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Porcine Epidemic Diarrhea (PED) Infection, Diagnosis and Vaccination: A Mini Review

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Abstract

Porcine epidemic diarrhea virus (PEDV) is a main etiology causing severe enteric disease in piglets with clinical signs of anorexia, vomiting, diarrhea and dehydration resulting in loss of condition and death within a few days. Historically, PED is one of major causes of loss in swine and remains prevalent in some parts of the world. Even with increase in the available tests for PED diagnosis, which include histological diagnosis; virological diagnosis and serological diagnosis, there is no vaccine or specific treatment for this disease yet. In this mini review, the overview and current situation of PED is described with updated techniques, in an effort to comprehensively discuss and understand the disease characteristics.

Keywords: Porcine epidemic diarrhea, PED, PED virus, Enteric disease

Introduction

Porcine epidemic diarrhea (PED) is a non-zoonotic viral disease of swine caused by a coronavirus and characterized by dramatic watery diarrhea and weight loss in swine with high mortality to neonatal piglets[1,2]. Pigs of all age can be infected by PED virus from neonates to sows or boars but the fatality rate of PED in swine depends on age[3]. The morbidity and mortality of PED in 10-day-old piglets are less than piglets under 5 days old that may reach 100% in death because of severe diarrhea and dehydration[4]. The older pigs are able to recover within 7 to 10 days but can re-infect within five months[4].

The fecal-oral route is perhaps only transmission of PED and no other vector or reservoir in disease spreading[5]. However, the disease is highly contagious and rapidly infect throughout the year to both industrial and small pig farms. Swine (*Sus scrofa*) are the only natural hosts for PED virus infection. Kamau *et al*. (2010) investigated the ability of using specific pathogen free mice as a potential vector in PED virus transmission. Nevertheless, molecular tests and serological data showed no antibodies detection so mice and rat were not susceptible vectors of the PED transmission[6]. There are 2 forms of PED disease: the PED type I, which is only infected in weaning pigs, and the PED type II affects pigs of all age[7].

The main etiologic agent of the disease is porcine epidemic diarrhea virus (PEDV) which was firstly reported in Europe in 1971 and was identified as coronavirus-like strain CV777 from pigs with watery diarrhea in Belgium and United Kingdom in 1978[8-10]. After that the disease widely spread throughout many swine-raising countries in Western Europe: Hungary (1981); Germany (1981); to Asian countries: Japan (1982); South Korea (2000); Taiwan (2013) and North America (2013)[11]. During the 1970s and 1990s, a few severe PED outbreaks have been reported in Europe[12]. Nevertheless, PEDV infection nowadays has become epidemic in Asia pig industries, consist of China, Japan, South Korea, Vietnam, Thailand, the Philippines and Taiwan[13]. In China, a large-scale diarrhea outbreak was reported in the end of 2010 with the confirmation of PEDV in pig population that over 1,000,000 piglets died with a mortality rate of 80%-100% and resulted

enormous economic losses[14,15]. In South Korea, the PEDV was firstly described in 1992 and re-emerged as a severe outbreak during 2013 with considerable variants that were different from previous Korean isolates or vaccine strains[16,17]. A massive PED outbreak suddenly occurred in the North American pig farm in April 2013 and rapidly spread across the country also to countries sharing the same border: Canada (2014) and Mexico (2013); causing high rates of mortality and huge economic losses[2,3].

Genetic Structure and Characteristics of PEDV

Although the PED disease has similarly clinical signs to transmissible gastro-enteritis (TGE) including anorexia; vomiting; diarrhea and dehydration, the causative agent PED virus is antigenically distinguishable from TGE virus (TGEV) and haemagglutinating encephalomyelitis virus (HEV)[4]. The PED virus belongs to the member of genus *Alphacoronavirus* in the family *Coronaviridae* which constitutes the order *Nidovirales* and causes acute diarrhea in human and animals, especially fatal to newborn individuals[18,19]. Under the electron microscopy (EM), the characteristic appearance of PEDV contains an opaque and pleomorphic body about 90 to 190 nm diameter in range, with an electron-dense core, a large fringe and bulb-shaped projections of approximately 20 nm but the detailed internal structure of PED virus remains unknown[1,7]. The PED virus adapts culture to be stable at wide range of pH from 5.0 to 9.0 at 4°C and still keeps infectivity between pH 6.5 and 7.5 at 37°C[1,20]. However, this virus is sensitive to high temperature when heating to or over 60°C for 30 minutes and to chemical reagents such as ether and chloroform[1]. Besides, most disinfectants are effective to against PEDV, consisting of cresol, formalin (1%), anhydrous sodium carbonate (4%), sodium hydroxide (2%), iodophors (1%) in phosphoric acid, ionic and non-ionic detergents. More than 10 years after the first identification of PEDV, the effort of growing PEDV in cell culture was successfully proved in the study of Hofmann M. and Wyler R. (1988)[21,22]. The Vero cell (derived from African green monkey kidney) culture with the presence of trypsin was susceptible for PED virus propagation. In another research, Shibata *et al*. (1999) described additional methods to isolate

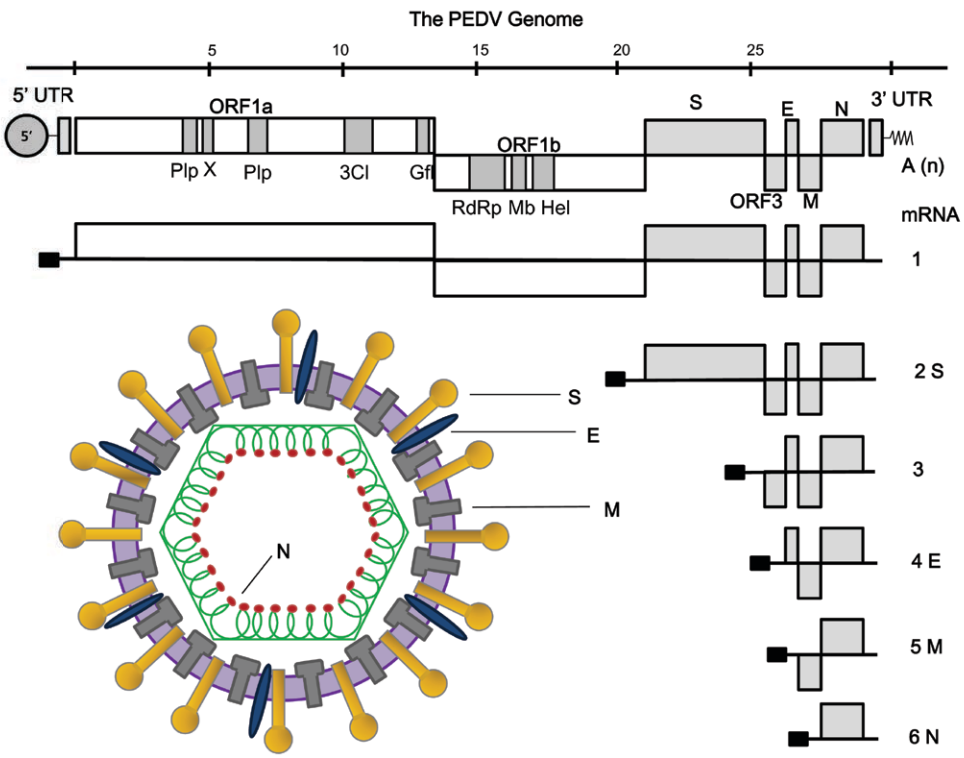

Figure 1. A schematic model of the PEDV genome consists of 7 open reading frames (ORFs) that represented as boxes: ORF1a, ORF1b, S (spike protein), ORF3, E (peplomer protein), M (membrane protein), N (nuclocapsid protein). The domains located in ORF1 are shown: the papain-like proteinase (Plp), the X domain (X), the poliovirus 3C-like proteinase (3C1) and the growth factor-like domain (Gfl) in the ORF1a; the RNA-dependent RNA polymerase (RdPp), the metal ion-binding-domain (Mb), and the helicase (Hel) in the ORF1b[8,25,62].

PEDV using pig bladder cell- and kidney cell-derived cultures, also in the presence of trypsin in the medium because trypsin allowed the *in vitro* propagation of several enteric viruses and facilitated coronavirus growth as an important ingredient in the cell culture media[23].

The PEDV is an enveloped virus with a single-stranded positive-sense RNA genome with approximately 28,000 bases in size with a 5′ cap and a 3′ polyadenylated tail (Poly A)[9,24]. The PEDV genome comprises at least 7 open reading frames (ORFs) that encode three non-structural proteins (ORF1a, ORF1b and ORF3) and four structural proteins (ORF2, ORF4-6)[1,8,25] (Figure 1). The viral genome organization followed a conserved gene order: an untranslated region (UTR) at 5′-end; the large ORF1a and 1b that cover 70% at the 5′-end of genome for polymerase genes; a set of four genes encoding the structural proteins: 150-220 kDa glycosylated spike protein (S), 7 kDa envelope protein (E), 27-36 kDa membrane protein (M) and 58 kDa nucleocapsid protein (N); and a 3′UTR[1,8,26]. Table 1 discusses the physical and structural characteristics of Porcine Epidemic Diarrhea Virus (PEDV) as compared to Transmissible Gastro-Enteritis Virus (TGEV) and Porcine Reproductive and Respiratory Syndrome Virus (PRRSV).

The Non-structural Proteins: ORF1a, ORF1b and ORF3

Based on all sequences of coronaviruses, the ORF1a (nt 297-12650) and ORF1b (nt 12605-20641) shared slightly overlapping sequences at a possible ribosomal frameshift (RFS) site[7,8]. The ORF1a translated for a replicase polyprotein 1a (pp1a) which could be extended into pp1ab at C-terminal of RFS site and encoded for several functional motifs such as three protease: papain-like proteinase (Plp), X domain (X), poliovirus 3C-like proteinase (3C1) and one growth factor-like motif (Gfl), whereas there were a metal ion binding domain (Mb), a RNA-dependent RNA polymerase domain (RdRp) and a helicase motif (Hel) that could be encoded within ORF1b by analogy to amino acid sequences of other coronaviruses[25]. The post-translational cleavage of pp1a and pp1ab resulted in a group of 16 non-structural proteins (nsp1-16) by internal proteases. The ORF3 is a functional accessory gene which located between S and E structural protein. It is conserved among canine coronavirus type I and encodes a glycoprotein gp3 that is concerned to virulence of PEDV in the natural host and to be functional as an ion channel but not generally related to viral replication in cultured cells[1,27,28]. Song *et al*. (2002) analyzed ORF3 in restriction fragment length polymorphism (RFLP) to differentiate the Korean PEDV, KPEDV-9 field strains from wild-type strains for application in vaccine against PEDV infection[28]. This study suggested that the loss of ORF3 production or the changes in sequence of ORF3 resulted in unexpected consequence of the adaption of PEDV in cell culture.

The Structural Proteins: E, M, S and N Protein

The E Protein

The E proteins, one of four structural components in PEDV, are recognized as a small, hydrophobic transmembrane protein of coronaviruses with 76-109 amino acids (aa) in length that are rather divergent among different coronavirus strains[29,30]. It was found that they play a remarkable role on the morphogenesis and viral assembly where they could prompt the curvature of membrane as well as assist in membrane scission[31,32]. Besides, the E proteins without modifications are also integrated into the endoplasmic reticulum (ER) membrane where they are independent of inducing ER stress[33]. Another investigation of E protein expression in *Escherichia coli* and mammalian cells demonstrate the crucial function in altering membrane permeability[34] and apoptosis promotion[35,36]. Additionally the E proteins are observed the interaction with host-cell proteins and *in vitro* ion channel activity which is more selective for monovalent cations and is blocked by hexamethylene amiloride in other coronaviruses[37]. The co-expression of E protein and a major membrane M glycoprotein, via their cytoplasmic domains localizing to pre-Golgi membranes, allows the generation of coronavirus-like pseudoparticles which were identified same size with TGEV virions[33,38]. In recent studies of other coronaviruses, the absence of E proteins leads to two actions: non-infectious virions and the inhibition of full virus growth[39].

The Membrane M Protein

The membrane M proteins, the most abundant component of coronavirus envelope, have potential role in virus assembly. It is a glycoprotein type III with molecular weight in 27-36 kDa, consisting of triple-spanning transmembrane segments which flanked by one short N-terminal ectodomain on the outside of virus and one long C-terminal domain in the cytoplasm[40,41]. When expressed in the absence of other viral proteins, M protein tends to accumulate in the Golgi apparatus as detergent-insoluble, heterogeneous complexes in polymeric structures[42,43]. By employing coimmunoprecipitation and immunofluorescence colocalization analysis in mouse hepatitis virus (MHV), M protein complexes appeared to immediately interact with the spike (S) proteins after their synthesis, forming heteromultimeric complexes in the pre-Golgi membranes[42,44,45].

Table 1. The physical and structural characteristics of Porcine Epidemic Diarrhea Virus (PEDV) as compared to Transmissible Gastro-Enteritis Virus (TGEV) and Porcine Reproductive and Respiratory Syndrome Virus (PRRSV)

	PEDV	TGEV	PRRSV
Classification	The member of the order *Nidoviracles*; family *Coronaviridae*; genus *Alphacoronavirus*	The member of the order *Nidoviracles*; family *Coronaviridae*; genus *Alphacoronavirus*	The member of the order *Nidovirales*; family *Arteriviridae*; genus *Arterivirus*
Temperature and pH Stability	PEDV remained moderately stable at 4°C (pH 5.0-9.0) and at 37°C (pH 6.5-7.5), but totally lost their infectivity at $\geq$60°C for 30 min.	TGEV can keep stability at 37°C (pH 4.0-8.0) for 1 hour and at 4°C (pH 5.0-8.0)	PRRSV are stable at 4°C for 1 week, at 37°C for 3 hours to 2 days but completely inactivated at 56°C in 30 min or lose 90% of their infectivity at pH $<$ 6 or 7.5 $<$ pH
Genome	Enveloped virus with a single-stranded positive-sense RNA genome with approximately 28 kbs in size	Enveloped RNA virus contains a single-stranded positive-sense genome with size ranges from approximately 28.6 kbs	Enveloped RNA virus, PRRSV genome contains a single-stranded, positive-sense RNA with approximate size of 15 kbs
Proteins	The genomic RNA contains 7 ORFs encoding 3 nonstructural proteins (ORF1a, ORF1b and ORF3) and 4 structural proteins (ORF2, ORF4-6)	The 7 ORFs located in genome, consisting of 3 nonstructural proteins and 4 structural proteins (S spike, E envelope, M membrane and N nuclecocapsid protein)	The genome comprises 8 ORFs: ORF1a and 1b; 3 major proteins GP5 glycoprotein, M matrix and N nucleocapsid protein; and 3 minor proteins: GP2, GP3, GP4.
Host cell receptor	The aminopeptidase N	The aminopeptidase N	The porcine Sialo adhesion (pSn)
Diagnosis	Histological diagnosis by HE stain, IHC using anti-PEDV rabbit serum. Virological diagnosis by virus isolation, IFA and gene detection employing RT-PCR, real-time RT-PCR. Serological diagnosis using neutralization test.	The RT-PCR or real-time RT-PCR	Virus isolation, RT-PCR, antibody test by IFA, ELISA
Vaccine	Oral vaccination with attenuated vaccine PPEDV-9 strain (Korea), P-5V and 96-P4C6 strain (Japan), CV777-attenuated or inactivated vaccines (China).	Live attenuated vaccine	Live attenuated vaccine, DNA vaccine and recombinant DNA vector vaccine.
References	29, 79-82	22, 80, 83	84-86

Additional studies recently provided proof for the existence of M-M interactions which were observed that assembly-incompetent M could be rescued into virus-like particles (VLPs) by assembly-competent one[42]. It is indicated that the self-association of coronaviruses envelope glycoproteins plays an essential role in arranging of the viral membrane proteins and is thought to drive virion envelope assembly but do not determine the viral budding site at the pre-Golgi membrane. Another function of PEDV M protein is useful in developing epitope-based vaccines. Zhang *et al*. (2012) identified epitopes on the PED virus M protein, highly conserved 4D4-defined epitope, to study the antigenic properties of this protein[40]. This epitope could be a candidate for development of protective antibodies for PED because it was recognized by positive serum of different PEDV strains, comparing to TGEV-positive serum.

The Spike S Protein

Among PEDV structural proteins, the S protein is a major type I membrane glycoprotein on virion surface, with average 1,300aa in length and 150-220 kDa in weight[46]. This trimeric spike protein forms the characteristic peplomers (surface antigen) and consists of a signal peptide (1-18aa), four neutralizing epitopes (499-638, 748-755, 764-711, and 1,368-1,374aa), a transmembrane domain (1,334-1,356aa), and a short cytoplasmic tail[2]. The S protein can also have potential

N-glycosylation sites and can be cleaved by exogenous or host cell protease into S1 (1-789aa) and S2 (790-1,383aa) domains[47]. The S1 domain is composed of a binding domain for host cell receptors whilst the S2 domain, involved in virus-cell attachment and in both virus-cell and cell-cell fusion, could be divided into three domains: a large ectodomain, a single transmembrane (TM) region and a cytoplasmic tail (CT) region[48]. The large ectodomain of S2 subunit was an identified role in membrane fusion activity and composed of a protease cleavage site, a putative fusion peptide and two heptad repeat (HR) region[48-50]. A few studies reported that The TM region with aromatic domain and the CT region carrying the cysteine-rich domain were concerned to regulate the fusogenic activities[51,52]. Further, another important role of CT region of the PEDV spike protein, which was determined by a signal: a dibasic (KxxHxx-COOH), was accountable to retrieve this protein in the endoplasmic reticulum-Golgi intermediate compartment (ERGIC), whereas the S protein with a mutation (H → R) or lack of this motif resulted in enhanced cell surfaces expression of S protein. Nevertheless the role of a potential tyrosine-based (YxxF motif) signal of CT region remains unknown[53-55]. The interaction between S proteins and M or E proteins (as mentioned before), which is essential for virus assembly into those intracellular vesicles, involved in the localization of S protein in the ERGIC.

Some studies showed that the S protein were not essential for budding of coronavirus particle but needed for infectivity. By culturing coronaviruses MHV A59 in the presence of antibiotic tunicamycin, Rottier *et al*. (1982) found that the spikeless and noninfectious virions were produced[56]. Additionally, Luytjes *et al*. (1996) conducted biochemical analysis and electron microscopy to survey the characteristic of temperature-sensitive mutants of coronavirus MHV A59 and found that the temperature-sensitive mutant S protein at 39.5°C (non-permissive temperature) were fail to incorporate into virion particles and to mature to Golgi membrane[57]. However, the synthesis of the M protein and the nucleocapsid N protein of mutant viruses was not sensitive to temperature.

It is presumed that the S protein is the most antigenic of the PEDV proteins because of containing virus-neutralizing epitopes[58]. Chang *et al*. (2002) targeted the S protein as a primary antigen to identify alternative epitope region for designing an effective vaccine against coronaviruses[59]. Although the PED virus and TGE virus are serological distinct, this study based on the CO-26K, a collagenase-digested fragment, of the TGEV S protein to find the critical virus-neutralizing epitope located in the S protein of PEDV. By analyzing the neutralizing activity of the polyclonal antisera and comparing the sequences of COE (CO-26K equivalent) gene of spike protein among PEDV strains (PEDV CV777 strain, Korean isolate and PEDV Brl/87), this study suggested that the COE region of PEDV spike protein contained an important virus-neutralizing epitope with no cross-neutralization (based on very low homology in the same region comparing with feline infectious peritonitis virus, FIPV and TGEV).

The Nucleocapsid N Protein

The PEDV N protein is a phosphoprotein which is associated with RNA genome and indicates a basic structure for the helical nucleocapsid[1]. The encoded polypeptides range of PEDV N protein is from 377 to 455 amino acids and has the similar physical properties with the other members of the family *Coronaviridae*[60]. Nevertheless one unique sequence with a 40-residue-long insertion in the central position of PEDV N protein molecule could reflect recombinant event or stuttering of the viral polymerase. This sequence is particularly rich in arginine (Arg), serine (Ser) and asparagine (Asp) residues, and presents in PED virus N protein with no counterpart in the remainder of Coronaviruses. The N protein can be an alternative target to accurately diagnosis PEDV in early infection. Furthermore, Xu *et al*. (2013) generated a specific cell line IEC (porcine intestinal epithelial cell) expressed PEDV N protein as well as investigated the function and localization of N protein[60,61]. The study suggested that PEDV N protein affected on cycle progression, interleukin-8 (IL-8) expression, cell growth and survival. Particularly the PEDV N protein was identified in the ER membrane, induced cell cycle prolongation at the S-phage, which is associated with a low level of cyclin A transcription and increased in cyclin A degradation, and was responsible for ER stress, which included the initiation of an inflammatory react via activation of NF-kB, as well as up-regulation interleukin-8 expression[60,61]. This study was the first report to demonstrate novel functions of PEDV N protein that was useful in investigating the molecular mechanisms and PEDV pathogenesis.

Based on the partial sequence analysis of S, M and ORF3 genes, the genetic and phylogenetic relation of PEDV isolates were determined[26,28,46]. By phylogenetic analysis of S glycoprotein genes including epitope region, the diversity of Koreans PEDV strains were reported and divided into groups and subgroups. There were three groups of Korean PEDV isolates: the G1 group were highly homologous to reference PEDV strains (CV777, JS-2004-2, P-5V, SM98-1, KPED-9, parent and attenuated DR13) but did not have specific nucleotides and amino acids comparing with other groups G2 and G3 Korean PEDV isolates, including

highly homologous Spk1 and Chinju99[29]. Otherwise the subgroup G1-1 Korean PEDV isolates had unique specific nucleotide sequences and had the DNA sequence identities 95.3%-97.9%, 93.6-96.6%, 93.5-96.6%, and 88.7-90.7% with the subgroup G1-2, G1-3 and the group G2, G3 Korean PEDV isolates respectively. The study with analysis of S glycoprotein genes demonstrated the Korean PEDV strains were genetically diverse both among themselves and to other foreign reference PEDV strains.

PED Diagnostic Techniques

The PED diagnosis cannot be determined only on the epidemiological investigation, the basic of clinical signs and the histopathological lesions. Due to the dependence of PED clinical signs on the age of swine, the presence of secondary infection and the immunological status of the pigs, as well as the similarities in characteristics of PED syndromes from other causative agents of diarrhea such as TGEV, rotavirus, bacteria (*E. coli*, *Salmonella* sp., *Clostridium* spp., etc.) or by parasites, the laboratory examinations are required to identify and confirm the PEDV infection. Many techniques are available for the detection of PEDV from fecal materials, small infected intestines sample, including reverse transcript polymerase chain reaction (RT-PCR), direct immunofluorescence tests (IF), indirect fluorescence antibody tests (IFA), immunohistochemistry techniques (IHC), in situ hydridization, electron microscopy and enzyme-linked immunosorbent assays (ELISA)[2]. The popular diagnostic methods used to detect PED viral antigen, are immunofluorescence test while the most commonly applicable tests to detect PEDV antibodies in serum and antigen in feces are enzyme-linked immunosorbent assays.

Electron Microscopy (EM), Virus Neutralization (VN) Test, Immunofluorescence (IFA) and Immunohistochemical (IH) Assay

Of all available techniques to detect the causative agents of PED, electron microscopy (EM) is the least sensitive technique. Sueyoshi *et al.* (1995) evaluated the PEDV-infected lesions using transmission EM (TEM) technique, nevertheless the result showed a mix of coronaviruses within the cytoplasm and microvilli of epithelial cells in small intestine[63]. After that they carried out a streptavidin-biotin (SAB) technique to detect the specific viral antigen of PED in the cytoplasm of enterocytes, with light microscopy. This method examined formalin-fixed materials with a serial of washing step and no cryostat that demonstrated adequately detailed diagnosis methods for outbreak of PEDV occurred in Japan. In case the spikes of virus were lost or not clearly visible, the TEM results were inconclusive to confirm that the causative agents were PEDV or viruses with the similar size and morphology, especially TGEV. For the reason that the EM technique is not sensitive and specific, it could not be applied to differentiate PED virus from the remainder of coronaviruses, especially TGE virus.

Shibata *et al.* (1999) employed virus neutralization (VN) test and immunofluorescence assay (IFA) to detect antibodies against PEDV as well as the immunohistochemical examination for the detection of PEDV antigens in the enterocytes of infected pigs by avidin-biotin (AB) technique[12]. The paper determined the isolation of PEDV using porcine cell cultures (as mentioned before), whereas established the SB1 and SB2 cells derived from the epithelial cells of cesarean-derived colostrum-deprived (CDCD) pig bladder and the SK cells prepared from the CDCD pig kidney for different passage state, instead of using popular methods with Vero cells. Furthermore, this study discovered the effect of different pig age on the disease, in particular, the severe clinical signs were only found in two-day and one-week old piglets. So this research concluded that there was an age-dependent resistance to PED virus in swine. Until now, the Vero cell lines are the most popular and effective to isolate PED virus. Pan *et al.* (2012) used the Vero cell cultures to isolate CHGD-01 PEDV strain as well as employed direct immunofluorescence assay and EM technique to investigate its specific cytopathic effects in the outbreak of diarrhea in Guangdong (South China) swine[18]. The study also reported the susceptibility of Vero cells to different PEDV strains because the characteristic CPE caused by PEDV isolate in this research was not confirmed until seven passages of Vero cells. Based on phylogenetic analysis of the whole genome, the CHGD-01 isolates were placed in a cluster with two other Chinese strains, sharing almost 98% of nucleotide sequence identities. However, this recent Chinese strain might have originated from the Korean PEDV strains KNU 0802, based on amino acid sequence analysis of viral spike protein gene.

Molecular Tests for PEDV Detection: Reverse-transcription PCR and Enzyme-linked Immunosorbent Assays (ELISA)

The RT-PCR could be applied for a rapid detection of PEDV infection without showing any cross reaction with TGEV or porcine rotavirus. Kweon *et al.* (1996) designed three primer sequences from the membrane protein (M) gene of PEDV for RT-PCR test[64]. The result of this study proved that a positive DNA band of KPEDV-9 M gene could be early detected from fecal specimens at 24 h post-infection in experimentally inoculated pigs. The research from Ishikawa *et al.*

(1997) was in the close agreement with the conclusion of RT-PCR as a practical, rapid and specific method for detecting PEDV in swine[65]. The study developed a RT-PCR detection system by using primers to amplify an 854 bp fragment of M protein gene of PEDV. The RT-PCR system in this study could effectively detect PEDV RNA from viral mixtures or small intestinal/fecal samples in very low number of virus within short time. The virus isolation and the SAB technique were employed to confirm PEDV positive results by RT-PCR, based on virus and its antigen detection. Moreover, a serial dilution of PEDV JMe2 strain for RT-PCR was conducted to evaluate the ability of this assay to amplify viral RNA from small intestinal samples and fecal specimens as well as to eliminate the inhibitors of RT-PCR in specimens. Previous studies have showed difference in the sensitivity of RT-PCR assay. This could be explained by the difference in RNA extract processes, RT-PCR conditions or the presence of inhibitors in the intestinal and fecal samples that could affect the sensitivity of RT-PCR. There were many factors in the intestinal and fecal specimen that could inhibit the activity of thermostable polymerase in enzymatic reaction of PCR amplification, especially the presence of bilirubin and bile salts[66,67]. To enhance the specificity and sensitivity of RT-PCR, the extraction process of viral RNA and PCR conditions need to be optimized and chemical reagents that prevented the inhibition factors in raw materials, were advised to use. In the reverse transcription step, the RT-PCR also revealed high potential in cross contamination during mass screening, however.

Kim *et al*. (2007) improved the molecular detection methods of PEDV by generating a multiplex real-time RT-PCR employing 2 sets of primers and different probes labeling with reporter dyes in a single reaction tube[68]. The primers and probes were designed and synthesized base on conserved sequence of the nucleocapsid N genes from a number of strains of TGEV and PEDV, and were labeled with specific dyes for each virus: 5′-reporter dye FAM and 3′-quencher BHQ1 for TGEV, 5′-reporter dye Cy-5 and 3′-quencher BHQ2 for PEDV. The selection of primers and probes which based on the highly conversed regions of nuclecapsid gene of the CV777 strain of PEDV and the Purdue 46-MAD strain of PEDV as well as using the same concentrations for both of them could optimize this assay. The multiplex real-time RT-PCR was able to differentially detect and qualify the PEDV and TGEV rapidly from both experimentally and naturally infected pigs, with no cross-reaction between diarrhea-causing viruses. This assay also saved the time-consuming comparing to complete RT-PCR-based dot blot hybridization and minimized the rate of contamination by reducing the number of experimental steps. Hence, the RT-PCR-based technique was considered as a useful and practical method for rapid, sensitive and specific detection of PEDV infection in fecal samples from live pigs, without killing.

The study of Carvajal *et al*. (1995) was one of the first researches that focused on detection of PEDV antigen and antibodies[69]. The study generated simultaneously 2 separate blocking enzyme-linked immunosorbent assays (ELISAs) and an indirect fluorescence test (IFT) in order to detect both PEDV antigen in fecal samples and PEDV antibodies in serum, using monoclonal antibodies (MAb) as capture and reporter agents. The results indicated that the antibodies in both natural and experimental PEDV infections were detected by ELISA in shorter time than by IFT (3-5 days sooner). Hence the MAb-based ELISA was considered higher sensitive method allowing the diagnosis of PEDV infection in diarrhea endemic and on farm. However, there was unknown reason between ELISA-negative but IFT-positive results that remained in this study. In another research, the competitive blocking ELISA (CB-ELISA) was generated to identify PEDV in culture medium and fecal samples, evolving non-conjugated monoclonal antibodies to membrane M protein of PEDV. This study also showed the low correlation of sensitivity between the EM technique and the CB-ELISA that only 3/15 fecal samples was positive with coronavirus by EM examination while the negative results of CB-ELISA was 14% (9/65 fecal samples). Hou *et al*. (2007) based on recombinant 48 kDa nucleocapsid (N) protein of PEDV as a useful antigen to develop an ELISA (rnELISA) for detecting antibodies to PEDV[18]. The RT-PCR was conducted to amplify nucleocapsid N protein gene from the PEDV Korean strain, using forward and reverse primer containing restriction enzyme sites (*Bam*HI and *Sac*I). After subcloning into the prokaryotic expression vector pQE-30, the recombinant plasmid pQE30-PN was transformed into host cell (*Escherichia coli*) to produce the PEDV soluble nucleocapsid proteins with an N-terminal His6-tag of 442 amino acids. The recombinant N proteins were purified by affinity chromatography in Ni-nitrilotriacetic acid (Ni-NTA) agarose and were analyzed by SDS-PAGE and Western blotting with anti-His-tag monoclonal antibody before applying to rnELISA for PEDV detection. Besides, the limitation of rnELISA was determined by a cut-off value that was calculated as absorbance values among 80 serum samples from field, based on the mean value plus two SDs (standard deviation). In the additional confirmation from RT-PCR and the serum neutralization (SN) tests, the recombinant N protein IgG ELISA obtained 98% sensitivity (among 103 clinical PEDV-infected sam-

ples) and 98.7% specificity (among 80 PEDV-free samples) compared to RT-PCR in methodologies as well as this recombinant protein-based serological test only reached 8.5% false-positive results by antibody detection in 18 of 213 SN negative results. The study indicated that the nucleocapsid N protein might be a sensitive antigen for the serological diagnosis of PEDV and the rnELISA had high sensitivity and specificity for a rapid and simple method for the large-scale detection of PEDV.

The correlation between the DAS-ELISA and the RT-PCR was also demonstrated by examining 506 specimens of pig herd from different farms in the Po valley (Italy), according to the study of Sozzi *et al.* (2009)[70]. The double antibody sandwich (DAS) ELISA was generated for detection of PEDV in both swine intestinal and fecal samples, using monoclonal antibodies. The six MAbs specific for PEDV, which were generated and characterized from the screening of forty hybridomas by indirect IF and indirect ELISA using non-infected and PEDV-infected cells, were purified and modified with horseradish peroxidase (HRPO) conjugation before applying to DAS-ELISA. The six selected MAbs were surveyed the intensity and specificity in different combination, i.e. 1F12 as conjugated MAb and MAb 4C3 as antigen catching antibody, for the best catcher and tracer in DAS-ELISA. The comparison of DAS-ELISA and RT-PCR indicated the complicated correlation between intestinal and fecal samples. For the fecal examining, the high kappa statistic value suggested an almost perfect agreement in two methods that only 2 of 215 samples were ELISA-negative but RT-PCR-positive identification. This could be explained that the clinical samples were collected in the recovery period of disease with the low viral titres and the forms of specific antibody in faeces were immunocomplexes. However the intestinal examination by two methods gave a disagreement because there were 7 samples of RT-PCR negative but ELISA positive. The disagreement might be due to the non-specific binding of antibodies in ELISA reaction and the available inhibitors of PCR assay. These finding agreed with previous studies that serological test-ELISAs were rapid and sensitive for the screening of a large number of specimens so the PEDV-ELISAs were suitable and effective in controlling PED disease during epizootic outbreaks.

Comparison of Various PEDV Detection Methods

In a research by Guscetti *et al.* (1998), four methods were compared for diagnosis of wild-type PEDV: an immunohistochemical detection using formalin-fixed tissues, a direct immunofluorescence assay using cryostat sections, an ELISA and a PCR method[71]. This study showed that the ELISA results confirmed a sensitive and reliable detection in fecal material for PEDV but the presence of false positive reactions might affect the results when using for large number of specimen samples or samples from different infected animals in clinical practice. The PCR methods were ineffective in this study because wrong negative results were indicated by the other methods but the virus purification step before RNA extraction could enhance this method. According to conclusion of this research, the IHC and IF methods, which were used to detect viral antigen from gut tissues, demonstrated very high sensitivity and reliability, allowing detection for more than 75% enterocytes were positive for viral antigen by IHC in the mid-jejunum particularly. In contrast, the RT-PCR used in the recent study was indicated to be a simple, rapid, specific and sensitive method for the identification of PEDV from fecal samples. Kim and Chae (2012) used and compared reverse-transcription polymerase chain reaction (RT-PCR), immunohistochemistry (IH) technique, and in situ hybridization for detection of PEDV in fifteen experimental PEDV-infected pigs and 94 samples of diarrheic piglets[72]. For the intestinal and fecal samples from experimental PEDV-inoculated pigs, the RT-PCR could detect viral antigen and nucleic acid in all samples (15/15) while the IH and in situ hybridization could not identify the causative agent in 1/15 and 2/15 samples, respectively. For PEDV detection of diarrheic pigs in fecal samples, 63/94 samples were positive and 15/94 samples were negative for causative PEDV by all 3 methods, indicating a high agreement and correlation (83%) among these methods. The high rate of agreement demonstrated that the 3 methods could be applied independently to accumulate an accurate diagnosis for PEDV infection. This study also indicated how to choose the suitable technique to diagnose PEDV depends on the available types of sample. The RT-PCR test was recommended for virus detection if only fecal samples were provided whilst the immunohistochemistry (Figure 2) and in situ hybridization were suggested for more sensitive detection of PEDV in formalin-fixed intestinal samples[72]. Due to the special features such as rapid detection, high sensitivity and specificity, and cost-effectiveness, the RT-PCR is considered as an important and successful test for detection of PEDV both laboratory and field virus strains.

Vaccination

Vaccination is one of the traditional and required methods to prevent and control PED virus infection in swine. Nowadays, the PED epidemic outbreaks, which neonatal pigs are involved, tend to be more serious

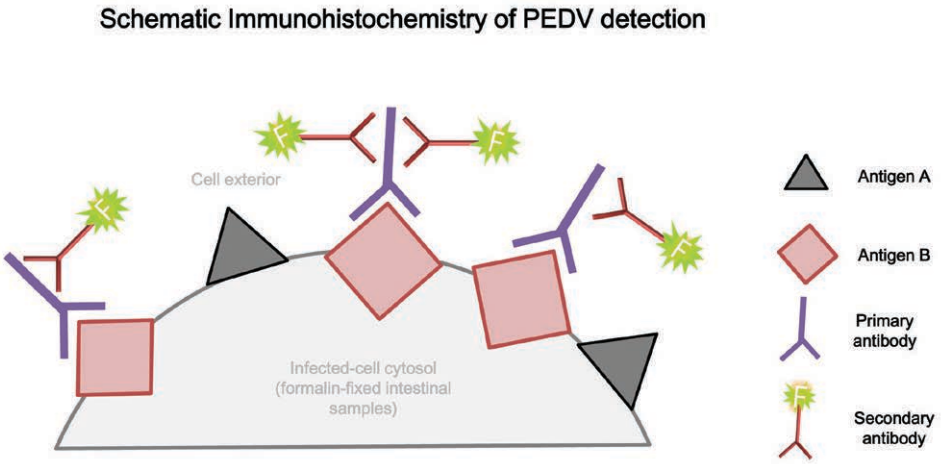

Figure 2. The immunohistochemistry is recommended for confirming the diagnosis of PEDV infection from available formalin-fixed intestinal samples with specificity and sensitivity, and for cellular detail and histologic architecture of infected cells.

and dangerous in Asia countries than in Europe and others with the sufficient economic loss. Therefore, several trials of PEDV vaccine were mainly researched and developed in Asia countries such as attenuated vaccine PPEDV-9 strain in Korea, P-5V and 96-P4C6 strain in Japan, CV777-attenuated or inactivated vaccines in China[73-75]. In sow, neonatal pigs are normally protected until they are 13-day-old by a transfer of IgG maternal antibodies from colostrum and milk of immune mothers[1]. Beside the 60% of IgG accounting in colostrum immunoglobulin content, there are IgM and especially IgA which is more resistant and has high virus neutralizing activity than IgG[76]. However, these antibodies from passive immunity are not able to prevent the intestinal infection of PEDV.

In South Korea, researchers investigated attenuation of KPEDV-9 isolate via serial passages in Vero cell cultures (up to 93 passages) and its immunoprophylactic effects in pregnant sows[74]. The piglets intramuscularly or orally inoculated with attenuated virus of high passage level did not reveal any severe symptoms of diarrhea or death comparing to the animal with wild PEDV-infection, so an alternative vaccine might be developed from attenuated virus deriving from serial passage of PEDV. This study also indicated that the vaccinated pregnant sows by intramuscular inoculation had the low mortality rate of piglets. Another researcher group generated a Vero cell attenuated PEDV DR13 which was differed from wild-type PEDVs by a restriction fragment length polymorphism (RFLP) pattern[77]. The study compared the ability of the cell attenuated DR13 virus after inoculation via oral (O) and intramuscular (IM) routes in late-term pregnant sows to protect neonatal pigs against PED, employing the SN test and ELISA. The experiment of the immunoprophylactic effect in pregnant pigs showed the similar agreement with the research by Kweon *et al*. (1999) in decreasing mortality of piglets but cell-adapted DR13 vaccination in this study through oral route[74]. Previously, PEDV DR13 was also isolated in Vero cells through serial passage at level 100 and was analyzed for differentiation from other Korean field strain via RT-PCR RFLP with *Hind*III and *Xho*II enzymes. Moreover, the investigation of cell-adapted DR13 indicated the reduced virulence after high serial passage as well as its immune response in 14-day-old piglets and pregnant sows. The attenuation and safety of high serial passage DR13 after oral vaccination were also discussed in this study.

Hou *et al*. (2007) targeted the nucleocapsid N protein of PEDV to develop a recombinant vaccine trial by employing a surface antigen display system on lactobacilli[78]. This system used the poly-γ-glutamate A (pgsA) protein, which is a synthetase complex of *Bacillus subtilis*, as the transmembrane anchor to express heterologous antigens in the surface of *Lactobacillus casei*525. The live and dead *L. casei* anchoring nucleocapsid protein of PEDV were vaccinated to two animal models: intranasal and oral inoculation of mouse, oral inoculation in pregnant sows that induced the systemic and local immune responses in both. The rnELISA were carried out to indicate the high levels of serum IgG and mucosal IgA after inoculation. Moreover, the IgG levels of piglets were highly increased after receiving colostrum secreted from vaccinated sows with recombinant *L. casei*. The surface antigen expression system on LAB (lactic acid bacteria) was potential for vaccine applications to against PED but

limited in the display size of foreign antigens.

Perspective

The PEDV infection cause acute and severe enteritis with clinical symptoms: diarrhea and vomit, followed by extensive dehydration which is the main reason of death in neonatal pigs. This viral infection had a significant impact on the economy of the European and Asia pig industries for the last three decades. The disappearance and re-emergence of epidemic PED demonstrated that the current vaccine application, the detection methods, the biosecurity system are old-fashioned and not effective in control and treatment of PEDV infection. Comprehensive knowledge of the pathogenic characteristics of epidemic and endemic PEDV strains is required to generate effective tests for PEDV detection in all affected countries and to develop suitable vaccines to different PEDV strain for all regions.

Over the geographic limitation, a number of PED discoveries about the epidemiology, the virology, the pathogenesis, the immunology, the vaccinology as well as several detection methods such as histological examination, virological and serological diagnosis have been gained and upgraded[1,2,20]. The potential diagnosis of PEDV should not only be rapid and robust but also cost-effective and able to detect a large number of samples in field. New researches and experiments are required to investigate cross-protection between field viruses and vaccine strains. More significant measures (such as physicochemical, genetic and antigenic analyses) need to be established to control and prevent the PEDV infection. Lastly the combined applications of early detection, vaccination, biosecurity programs and management play a major role in prevent and control PED. This review will provide basic and applied understanding of pathogenic PEDV as well as the extensive discussion about the recent PED circumstance with modern and popular methods to against PEDV.

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