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Review

Porcine epidemic diarrhea virus: An overview of current virological and serological diagnostic methods



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ABSTRACT

Porcine epidemic diarrhea virus (PEDV) is the causative agent of an acute, highly contagious, and severe enteric disease that leads to high mortality rates in suckling piglets. Therefore, accurate diagnosis of PEDV infection is critical for the implementation of control measures for the virus. Many diagnostic tests have been recently developed and are currently available for the detection of PEDV, its proteins or nucleic acid, including virus isolation, immunofluorescence (IF) or immunohistochemistry (IHC), polymerase chain reaction (PCR) and isothermal amplification assays. Additionally, several serological assays have been developed and are currently used for the detection of antibodies against PEDV. Molecular assays such as real-time reverse transcriptase-PCR (rRT-PCR) became the methods of choice for the diagnosis of PEDV infection, providing sensitive, specific and rapid detection of the virus RNA in clinical samples. Whereas serological assays have been widely used to monitor prior exposure to the virus and to evaluate the efficacy of novel vaccine candidates or vaccination strategies. Here we discuss the properties of current PEDV diagnostic assays and prospects for improving diagnostic strategies in the future.

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1. Introduction

Porcine epidemic diarrhea virus (PEDV), a member of the genus *Alphacoronavirus* of the family *Coronaviridae* (Jung and Saif, 2015; Lee, 2015; Song and Park, 2012), was initially identified in Europe in 1978 as the causative agent of porcine epidemic diarrhea (PED), an acute, highly contagious, and severe enteric disease affecting swine (Pensaert and de Bouck, 1978). PED is characterized by severe diarrhea, vomiting, and dehydration followed by high mortality in suckling piglets (50–100%). Animals of all ages are susceptible to PEDV; however, older pigs usually present a milder form of the disease with lower mortality rates (Alvarez et al., 2015; Annamalai et al., 2015).

Since its initial detection in Europe, PEDV has spread to several of the major swine producing areas of the world, including Asia and most recently North America, causing significant economic losses to the swine industry (Lee, 2015; Song and Park, 2012). In Asia, the virus was first detected in Japan in 1982, spreading to several neighboring countries, including Korea, China, Thailand, Taiwan and Vietnam, where it remains endemic to date (Chen et al., 2010; Lee, 2015; Olanratmanee et al., 2010; Sun et al., 2015). In 2013, PEDV was introduced in North America, being initially detected in the US and subsequently spreading to Canada and Mexico (Ojic et al., 2015; Stevenson et al., 2013). After its introduction in the US, PEDV was rapidly disseminated across the country, causing the deaths of over 7 million piglets (~10% of the country's swine population).

The PEDV genome consists of a large (~28 kb) single-stranded, positive sense RNA molecule that contains seven open reading frames (ORF1a, ORF1b, and ORF2-6). ORFs 1a and 1b encode large polyproteins (pp1a and pp1b), which are cleaved by viral encoded proteases into 16 non-structural proteins (nsp1-nsp16) involved in basic mechanisms of viral RNA transcription and replication (Lee, 2015; Song and Park, 2012). ORFs2-6 encode four structural proteins, including the Spike (S) glycoprotein, membrane (M) protein, envelope (E) protein, and the nucleocapsid (N) protein, and one accessory protein ORF3 (Huang et al., 2013; Lee, 2015; Song and Park, 2012). Among the structural proteins, the S glycoprotein plays an important role in receptor binding, cell membrane fusion and entry, and is the main target of neutralizing antibodies against PEDV (Cruz et al., 2008; Sun et al., 2008; Wicht et al., 2014). Notably, the S gene has been frequently targeted in epidemiological studies involving phylogenetic analysis and in a number of molecular and serologic diagnostic assays for PEDV (Gerber et al., 2014a; Huang et al., 2013; Kim et al., 2015; Li et al., 2015; Park et al., 2007; Paudel et al., 2014b). Additionally, the M and N proteins have been targeted for the development of molecular and serological diagnostic assays for PEDV (Ishikawa et al., 1997; Okda et al., 2015; Wicht et al., 2014).

Outbreaks of watery diarrhea concurrent with high mortality rates in suckling piglets are usually the first signs that PEDV might be circulating within a swine population. However, given the similarities of these clinical manifestations with other enteric pathogens of swine, including the closely related coronaviruses, transmissible gastroenteritis virus (TGEV) and porcine deltacoronavirus (PDCoV) (Chen et al., 2015; Kim et al., 2000a,b; Song et al., 2015; Wang et al., 2014a), a diagnosis of PEDV cannot be made based on the clinical signs and histological changes observed in the gastrointestinal tract. A positive PEDV diagnosis involves the detection and identification of PEDV-specific proteins or its RNA genome in clinical samples from affected animals. Conventional PEDV diag-

nostic methods are based on the detection of the virus, its nucleic acid, viral proteins or antibodies induced in response to infection. While the diagnosis of a PEDV outbreak is usually best obtained by virological detection methods, serological assays provide important information regarding previous exposure to the virus and the immune status of a given animal and/or herd. Here we present a brief overview of virological and serological diagnostic methods that allow rapid and accurate detection of PEDV and assessment of the host responses to infection.

2. PEDV infection dynamics and host responses to infection

After fecal-oral transmission, PEDV replicates in epithelial cells lining the small intestine, resulting in rapid necrosis and destruction of infected cells (Jung et al., 2015; Thomas et al., 2015). Following an incubation period that varies from one day in 1-day-old nursing piglets to 3–6 days in 3-week-old weaned piglets, characteristic clinical signs of PED, including diarrhea and vomiting, are observed and usually persist for approximately 5–10 days (Jung et al., 2015; Madson et al., 2016, 2014; Stevenson et al., 2013). Fecal shedding of PEDV occurs shortly after infection (1–3 days), and the virus RNA has been detected in feces for up to 24–30 days post-infection (pi) (Thomas et al., 2015). A short viremic phase has been described between one and five days pi in suckling piglets (Jung et al., 2015; Madson et al., 2016). Viral antigens have been detected in enterocytes in the duodenum and ileum as early as 12 h pi in 1-day old piglets (Madson et al., 2016), and through day 14 pi in 3-week-old weaned pigs (Madson et al., 2014).

Antibodies are first detected in serum between days 6 and 14 post-primary infection with PEDV (Okda et al., 2015). Notably, antibody responses generated against PEDV N and S proteins, the two major structural proteins of the virus, present different magnitude and dynamics (Fig. 1). While N-specific IgM antibodies peak on day 7 pi, S IgM responses peak on day 14 pi declining to pre-infection levels between days 14 and 21 pi. Whereas N- and S-specific IgG antibodies are first detected on day 7 pi, with anti-N IgG antibodies peaking on day 21 pi, and S IgG responses peaking on day 14 pi. Both N- and S-IgG antibody levels begin to decline around day 21 pi, whereas N IgG responses seem to be sustained for up to 43 dpi. Notably, S-specific IgA responses present a similar kinetics to S-IgG responses, with a low level of antibodies being detected on day 7, peaking on day 14 pi, with sustained levels for up to 6 months (Ouyang et al., 2015).

PEDV specific antibodies have also been detected in oral fluids using IgG or IgA isotype ELISAs (Bjstrom-Kraft et al., 2016; Gimenez-Lirola et al., 2016). Notably, IgA seems to be the predominant antibody isotype in oral fluids, with antibody levels increasing through 100 days post-infection. While IgG antibody levels peaked around day 14 post-infection gradually declining thereafter (Bjstrom-Kraft et al., 2016).

Neutralizing PEDV antibodies (NABs) are first detected between days 7 and 14 pi, peaking on day 21 pi (Ouyang et al., 2015; Thomas et al., 2015). Notably, NABs seem to persist for at least 6 months post infection (Fig. 1) (Clement et al., 2016; Ouyang et al., 2015). These aspects of PEDV infection and host responses to the virus provide important information that should be considered prior to diagnostic sample collection and testing.

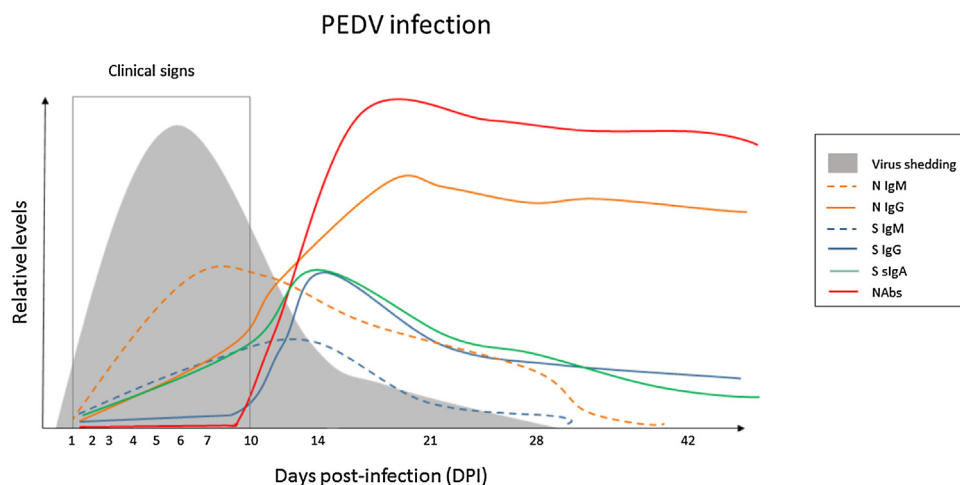


Fig. 1. Porcine epidemic diarrhea virus infection dynamics and host responses to infection. Schematic representation of the course of PEDV infection highlighting time of clinical signs, virus shedding in feces and the kinetics of serum antibody responses against the virus. N IgM: nucleoprotein specific serum IgM responses; N IgG: nucleocapsid specific serum IgG responses; S IgM: spike specific serum IgM responses; S IgG: spike specific serum IgG responses; S IgA: spike specific serum IgA responses; NAbs: serum neutralizing antibody responses. The information presented was collected from several PEDV experimental studies performed with naïve animals of different age groups. It is important to note that a short viremic phase (1–3 days) that has been described in neonatal piglets is not depicted in the graph.

3. PEDV diagnosis

Current PEDV diagnostic assays can be divided in two categories: virological and serological methods. Virological methods target detection of the virus, its nucleic acid and viral proteins, while serological assays detect antibodies induced in response to infection. Following the introduction of PEDV in the US in 2013, a new array of improved diagnostic reagents and assays were developed for rapid and specific detection of PEDV. Here we present a brief overview of the properties of these assays. A summary of current PEDV diagnostic assays and their properties are presented in Table 1.

3.1. Virological assays for the detection of PEDV and/or viral antigens

3.1.1. Virus isolation

Vero cells are the most widely used cell line for isolation and propagation of PEDV (Chen et al., 2014; Jung and Saif, 2015; Oka et al., 2014). Typical cytopathic effect (CPE) induced by PEDV replication in cell culture is characterized by cell fusion, syncytia formation and eventually cell detachment (Chen et al., 2014; Oka et al., 2014) (Fig. 2). Isolation of PEDV in cell culture is usually confirmed by immunofluorescence (Fig. 2) and/or RT-PCR (Chen et al., 2014; Oka et al., 2014).

Replication of PEDV in cell culture is dependent on the presence of exogenous trypsin in the cell culture medium (Chen et al., 2014; Oka et al., 2014; Wicht et al., 2014). The proteolytic activity of trypsin leads to cleavage of the S glycoprotein into the S1 and S2 subunits, resulting in enhanced virus-to-cell and cell-to-cell fusion and increased virus infectivity and release from infected cells (Wicht et al., 2014). Therefore, exogenous trypsin has to be added to the medium for isolation of PEDV in cell culture.

The dependence on exogenous trypsin and the lack of a better cell culture system, make the isolation of PEDV in cell culture difficult. Two recent studies undertook a comprehensive approach to optimize the isolation of PEDV in cell culture (Chen et al., 2014; Oka et al., 2014). However, from a total of 138 clinical samples that were positive for PEDV by RT-PCR, only 13 PEDV isolates were recovered in Vero cells (Chen et al., 2014; Oka et al., 2014). All these isolates were obtained from intestinal contents (Oka et al., 2014) or intestinal homogenates (Chen et al., 2014), and no virus was recovered

from rRT-PCR positive fecal samples (Chen et al., 2014). Recently, however, Chen and collaborators (Chen et al., 2016a) reported the isolation of three PEDV strains from rRT-PCR fecal samples. These observations indicate a low diagnostic sensitivity of PEDV isolation methods, and highlight the need for improved cell culture systems for the isolation PEDV *in vitro*. Primary intestinal epithelial cell cultures or cell lines expressing the PEDV receptor, porcine aminopeptidase N (APN) (Li et al., 2007; Nam and Lee, 2010; Shan et al., 2015), could be valuable alternatives and potentially increase the success rate of PEDV isolation in cell culture (Liu et al., 2015).

3.1.2. Immunofluorescence assay (IF)

The immunofluorescence (IF) assay has been used to detect PEDV antigens in cell culture to confirm virus isolation (Chen et al., 2014; Oka et al., 2014), or in cryosections of intestinal tissue to detect virus antigens in infected enterocytes (Debouck et al., 1981; Guscetti et al., 1998; Jung and Saif, 2015; Jung et al., 2014). The principle of the assay consists of the immune-detection of viral antigens (antibody-antigen reaction) in cell culture or ultrathin cryosections of tissues from pigs suspected of being infected with PEDV. Intracellular viral antigens are detected using fluorophore-conjugated PEDV-specific antibodies. Fluorescent viral antigens are visualized in the cytoplasm of infected cells under a fluorescent microscope. PEDV antigens have been detected by IF in villous epithelial cells throughout the small intestine during various stages of infection, including at early stages involving the incubation period of the disease (first 12–24 h pi). Viral antigens have been detected in intestinal samples for up to 72 h after the onset of clinical signs by the IF assay (Debouck et al., 1981; Guscetti et al., 1998; Jung et al., 2014).

3.1.3. Immunohistochemistry (IHC)

Immunohistochemistry (IHC) has been used for the detection of PEDV antigens in intestinal tissues (Fig. 3) (Guscetti et al., 1998; Kim et al., 1999; Madson et al., 2016; Stevenson et al., 2013). IHC is based on the detection of PEDV antigens in tissue sections by means of immunological (antibody-antigen) and chemical (enzyme-substrate) reactions (Ramos-Vara, 2011). Viral antigens are detected using PEDV-specific antibodies that are subsequently detected using an enzyme-linked secondary antibody followed by chemical substrate development (Guscetti et al., 1998; Ramos-Vara, 2011). Similar to the results obtained via IF, PEDV antigens

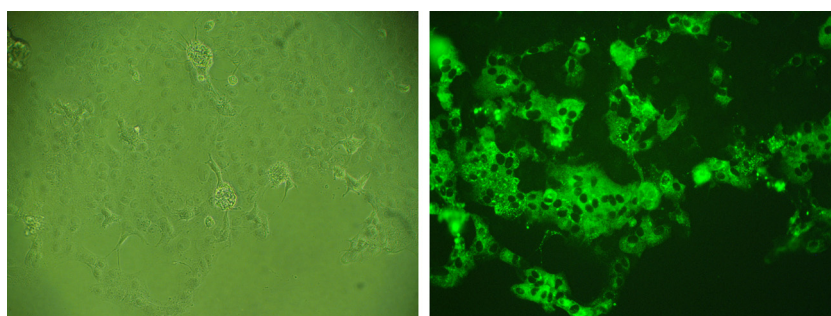


Fig. 2. Isolation of PEDV in cell culture. (A) Cytopathic effect characterized by syncytia and cell detachment induced by PEDV isolate SD15-134 in Vero cells at 48 h post-infection (passage 2). (B) Immunofluorescence confirming isolation of PEDV isolate SD15-134 in Vero cells at 48 h pi (anti-PEDV N protein MA6 SD6-29-FITC).

Table 1
Properties of PEDV diagnostic assays.

Assay Category	Assay	Principle	Appropriate Samples	Relative Analytical Sensitivity ^a	References
Detection of viable virus	Virus isolation	Isolation of infectious PEDV in cell culture	Intestine	+/- ^b	Chen et al. (2014); Oka et al. (2014)
	IF	Detection of PEDV antigens in cell culture or tissues using antibodies	Intestinal contents	+++	Debouck et al. (1981); Jung et al. (2014)
	IHC	Detection of PEDV antigens using antibodies in tissues	Cell culture	+++	Guscetti et al. (1998)
Detection of viral antigens	Ag ELISA	Detection/capture of PEDV antigens on a solid phase using a pair of antibodies	Intestine	+++	Callebaut et al. (1982); Carvajal et al. (1995)
	RT-PCR	Primer specific amplification of PEDV nucleic acid	Feces	+++	Ishikawa et al. (1997); Kubota et al. (1999)
Detection of viral RNA	rRT-PCR	Primer specific amplification and fluorescent probe-based detection of PEDV RNA	Rectal swabs	+++	Kim et al. (2007); Miller et al. (2016); Yoon (2015)
	LAMP-RT-PCR	Isothermal amplification of PEDV RNA using specific primers	Feces	+++++	Ren and Li (2011); Yu et al. (2015)
	VN/FFN ^d	Detection of antibodies capable of neutralizing PEDV infectivity	Intestine	+++	
Detection of host antibody responses	IFA	Detection of serum antibodies tested in PEDV infected cell culture	Intestinal contents	++	Clement et al. (2016); Okda et al. (2015)
	iELISA ^e	Two-step detection of serum antibodies with antigen immobilized into a solid phase and anti-swine secondary antibody	Serum	++	Okda et al. (2015); Clement et al. (2016); Thomas et al. (2015)
	bELISA	Competing antibody mixed with serum and compete for antigen on the solid phase		+++	Okda et al. (2015); Li et al. (2015)
	FMIA	Detection of PEDV antibodies by antigen coupled to fluorescent beads		+++	Carvajal et al. (1995); Okda et al. (2015)
					Okda et al. (2015)

^a Relative analytical sensitivity based on comparison with other assays in the same category. Increased number of + represents increased assay sensitivity.

^b VI has a very low sensitivity as shown in Chen et al. (2014) and Oka et al. (2014).

^c Typically tested by rRT-PCR.

^d NAbs can also be detected in colostrum and milk samples.

^e Has been used to detect antibodies in oral fluid.

have been detected by IHC prior to the development of clinical signs (1 day pi) and up to day 14 pi (Guscetti et al., 1998; Madson et al., 2016). The major advantage of using *in situ* detection methods such as IF and IHC for the diagnosis of PEDV is that they allow the visualization of target cells and the tissue distribution of viral antigens during the course of infection (Duraiyan et al., 2012). Additionally, IHC is performed in formalin fixed paraffin-embedded

tissues, which allows long-term storage and retrospective testing of archived tissue samples.

3.1.4. Antigen ELISA

Porcine epidemic diarrhea virus (PEDV) antigens can be detected in feces using antigen capture ELISAs (Callebaut et al., 1982; Carvajal et al., 1995; Rodák et al., 2005). PEDV antigen capture or sandwich ELISAs are based on the use of a pair of PEDV-specific antibodies to capture and detect virus antigens onto a solid phase

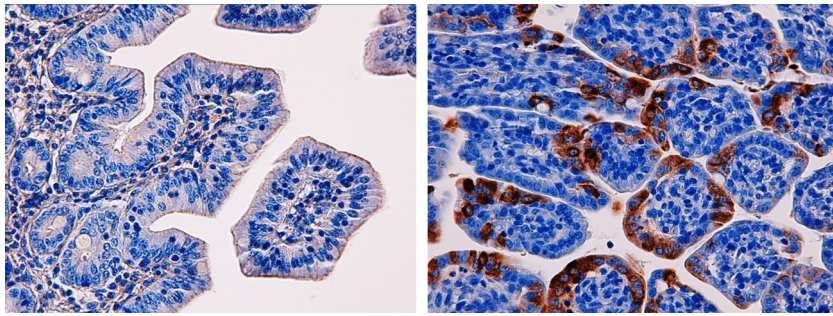


Fig. 3. Immunohistochemistry detection of PEDV antigens in small intestine epithelial enterocytes. Brown staining indicates PEDV N antigen in the cytoplasm of infected enterocytes (right panel). Antigen was detected with anti-N protein MAb SD6-29 followed by development with chromogenic substrate. Left panel: negative control.

(Aydin, 2015; Callebaut et al., 1982; Carvajal et al., 1995; Rodák et al., 2005). The solid phase (polystyrene, polyvinyl or polypropylene microplate well) is coated with the PEDV-specific capture antibody and incubated with the sample, when the PEDV antigens present in the sample bind the capture antibody. The detecting antibody will bind to the virus antigen, and the reaction is developed by adding an enzyme-linked secondary antibody, followed by a chemical substrate (Aydin, 2015). PEDV capture ELISAs have been developed for detection of PEDV in fecal samples (Callebaut et al., 1982; Rodák et al., 2005; Sozzi et al., 2010); however, detection of the virus in clinical samples may be affected by several factors (Rodák et al., 2005), including the timing of sample collection and the conditions of sample storage and shipping to the laboratory (*i.e.* temperature) (Jung and Chae, 2004; Rodák et al., 2005). Fecal shedding of PEDV, for example, was consistently detected by antigen capture ELISA during the acute phase of the disease, but much less frequently during the incubation period or the recovery phase (Callebaut et al., 1982; Sozzi et al., 2010). It is advisable, therefore, that samples are collected during the acute phase of infection, shortly after the onset of the clinical signs (Sozzi et al., 2010), and shipped on ice to the laboratory (Jung and Chae, 2004).

3.2. Polymerase chain reaction (PCR)-based assays for detection of PEDV nucleic acid

Given the diagnostic sensitivity and specificity and rapid turnaround of results, molecular diagnostic assays, such as conventional and real-time reverse transcriptase-polymerase chain reaction (rRT-PCR) assays, became the methods of choice for the diagnosis of PEDV (Gou et al., 2015; Kim et al., 2007; Song et al., 2006; Zhao et al., 2014). Most veterinary diagnostic laboratories in the US, report rRT-PCR results within 24 h of receiving the clinical samples, thus, allowing for rapid implementation of intervention and control measures to reduce the risk of further dissemination of the virus.

PCR-based assays can be applied to detect the PEDV genome in feces, rectal swabs or intestinal samples from animals suspected of being infected with the virus (Chen et al., 2014; Jung et al., 2015). Additionally, oral fluid has been routinely used for diagnosis of PEDV infection (Bjstrom-Kraft et al., 2016; Gimenez-Lirola et al., 2016), and feed samples have been used on rRT-PCR based epidemiological investigations for PEDV (Bowman et al., 2015; Dee et al., 2014; Gerber et al., 2014b; Pasick et al., 2014; Scott et al., 2016; Yoon, 2015). PCR-based assays provide more sensitive, specific and rapid alternatives to virus isolation or other antigen detection methods, such as IF, IHC and antigen capture ELISA; however, since the PCR detects the viral nucleic, it may be positive when no infectious virus is present in the clinical sample.

When designing primers for detection of PEDV, it is important to select areas of the virus genome that allow the detection of most

PEDV strains circulating in the field (*i.e.* PEDV prototype, S-indel) (Oka et al., 2014), and the differentiation from other closely related viruses, including TGEV and PDCoV. Most PCR based assays for PEDV have been designed against the genes encoding one of the structural proteins of the virus, N, M or S (Ben Salem et al., 2010; Ishikawa et al., 1997; Jung et al., 2003; Miller et al., 2016; Song et al., 2006; Zhao et al., 2014). While these genes represent good diagnostic targets, it is important to consider their genetic variability (Jarvis et al., 2016) when designing new assays. The S gene, for example, has been shown to be one of the most variable regions of PEDV genome (Chen et al., 2013; Jarvis et al., 2016), with several variant strains containing deletions in the S1 region being reported in the US and in China (Jarvis et al., 2016; Oka et al., 2014; Zhang et al., 2015). Therefore, it is important to conduct extensive testing of all new PCR-based assays intended for use in PEDV diagnosis (Miller et al., 2016). A summary of recent PCR-based assays developed for the detection of PEDV is presented in Table 2.

3.2.1. Conventional RT-PCR

Several conventional RT-PCR assays have been developed for the detection of PEDV (Ishikawa et al., 1997; Kubota et al., 1999; Kweon et al., 1997). These assays consist of the amplification of a target region of the PEDV genome using virus specific primers in RT-PCR reactions, followed by electrophoresis and visualization of amplicons in agarose gels (Ishikawa et al., 1997; Kubota et al., 1999; Kweon et al., 1997). A few studies have also described the development of multiplex RT-PCR assays for detection and differentiation of several porcine enteric viruses, including PEDV, TGEV and porcine rotaviruses (group A) (Ben Salem et al., 2010; de Arriba et al., 2002; Jung et al., 2003; Kim et al., 2000a,b). These assays have now mostly been replaced by more sensitive and rapid real-time RT-PCR assays (rRT-PCR).

3.2.2. Real-time RT-PCR (rRT-PCR)

There are two common methods for the detection of amplicons in rRT-PCR: (1) non-specific fluorescent dyes that intercalate with double-stranded DNA, and (2) sequence-specific oligonucleotide probes that are labeled with a fluorescent reporter allowing the detection after hybridization of the probe with its target sequence (Arya et al., 2005). Given the higher diagnostic specificity of probe-based assays, they are usually the best choice for molecular diagnostics. The increase in fluorescence emission by the fluorescent probe during extension of the target sequence is detected in real time by the thermocycler (Arya et al., 2005).

The major advantages of rRT-PCR assays are the analytical sensitivity and the ability to detect the target sequences simultaneously with the amplification reaction (Arya et al., 2005). Additionally, rRT-PCR assays allow high throughput testing and process automation. Another advantage of the rRT-PCR is its multiplexing capability, which allows simultaneous testing and discrimination of differ-

Table 2
Summary of rRT-PCR assays for PEDV diagnosis.^a

Detection method	Target	Analytical Sensitivity ^b	Reference
RT-PCR	M protein	10 ¹ TCID ₅₀ /mL	Kim et al. (2000a,b) Kim et al. (2001)
	S protein	10 ¹ TCID ₅₀ /mL	
rRT-PCR	N protein	10 ^{-2.2} TCID ₅₀ /mL	Miller et al. (2016) Pasick et al. (2014) Jung et al. (2014) Kim et al. (2007) Miller et al. (2016) Zhao et al. (2014) Zhao et al. (2013) Wang et al. (2014a,b)
		– ^c	
	S protein	4.8 log ₁₀ (feces) and 3.8 log ₁₀ (serum) GE/mL	
		1 TCID ₅₀ /mL	
LAMP-RT-PCR	M and S proteins	10 ^{-0.2} TCID ₅₀ /mL	
		5 × 10 ² DNA copies/μL	
	M protein	2.1 × 10 ³ DNA copies/μL	
LAMP-RT-PCR	N protein	1 DNA copy/μL	Yu et al. (2015) Ren and Li (2011)
		10 ⁻¹ TCID ₅₀ /mL (10 ^{0.75} TCID ₅₀ /mL)	

^a Assays shown represent only a fraction of the PCR-based assays for PEDV.

^b Sensitivity units were obtained from original article describing each assay.

^c No sensitivity data available.

ent strains of PEDV (Zhao et al., 2014) or of other enteric viral pathogens of swine, including the closely related coronaviruses TGEV and PDCoV (Kim et al., 2007; Song et al., 2006; Zhao et al., 2014). Recently, a multiplex rRT-PCR assay developed for simultaneous detection of PEDV, TGEV, group A rotaviruses, and porcine circovirus 2 (PCV2) revealed that PEDV is the most common viral cause of enteric infections in China (42.1%), whereas the most common co-infection found in the study involved PEDV and PCV2 (9%) (Zhao et al., 2014). This study is a clear example of the utility and versatility of multiplex rRT-PCR assays for the diagnosis of enteric infections in swine.

After the introduction of PEDV in the US in 2013 (Chen et al., 2014; Stevenson et al., 2013), several PCR-based assays were developed for the diagnosis of PEDV infection (Stevenson et al., 2013). Initially, gel-based PCR assays were used; however, these were quickly replaced by more sensitive and high throughput rRT-PCR assays (Wang et al., 2014b). With the introduction of PDCoV in the US swine population in 2014 (Wang et al., 2014a), multiplex rRT-PCR assays were developed to allow simultaneous testing and discrimination between the two swine enteric coronaviruses. At the SDSU Animal Disease and Research Diagnostic Laboratory (ADRDL), for example, a multiplex rRT-PCR assay for simultaneous detection of PEDV, PDCoV and TGEV has been used routinely since July 2014. Since then, approximately 22,000 samples from about 25 US states have been tested using this multiplex assay. Of these 12% were positive for PEDV, 2.2% were positive for PDCoV and 1.8% were positive for both PEDV and PDCoV. No TGEV positive samples were detected from July 2014 to March 2016. A summary of the multiplex rRT-PCR results for individual years (2014, 2015 and 2016) is presented in Fig. 4. Notably, similar cumulative detection rates are observed at national level, with 12.3% of accessions tested since 2014 being positive for PEDV and 2.2% testing positive for PDCoV (www.aphis.usda.gov/animal-health/secld).

Many other rRT-PCR assays have been developed for the diagnosis of PEDV by research groups around the world, and both diagnostic and analytical sensitivity and specificity of these assays have been extensively evaluated using experimental and/or clinical samples (Kim et al., 2007; Miller et al., 2016; Wang et al., 2014b; Zhao et al., 2014). Recently, Miller and collaborators evaluated two rRT-PCR assays targeting the N and the S gene of PEDV (Miller et al., 2016). The detection limit of these rRT-PCRs were 10⁻⁶ and 10⁻⁵ for the N and S assays, which corresponds to a virus suspension containing ~10^{-2.2} and ~10^{-0.2} TCID₅₀/mL of PEDV, respectively (Miller et al., 2016). Additionally, from a total of 1064 fecal swabs that were subjected to these rRT-PCR assays, 354 samples tested positive by the N assay, while 349 were positive by the S assay, indicating a slightly better performance of the N-based assay. Inter-

estingly, by inoculating 3-day-old piglets with a serial dilution of PEDV with known Cq values, the authors were able to determine the infectious dose of the virus (10^{1.3} and 10^{2.3} TCID₅₀/mL) and correlate it to the Cq values obtained by the N rRT-PCR assay (24.14 [±0.02] and 27.28 [±0.07], respectively) (Miller et al., 2016).

In addition to rRT-PCR assays developed by research groups and diagnostic laboratories, multiple commercial kits were developed and are currently available, including the multiplex assay for PEDV, TGEV and PDCoV used at the SD ADRDL (EZ-PED/TGE/PDCoV MPX 1.0; Tetracore Inc., Rockville, MD). These assays represent important tools for rapid and accurate detection of PEDV infection.

3.2.3. Isothermal amplification assays

Loop-mediated isothermal amplification (LAMP) is a simple, specific and cost-effective nucleic acid amplification method (Notomi et al., 2015), that has been recently applied for infectious disease diagnosis, including for the detection of PEDV (Notomi et al., 2015; Ren and Li, 2011; Yu et al., 2015). The assay uses four to six primers that recognize six to eight regions of target DNA in association with the Bst DNA polymerase, which presents strand-displacement activity (Notomi et al., 2015; Ren and Li, 2011). The synchronized binding of the primers to the target sequences maintains the specificity of the assay (Notomi et al., 2015; Ren and Li, 2011). Notably, the amplification step can be performed under a single temperature (isothermal conditions), resulting in the synthesis of large amounts of nucleic acid (Notomi et al., 2015; Ren and Li, 2011). The amplification reactions can be performed using inexpensive equipment (*i.e.* water bath), without the need for thermocyclers (Notomi et al., 2015; Oura et al., 2013). Furthermore, the addition of a fluorescent dye in the reaction mix allows the assessment of the results by simple visual inspection (Tomita et al., 2008). To date, two isothermal amplification assays have been designed and evaluated for the diagnosis of PEDV (Ren and Li, 2011; Yu et al., 2015).

The RT-LAMP developed by Ren and Li (Ren and Li, 2011) was designed to amplify the N gene of PEDV. The analytical sensitivity of the assay was assessed with experimental samples and compared to the sensitivity of a conventional RT-PCR assay and an antigen capture ELISA. The detection limit of the RT-LAMP was lower than that of the RT-PCR (10^{0.75} vs 10^{2.25} TCID₅₀/mL, respectively), and that of the antigen capture ELISA (1 × 10⁻³ μg PEDV particles vs 1 × 10⁻⁴ μg PEDV particles). Comparisons among all three assays in clinical samples revealed similar diagnostic sensitivity and specificity (Ren and Li, 2011).

Recently Yu and collaborators developed and optimized a real-time RT-LAMP (rRT-LAMP) for the detection of PEDV (Yu et al., 2015). In this study the authors evaluated five sets of primers tar-

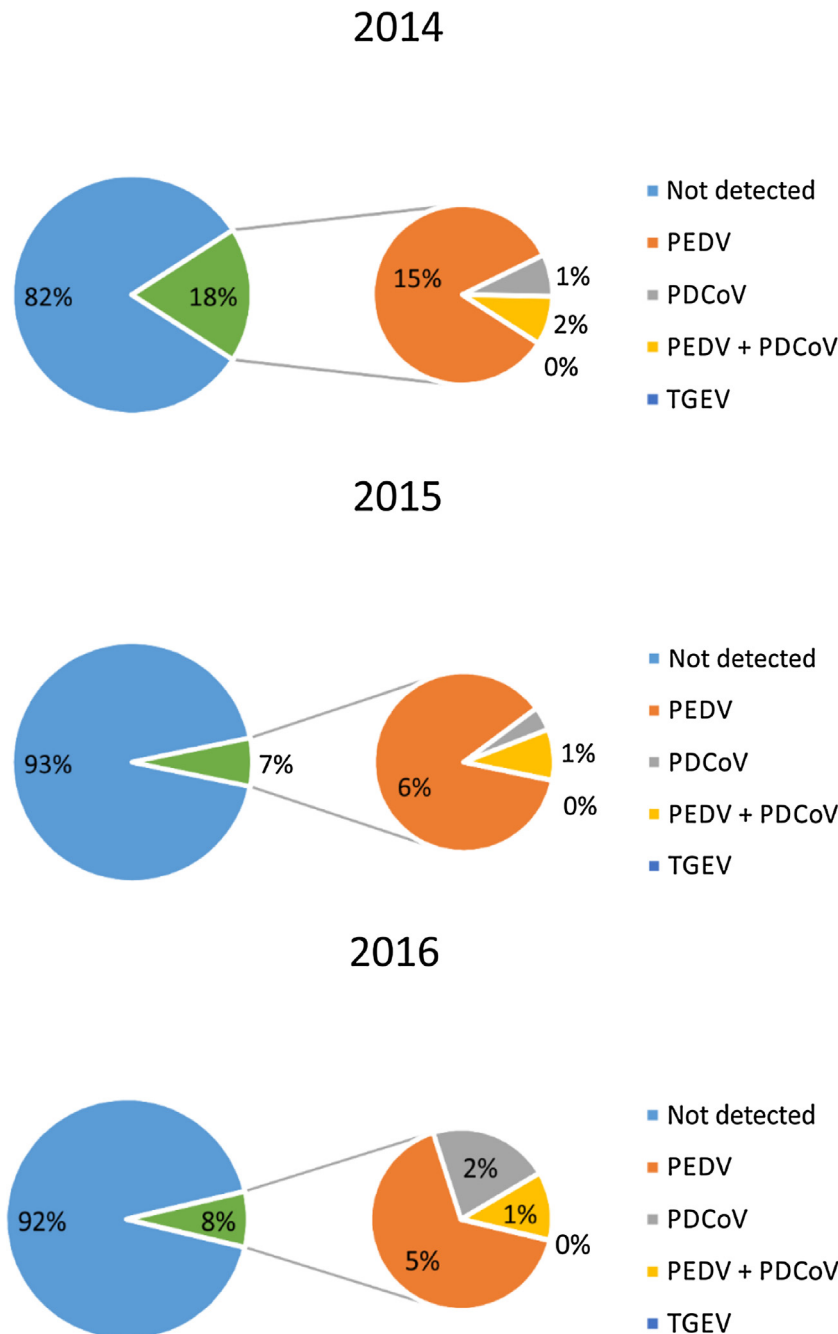


Fig. 4. Multiplex rRT-PCR results for PEDV, PDCoV and TGEV. Results represent clinical samples submitted to routine diagnostic investigation at the SD ADRDL between July 2014 and March 2016. Overall percentage of rRT-PCR positive samples are presented on the plots on the left (green) and individual percentages for PEDV (orange), and PDCoV (gray), or co-infection with PEDV plus PDCoV (golden yellow) are presented on the plots on the right. Not detected: rRT-PCR assay was negative for all three pathogens (PEDV, PDCoV and TGEV).

getting the M gene of PEDV. They have used a loopamp real-time turbidimeter to detect the amplification of the target sequence. The analytical sensitivity of the assay was determined and compared to a conventional and a real-time RT-PCR by testing 10-fold serial dilutions of a viral stock containing a known amount of PEDV. The detection limits of the rRT-LAMP and the rRT-PCR were identical with both assays detecting 10^{-1} TCID₅₀/mL, whereas the detection limit of the conventional RT-PCR was 100-fold higher (10^1 TCID₅₀/mL) than the other two assays. The diagnostic and analytical sensitivity and specificity of RT-LAMP-based assays coupled with their low cost and efficiency make this method an alternative

for PEDV diagnosis, if thermal cyclers or real-time instrumentation are not available (Yu et al., 2015).

3.3. Serological assays for detection of PEDV specific antibodies

Several assays have been developed for the detection of antibodies against PEDV in serum, colostrum, milk, feces and oral fluid including indirect fluorescent antibody assay (IFA), virus neutralization assays, enzyme-linked immunosorbent assay (ELISA), and fluorescent microsphere immunoassay (FMIA) (Gerber and Opriessnig, 2015; Li et al., 2015; Okda et al., 2015; Rodák et al., 2005). Serological assays represent important tools to evaluate

antibody responses to PEDV infection, to determine prior exposure of a given herd to the virus, or yet to evaluate the efficacy of vaccines and vaccination strategies. Detection of PEDV specific antibodies is also used to assess sow immunity, which might be helpful in predicting neonatal protection. To date, only one serotype of PEDV has been identified and current serological assays for PEDV have been shown to detect antibodies against various PEDV strains, including historical and contemporary prototype and S-indel strains (Chen et al., 2016b; Lin et al., 2015). Notably, cross reaction between hyperimmune serum generated against TGEV strain Miller and four strains of PEDV was observed in IFA assays; however, no cross neutralizing activity was detected (Chen et al., 2016b; Lin et al., 2015). In the following sections, we present a brief overview of the serological assays currently available for PEDV.

3.3.1. Indirect immunofluorescence assay (IFA)

Indirect immunofluorescence assay (IFA) has been routinely used to detect antibodies against PEDV and to evaluate the immune status of affected herds (Madson et al., 2014; Thomas et al., 2015). The IFA assay is based on an antibody-antigen reaction. Usually, PEDV infected Vero cell cultures are used as antigen substrates to detect antibodies present in serum samples from suspect animals. If the sample is positive, the PEDV specific antibodies bind to the antigens immobilized in the solid phase. In a second step, these antibodies are detected with fluorophore-labeled anti-porcine secondary antibodies and visualized under a fluorescence microscope (Thomas et al., 2015; Clement et al., 2016). The IFA assay presents specificity comparable to ELISA and FFN assays and has the advantage of being less time consuming and easier to perform (Lee, 2015). IFA can also be used for qualitative (positive or negative) or quantitative (antibody titer) evaluation of PEDV-specific antibodies in serum. To determine the titers of antibodies, serial two-fold dilutions of serum are incubated with PEDV infected cells, and the antibody titers are determined as the reciprocal of the highest dilution of serum in which specific fluorescent signal is observed. PEDV-specific antibodies are first detected by IFA by 7–14 days post-exposure, and titers seem to be maintained for at least 43 days pi when a decline in the detectable levels of antibodies is observed. Despite being a useful assay for detection of antibodies during the acute stage of infection, the IFA is not the test of preference to determine prior exposure to PEDV due to the lower analytical sensitivity of the assay. Additionally, interpretation of endpoint IFA antibody titers is more subjective than other PEDV antibody detection assays.

3.3.2. Virus neutralization assays

Virus neutralization assays (VNs) are widely used for detection of PEDV specific antibodies (Okda et al., 2015). Virus neutralization is defined as the reduction in viral infectivity mediated by antibodies (neutralizing antibodies [NAbs]) (Klasse, 2014). By binding to the virions, NAbs block one or more steps of the viral replication cycle (*i.e.* entry, binding to cellular receptors, uncoating), and/or cause aggregation or lysis of virus particles, thus neutralizing or reducing virus infectivity (Klasse, 2014). Historically, CPE-based VNs have been used to detect NAbs against PEDV (Paudel et al., 2014a,b). However, interpretation of endpoint neutralization titers based on CPE is complicated by the fact that the trypsin added in the culture media leads to cell rounding and detachment, thus impairing precise differentiation between virus- and trypsin-induced changes in cellular morphology (Okda et al., 2015).

Recently, neutralization assays using a fluorescent focus neutralization (FFN) format have been developed for PEDV (Okda et al., 2015; Thomas et al., 2015). The FFN allows for rapid assessment of NAbs titers (~30 h) against PEDV, because viral antigens can be detected earlier than virus induced CPE in infected cells. To detect the presence of PEDV neutralizing antibodies using the FFN assay, serial dilutions of serum samples are incubated with a constant

amount of PEDV, thus allowing the antibodies to bind to the virus. The antibody-virus mixture is transferred to susceptible Vero cell cultures and PEDV infectivity is assessed by using an anti-PEDV fluorophore-conjugated antibody (Okda et al., 2015; Thomas et al., 2015). NAb titers are determined as the reciprocal of the highest serum dilution resulting in 90% or greater reduction in fluorescent foci relative to negative control samples (Okda et al., 2015). Neutralizing antibodies against PEDV have been detected by day 7–14 pi (Okda et al., 2015; Thomas et al., 2015) in experimentally infected animals, with high titers of NAbs being detected for at least six months post-natural infection (Clement et al., 2016; Ouyang et al., 2015).

The FFN assay has also been optimized for the detection and quantitation of PEDV NAbs in colostrum and milk samples (Clement et al., 2016; Okda et al., 2015). This is important to monitor the transfer of maternal immunity to suckling piglets. The levels of NAbs detected in colostrum are higher (four-fold) than those detected in serum. Whereas, levels of NAbs detected in milk are similar to those detected in serum (Clement et al., 2016; Okda et al., 2015). Given that VN or FFN assays detect functional antibodies capable of neutralizing PEDV infectivity, these assays have been frequently used in studies evaluating novel vaccine candidates for PEDV (Collin et al., 2015; Goede et al., 2015; Liu et al., 2012; Paudel et al., 2014a). Additionally, NAbs are very specific, and VN assays are capable of discriminating between serologic responses induced against PEDV and the closely related TGEV (Chen et al., 2016b; Lin et al., 2015).

3.3.3. ELISAs

Two types of ELISAs have been used for the detection of antibodies against PEDV, including the indirect (Fan et al., 2015; Gerber and Opriessnig, 2015; Gerber et al., 2014a; Hofmann and Wyler, 1990; Hou et al., 2007; Li et al., 2015; Okda et al., 2015) and the competitive or blocking-ELISA (Carvajal et al., 1995; Okda et al., 2015; van Nieuwstadt and Zetstra, 1991). The indirect ELISAs have been developed based on either whole virus preparations (Hofmann and Wyler, 1990; Oh et al., 2005; Thomas et al., 2015) or recombinant viral proteins (S, N and M) expressed in bacteria (Gerber and Opriessnig, 2015; Gerber et al., 2014a; Hou et al., 2007; Li et al., 2015; Okda et al., 2015; Paudel et al., 2014b). In this assay, the viral antigen is immobilized on a solid polystyrene surface, and incubated with the test sample to allow the antibody-antigen complex to form. The reaction is developed by using an enzyme-linked secondary antibody coupled with a colorimetric substrate (Aydin, 2015).

The PEDV blocking or competitive ELISAs are based on the use of PEDV-specific monoclonal or polyclonal antibodies (Carvajal et al., 1995; Okda et al., 2015; van Nieuwstadt and Zetstra, 1991) that, when added to the antigen coated microplates together with the test sample, compete with the antibodies present in the serum for the antigen immobilized in the wells (Aydin, 2015). The reaction is developed as described above for the indirect ELISA, with the addition of an enzyme-linked secondary antibody followed by an enzyme-substrate colorimetric reaction. The main advantage of the blocking ELISA is its increased specificity when compared to the indirect ELISA (Aydin, 2015); however, the assay specificity seems to be dependent on the isotype of the blocking antibody and its specificity for the target antigen (Carvajal et al., 1995).

Recently, the SDSU ADRDL developed several serological assays for the detection of antibodies against PEDV, including an indirect and a blocking ELISA based on the N protein (Okda et al., 2015). These have been evaluated using experimental and clinical samples and presented similar diagnostic sensitivity and specificity (97.9 and 97.6% for the iELISA; and 98.2 and 98.0% for the bELISA, respectively) (Okda et al., 2015). Both assays detected N-specific IgG antibodies between days 9 and 14 pi, with peak antibody lev-

els being detected on day 21 pi. On day 43 pi, N-specific antibodies were still detected by both assays; however a slight decline in the antibody levels was observed after day 21 pi (Okda et al., 2015).

Indirect ELISAs have been recently developed to assess the presence of PEDV-specific IgG and IgA antibodies in oral fluids (Bjuström-Kraft et al., 2016; Gimenez-Lirola et al., 2016). Notably, levels of PEDV specific IgA antibodies in oral fluid seem to increase through ~100 days post-infection (Bjuström-Kraft et al., 2016; Gimenez-Lirola et al., 2016). This observation makes oral fluid a good sample to monitor prior herd exposure to PEDV.

3.3.4. Fluorescent microsphere immunoassay (FMIA)

The fluorescent microsphere immunoassay (FMIA) is based on a fluidic fluorescent microsphere laser scanning system that has several advantages over the ELISA, including analytical sensitivity, higher sample throughput, and the ability to multiplex and detect antibodies to multiple pathogens simultaneously (Christopher-Hennings et al., 2013). The FMIA is based on coupling a specific viral antigen to fluorescent microspheres; then, antigen-specific antibodies are detected using a biotin/streptavidin/fluorophore detection system. Antigen-antibody reactions are detected using dual-laser instruments (i.e. Bio-Plex 200, Bio-Rad) and results expressed as median fluorescent intensity (MFI) (Okda et al., 2015).

Recently, a FMIA based on the N protein of PEDV was developed for the detection of PEDV specific antibodies in serum (Okda et al., 2015). This assay was evaluated using a large number of experimental and clinical samples and the results obtained by the FMIA presented a high correlation with those obtained by indirect and blocking ELISAs and by the IFA test (kappa scores >0.91) (Okda et al., 2015). Additionally, diagnostic sensitivity and specificity were 98.2% and 99.2%, respectively (Okda et al., 2015). PEDV N-specific antibodies were detected as early as 6 days pi, peaking on day 21 pi and being detected up to day 43 pi (Okda et al., 2015).

4. Conclusions

Rapid and accurate diagnosis of PEDV infection are critical for the implementation of effective disease control measures. Thus, reliable virological and serological diagnostic assays are extremely important. While virological tests allow the rapid identification of PEDV and its differentiation from other enteric pathogens of swine, serological assays provide valuable information regarding prior exposure to PEDV and the prevalence of infection. Additionally, serological screenings allow the evaluation of novel vaccines and vaccination strategies in the field. Immunoassays such as ELISAs and FMIAs also allow the evaluation of isotype-specific immune responses against PEDV. The introduction of PEDV in the US in 2013 and its re-emergence in Asia have resulted in the development of a broad array of improved diagnostic assays to detect the virus or to evaluate the host responses to infection. As more reagents become available (i.e. monoclonal antibodies against S, N and M), we are likely to see the development of additional diagnostic approaches that may allow rapid on-site diagnosis of PEDV.

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References

Alvarez, J., Sarradell, J., Morrison, R., Perez, A., 2015. Impact of porcine epidemic diarrhea on performance of growing pigs. *PLoS One* 10, e0120532, <http://dx.doi.org/10.1371/journal.pone.0120532>.

- Annamalai, T., Saif, L.J., Lu, Z., Jung, K., 2015. Age-dependent variation in innate immune responses to porcine epidemic diarrhea virus infection in suckling versus weaned pigs. *Vet. Immunol. Immunopathol.* 168, 193–202, <http://dx.doi.org/10.1016/j.vetimm.2015.09.006>.
- Arya, M., Shergill, I.S., Williamson, M., Gommersall, L., Arya, N., Patel, N.R., 2005. Basic principles of real-time quantitative PCR. *Expert Rev. Mol. Diagn.* 5, 209, <http://dx.doi.org/10.1586/14737159.5.2.209>.
- Aydin, S., 2015. A short history, principles, and types of ELISA, and our laboratory experience with peptide/protein analyses using ELISA. *Peptides* 72, 4–15, <http://dx.doi.org/10.1016/j.peptides.2015.04.012>.
- Ben Salem, A.N., Chupin Sergei, A., Bjadovskaya Olga, P., Andreeva Olga, G., Mahjoub, A., Prokhvatilova Larissa, B., 2010. Multiplex nested RT-PCR for the detection of porcine enteric viruses. *J. Virol. Methods* 165, 283–293, <http://dx.doi.org/10.1016/j.jviromet.2010.02.010>.
- Bjuström-Kraft, Jordan, Gimenez-Lirola, Luis, Zimmerman, Jeffrey, Main, Rodger, Rademacher, C., 2016. Understanding PEDV shedding, immune response aids in implementing gilt acclimation [WWW Document]. *Natl. Hog Farmer* <http://nationalhogfarmer.com/pedv/understanding-pedv-shedding-immune-response-aids-implementing-gilt-acclimation>.
- Bowman, A.S., Krogwold, R.A., Price, T., Davis, M., Moeller, S.J., 2015. Investigating the introduction of porcine epidemic diarrhea virus into an Ohio swine operation. *BMC Vet. Res.* 11, 38, <http://dx.doi.org/10.1186/s12917-015-0348-2>.
- Callebaut, P., Debouck, P., Pensaert, M., 1982. Enzyme-linked immunosorbent assay for the detection of the coronavirus-like agent and its antibodies in pigs with porcine epidemic diarrhea. *Vet. Microbiol.* 7, 295–306.
- Carvajal, A., Lanza, I., Diego, R., Rubio, P., Cármenes, P., 1995. Evaluation of a blocking ELISA using monoclonal antibodies for the detection of porcine epidemic diarrhea virus and its antibodies. *J. Vet. Diagn. Invest.* 7, 60–64.
- Chen, J., Wang, C., Shi, H., Qiu, H., Liu, S., Chen, X., Zhang, Z., Feng, L., 2010. Molecular epidemiology of porcine epidemic diarrhea virus in China. *Arch. Virol.* 155, 1471–1476, <http://dx.doi.org/10.1007/s00705-010-0720-2>.
- Chen, J., Liu, X., Shi, D., Shi, H., Zhang, X., Li, C., Chi, Y., Feng, L., 2013. Detection and molecular diversity of spike gene of porcine epidemic diarrhea virus in China. *Viruses* 5, 2601–2613, <http://dx.doi.org/10.3390/v5102601>.
- Chen, Q., Li, G., Stasko, J., Thomas, J.T., Stensland, W.R., Pillatzki, A.E., Gauger, P.C., Schwartz, K.J., Madson, D., Yoon, K.-J., Stevenson, G.W., Burrough, E.R., Harmon, K.M., Main, R.G., Zhang, J., 2014. Isolation and characterization of porcine epidemic diarrhea viruses associated with the 2013 disease outbreak among swine in the United States. *J. Clin. Microbiol.* 52, 234–243, <http://dx.doi.org/10.1128/JCM.02820-13>.
- Chen, Q., Gauger, P., Stafne, M., Thomas, J., Arruda, P., Burrough, E., Madson, D., Brodie, J., Magstadt, D., Derscheid, R., Welch, M., Zhang, J., 2015. Pathogenicity and pathogenesis of a United States porcine deltacoronavirus cell culture isolate in 5-day-old neonatal piglets. *Virology* 482, 51–59, <http://dx.doi.org/10.1016/j.virol.2015.03.024>.
- Chen, Q., Thomas, J.T., Giménez-Lirola, L.G., Hardham, J.M., Gao, Q., Gerber, P.F., Opriessnig, T., Zheng, Y., Li, G., Gauger, P.C., Madson, D.M., Magstadt, D.R., Zhang, J., 2016a. Pathogenesis comparison between the United States porcine epidemic diarrhoea virus prototype and S-INDEL-variant strains in conventional neonatal piglets. *J. Gen. Virol.* 97, 1107–1121, <http://dx.doi.org/10.1099/jgv.0.000419>.
- Chen, Q., Thomas, J.T., Giménez-Lirola, L.G., Hardham, J.M., Gao, Q., Gerber, P.F., Opriessnig, T., Zheng, Y., Li, G., Gauger, P.C., Madson, D.M., Magstadt, D.R., Zhang, J., 2016b. Evaluation of serological cross-reactivity and cross-neutralization between the United States porcine epidemic diarrhea virus prototype and S-INDEL-variant strains. *BMC Vet. Res.* 12, 70, <http://dx.doi.org/10.1186/s12917-016-0697-5>.
- Christopher-Hennings, J., Araujo, K.P.C., Souza, C.J.H., Fang, Y., Lawson, S., Nelson, E.A., Clement, T., Dunn, M., Lunney, J.K., 2013. Opportunities for bead-based multiplex assays in veterinary diagnostic laboratories. *J. Vet. Diagn. Invest.* 25, 671–691, <http://dx.doi.org/10.1177/1040638713507256>.
- Clement, Travis, Singrey, Aaron, Lawson, Steven, Okda, Faten, Nelson, Julie, Diel, Diego, Nelson, Eric A., Christopher-Hennings, J., 2016. Measurement of neutralizing antibodies against porcine epidemic diarrhea virus in sow serum, colostrum, and milk samples and piglet serum samples after feedback. *J. Swine Heal. Prod.* 24, 1–10.
- Collin, E.A., Anbalagan, S., Okda, F., Batman, R., Nelson, E., Hause, B.M., 2015. An inactivated vaccine made from a U.S. field isolate of porcine epidemic disease virus is immunogenic in pigs as demonstrated by a dose-titration. *BMC Vet. Res.* 11, 62, <http://dx.doi.org/10.1186/s12917-015-0357-1>.
- Cruz, D.J.M., Kim, C.-J., Shin, H.-J., 2008. The GPRLQPY motif located at the carboxy-terminal of the spike protein induces antibodies that neutralize porcine epidemic diarrhea virus. *Virus Res.* 132, 192–196, <http://dx.doi.org/10.1016/j.virusres.2007.10.015>.
- Debouck, P., Pensaert, M., Coussement, W., 1981. The pathogenesis of an enteric infection in pigs, experimentally induced by the coronavirus-like agent, CV 777. *Vet. Microbiol.* 6, 157–165, [http://dx.doi.org/10.1016/0378-1135\(81\)90007-9](http://dx.doi.org/10.1016/0378-1135(81)90007-9).
- Dee, S., Clement, T., Schelkopf, A., Nerem, J., Knudsen, D., Christopher-Hennings, J., Nelson, E., 2014. An evaluation of contaminated complete feed as a vehicle for porcine epidemic diarrhea virus infection of naïve pigs following consumption via natural feeding behavior: proof of concept. *BMC Vet. Res.* 10, 176, <http://dx.doi.org/10.1186/PREACCEPT-5213019221299203>.
- de Arriba, M.L., Carvajal, A., Pozo, J., Rubio, P., 2002. Isotype-specific antibody-secreting cells in systemic and mucosal associated lymphoid tissues and antibody responses in serum of conventional pigs inoculated with PEDV. *Vet. Immunol. Immunopathol.* 84, 1–16.

- Duraiyan, J., Govindarajan, R., Kaliyappan, K., Palanisamy, M., 2012. Applications of immunohistochemistry. *J. Pharm. Bioallied Sci.* 4, S307–S309, <http://dx.doi.org/10.4103/0975-7406.100281>.
- Fan, J.-H., Zuo, Y.-Z., Shen, X.-Q., Gu, W.-Y., Di, J.-M., 2015. Development of an enzyme-linked immunosorbent assay for the monitoring and surveillance of antibodies to porcine epidemic diarrhea virus based on a recombinant membrane protein. *J. Virol. Methods* 225, 90–94, <http://dx.doi.org/10.1016/j.jviromet.2015.07.021>.
- Gerber, P.F., Opriessnig, T., 2015. Detection of immunoglobulin (Ig) A antibodies against porcine epidemic diarrhea virus (PEDV) in fecal and serum samples. *MethodsX* 2, 368–373, <http://dx.doi.org/10.1016/j.mex.2015.10.001>.
- Gerber, P.F., Gong, Q., Huang, Y.-W., Wang, C., Holtkamp, D., Opriessnig, T., 2014a. Detection of antibodies against porcine epidemic diarrhea virus in serum and colostrum by indirect ELISA. *Vet. J.* 202, 33–36, <http://dx.doi.org/10.1016/j.tvjl.2014.07.018>.
- Gerber, P.F., Xiao, C.-T., Chen, Q., Zhang, J., Halbur, P.G., Opriessnig, T., 2014b. The spray-drying process is sufficient to inactivate infectious porcine epidemic diarrhea virus in plasma. *Vet. Microbiol.* 174, 86–92, <http://dx.doi.org/10.1016/j.jvetmic.2014.09.008>.
- Gimenez-Lirio, L., Kraft, J.B., Woodard, J.B., Wang, C., Baum, D., Zimmerman, J., Main, R., 2016. PEDV antibody responses in fecal and oral fluid specimens. In: *American Association of Swine Veterinarians, New Orleans* (p. 64).
- Goede, D., Murtaugh, M.P., Nerem, J., Yeske, P., Rossow, K., Morrison, R., 2015. Previous infection of sows with a mild strain of porcine epidemic diarrhea virus confers protection against infection with a severe strain. *Vet. Microbiol.* 176, 161–164, <http://dx.doi.org/10.1016/j.jvetmic.2014.12.019>.
- Gou, H., Deng, J., Wang, J., Pei, J., Liu, W., Zhao, M., Chen, J., 2015. Rapid and sensitive detection of porcine epidemic diarrhea virus by reverse transcription loop-mediated isothermal amplification combined with a vertical flow visualization strip. *Mol. Cell. Probes* 29, 48–53, <http://dx.doi.org/10.1016/j.mcp.2014.11.004>.
- Guscetti, F., Bernasconi, C., Tobler, K., Van Reeth, K., Pospischil, A., Ackermann, M., 1998. Immunohistochemical detection of porcine epidemic diarrhea virus compared to other methods. *Clin. Diagn. Lab. Immunol.* 5, 412–414.
- Hofmann, M., Wyler, R., 1990. Enzyme-linked immunosorbent assay for the detection of porcine epidemic diarrhea coronavirus antibodies in swine sera. *Vet. Microbiol.* 21, 263–273.
- Hou, X.-L., Yu, L.-Y., Liu, J., 2007. Development and evaluation of enzyme-linked immunosorbent assay based on recombinant nucleocapsid protein for detection of porcine epidemic diarrhea (PEDV) antibodies. *Vet. Microbiol.* 123, 86–92, <http://dx.doi.org/10.1016/j.jvetmic.2007.02.014>.
- Huang, Y.-W., Dickerman, A.W., Piñeyro, P., Li, L., Fang, L., Kiehne, R., Opriessnig, T., Meng, X.-J., 2013. Origin, evolution, and genotyping of emergent porcine epidemic diarrhea virus strains in the United States. *MBio* 4, <http://dx.doi.org/10.1128/mBio.00737-13>, e00737-13.
- Ishikawa, K., Sekiguchi, H., Ogino, T., Suzuki, S., 1997. Direct and rapid detection of porcine epidemic diarrhea virus by RT-PCR. *J. Virol. Methods* 69, 191–195.
- Jarvis, M.C., Lam, H.C., Zhang, Y., Wang, L., Hesse, R.A., Hause, B.M., Vlasova, A., Wang, Q., Zhang, J., Nelson, M.L., Murtaugh, M.P., Marthaler, D., 2016. Genomic and evolutionary inferences between American and global strains of porcine epidemic diarrhea virus. *Prev. Vet. Med.* 123, 175–184, <http://dx.doi.org/10.1016/j.prevetmed.2015.10.020>.
- Jung, K., Chae, C., 2004. Effect of temperature on the detection of porcine epidemic diarrhea virus and transmissible gastroenteritis virus in fecal samples by reverse transcription-polymerase chain reaction. *J. Vet. Diagn. Invest.* 16, 237–239.
- Jung, K., Saif, L.J., 2015. Porcine epidemic diarrhea virus infection: etiology, epidemiology, pathogenesis and immunoprophylaxis. *Vet. J.* 204, 134–143, <http://dx.doi.org/10.1016/j.tvjl.2015.02.017>.
- Jung, K., Kim, J., Kim, O., Kim, B., Chae, C., 2003. Differentiation between porcine epidemic diarrhea virus and transmissible gastroenteritis virus in formalin-fixed paraffin-embedded tissues by multiplex RT-nested PCR and comparison with in situ hybridization. *J. Virol. Methods* 108, 41–47.
- Jung, K., Wang, Q., Scheuer, K.A., Lu, Z., Zhang, Y., Saif, L.J., 2014. Pathology of US porcine epidemic diarrhea virus strain PC21A in gnotobiotic pigs. *Emerg. Infect. Dis.* 20, 662–665.
- Jung, K., Annamalai, T., Lu, Z., Saif, L.J., 2015. Comparative pathogenesis of US porcine epidemic diarrhea virus (PEDV) strain PC21A in conventional 9-day-old nursing piglets vs 26-day-old weaned pigs. *Vet. Microbiol.* 178, 31–40, <http://dx.doi.org/10.1016/j.jvetmic.2015.04.022>.
- Kim, O., Chae, C., Kweon, C.H., 1999. Monoclonal antibody-based immunohistochemical detection of porcine epidemic diarrhea virus antigen in formalin-fixed, paraffin-embedded intestinal tissues. *J. Vet. Diagn. Invest.* 11, 458–462.
- Kim, L., Hayes, J., Lewis, P., Parwani V. A., Chang, K.O., Saif, L.J., 2000a. Molecular characterization and pathogenesis of transmissible gastroenteritis coronavirus (TGEV) and porcine respiratory coronavirus (PRCV) field isolates co-circulating in a swine herd. *Arch. Virol.* 145, 1133–1147.
- Kim, O., Choi, C., Kim, B., Chae, C., 2000b. Detection and differentiation of porcine epidemic diarrhoea virus and transmissible gastroenteritis virus in clinical samples by multiplex RT-PCR. *Vet. Rec.* 146, 637–640.
- Kim, S.Y., Song, D.S., Park, B.K., 2001. Differential detection of transmissible gastroenteritis virus and porcine epidemic diarrhea virus by duplex RT-PCR. *J. Vet. Diagn. Invest.* 13, 516–520.
- Kim, S.-H., Kim, I.-J., Pyo, H.-M., Tark, D.-S., Song, J.-Y., Hyun, B.-H., 2007. Multiplex real-time RT-PCR for the simultaneous detection and quantification of transmissible gastroenteritis virus and porcine epidemic diarrhea virus. *J. Virol. Methods* 146, 172–177, <http://dx.doi.org/10.1016/j.jviromet.2007.06.021>.
- Kim, S.-H., Lee, J.-M., Jung, J., Kim, I.-J., Hyun, B.-H., Kim, H.-I., Park, C.-K., Oem, J.-K., Kim, Y.-H., Lee, M.-H., Lee, K.-K., 2015. Genetic characterization of porcine epidemic diarrhea virus in Korea from 1998 to 2013. *Arch. Virol.* 160, 1055–1064, <http://dx.doi.org/10.1007/s00705-015-2353-y>.
- Klasse, P.J., 2014. Neutralization of virus infectivity by antibodies: old problems in new perspectives. *Adv. Biol.* 24, <http://dx.doi.org/10.1155/2014/157895>.
- Kubota, S., Sasaki, O., Amimoto, K., Okada, N., Kitazima, T., Yasuhara, H., 1999. Detection of porcine epidemic diarrhea virus using polymerase chain reaction and comparison of the nucleocapsid protein genes among strains of the virus. *J. Vet. Med. Sci.* 61, 827–830.
- Kweon, C.H., Lee, J.G., Han, M.G., Kang, Y.B., 1997. Rapid diagnosis of porcine epidemic diarrhea virus infection by polymerase chain reaction. *J. Vet. Med. Sci.* 59, 231–232.
- Lee, C., 2015. Porcine epidemic diarrhea virus: an emerging and re-emerging epizootic swine virus. *Viol. J.* 12, 193, <http://dx.doi.org/10.1186/s12985-015-0421-2>.
- Li, B.X., Ge, J.W., Li, Y.J., 2007. Porcine aminopeptidase N is a functional receptor for the PEDV coronavirus. *Virology* 365, 166–172, <http://dx.doi.org/10.1016/j.viro.2007.03.031>.
- Li, Y., Zheng, F., Fan, B., Muhammad, H.M., Zou, Y., Jiang, P., 2015. Development of an indirect ELISA based on a truncated S protein of the porcine epidemic diarrhea virus. *Can. J. Microbiol.* 61, 811–817, <http://dx.doi.org/10.1139/cjm-2015-0213>.
- Lin, C.-M., Gao, X., Oka, T., Vlasova, A.N., Esseili, M.A., Wang, Q., Saif, L.J., 2015. Antigenic relationships among porcine epidemic diarrhea virus and transmissible gastroenteritis virus strains. *J. Virol.* 89, 3332–3342, <http://dx.doi.org/10.1128/JVI.03196-14>.
- Liu, D., Ge, J., Qiao, X., Jiang, Y., Liu, S., Li, Y., 2012. High-level mucosal and systemic immune responses induced by oral administration with *Lactobacillus*-expressed porcine epidemic diarrhea virus (PEDV) S1 region combined with *Lactobacillus*-expressed N protein. *Appl. Microbiol. Biotechnol.* 93, 2437–2446, <http://dx.doi.org/10.1007/s00253-011-3734-0>.
- Liu, C., Tang, J., Ma, Y., Liang, X., Yang, Y., Peng, G., Qi, Q., Jiang, S., Li, J., Du, L., Li, F., 2015. Receptor usage and cell entry of porcine epidemic diarrhea coronavirus. *J. Virol.* 89, 6121–6125, <http://dx.doi.org/10.1128/JVI.00430-15>.
- Madson, D.M., Magstadt, D.R., Arruda, P.H.E., Hoang, H., Sun, D., Bower, L.P., Bhandari, M., Burrough, E.R., Gauger, P.C., Pillatzki, A.E., Stevenson, G.W., Wilberts, B.L., Brodie, J., Harmon, K.M., Wang, C., Main, R.G., Zhang, J., Yoon, K.J., 2014. Pathogenesis of porcine epidemic diarrhea virus isolate (US/Iowa/18984/2013) in 3-week-old weaned pigs. *Vet. Microbiol.*, <http://dx.doi.org/10.1016/j.jvetmic.2014.09.002>.
- Madson, D.M., Arruda, P.H.E., Magstadt, D.R., Burrough, E.R., Hoang, H., Sun, D., Bower, L.P., Bhandari, M., Gauger, P.C., Stevenson, G.W., Wilberts, B.L., Wang, C., Zhang, J., Yoon, K.J., 2016. Characterization of porcine epidemic diarrhea virus isolate US/Iowa/18984/2013 infection in 1-day-old cesarean-derived colostrum-deprived piglets. *Vet. Pathol.* 53, 44–52, <http://dx.doi.org/10.1177/0309858115591080>.
- Miller, L.C., Crawford, K.K., Lager, K.M., Kellner, S.G., Brockmeier, S.L., 2016. Evaluation of two real-time polymerase chain reaction assays for Porcine epidemic diarrhea virus (PEDV) to assess PEDV transmission in growing pigs. *J. Vet. Diagn. Invest.* 28, 20–29, <http://dx.doi.org/10.1177/1040638715621949>.
- Nam, E., Lee, C., 2010. Contribution of the porcine aminopeptidase N (CD13) receptor density to porcine epidemic diarrhea virus infection. *Vet. Microbiol.* 144, 41–50, <http://dx.doi.org/10.1016/j.jvetmic.2009.12.024>.
- Notomi, T., Mori, Y., Tomita, N., Kanda, H., 2015. Loop-mediated isothermal amplification (LAMP): principle, features, and future prospects. *J. Microbiol.* 53, 1–5, <http://dx.doi.org/10.1007/s12275-015-4656-9>.
- Oh, J.S., Song, D.S., Yang, J.S., Song, J.Y., Moon, H.J., Kim, T.Y., Park, B.K., 2005. Comparison of an enzyme-linked immunosorbent assay with serum neutralization test for serodiagnosis of porcine epidemic diarrhea virus infection. *J. Vet. Sci.* 6, 349–352.
- Ojkic, D., Hazlett, M., Fairles, J., Marom, A., Slavic, D., Maxie, G., Alexandersen, S., Pasick, J., Alsop, J., Burlatschenko, S., 2015. The first case of porcine epidemic diarrhea in Canada. *Can. Vet. J. La Rev. vétérinaire Can.* 56, 149–152.
- Oka, T., Saif, L.J., Marthaler, D., Esseili, M.A., Meulia, T., Lin, C.-M., Vlasova, A.N., Jung, K., Zhang, Y., Wang, Q., 2014. Cell culture isolation and sequence analysis of genetically diverse US porcine epidemic diarrhea virus strains including a novel strain with a large deletion in the spike gene. *Vet. Microbiol.* 173, 258–269, <http://dx.doi.org/10.1016/j.jvetmic.2014.08.012>.
- Okda, F., Liu, X., Singrey, A., Clement, T., Nelson, J., Christopher-Hennings, J., Nelson, E.A., Lawson, S., 2015. Development of an indirect ELISA, blocking ELISA, fluorescent microsphere immunoassay and fluorescent focus neutralization assay for serologic evaluation of exposure to North American strains of porcine epidemic diarrhea virus. *BMC Vet. Res.* 11, 180, <http://dx.doi.org/10.1186/s12917-015-0500-z>.
- Olanratmanee, E.-O., Kunavongkrit, A., Tummaruk, P., 2010. Impact of porcine epidemic diarrhea virus infection at different periods of pregnancy on subsequent reproductive performance in gilts and sows. *Anim. Reprod. Sci.* 122, 42–51, <http://dx.doi.org/10.1016/j.anireprosci.2010.07.004>.
- Oura, C.A.L., Edwards, L., Batten, C.A., 2013. Virological diagnosis of African swine fever—comparative study of available tests. *Virus Res.* 173, 150–158, <http://dx.doi.org/10.1016/j.virusres.2012.10.022>.
- Ouyang, K., Shyu, D.-L., Dhakal, S., Hiremath, J., Binjawadagi, B., Lakshmanappa, Y.S., Guo, R., Ransburgh, R., Bondra, K.M., Gauger, P., Zhang, J., Specht, T.,

- Gilbertie, A., Minton, W., Fang, Y., Renukaradhya, G.J., 2015. Evaluation of humoral immune status in porcine epidemic diarrhoea virus (PEDV) infected sows under field conditions. *Vet. Res.* 46, 140, <http://dx.doi.org/10.1186/s13567-015-0285-x>.
- Park, S.-J., Song, D.-S., Ha, G.-W., Park, B.-K., 2007. Cloning and further sequence analysis of the spike gene of attenuated porcine epidemic diarrhoea virus DR13. *Virus Genes* 35, 55–64, <http://dx.doi.org/10.1007/s11262-006-0036-1>.
- Pasick, J., Berhane, Y., Ojkic, D., Maxie, G., Embury-Hyatt, C., Swekla, K., Handel, K., Fairles, J., Alexandersen, S., 2014. Investigation into the role of potentially contaminated feed as a source of the first-detected outbreaks of porcine epidemic diarrhoea in Canada. *Transbound. Emerg. Dis.* 61, 397–410, <http://dx.doi.org/10.1111/tbed.12269>.
- Paudel, S., Park, J.E., Jang, H., Hyun, B.H., Yang, D.G., Shin, H.J., 2014a. Evaluation of antibody response of killed and live vaccines against porcine epidemic diarrhoea virus in a field study. *Vet. Q* 34, 194–200, <http://dx.doi.org/10.1080/01652176.2014.973999>.
- Paudel, S., Park, J.E., Jang, H., Shin, H.J., 2014b. Comparison of serum neutralization and enzyme-linked immunosorbent assay on sera from porcine epidemic diarrhoea virus vaccinated pigs. *Vet. Q* 34, 218–223, <http://dx.doi.org/10.1080/01652176.2014.979512>.
- Pensaert, M.B., de Bouck, P., 1978. A new coronavirus-like particle associated with diarrhoea in swine. *Arch. Virol.* 58, 243–247, <http://dx.doi.org/10.1007/BF01317606>.
- Ramos-Vara, J.A., 2011. Principles and methods of immunohistochemistry. *Methods Mol. Biol.* 691, 83–96, http://dx.doi.org/10.1007/978-1-60761-849-2_5.
- Ren, X., Li, P., 2011. Development of reverse transcription loop-mediated isothermal amplification for rapid detection of porcine epidemic diarrhoea virus. *Virus Genes* 42, 229–235, <http://dx.doi.org/10.1007/s11262-011-0570-3>.
- Rodák, L., Valíček, L., Smíd, B., Nevoránková, Z., 2005. An ELISA optimized for porcine epidemic diarrhoea virus detection in faeces. *Vet. Microbiol.* 105, 9–17, <http://dx.doi.org/10.1016/j.vetmic.2004.09.020>.
- Scott, A., McCluskey, B., Brown-Reid, M., Grear, D., Pitcher, P., Ramos, G., Spencer, D., Singrey, A., 2016. Porcine epidemic diarrhoea virus introduction into the United States: root cause investigation. *Prev. Vet. Med.* 123, 192–201, <http://dx.doi.org/10.1016/j.prevetmed.2015.11.013>.
- Shan, Z., Yin, J., Wang, Z., Chen, P., Li, Y., Tang, L., 2015. Identification of the functional domain of the porcine epidemic diarrhoea virus receptor. *J. Gen. Virol.* 96, 2656–2660, <http://dx.doi.org/10.1099/vir.0.000211>.
- Song, D., Park, B., 2012. Porcine epidemic diarrhoea virus: a comprehensive review of molecular epidemiology, diagnosis, and vaccines. *Virus Genes* 44, 167–175, <http://dx.doi.org/10.1007/s11262-012-0713-1>.
- Song, D.S., Kang, B.K., Oh, J.S., Ha, G.W., Yang, J.S., Moon, H.J., Jang, Y.-S., Park, B.K., 2006. Multiplex reverse transcription-PCR for rapid differential detection of porcine epidemic diarrhoea virus, transmissible gastroenteritis virus, and porcine group A rotavirus. *J. Vet. Diagn. Invest.* 18, 278–281.
- Song, D., Zhou, X., Peng, Q., Chen, Y., Zhang, F., Huang, T., Zhang, T., Li, A., Huang, D., Wu, Q., He, H., Tang, Y., 2015. Newly emerged porcine deltacoronavirus associated with diarrhoea in swine in China: identification prevalence and full-length genome sequence analysis. *transbound. Emerg. Dis.* 62, 575–580, <http://dx.doi.org/10.1111/tbed.12399>.
- Sozzi, E., Luppi, A., Lelli, D., Martin, A.M., Canelli, E., Brocchi, E., Lavazza, A., Cordioli, P., 2010. Comparison of enzyme-linked immunosorbent assay and RT-PCR for the detection of porcine epidemic diarrhoea virus. *Res. Vet. Sci.* 88, 166–168, <http://dx.doi.org/10.1016/j.rvsc.2009.05.009>.
- Stevenson, G.W., Hoang, H., Schwartz, K.J., Burrough, E.R., Sun, D., Madson, D., Cooper, V.L., Pillatzki, A., Gauger, P., Schmitt, B.J., Koster, L.G., Killian, M.L., Yoon, K.J., 2013. Emergence of Porcine epidemic diarrhoea virus in the United States: clinical signs, lesions, and viral genomic sequences. *J. Vet. Diagn. Invest.* 25, 649–654, <http://dx.doi.org/10.1177/1040638713501675>.
- Sun, D., Feng, L., Shi, H., Chen, J., Cui, X., Chen, H., Liu, S., Tong, Y., Wang, Y., Tong, G., 2008. Identification of two novel B cell epitopes on porcine epidemic diarrhoea virus spike protein. *Vet. Microbiol.* 131, 73–81, <http://dx.doi.org/10.1016/j.vetmic.2008.02.022>.
- Sun, D., Wang, X., Wei, S., Chen, J., Feng, L., 2015. Epidemiology and vaccine of porcine epidemic diarrhoea virus in China: a mini-review. *J. Vet. Med. Sci.*, <http://dx.doi.org/10.1292/jvms.15-0446>.
- Thomas, J.T., Chen, Q., Gauger, P.C., Giménez-Lirola, L.G., Sinha, A., Harmon, K.M., Madson, D.M., Burrough, E.R., Magstadt, D.R., Salzbrenner, H.M., Welch, M.W., Yoon, K.-J., Zimmerman, J.J., Zhang, J., 2015. Effect of porcine epidemic diarrhoea virus infectious doses on infection outcomes in naïve conventional neonatal and weaned pigs. *PLoS One* 10, e0139266, <http://dx.doi.org/10.1371/journal.pone.0139266>.
- Tomita, N., Mori, Y., Kanda, H., Notomi, T., 2008. Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. *Nat. Protoc.* 3, 877–882, <http://dx.doi.org/10.1038/nprot.2008.57>.
- Wang, L., Byrum, B., Zhang, Y., 2014a. Detection and genetic characterization of deltacoronavirus in pigs, Ohio USA. *Emerg. Infect. Dis.* 20, 1227–1230, <http://dx.doi.org/10.3201/eid2007.140296>.
- Wang, L., Zhang, Y., Byrum, B., 2014b. Development and evaluation of a duplex real-time RT-PCR for detection and differentiation of virulent and variant strains of porcine epidemic diarrhoea viruses from the United States. *J. Virol. Methods* 207, 154–157, <http://dx.doi.org/10.1016/j.jviromet.2014.07.005>.
- Wicht, O., Li, W., Willems, L., Meuleman, T.J., Wubbolts, R.W., van Kuppeveld, F.J.M., Rottier, P.J.M., Bosch, B.J., 2014. Proteolytic activation of the porcine epidemic diarrhoea coronavirus spike fusion protein by trypsin in cell culture. *J. Virol.* 88, 7952–7961, <http://dx.doi.org/10.1128/JVI.00297-14>.
- van Nieuwstadt, A.P., Zetstra, T., 1991. Use of two enzyme-linked immunosorbent assays to monitor antibody responses in swine with experimentally induced infection with porcine epidemic diarrhoea virus. *Am. J. Vet. Res.* 52, 1044–1050.
- Yoon, K.-J., 2015. Oral Fluid Testing for Efficient PEDV Surveillance and Control [WWW Document] (accessed 01.01.16.) <https://www.aasv.org/news/story.php?id=8120>.
- Yu, X., Shi, L., Lv, X., Yao, W., Cao, M., Yu, H., Wang, X., Zheng, S., 2015. Development of a real-time reverse transcription loop-mediated isothermal amplification method for the rapid detection of porcine epidemic diarrhoea virus. *Virol. J.* 12, 76, <http://dx.doi.org/10.1186/s12985-015-0297-1>.
- Zhang, X., Pan, Y., Wang, D., Tian, X., Song, Y., Cao, Y., 2015. Identification and pathogenicity of a variant porcine epidemic diarrhoea virus field strain with reduced virulence. *Virol. J.* 12, 88, <http://dx.doi.org/10.1186/s12985-015-0314-4>.
- Zhao, J., Shi, B., Huang, X., Peng, M., Zhang, X., He, D., Pang, R., Zhou, B., Chen, P., 2013. A multiplex RT-PCR assay for rapid and differential diagnosis of four porcine diarrhoea associated viruses in field samples from pig farms in East China from 2010 to 2012. *J. Virol. Methods* 194, 107–112, <http://dx.doi.org/10.1016/j.jviromet.2013.08.008>.
- Zhao, P., Bai, J., Jiang, P., Tang, T., Li, Y., Tan, C., Shi, X., 2014. Development of a multiplex TaqMan probe-based real-time PCR for discrimination of variant and classical porcine epidemic diarrhoea virus. *J. Virol. Methods* 206, 150–155, <http://dx.doi.org/10.1016/j.jviromet.2014.06.006>.