, Mason HS. Expression of an immunogenic Ebola immune complex in Nicotiana benthami. Plant Biotechnol J 2011; 9: 807–816

[21] Lai H, He J, Engl M, Diamond MS, Chen Q. Robust production of virus-like particles and monoclonal antibodies with geminivirus replicon vectors in lettuce. Plant Biotechnol J 2012; 10: 95–104

[22] Lai H, He J, Hurtado J, Stahnke J, Fuchs A, Melihope E, Gorlatov S, Loos A, Diamond MS, Chen Q. Structural and functional characterization of an anti-West Nile virus monoclonal antibody and its single-chain variant produced in glycoengineered plants. Plant Biotechnol J 2014; 12: 1098–1107

[23] Lee C. Porcine epidemic diarrhoea virus: An emerging and re-emerging epizootic swine virus. Virol J 2015; 12: 193

[24] Song D, Park B. Porcine epidemic diarrhoea virus: a comprehensive review of molecular epidemiology, diagnosis, and vaccines. Virus Genes 2012; 44: 167–175

[25] Sun KQ, Cai Rj, Chen YQ, Liang PS, Chen DK, Song CX. Outbreak of porcine epidemic diarrhoea in suckling piglets, China. Emerg Infect Dis 2012; 18: 161–163

[26] Song DS, Yang JS, Oh JS, Han JH, Park BK. Differentiation of a Vero cell adapted porcine epidemic diarrhoea virus from Korean field strains by restriction fragment length polymorphism analysis of ORF 3. Vaccine 2003; 21: 1833–1842

[27] Baek PS, Choi HW, Lee S, Yoon J, Lee JY, Lee DS, Lee S, Lee C. Efficacy of an inactivated genotype 2b porcine epidemic diarrhoea virus vaccine in neonatal piglets. Vet Immunol Immunopathol 2016; 174: 45–49

[28] Song D, Moon H, Kang B. Porcine epidemic diarrhoea: a review of current epidemiology and available vaccines. Clin Exp Vaccine Res 2015; 4: 166–176

[29] Cao L, Zin Q, Ge X, Yin X, Xia C, Bu RE, Fang Y, Liu J, Gao Y, Ren X. Generation of a monoclonal antibody to S1 protein of porcine epidemic diarrhoea virus. Monoclon Antib Immunodig Immunother 2013; 32: 371–374

[30] Zhu Q, Guo D, Feng L, Sun D. Expression and purification of the scFv from hybridoma cells secreting a monoclonal antibody against S PROTEIN of PEDV. Monoclon Antib Immunodig Immunother 2013; 32: 41–46

[31] Van Nieuwstadt AP, Zetsstra T. Use of two enzyme-linked immunosorbent assays to monitor antibody responses in swine with experimentally
induced infection with porcine epidemic diarrhea virus. Am J Vet Res 1991; 52: 1044–1050

[35] Lee HM, Lee BJ, Tae JH, Kweon CH, Lee YS, Park JH. Detection of porcine epidemic diarrhea virus by immunohistochemistry with recombinant antibody produced in phages. J Vet Med Sci 2000; 62: 333–337

[36] Zhang Y, Yao Y, Gao X, Wang Y, Jia X, Xiao Y, Wang T, Li X, Tian K. Development of a neutralizing monoclonal antibody against porcine epidemic diarrhea virus S1 protein. Monoclon Antib Immunodiagn Immunother 2016; 35: 37–40

[37] Wang J, Chen J, Wei F, Dong Y, Zhu L, Han W, Wang L, Shen Z. Prokaryotic expression of truncated S1 protein of porcine epidemic diarrhea virus and production of monoclonal antibodies to recombinant protein. Monoclon Antib Immunodiagn Immunother 2015; 34: 327–333

[38] Pan X, Kong N, Shan T, Zheng H, Tong W, Yang S, Li G, Zhou E, Tong G. Monoclonal antibody to N protein of porcine epidemic diarrhea virus. Monoclon Antib Immunodiagn Immunother 2015; 34: 51–54

[39] Diamos AG, Rosenthal SH, Mason HS. 5' and 3' untranslated regions strongly enhance performance of geminiviral replicons in Nicotiana benthamiana leaves. Front Plant Sci 2016; 7: 200

[40] Rattanapisit K, Cho MH, Bhoo SH. Lysine 206 in Arabidopsis phytochrome A is the major site for ubiquitin-dependent protein degradation. J Biochem 2016; 159: 161–169

[41] Langel SN, Paim FC, Lager KM, Vlasova AN, Saif LJ. Lactogenic immunity and vaccines for porcine epidemic diarrhea virus (PEDV): historical and current concepts. Virus Res 2016; 226: 93–107

[42] Brandzaeg P. Secretory immunity with special reference to the oral cavity. J Oral Microbiol 2013; DOI: 10.3402/jom.v5i0.20401

[43] Paul M, Reljic R, Klein K, Drake PM, van Dolleweerd C, Pabst M, Windwarder M, Arcalis E, Stoger E, Altmann F, Cosgrove C, Bartoff A, Baden S, Ma JK. Characterization of a plant-produced recombinant human secretory IgA with broad neutralizing activity against HIV. MAb 2014; 6: 1585–1597

[44] Hiatt A, Ma JK. Monoclonal antibody engineering in plants. FEBS Lett 1992; 307: 71–75

[45] Ma JK, Lehner T, Stabila P, Fux CI, Hiatt A. Assembly of monoclonal antibodies with IgG1 and IgA heavy chain domains in transgenic tobacco plants. Eur J Immunol 1994; 24: 131–138

[46] Both L, van Dolleweerd C, Wright E, Banyard AC, Bulmer-Thomas B, Selden D, Altmann F, Fooks AR, Ma JK. Production, characterization, and antigen specificity of recombinant 62-71-3, a candidate monoclonal antibody for rabies prophylaxis in humans. FASEB J 2016; 27: 2055–2065