Indian Journal of Animal Sciences **94** (5): 401–405, May 2024/Article
https://doi.org/10.56093/ijans.v94i5.131797

Development of one-step reverse transcription PCR assay for detection of porcine epidemic diarrhoea virus in pigs

FATEH SINGH[1]✉, KATHERUKAMEM RAJUKUMAR[1], DHANAPAL SENTHILKUMAR[1], GOVINDARAJULU VENKATESH[1], SHASHI BHUSHAN SUDHAKAR[1], VIJENDRA PAL SINGH[2] and ANIKET SANYAL[1]

ICAR-National Institute of High Security Animal Diseases, Bhopal, Madhya Pradesh 462 021 India

Received: 29 December 2022; Accepted: 1 April 2024

ABSTRACT

The present study aimed to develop an in-house one-step reverse transcription (RT) PCR assay as a diagnostic preparedness for the detection of porcine epidemic diarrhoea virus (PEDV) in pigs. Primers and gene construct targeting the nucleoprotein gene of PEDV were designed and synthesised. *In vitro* transcribed (IVT) RNA synthesised from linearised plasmid DNA containing the gene of interest was used as the positive control for the development of the RT-PCR assay. The RT-PCR protocol was optimised using different concentrations of molecular reagents, the gradient of annealing temperatures and other thermal cycling conditions. Analytical sensitivity of the RT-PCR assay was determined using 10-fold serial dilutions of the IVT-RNA directly and of the RNA extracted from swine faeces spiked with the IVT-RNA. The developed RT-PCR assay had analytical sensitivity of 939 and 2682 RNA copies at 10^{-7} and 10^{-6} dilutions in IVT-RNA directly and RNA extracted from spiked faeces, respectively. The RT-PCR assay was found to be specific for PEDV, without any amplification for classical swine fever virus, swine influenza virus, porcine reproductive and respiratory syndrome virus and transmissible gastroenteritis virus. All the known negative field faecal samples (n=126) of pigs tested negative by the developed RT-PCR. The one-step RT-PCR assay developed in the present study will be highly useful in specific diagnosis of the disease in the event of its future ingression, and will also aid in monitoring of PED in Indian swine population.

Keywords: Pigs, Porcine epidemic diarrhoea virus, Reverse transcription PCR

Porcine epidemic diarrhoea (PED) is a highly contagious non-zoonotic enteric viral disease of pigs caused by PED virus (PEDV), the *Alphacoronavirus* of the family Coronaviridae and characterised by watery diarrhoea, dehydration and weight loss (Saif *et al.* 2012). PEDV can infect pigs of all ages with a very high morbidity and mortality that can reach up to 100% in neonatal piglets. PED was firstly recorded during early 1970s in United Kingdom. Since then, several epidemics of PED have been reported from different continents. In Asia, PED has been reported from Japan (Lee 2015, Diep *et al.* 2018a), South Korea (Lee 2015, Jang *et al.* 2023), Thailand (Puranaveja *et al.* 2009, Stott *et al.* 2017), Vietnam (Diep *et al.* 2018b), China (Li *et al.* 2021, Zhang *et al.* 2023), Taiwan (Lin *et al.* 2014, Tsai *et al.* 2020) and Philippines (Paraguison-Alili and Domigo 2016).

Present address: [1]ICAR-National Institute of High Security Animal Diseases, Bhopal, Madhya Pradesh. [2]College of Veterinary and Animal Sciences, Rani Lakshmi Bai Central Agricultural University, Jhansi, Uttar Pradesh. ✉Corresponding author email: fateh.ars07@gmail.com

PED is a transboundary viral disease that can readily spread to neighbouring or distant countries even across continents. Transmission of PEDV among pigs occurs usually by a direct faecal-oral route or by indirect routes. Except for one report on seroprevalence of PED from Assam (Barman *et al.* 2003), there is no information so far on its clinical existence in Indian pig population. Due to high prevalence of PED in China and other Asian countries, north-eastern states of India are at high risk for transboundary introduction of this disease.

There is no specific treatment other than symptomatic treatment of diarrhoea, dehydration and control of secondary infections. Rapid and specific diagnosis coupled with implementation of appropriate biosecurity measures are known to be effective in controlling PED in endemic countries, and to prevent its introduction from disease-prevailing countries to disease-free countries.

In view of the possible transmission and/ or emergence due to non-rigid human and animal movement across the north-eastern border into India, there was a need to develop a diagnostic preparedness for rapid diagnosis of PED to implement suitable control measures. Therefore, the

present study was aimed to develop a sensitive one-step reverse transcription PCR assay for rapid diagnosis of PED in pigs.

MATERIALS AND METHODS

Designing of primers and synthetic construct: The one-step reverse transcription (RT)-PCR assay was developed and validated as per the methods described by Song *et al.* (2006) and Niu *et al.* (2022).

Nucleoprotein (N) gene was selected based on its conserved nature across various strains of PEDV for designing the primers and synthetic gene construct. N gene sequences of PEDV are available in the NCBI GenBank database were aligned and the primers were designed using Primer3 (v. 0.4.0) online software tool (Koressaar and Remm 2007, Untergasser *et al.* 2012). To include variable nucleotides for wider coverage, the degenerate nucleotides were added in the primer sequences at the variable sites. Two sets of primers were designed. Set 1 included forward primer– GGGTGCCATTATCCCTCTATG, reverse primer– CTCCACGACCCTGGTTRTTT with an expected product size of 519 bp. Set 2 included forward primer-GGGTGTTTTCTGGGTTGCTA, reverse primer-GATTTAAGGGCATCCTTGACAG with an expected product size of 417 bp. N gene construct of 798 bp (position 1-798) size was designed. The primers and synthetic gene construct were synthesised commercially (Eurofins Genomics India Pvt. Ltd., Bengaluru, India).

Sub-cloning of gene construct: Synthetic gene construct was sub-cloned in pTZ57R/T vector. Briefly, the N gene of PEDV in the synthetic construct was released by restriction endonuclease (RE) digestion using *Eco*RI and *Hind*III. The released insert was gel purified and ligated into pTZ57R/T vector (Thermo Fisher Scientific, Waltham, Massachusetts, USA). After ligation, it was transformed into *Escherichia coli* JM109 competent cells and grown overnight in Luria-Bertani (LB) agar plate containing ampicillin (100 µg/ mL) at 37°C. Recombinant colonies (white) were picked and tested for the gene insert by PCR. Positive colonies were selected and propagated in LB broth containing ampicillin (100 µg/ mL) in a shaking incubator at 37°C. The plasmid DNA was extracted from the recombinant colonies using PureYield Plasmid Miniprep System (Promega Corporation, Madison, USA) and linearised by RE digestion using *Eco*RI.

In vitro transcription (IVT) of RNA: Linearised DNA was precipitated using 0.5 M EDTA, 3M sodium acetate in absolute ethanol. The precipitated DNA was purified by the Phenol-chloroform method (Sambrook and Russell 2001). Purified DNA was checked for the presence of N gene by PCR before proceeding for IVT-RNA synthesis. IVT-RNA was synthesised using T7 RNA polymerase (mMESSAGE mMACHINE™ T7 Transcription Kit, Invitrogen, USA) (Milligan *et al.* 1987). RNA was purified by Phenol: Chloroform: Isopropanol method (Sambrook and Russell 2001). Pelleted RNA was dissolved in nuclease free water. The IVT-RNA was treated with 8 U of DNase (TURBO™ DNase, Invitrogen, USA) and incubated at 37°C for 75 min to digest the residual DNA. DNase was inactivated by treating with ammonium acetate.

Testing of IVT-RNA: The presence of specific RNA (PEDV N gene) was assessed by performing one-step RT-PCR. The RT-PCR product was analysed by agarose gel electrophoresis. The RT-PCR product in excised gel was purified by QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) following the Manufacturer's instructions and subjected to nucleotide sequencing by Sanger's method for confirmation. The absence of residual DNA in IVT-RNA was confirmed by performing PCR.

Quantification of IVT-RNA: The IVT-RNA was quantified by Qubit 4 Fluorometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Quantified RNA was expressed in copy number. The IVT-RNA was diluted 10-fold in nuclease free water and stored in small aliquots at -80°C.

Optimisation of RT-PCR assay: One-step RT-PCR amplification was performed by adding 4 µL of AMV/ *Tfl* 5× reaction buffer, 0.2 mM dNTPs, 0.75 mM MgSO4, AMV reverse transcriptase (2 U), *Tfl* DNA polymerase (2 U) using Access RT-PCR System (Promega Corporation, Madison, USA), 10-20 picomoles of each primer and 2 µL of IVT-RNA of different 10-fold dilutions as template for 20 µL reactions. The RT-PCR protocol was optimised with varying annealing temperatures (47-57°C), thermal cycling conditions (35 and 39 cycles) (Table 1). The RT-PCR products were analysed by agarose gel electrophoresis.

Table 1. RT-PCR thermal cyclic conditions used for optimisation

Thermal cycler steps	Temperature (°C)	Time	Cycle (n)
cDNA synthesis	45	30 min	01
Initial denaturation	95	10/5/3 min	01
Cyclic denaturation	95	30/20 s	35/39
Annealing	47-57	30 s	
Extension	72	45/30 s	
Final extension	72	7/5 min	01

Spiking of swine faecal samples with IVT-RNA: For faecal spiking, 20% (w/v) homogenates of swine faeces (previously tested negative for PEDV genome) were made in sterile phosphate buffered saline and clarified by centrifugation. The clarified faecal homogenates (140 µL) were spiked with 10 µL of the IVT-RNA of each 10-fold dilution. The RNA was extracted from the spiked faecal samples using QIAamp Viral RNA Kit (Qiagen, Hilden, Germany). The extracted RNA was tested for the presence of PEDV N gene by performing RT-PCR as described above.

Analytical sensitivity and analytical specificity of RT-PCR assay: Analytical sensitivity of the optimised RT-PCR assay was determined based on end point detection of RNA template directly from IVT-RNA and from RNA extracted after faecal spiking. Analytical sensitivity was calculated

using the formula given here.

$$\text{Moles of ssRNA (mol)} = \frac{\text{mass of ssRNA (g)}}{\text{(length of ssRNA (nt)} \times 321.47 \text{ g/mol)} + 18.02 \text{ g/mol)}}$$

$$\text{RNA copy number} = \text{moles of ssRNA} \times 6.02214 \times 10^{23} \text{ molecules/mol}$$

The analytical specificity of the optimised RT-PCR assay was assessed using known RNA template specific to classical swine fever virus (CSFV), swine influenza virus (SIV), porcine reproductive and respiratory syndrome virus (PRRSV) (genotype 1 (EU) and 2 (NA)) and transmissible gastroenteritis virus (TGEV). Moreover, 126 faecal samples collected from pigs (seronegative to PED) were also tested by the optimised RT-PCR protocol.

RESULTS AND DISCUSSION

Sub-cloning of PEDV gene construct in pTZ57R/T vector was done successfully. PCR amplification carried out with the plasmid DNA extracted from recombinant colonies confirmed the presence of PEDV N gene insert (Supplementary Fig. 1). Based on the results of initial evaluation of primers for optimum amplification, primer set 2 was selected for further optimisation of amplification reactions by RT-PCR. The presence of amplification by RT-PCR, and the absence of amplification by PCR indicated the absence of DNA impurity in IVT-RNA. The RT-PCR product was confirmed to be PEDV N gene (gene construct) by Sanger's sequencing. Synthesised IVT-RNA was quantified as 2 µg per mL. Each µL (2 ng) of the IVT-RNA was estimated to contain 7.796 femtomole (7.796×10^{-15} mole) equal to 4.695×10^9 copies of RNA.

The RT-PCR protocol was optimised using different annealing temperatures. There was no difference in band intensity on annealing temperatures ranging from 47°C to 57°C. The RT-PCR reactions showed amplification up to 10^{-6} and 10^{-7} dilutions of IVT-RNA template after 35 and 39 cycles, respectively (Fig. 1). An annealing temperature of 52°C, and 39 amplification cycles were selected as optimum for the RT-PCR protocol. Optimised one-step RT-PCR conditions are as follows: reverse transcription of RNA at 45°C for 30 min followed by denaturation at 95°C for 3 min which was then succeeded by 39 amplification cycles of denaturation at 95°C for 20 s, annealing at 52°C for 30 sec, extension at 72°C for 30 s and the final extension at 72°C for 5 min. The optimised protocol of the developed RT-PCR assay took 1 h 45 min for the completion of amplification including initial cDNA synthesis step. The developed RT-PCR assay revealed an analytical sensitivity of 939 RNA copies showing visible band of amplification at 10^{-7} dilution of IVT-RNA (2 µL) directly. After spiking of faecal samples with IVT-RNA, the analytical sensitivity was found to be 2682 RNA copies at 10^{-6} dilution of RNA template (Fig. 2). The RNA copies in different dilutions of IVT-RNA and of RNA extracted after faecal spiking are shown in Table 2. There was no amplification with the RNA templates of CSFV, SIV, PRRSV and TGEV, showing specificity of the developed RT-PCR assay for the detection of PEDV genome (Supplementary Fig. 2). Moreover, all clinically known negative field samples were tested negative by the developed RT-PCR assay.

PED is a transboundary disease, which can be readily spread to neighbouring or distant countries even across the continents. Since PED is not a notifiable disease to World Organization for Animal Health (WOAH/formerly OIE),

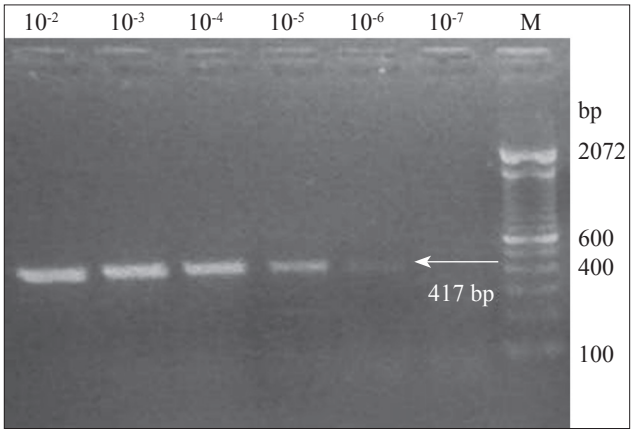

Fig. 2. RT-PCR amplification using different dilutions of RNA extracted after spiking of faecal samples.

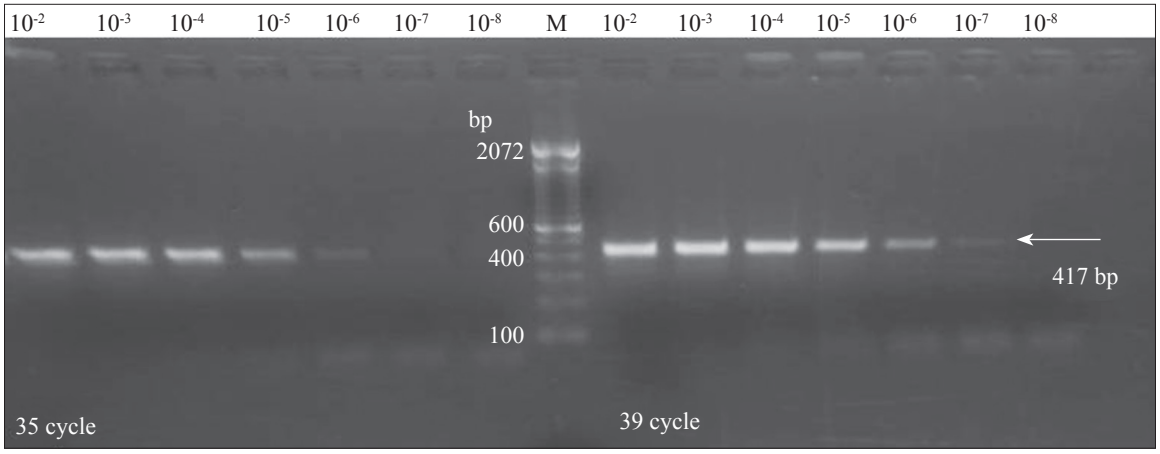

Fig. 1. RT-PCR amplification using serial dilutions of IVT-RNA specific to PEDV N gene.

Table 2. RNA copies in different dilutions of IVT-RNA and of RNA extracted after faecal spiking

Serial dilution	Direct IVT-RNA	RNA from spiked faeces
	RNA copies per reaction	RNA copies per reaction
Undiluted	9.39×10^9	2.682×10^9
10^{-1}	9.39×10^8	2.682×10^8
10^{-2}	9.39×10^7	2.682×10^7
10^{-3}	9.39×10^6	2.682×10^6
10^{-4}	9.39×10^5	2.682×10^5
10^{-5}	9.39×10^4	2.682×10^4
10^{-6}	9.39×10^3	$^*2.682 \times 10^3$
10^{-7}	$^*9.39 \times 10^2$	2.682×10^2
10^{-8}	9.39×10^1	2.682×10^1

*Detection limit.

quarantine and biosecurity measures might not be properly implemented and that could facilitate PEDV transmission between countries (Lee 2015). On account of the endemic occurrence of PED in neighbouring countries like China and due to non-rigid human and animal movement across international borders, especially in north-eastern states, there is a high risk of PEDV ingression into India. Although, there is no information on clinical existence of PED in Indian pig population except a report on its serological evidence from Assam state (Barman *et al.* 2003), an active surveillance involving testing of target pig population for genomic detection of the causative virus (PEDV) by RT-PCR may give deep insights into current status of PED in India. It may also help to devise suitable and effective control strategies in proactive ways to control the disease before its possible clinical occurrence in future (Olech 2022).

An accurate and confirmatory diagnosis is the prerequisite for the control or eradication of any infectious disease. Though the presumptive diagnosis of PED can be made on the basis of history and clinical signs of the disease or by characteristic pathological findings at necropsy, the clinical pictures of endemic PED are not so characteristic to make a confirmatory diagnosis. Moreover, three of the viral species of the genus *Alphacoronavirus*, viz. PEDV, TGEV and swine acute diarrhoea syndrome coronavirus (SADS-CoV), and one species of the genus *Deltacoronavirus,* the porcine deltacoronavirus (PDCoV) have affinity to infect pig enterocytes developing enteric disease with acute diarrhoea and vomiting, and thus, grouped as swine enteric coronaviruses (Wang *et al.* 2019, Yang *et al.* 2020). These four porcine enteric coronaviruses produce indistinguishable clinical signs, with a high morbidity and mortality in the infected pigs and thus, must be differentiated with each other. Therefore, a differential diagnosis is important and critical to control viral epidemic diarrhoeas in pigs. Moreover, the PED must also be differentiated from other diseases with similar clinical manifestations such as rotavirus diarrhoea, infections due to several bacteria (*Escherichia coli*, *Clostridium* spp., *Salmonella* spp., *Brachyspira* spp., *Lawsonia intracellularis*, etc.) or by parasites (*Cystoisospora suis*, *Cryptosporidium* spp., nematodes, etc.) (Saif *et al.* 2012, OIE 2014). Hence, confirmatory diagnosis of PED must be established, which requires laboratory testing using one or more specific diagnostic techniques. By virtue of high sensitivity and specificity, molecular tests such as RT-PCR are most commonly used for PED diagnosis in outbreak situations, as well as for culling or quarantine purposes (OIE 2014, Lee 2015). The RT-PCR is very useful for testing of weaned and older pigs, which have low virus load, exhibit mild clinical signs, or possess less prominent lesions (Olech 2022).

PCR-based techniques have practical advantage by virtue of reasonable specificity and sensitivity for testing different clinical specimens or infectious materials, such as faeces, rectal swabs, intestinal samples, oral fluid, etc. (Jung and Saif 2015, Diel *et al.* 2016). A number of one-step or nested RT-PCR assays have been developed for the detection of PEDV earlier (Zhou *et al.* 2017, Olech 2022).

The current study presents a sensitive and specific RT-PCR assay developed for detection of PEDV genome in pigs. The developed RT-PCR assay is rapid, which can be performed using nucleic acid derived from the clinical faecal samples or from the intestinal tissues of the dead pigs. The infrastructure for conventional RT-PCR can be easily established in peripheral diagnostic laboratories. The one-step RT-PCR assay developed in the present study will be useful for diagnosis of PED in the events of its future ingression, and also for further monitoring of PED in Indian pig population.

ACKNOWLEDGEMENTS

The authors are thankful to the Director, ICAR-National Institute of High Security Animal Diseases, Bhopal and the Indian Council of Agricultural Research, New Delhi for providing funding support to carry out the present research work under the institutional project entitled Diagnostic preparedness for porcine epidemic diarrhoea and transmissible gastroenteritis in pigs (Project code IXX 13779).

REFERENCES

Barman N N, Barman B, Sharma D K and Pensaert M B. 2003. Prevalence of rotavirus, transmissible gastroenteritis virus and porcine epidemic diarrhoea virus antibodies in pigs of Assam. *The Indian Journal of Animal Sciences* **73**(6): 576–78.

Diel D G, Lawson S, Okda F, Singrey A, Clement T, Fernandes M H V, Christopher-Hennings J and Nelson E A. 2016. Porcine epidemic diarrhea virus: An overview of current virological and serological diagnostic methods. *Virus Research* **226**: 60–70.

Diep N V, Sueyoshi M, Izzati U, Fuke N, Teh A P, Lan N T and Yamaguchi R. 2018b. Appearance of US like porcine epidemic diarrhoea virus (PEDV) strains before US outbreaks and genetic heterogeneity of PEDVs collected in Northern Vietnam during 2012–2015. *Transboundary and Emerging Diseases* **65**(1): e83-93.

Diep N V, Sueyoshi M, Norimine J, Hirai T, Myint O, Teh A P P, Izzati U Z, Fuke N and Yamaguchi R. 2018a. Molecular

characterization of US-like and Asian non-S INDEL strains of porcine epidemic diarrhoea virus (PEDV) that circulated in Japan during 2013–2016 and PEDVs collected from recurrent outbreaks. *BMC Veterinary Research* **14**(1): 96.

Jang G, Lee D, Shin S, Lim J, Won H, Eo Y, Kim CH and Lee C. 2023. Porcine epidemic diarrhea virus: An update overview of virus epidemiology, vaccines, and control strategies in South Korea. *Journal of Veterinary Science* **24**(4): e58.

Jung K and Saif L J. 2015. Porcine epidemic diarrhea virus infection: Etiology, epidemiology, pathogenesis and immunoprophylaxis. *The Veterinary Journal* **204**(2): 134–43.

Koressaar T and Remm M. 2007. Enhancements and modifications of primer design program Primer3. *Bioinformatics* **23**(10): 1289–91.

Lee C. 2015. Porcine epidemic diarrhea virus: An emerging and re-emerging epizootic swine virus. *Virology Journal* **12**: 193.

Li D, Li Y, Liu Y, Chen Y, Jiao W, Feng H, Wei Q, Wang J, Zhang Y and Zhang G. 2021. Isolation and identification of a recombinant porcine epidemic diarrhea virus with a novel insertion in S1 domain. *Frontiers in Microbiology* **12**: 667084.

Lin C N, Chung W B, Chang S W, Wen C C, Liu H, Chien C H and Chiou M T. 2014. US-like strain of porcine epidemic diarrhoea virus outbreaks in Taiwan, 2013–2014. *The Journal of Veterinary Medical Science* **76**(9): 1297–99.

Milligan J F, Groebe D R, Witherell G W and Uhlenbeck O C. 1987. Oligoribonucleotide synthesis using T7 RNA polymerase and synthetic DNA template. *Nucleic Acids Research* **15**(21): 8783–98.

Niu J W, Li J H, Guan J L, Deng K H, Wang X W, Li G, Zhou X, Xu M S, Chen R A, Zhai S L and He D S. 2022. Development of a multiplex RT-PCR method for the detection of four porcine enteric coronaviruses. *Frontiers in Veterinary Science* **9**: 1033864.

OIE (World Organization for Animal Health). 2014. Infection with porcine epidemic diarrhea virus, pp. 1–4. OIE Technical Factsheet.

Olech M. 2022. Current state of molecular and serological methods for detection of porcine epidemic diarrhea virus. Pathogens **11**(10): 1074.

Paraguison-Alili R and Domigo C Y J. 2016. Phylogenetic tracking of current porcine epidemic diarrhea virus (PEDV) strains in the Philippines. *Archives of Virology* **161**(9): 2601–04.

Puranaveja S, Poolperm P, Lertwatcharasarakul P, Kesdaengsakonwut S, Boonsoongnern A, Urairong K, Kitikoon P, Choojai P, Kedkovid R, Teankum K and Thanawongnuwech R. 2009. Chinese-like strain of porcine epidemic diarrhea virus, Thailand. *Emerging Infectious Diseases* **15**(7): 1112–15.

Saif L J, Pensaert M B, Sestak K, Yeo S G and Jung K. 2012. Coronaviruses, pp 501–24. Diseases of Swine. 10th Ed. John Wiley & Sons, Inc, New York, USA.

Sambrook J and Russell D W. 2001. Commonly used Techniques in Molecular Cloning. 3rd Ed, 3. CSHL Press, New York.

Song D S, Kang B K, Oh J S, Ha G W, Yang J S, Moon H J, Jang Y S and Park B K. 2006. Multiplex reverse transcription-PCR for rapid differential detection of porcine epidemic diarrhea virus, transmissible gastroenteritis virus, and porcine group A rotavirus. *Journal of Veterinary Diagnostic Investigation* **18**(3): 278–81.

Stott C J, Temeeyasen G, Tripipat T, Kaewprommal P, Tantituvanont A, Piriyapongsa J and Nilubol D. 2017. Evolutionary and epidemiological analyses based on spike genes of porcine epidemic diarrhea virus circulating in Thailand in 2008–2015. *Infection, Genetics and Evolution* **50**: 70–76.

Tsai K J, Deng M C, Wang F I, Tsai S H, Chang C, Chang C Y and Huang Y L. 2020. Deletion in the S1 region of porcine epidemic diarrhea virus reduces the virulence and influences the virus-neutralizing activity of the antibody induced. *Viruses* **12**(12): 1378.

Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth B C, Remm M and Rozen S G. 2012. Primer3 - New capabilities and interfaces. *Nucleic Acids Research* **40**(15): e115.

Wang Q, Vlasova A N, Kenney S P and Saif L J. 2019. Emerging and re-emerging coronaviruses in pigs. *Current Opinion in Virology* **34**: 39–49.

Yang Y L, Yu J Q and Huang Y W. 2020. Swine enteric alphacoronavirus (swine acute diarrhea syndrome coronavirus): An update three years after its discovery. *Virus Research* **285**: 198024.

Zhang H, Zou C, Peng O, Ashraf U, Xu Q, Gong L, Fan B, Zhang Y, Xu Z, Xue C, Wei X, Zhou Q, Tian X, Shen H, Li B, Zhang X and Cao Y. 2023. Global dynamics of porcine enteric coronavirus PEDV epidemiology, evolution, and transmission. *Molecular Biology and Evolution* **40**(3): msad052.

Zhou X, Zhang T, Song D, Huang T, Peng Q, Chen Y, Li A, Zhang F, Wu Q, Ye Y and Tang Y. 2017. Comparison and evaluation of conventional RT-PCR, SYBR green I and TaqMan real-time RT-PCR assays for the detection of porcine epidemic diarrhea virus. *Molecular and Cellular Probes* **33**: 36–41.