Porcine Epidemic Diarrhea Virus

Zayn Khamis and Rima Menassa

Abstract Porcine epidemic diarrhea virus (PEDv) causes disease and mortality to piglets worldwide. Most vaccines used to combat the disease have been ineffective live attenuated virus vaccines. Research has emerged showing both the spike (S) and membrane (M) proteins of the virus have potential for use as subunit vaccines. This research has been largely undertaken using plants as expression platforms, with some promising candidates having emerged.

Keywords Porcine epidemic diarrhea virus · Recombinant protein
Subunit vaccine · Coronavirus · Plant biotechnology

1 Disease Symptoms and Occurrence

Porcine epidemic diarrhea virus (PEDv) is a coronavirus that causes porcine epidemic diarrhea (PED) in pigs. Its high mortality rate for piglets, at 90–95% (Stevenson et al. 2013) is a key distinguishing factor between PEDv and the similar transmissible gastroenteritis virus (TGEV). In suckling piglets, the PEDv incubation period is around two days, varying from 1 to 8 days. Diarrhea and vomiting can develop within 24 h, and as a result dehydration, anorexia and severe weight loss occur. In older pigs and sows, morbidity varies, and the period between onset and end of clinical symptoms is 3–4 weeks (Lee 2015; Stevenson et al. 2013).

The first known PEDv outbreaks occurred in Europe in the 1970s and 1990s, including in Belgium (Pensaert and de Bouck 1978) and Hungary (Nagy et al. 1996). While PEDv has since posed less of a threat to Europe, it has re-emerged in Italy (Martelli et al. 2008), Germany (Hanke et al. 2015), and France (Grasland

Z. Khamis · R. Menassa (✉)
Agriculture and Agri-Food Canada, London Research and Development Centre, London, ON, Canada
e-mail: rima.menassa@agr.gc.ca

Z. Khamis · R. Menassa
Department of Biology, The University of Western Ontario, London, ON, Canada

J. MacDonald (ed.), *Prospects of Plant-Based Vaccines in Veterinary Medicine*,
https://doi.org/10.1007/978-3-319-90137-4_12

et al. 2015) affecting pigs of all ages. PEDv spread to Asia in the early 1980s, where it was first detected in Japan in 1982, and then in South Korea, China, and Thailand (Chen et al. 2014). Since October 2010 China has seen a severe outbreak of PEDv, resulting in high porcine mortality rates and economic losses (Sun et al. 2012). PEDv was first detected in the United States (U.S.) in May 2013 (Stevenson et al. 2013) and then in Canada in January 2014 (Ojkic et al. 2015). These two countries have experienced severe economic losses due to the death of millions of suckling piglets and to diarrhea-derived weight loss in fattening pigs (Chen et al. 2014). The North American serotypes are most closely related to a recently emerged Chinese serotype (Huang et al. 2013) and since the North American epidemic, they have spread to South Korea, Taiwan, and Japan (Lee 2015).

While a few vaccines are available against PEDv, those targeting the Asian strains are not effective against the North American strains, while the effectiveness of a conditionally approved vaccine in the U.S. is unknown (see below). By vaccinating sows with an effective vaccine, the suckling piglets can receive lactogenic immunity through IgA antibodies secreted into the milk (Bae et al. 2003), and may be spared from vertical transmission of PEDv from sow milk (Sun et al. 2012).

2 Mechanisms of Infection

PEDv primarily enters pigs' bodies through the fecal-oral route, although airborne transmission may play a role (Alonso et al. 2014). Diarrhea and vomiting result in the spread of PEDv through contaminated environmental sources including pigs, trailers, clothing (Lowe et al. 2014), sow's milk (Sun et al. 2012), feed, and feed-supplements such as spray-dried porcine plasma (Pasick et al. 2014), highlighting the importance of biosecurity.

PEDv causes sickness through its actions in the intestines of pigs. The virus enters porcine enterocytes, which line the inner surface of the intestines, via interaction between the viral S protein and the enterocyte aminopeptidase N which acts as a cellular receptor for PEDv. Through this receptor PEDv enters the enterocyte cells, where new virions assemble by budding through the endoplasmic reticulum and Golgi apparatus membranes (Ducatelle et al. 1981; Li et al. 2007).

Once PEDv enters enterocytes it causes them to undergo acute necrosis (Jung et al. 2014). PEDv also causes a reduction in the number of goblet cells, the cells which secrete mucins to defend against microbial infection (Jung and Saif 2015). Cytolysis additionally leads to shortening and severe atrophy of intestinal villi, and causes the tips of villi to erode or become covered with attenuated epithelial cells (Jung et al. 2014; Stevenson et al. 2013). The atrophy of intestinal villi results in microscopic lesions, which are typical of an enteritic infection, (Sueyoshi et al. 1995). PEDv also results in swollen cells, and the detachment of cells from adjacent cells and from the membranes of the basal surfaces (Stevenson et al. 2013). These factors inhibit the pig's ability to absorb water and nutrients, and result in the malabsorption and diarrhea discussed above.

3 PEDv Vaccine Design and Plant-Made Candidates

To produce an effective vaccine against PEDv, it is important to understand the structure of the virus. The Coronavirinae subfamily consists of three genera: alphacoronavirus, betacoronavirus, and gammacoronavirus. PEDv is an enveloped alphacoronavirus encoded by a 28 kilobase single-stranded, positive-sense RNA genome (Song and Park 2012). Coronaviruses have the largest known RNA genomes of all viruses (King 2011). The PEDv genome has seven open reading frames (ORFs), which code for three non-structural polyproteins, and four structural proteins [spike (S), envelope (E), membrane (M) and nucleocapsid (N)]. These open reading frames are flanked by a 5′ cap and a 3′ polyadenylated tail (Figs. 1 and 2) (Song and Park 2012). The ORFs encoding the non-structural proteins consist of two overlapping open reading frames, ORF1a and ORF1b, encoding two polyproteins. These polyproteins are processed by three virus-encoded proteases, a 3C-like proteinase (3CLpro) and two papain-like proteinases (PLP) which results in 16 non-structural proteins required for genome replication and mRNA transcription (John et al. 2016; Prentice et al. 2004). The accessory protein ORF3 is a potassium ion channel, but its role is not well defined (Wang et al. 2012). Reports on transmissible gastroenteritis coronavirus (TGEV), another alphacoronavirus, indicate that S and E are only present in the virion in small quantities, with E estimated to occur 20 times in a virion (Godet et al. 1992). N and M occur in higher numbers, at a ratio of 1N:3M (King 2011). A recent study examining mouse hepatitis virus (MHV, a betacoronavirus), severe acute respiratory syndrome-coronavirus (SARS-CoV, a betacoronavirus), and feline coronavirus (FCoV, an alphacoronavirus), has determined that coronaviruses have approximately 1100 M dimers, 90 S trimers, and N proteins in a ratio from 3M:1N to 1M:1N (Neuman et al. 2011). Thus, M is the most abundant structural protein displayed at the viral surface.

Of the four structural proteins, S and M are the most antigenic. To date, the S protein has been the primary focus of subunit vaccine design due to its antigenicity, and the role it plays in viral entry, as it regulates interactions with host cell receptor protein, aminopeptidase N (Bosch et al. 2003). S contains three antigenic regions. The first epitope that was recognized spans amino acids 1495–1913. Called the CO-26K equivalent (COE), this epitope induced a neutralizing immune response and was identified through sequence homology with TGEV, which induces similar clinical symptoms in pigs (Chang et al. 2002). Subsequently, the motif spanning

Fig. 1 A schematic of the genome of PEDv. The first two ORFs from the 5′ end of the genome cover two thirds of the genome, and code for polyprotein 1a and polyprotein 1b, respectively. The next part of the genome codes for the S protein, then ORF3, E, M, and finally the N protein (Khamis 2016)

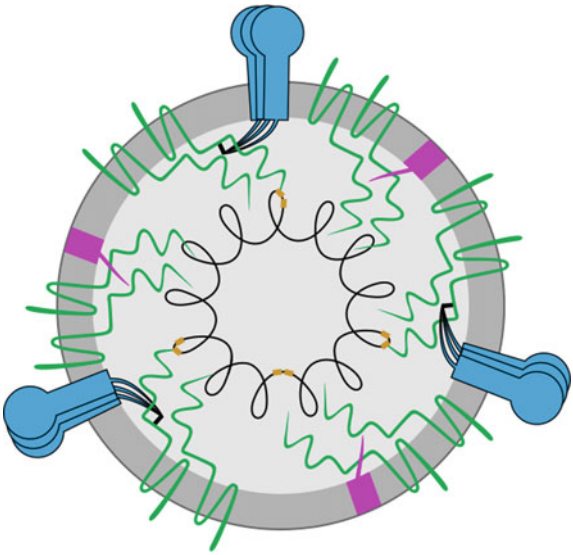

Fig. 2 Schematic of an assembled PEDv virion. The nucleocapsid protein (N, small yellow rectangles) forms a ribonucleoprotein complex with viral RNA (black line) inside the virion. The envelope protein (E, pink) is embedded in the membrane (darker grey) as is the membrane protein (M, green). The spike protein (S, blue) also embeds in the membrane, and forms surface projections, or 'spikes'. Stoichiometry not to scale. Figure modified from Khamis (2016)

amino acids 1368–1374, and the epitope region from amino acids 636–789 have also been found to induce the production of neutralizing antibodies (Cruz et al. 2008; Sun et al. 2006).

While S is antigenic, the use of S as a subunit vaccine poses challenges. *S* has been shown to be prone to mutations through serial passages (Sato et al. 2011), and has high genetic variability among PEDv strains–strains have even been found with 582 nucleotide deletions in the *S* gene (Masuda et al. 2015). Indeed, the variability of *S* makes it the gene of choice to study the genetic relatedness of different PEDv strains (Chen et al. 2014). The difference in amino acid sequence between the S protein of different strains can lead to different epitopes being presented, and may explain why previous vaccines have failed to provide effective immunity against infectious strains (Sun et al. 2012).

Nonetheless, due to the early recognition of the antigenic importance of the protein, PEDv recombinant protein production has focussed on S. Work has been done to produce S or S epitopes in *Escherichia coli* (Van Noi and Chung 2017), *Lactobacillus casei* (Ge et al. 2012), and via the Orf virus as a vaccine delivery vector (Hain et al. 2016). However, most recombinant PEDv S production has occurred in plants, with all of these plants producing S-COE. The protein yield has varied widely, as has the plant host utilized. Historically, tobacco has been the most established and developed platform for high-yield plant-based recombinant protein production (Conley et al. 2011). As such, it follows suit that five of the fourteen reports of S-COE production were carried out using tobacco (Table 1). Four of the reports expressed S-COE in a stable transgenic line, with yields between 0.1% of TSP (Kang et al. 2005b) and 2.1% of TSP, depending on codon optimization (Kang et al. 2005a), while one reported transient production at 5% of TSP (Kang et al. 2004).

Table 1 Production of S-COE in plants

Plant host	Transient or transgenic	Fusions	Yield	Promoter and/or enhancer used	References
Tobacco	Transgenic	–	10 mg/kg of protein per fresh weight[a]	2x35S, TOL	Bae et al. (2003)[b]
Tobacco	Transient	–	5% TSP	TMV RNA	Kang et al. (2004)
No-nicotine Tobacco	Transgenic	–	2.1% TSP	2x35S, TOL	Kang et al. (2005a)
Tobacco	Transgenic	–	0.1% TSP	2x35S, TOL	Kang et al. (2005b)
Potato	Transgenic	–	0.1% TSP	2x35S, TOL	Kim et al. (2005)
Duckweed	Transgenic	–	Not reported	35S	Ko et al. (2011)
Sweet potato	Transgenic	–	Not reported	35S	Yang et al. (2005)
Corn seed	Transgenic	–	0.122% TSP	2x35S, maize intron Hsp70	Kun et al. (2014)
Carrot	Transgenic	–	Not reported	2x35S, TOL	Kim et al. (2003)
Tobacco	Transgenic	LTB	1.6% TSP	Ubiquitin promoter	Kang et al. (2006)
Lettuce	Transgenic	CTB	0.0065% TSP	Ubiquitin promoter	Huy et al. (2011)
Lettuce	Transgenic	LTB	0.048% TSP	Ubiquitin promoter	Huy et al. (2009)
Rice endosperm	Transgenic	LTB	1.3% TSP	HMW-Bx17-p, Act1-i	Oszvald et al. (2007)
Rice endosperm	Transgenic	LTB	1.9% TSP	HMW-Bx17-p, Act1-i	Tamás (2010)
Rice calli	Transgenic	Co1	0.083% TSP	RAmy3D	Huy et al. (2012)[b]

2x, double-enhanced; 35S, cauliflower mosaic virus (CaMV) 35S promoter; Act1-I, rice actin first intron; Co1, M cell-targeting ligand; CTB, cholera toxin B subunit; HMW-Bx17-p, wheat high molecular weight glutenin subunit Bx17 endosperm-specific promoter; LTB, heat-labile enterotoxin B subunit of *Escherichia. coli*; RAmy3D, rice α-amylase 3D promoter; TOL, TMV Omega-prime leader, containing transcriptional and translational enhancer from the coat protein gene of TMV; TSP, total soluble protein; all yield values are highest levels reported

Table modified from Khamis (2016)

[a]Total soluble protein levels were not reported in this study

[b]Study also showed antibody production against protein

Many of the alternative plant choices resulted in yields that were either not quantified, or very low, despite often using the same promoters and enhancers as the tobacco studies. Like the tobacco studies, a double-enhanced 35S (2x35S) cauliflower mosaic virus (CaMV) promoter was used in carrot and potato

expression experiments. Expression in potato reached 0.1% of tuber TSP, while expression in carrot was not reported (Kim et al. 2003, 2005). Expression of S-COE has also been reported in *Lemna minor* [35S promoter, not quantified, (Ko et al. 2011)], sweet potato [35S promoter, not quantified (Yang et al. 2005)], and corn [2x35S, 0.122% TSP (Kun et al. 2014)].

The use of fusion proteins, particularly the heat-labile enterotoxin B subunit of *Escherichia coli* (LTB), have recently gained popularity in subunit vaccine design, and have been utilized in the production of S-COE. Three studies have expressed such fusions in rice, which has shown promise in reaching equivalent accumulation levels as tobacco. Rice was used to express S-COE both in the endosperm and in calli. While accumulation levels in the calli using the rice α-amylase 3D promoter (RAmy3D) only reached 0.083% for a S-COE-M cell-targeting ligand (Co1) fusion (Huy et al. 2012), accumulation levels in the endosperm for LTB-S-COE using the wheat high molecular weight glutenin subunit Bx17 endosperm-specific promoter (HMW-Bx17-p) and rice actin first intron (Act1-I) reached 1.3 and 1.9% (Oszvald et al. 2007; Tamás 2010). LTB-COE has also been produced in tobacco, reaching comparative levels of 1.6% TSP (Kang et al. 2006).

So far, only three studies have tested the immunogenicity of plant-produced S-COE. LTB-S-COE produced in tobacco was able to bind to the GM1-ganglioside intestinal membrane receptor (Kang et al. 2006). More directly, mice orally immunized with Co1-S-COE fusion protein produced in rice calli had threefold and eightfold higher levels of IgG and IgA secreting cells in their lymphocytes, respectively, compared to unimmunized mice (Huy et al. 2012). While this is encouraging, in order for a vaccine to be effective, the antibodies produced must be virus-neutralizing. Bae et al (2003) took this next step, showing that feeding ground lyophilized transgenic tobacco containing S-COE was able to induce an immune response in mice that could inhibit PEDv plaque formation by 49.7% in comparison to controls. While full immunity was not conferred by the mounted immune response, this study marks the most significant achievement to date in plant-based PEDv vaccine research.

M is the other antigenic protein of PEDv, and the most abundant component of the viral envelope (Utiger et al. 1995). In contrast to *S*, *M* is more conserved, showing 5.5% the number of mutations as *S* after 100 serial passages (Chen et al. 2014; Sato et al. 2011). The use of a protein that remains stable is important for subunit vaccine design to ensure that a vaccine can be used for a wide variety of strains and locations. The M protein of TGEV and SARS-CoV show virus-neutralizing activity in the presence of complement, the component of the immune system that enhances the ability of antibodies to clear pathogens. This virus-neutralizing activity was demonstrated to be higher or comparable to the neutralizing capacity of 8 individual S protein fragments (Pang et al. 2004; Woods et al. 1988).

Through sequence homology with infectious bronchitis virus (IBV, a gamma-coronavirus), M was found to have a B-cell epitope on its C-terminus from amino acids 195–200 (Zhang et al. 2012). However, it is likely that with further research sequence homology studies will find more epitopes on PEDv M. For example,

the M protein of SARS-CoV has B-cell epitopes on the N and C-termini (He et al. 2005), and two cytotoxic T-cell epitopes in the second and third transmembrane domain (Liu et al. 2010), while the M protein of MHV has a CD4+ T-cell epitope on its C-terminus (Xue et al. 1995).

Expression of M transiently in *N. benthamiana* as a fusion with elastin-like polypeptide (Khamis 2016) resulted in the production of virus-like particles (VLPs), an important development of subunit vaccine design. While subunit vaccines have previously failed to completely protect piglets from PEDv infection due to subpar immunogenicity, VLPs are more immunogenic because they resemble the native structure of the virion. Plant-made VLPs for influenza are shown to induce four- to six-fold higher levels of antibody response than fifty times more flu antigen not in VLPs (D'Aoust et al. 2008). The ability to produce PEDv VLPs using M may lead to the first commercial plant-produced PEDv vaccine.

4 Existing Commercial Vaccines

Much of the PEDv vaccine research has occurred in Asia, where outbreaks have been most severe, but none of the produced vaccines are completely effective against Asian PEDv strains (Song and Park 2012). Available Asian vaccines are based on strains that are genetically different from those sequenced in the U.S. that are currently causing epidemics globally (Huang et al. 2013). This has been on display in China, where vaccinated herds experienced PEDv breakouts that were found to be due to newer strains of the virus (Li et al. 2012). Two PEDv vaccines were given conditional licenses in the U.S. by the United States Department of Agriculture (USDA). The first was a vaccine originally produced by Harrisvaccines, Inc., and is based on their SirraVax^{SM} RNA platform. Using this platform, part of the RNA genome of a Venezuelan equine encephalitis alphavirus is replaced with a gene for PEDv S protein. After injection with genetic material, the pig's dendritic cells produce the S protein and an immune response is launched against the produced protein (Harrisvaccines, 2015). Merck Animal Health acquired Harrisvaccines in 2015 (Merck Animal Health 2015) but the product is still sold under a conditional license in the U.S., where safety and field trials are ongoing. The second conditionally licensed PEDv vaccine in the U.S. is an inactivated virus particle vaccine produced by Zoetis, Inc. (Zoetis 2016). Efficacy and potency studies are still in progress for the Zoetis vaccine, and duration of immunity has not been evaluated. It must be refrigerated, and used all at once when opened (Zoetis 2016). A third vaccine candidate is being developed by VIDO-Intervac (Vaccine and Infection Disease Organization—International Vaccine Centre) in Canada and is currently undergoing field testing. This vaccine candidate is a subunit S1 protein expressed in mammalian HEK-293 T cells, and although it induced production of neutralizing antibodies, it was not fully protective to suckling piglets (Makadiya et al. 2016).

None of the PEDv vaccines used commercially, at least in the U.S. and Canada, are made *in planta*. The ability to vaccinate orally through feeding plant tissue is important as (Song et al. 2007) demonstrated that oral vaccination was more effective than injection for their PEDv vaccine. When comparing oral to intramuscular administration of their attenuated virus vaccine, Song et al. found that more IgA's were produced by orally vaccinated pigs, and that the mortality rate for this group was 13% in comparison to 60% for the intramuscular group. Current vaccines on the market are also either based on S, which is prone to mutations, or are killed or live attenuated vaccines. Live attenuated vaccines present risks, as they can potentially mutate and become pathogenic again, and allow for genome segment re-assortment on farms—one Chinese PEDv strain is thought to have evolved from a live attenuated vaccine (Chen et al. 2010). Inactivated vaccines, in comparison, are safer, but have a high cost of production, and present concerns over the reliability of inactivation methods (Calvo-Pinilla et al. 2014). Both live attenuated and inactivated vaccines rule out the possibility of using "distinguish infected from vaccinated animals" (DIVA) assays, as they contain the entire virus. As such, the market has a need of an effective subunit vaccine based on the current infectious strains. Further research on current plant-made candidates could prove to fill this need.

5 Pathways to Commercialization

While proof of concept studies show that S-COE can be produced in plants, many of these studies show poor yields. Generally, even when higher yields are reported, the push forward to testing the immunogenicity of these vaccine candidates does not occur. There have been two instances where the direct immunogenicity of plant-produced S-COE has been tested. The first was incomplete, as it did not test whether the mounted immune response was virus-neutralizing (Huy et al. 2012). The other candidate that successfully took steps to test immunogenicity was the vaccine candidate produced by (Bae et al. 2003). However, while the immune response in mice inhibited PEDv plaque formation by 49.7% in comparison to controls, complete immunity was not achieved (Bae et al. 2003). Likely due to this, and due to the need for higher expression levels, no further efforts were made with this vaccine candidate.

Generally, even when higher expression levels have been obtained (Kang et al. 2004), immunogenicity was not tested, and research focused on increasing expression levels and not on producing an immunogenic commercial product. The focus on S-COE has left a gap in the research on plant production of other PEDv proteins. However, our recent results have shown that M can be produced *in planta* and that VLPs can be produced for PEDv (Khamis 2016). VLPs represent a step-forward for subunit vaccine design, demonstrating higher immunogenicity than regular subunit vaccines. In the future, advances in subunit vaccine design,

such as VLPs, and a commitment to improving yields and testing immunogenicity and protective immune responses to these VLPs may lead to the commercialization of a plant-produced subunit PEDv vaccine.

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