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Review

Lactogenic immunity and vaccines for porcine epidemic diarrhea virus (PEDV): Historical and current concepts



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ARTICLE INFO

Article history:

Received 5 April 2016

Received in revised form 17 May 2016

Accepted 18 May 2016

Available online 19 May 2016

Keywords:

Porcine epidemic diarrhea virus
Transmissible gastroenteritis virus
Lactogenic immunity
Maternal antibodies
Gut-mammary-secretory IgA axis
Swine

ABSTRACT

Morbidity, mortality, and loss of productivity from enteric diseases in neonatal piglets cost swine producers millions of dollars annually. In 2013–2014, the porcine epidemic diarrhea virus (PEDV) outbreak led to \$900 million to \$1.8 billion in annual losses to US swine producers. Passive lactogenic immunity remains the most promising and effective way to protect neonatal suckling piglets from enteric diseases like PEDV. Protecting suckling piglets through lactogenic immunity is dependent on trafficking of pathogen-specific IgA plasmablasts to the mammary gland and accumulation of secretory IgA (sIgA) antibodies in milk, defined as the gut-mammary-sIgA axis. Due to an impermeable placenta, piglets are born agammaglobulinic, and are highly susceptible to a plethora of infectious agents. They rely solely on colostrum and milk antibodies for maternal lactogenic immunity. Previous advances in the development of live and attenuated vaccines for another devastating diarrheal virus of pigs, transmissible gastroenteritis virus (TGEV), provide insights into the mechanisms of maternal immunity and piglet protection. In this chapter, we will review previous research on TGEV-induced lactogenic immunity to provide a historical perspective on current efforts for PEDV control and vaccines in the swine industry. Identifying factors that influence lactogenic immunity and the gut-mammary-sIgA axis may lead to improved vaccine regimens for PEDV and other enteric pathogens in gestating swine and improved overall herd immunity, swine health and industry productivity.

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Abbreviations: ASC, antibody secreting cells; CCL, chemokine ligand; CCR, chemokine receptor; M, membrane; MERS-CoV, Middle East respiratory syndrome coronavirus; MAdCAM-1, mucosal addressin cellular adhesion molecule 1; N, nucleocapsid; ORF, open reading frames; OLVE, oral live virus exposure; PNA, peripheral node addressin; PCD, piglet challenge day; PFU, plaque forming units; pIgR, polymeric immunoglobulin receptor; PEDV, porcine epidemic diarrhea virus; PRCV, porcine respiratory coronavirus; PID, post inoculation day; PPD, post partum day; sIgA, secretory IgA; SARS-CoV, severe acute respiratory syndrome-associated coronavirus; S, spike; SINDEL, spike insertion deletion; Th, T helper; TGEV, transmissible gastroenteritis virus; VCAM-1, vascular cellular adhesion molecule 1; VEE, Venezuelan equine encephalitis virus.

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<http://dx.doi.org/10.1016/j.virusres.2016.05.016>

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1. Introduction

In the 1970's a new alphacoronavirus, PEDV first emerged in fattening pigs in European swine herds. It then became widespread and was most severe in nursing piglets. No vaccines were used and PEDV cases became rare in Europe with only sporadic outbreaks. However, the re-emergence of PEDV outbreaks in multiple countries in Europe since 2014 has been reported recently (Bonioti et al., 2016; Dastjerdi et al., 2015; Dennis et al., 2015; Grasland et al., 2015; Martelli et al., 2008; Stadler et al., 2015; Theuns et al., 2015). Due to the PEDV outbreaks that occurred in Asian swine herds in the 1980's, the disease became widespread, and ultimately endemic (Song and Park, 2012). Subsequently, attenuated and inactivated PEDV vaccines were developed and were used widely in Asia. Nevertheless, since 2010, severe PEDV outbreaks with highly virulent PEDV strains have been reported throughout the continent. For unexplained reasons, vaccines based on European and other classical PEDV strains failed to control the more recent virulent PEDV strains in Asia (Song and Park, 2012). In 2013, PEDV emerged in the US as a new, devastating diarrheal disease in swine. PEDV spread rapidly throughout the US, infecting swine of all ages and causing major economic losses to the swine industry (Stevenson et al., 2013). It also spread to Canada and Mexico (Jung and Saif, 2015; Ojkic et al., 2015). Genetically US PEDV strains resemble the recent highly virulent strains from China (China AH2012 and CH/ZMZDY/11), but the origin of PEDV in the US remains unknown (Huang et al., 2013; Marthaler et al., 2013; Vlasova et al., 2014). The emergence and disease course of PEDV resembles that of another alphacoronavirus, TGEV that caused epizootic diarrhea outbreaks with severe losses in nursing pigs in the US in the 1960's–1980's (Saif et al., 2012). Although attenuated TGEV vaccines were developed and had some protective effects, the greatest impact on the epidemiology and severity of TGEV was the emergence of a respiratory variant of TGEV [porcine respiratory coronavirus (PRCV)] that emerged in the winter of 1983–1984 (Pensaert et al., 1986; Saif et al., 2012). The PRCV spread widely in swine in Europe, Asia and the US and has generally moderated the severity of TGEV and dramatically reduced and potentially eliminated the incidence of TGEV in certain regions (i.e. Western Europe). Due to the high virulence of PEDV and the naïve, immature immune system of the neonatal suckling piglet, passive lactogenic immunity to PEDV is critical for suckling piglet protection. It appears that, like for TGEV lactogenic immunity, the gut-mammary gland-sIgA axis may also play a key role in lactogenic immunity to PEDV (Bohl et al., 1972). Understanding the gut-mammary-sIgA link is critical to ensuring herd health and recovery during a PEDV outbreak and for vaccine design to prevent future PEDV outbreaks. In this review, we will focus on lactogenic immunity and the design of maternal vaccines for PEDV because like epizootic TGEV, PEDV is fatal in nursing piglets. Induc-

tion of lactogenic immunity to TGEV will be reviewed to provide a historical perspective for PEDV vaccines.

2. Passive lactogenic immunity and the 'gut-mammary-sIgA axis'

PEDV and TGEV are mainly localized intestinal infections, but with transient viremia based on viral RNA detection in the serum of young piglets (Jung et al., 2014). The severity of both infections is greatest in newborn piglets. Thus vaccination strategies for both diseases must focus on induction of mucosal immunity to protect the target intestinal enterocytes. This necessitates protective levels of mucosal immunity in neonates at birth and throughout the nursing period. There are two major issues for the direct vaccination of PEDV for neonatal piglets; (1) in seropositive sows, maternal antibodies may interfere with live oral vaccine-induced protection; and (2) in piglets, three weeks are needed for actual antibody production in piglets. Consequently immunity induced at mucosal sites in the pregnant sow and passively transferred to suckling piglets via colostrum and milk (lactogenic immunity) is crucial for immediate protection of neonates against enteric infections. As recognized in prior studies of passive immunity to TGEV, sows that recovered from TGEV infection protected their exposed litters against TGEV. This protection was associated with high levels of antibodies in the milk (lactogenic immunity), but not in the serum of the sows (Bohl et al., 1972; Bohl and Saif, 1975; Saif et al., 1972). However, the mechanisms to induce protective levels of milk antibodies and lactogenic immunity were unknown, which hindered the development of effective TGEV maternal vaccines. Likewise additional unanswered questions related to induction of lactogenic immunity to enteric pathogens may also impact efficacy of PEDV vaccines.

Several key immunologic findings emerged from the early work on TGEV vaccines (Saif, 1999; Saif et al., 2012). Due to the impermeable placenta of sows, piglets are born agammaglobulinic and rely solely on colostrum and milk antibodies for passive immunity (Saif and Jackwood, 1990). This leaves the newborn piglet highly susceptible to a plethora of infectious agents. In sows, IgG is dominant in colostrum and is transudated from sow serum (Klobasa et al., 1987). Newborn piglets acquire colostral antibodies (mainly IgG) via nursing. These immunoglobulins are transported across the piglet's intestinal epithelium only within the first 24–48 h after birth. During the next 2–3 days, in the transition to milk, sIgA becomes dominant and persists in milk throughout lactation. Thus IgG antibodies absorbed from sow colostrum provide piglets with serum antibodies that reflect the specificities of those in sow serum and prevent systemic infections. In contrast the IgA antibodies dominant in milk and function to provide local passive protection to the piglet intestinal tract. The enigma was to delineate the origin of the IgA immunocytes in the mammary gland that secrete sIgA anti-

bodies into the milk. This knowledge was critical to aid in the design of enteric vaccines to induce passive milk sIgA antibodies. Besides milk, sIgA is the dominant Ig at mucosal surfaces and in most mucosal secretions (Macpherson et al., 2008; Mantis et al., 2011). Its resistance to proteolytic enzymes affords sIgA a high level of stability in the gastrointestinal tract. TGEV research has provided both a basic understanding of the concept of lactogenic immunity, as well as vaccination strategies for its induction in seronegative pregnant swine. The discovery by Bohl and Saif of the gut-mammary-sIgA axis (trafficking of IgA immunocytes from the gut to the mammary gland) in swine in 1972 was a forerunner for the concept of a common mucosal immune system (Bohl et al., 1972; Saif, 1999; Saif and Bohl, 1983; Saif et al., 1972; Weisz-Carrington et al., 1978). It provided an explanation for why sows that were naturally infected or orally inoculated with live TGEV and recovered from the infection had high persisting levels of sIgA antibodies in milk that protected their piglets from TGE. However, sows systemically immunized with inactivated TGEV vaccines had mainly IgG antibodies in serum and colostrum that declined rapidly in milk and provided little lactogenic immunity to piglets. This discovery led to maternal vaccination strategies to induce mucosal passive immunity applicable to multiple species and multiple enteric pathogens, including PEDV.

2.1. Basic aspects of lymphocyte mucosal homing in swine

Lymphocytes are continuously circulating throughout the body between blood and lymphoid tissues to provide immune surveillance against invading pathogens and maintain homeostasis (Butcher and Picker, 1996; Gowans, 1959). Cells migrate ('home') in and out of secondary lymphoid organs by crossing specialized blood vessels known as high endothelial venules. B and T lymphocytes extravasate through high endothelial venules by a multistep process involving rolling, adhesion, transmigration and localization within their respective lymph node (Kunkel et al., 2003; Springer, 1995). Differential expression of surface ligands and receptors on lymphocytes and endothelial cells as well as expression of chemotactic factors and chemokines distinguish peripheral from mucosal homing sites. This distinction between systemic and mucosal lymphocyte homing is important to understand mucosal immune responses and vaccine design for systemic vs mucosal pathogens. For instance, control of peripheral migration is regulated by lymphocyte cellular ligands $\alpha_{4\beta 1}$, L-selectin and chemokine receptor (CCR)7 interacting with vascular cellular adhesion molecule 1 (VCAM-1), peripheral node addressin (PNAd) and chemokine ligands (CCL)21, respectively (Miyasaka and Tanaka, 2004). However, trafficking to mucosal tissues, specifically in the intestine, is largely regulated by $\alpha_{4\beta 7}$ interacting with mucosal addressin cellular adhesion molecule 1 (MAdCAM-1) (Briskin et al., 1997; Marui et al., 1993). Chemokines secreted from high endothelial venules and other tissues play a significant role in mucosal lymphocyte migration and define homing localities (i.e. tissue specificities of lymphocyte trafficking) (Miyasaka and Tanaka, 2004). To mediate the directed migration of lymphocytes, chemokines bind G protein-coupled receptors on the cellular surface, inhibiting adenylyl cyclases and mobilizing intracellular Ca^{2+} (Dixit and Simon, 2012). After receptor activation, chemokines bind to membrane-tethered and extracellular matrix-associated glycosaminoglycans on lymphoid organs or other tissues to create a chemokine gradient (Proudfoot et al., 2003). In the intestine, CCL25 and CCL28 are secreted by endothelial cells and bind to CCR9 and CCR10 on lymphocytes, respectively (Stenstad et al., 2006; Wang et al., 2000; Wurbel et al., 2007).

It is estimated that over 80% of IgA antibody-secreting cells (ASC) are located in gut associated lymphoid tissues (Suzuki et al., 2007; Macpherson and Slack, 2007). Therefore, initiating the specific trafficking signals needed for efficient migration of mucosal B cells in

swine is imperative for enteric disease prevention and clearance. For instance, in studies of intestinal infection by enteropathogenic rotavirus, only a short window of time exists (4–9 days after primary challenge, 3–7 days after secondary challenge) in which the trafficking of gut-derived lymphocytes and IgA ASC can be detected in blood. The transient trafficking of these cells in blood mirrors their stimulation in the gut and homing back to the intestine and other mucosal sites (Brown et al., 2000; Ward et al., 1996b; Yuan et al., 1996). These studies highlight the importance of identifying the window of time in swine when trafficking signals are initiated and when lymphocytes migrate from the stimulated gut into the circulation and traffic to other mucosal sites, like the mammary gland. In addition, homing markers like $\alpha_{4\beta 7}$ may be useful as a correlate of PEDV viral clearance, as appearance of $\alpha_{4\beta 7}^+$ memory B cells are correlated with rotavirus clearance in the gut of mice (Williams et al., 1998). Bourges and colleagues profiled the differential expression of these adhesion molecules and chemokines between different mucosal sites in swine (Bourges et al., 2007). Expression (as measured by mean fluorescence intensity) of $\alpha_{4\beta 1}$ on both lamina propria and intraepithelial CD3 T and IgA B lymphocytes was higher in the nasal mucosa and on peripheral blood lymphocytes when compared to $\alpha_{4\beta 7}$. However, the opposite was true in the intraepithelium of the intestine, where $\alpha_{4\beta 7}$ expression on CD3 T and IgA B lymphocytes was higher than $\alpha_{4\beta 1}$ expression. The differential expression between the nasal and small intestinal mucosa was also demonstrated for the vascular addressins measured. PNAd was expressed by blood vessels in the nasal lamina propria, but was not detected in intestinal blood vessels. The opposite was true for MAdCAM-1 where expression was detected in small intestinal, but not nasal lamina propria. This suggests that mucosal homing characteristics in the pig have tissue specificities and are comparable to what is seen in humans and mice (Butcher and Picker, 1996). In addition, mucosal chemokine expression and their associated receptors exhibit tissue specificities in swine as well. CCL25 exhibits low mRNA expression in respiratory tissues and higher expression in the intestine, particularly the small intestine. There are high levels of CCL28 expression in the small and large intestine as well as the trachea. While CCR9 expression is ubiquitous in both intestinal and respiratory tissues in swine, high CCR10 expression is localized to the small and large intestine, the mesenteric lymph nodes and the mammary gland. Evaluating the role of adhesion molecules and chemokines in healthy and infected swine is integral to understanding the disease pathogenesis and host immune response for development of more effective vaccines. Understanding lymphocyte trafficking and the signals involved in swine is integral to furthering our understanding of the immune response to enteric pathogens like PEDV. Optimizing vaccine efficacy to target the time when intestinal lymphocytes are most responsive to mucosal trafficking in swine will lead to enhanced lactogenic immunity and decreased morbidity and mortality in neonatal suckling piglets.

2.2. Homing of IgA plasmablasts from the intestine to the mammary gland in female swine

It is well documented that immunization of pregnant animals provides passive protection to suckling neonates against bacterial and viral infections (Bohl et al., 1972; Bohl and Saif, 1975; Bohl et al., 1974; Kortbeek-Jacobs et al., 1984; Lanza et al., 1995; Moon and Bunn, 1993; Saif et al., 1972; Saif and Fernandez, 1996; Saif et al., 1984; Wilson et al., 1972). Lactogenic immunity is described as the continuous supply of passively acquired immunoglobulins (IgG, IgM and sIgA) through the ingestion of colostrum and milk, but with IgG being the dominant isotype in colostrum and sIgA in milk (Klobasa et al., 1987). The most abundant antibody in gut secretions, sIgA, is generated by translocation of intestinal plasma

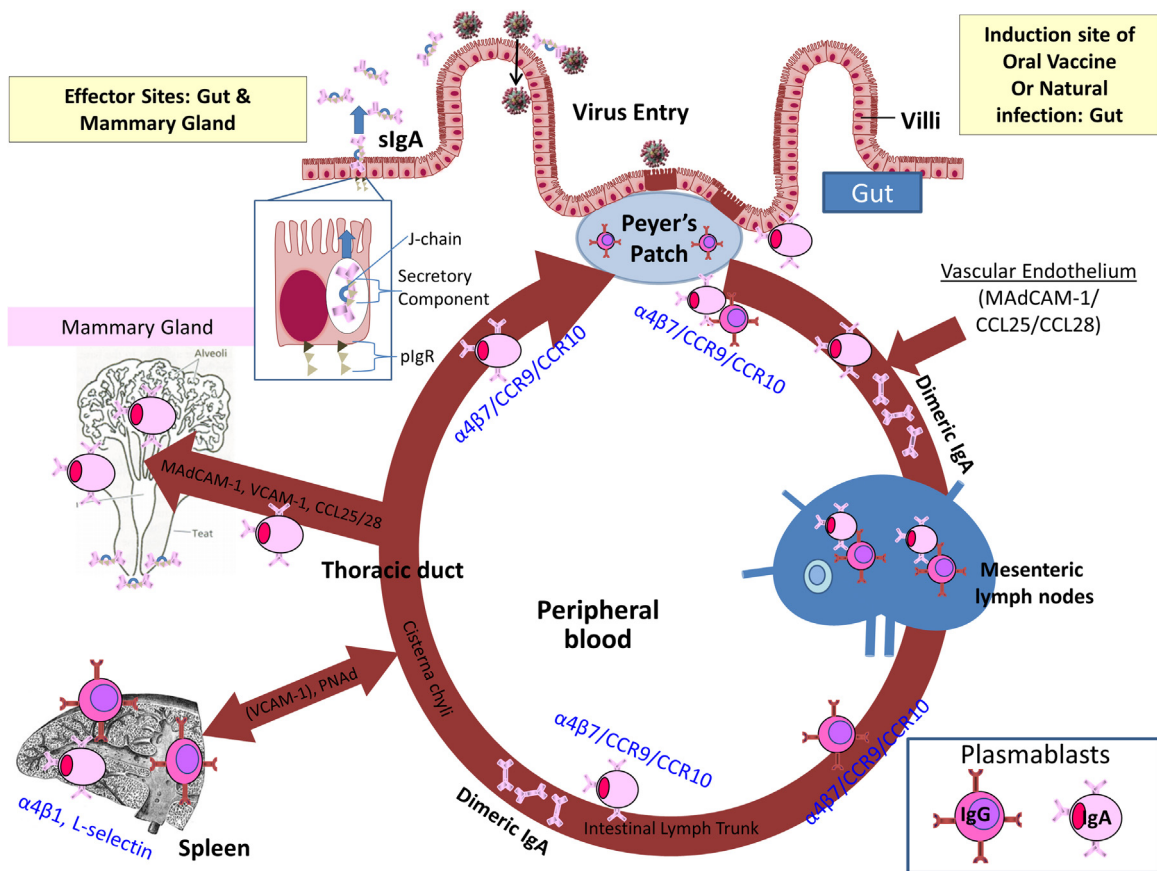


Fig. 1. Schematic to depict the gut-mammary-sIgA axis and trafficking molecules. Providing sufficient PEDV-specific immunity in colostrum/milk is dependent on trafficking of IgA plasmablasts (immature plasma cells) from the intestine to the mammary gland and accumulation of sIgA antibodies in milk. Trafficking of lymphocytes to mucosal tissues is largely regulated by $\alpha_4\beta_7$ interacting with MAdCAM-1 and CCR9/10 interacting with CCL25/28 while trafficking to systemic sites is regulated by $\alpha_4\beta_1$ and L-selection interacting with VCAM-1 and PNAAd, respectively. Adapted from (Chattha et al., 2015).

cell-produced dimeric IgA into the gut lumen via the polymeric immunoglobulin receptor on the basolateral surface of the epithelial cell (Fig. 1). Once in the lumen, sIgA provides immune protection and contributes to intestinal homeostasis (Macpherson et al., 2008; Mantis et al., 2011). After the gut-mammary-sIgA axis is initiated in the intestine by means of natural infection or oral vaccination, plasmablasts must traffic to the mammary gland to supply specific immunity via mammary secretions. As described earlier, migration of IgA plasmablasts and T cells in the intestine is mediated mostly by the surface integrin $\alpha_4\beta_7$ interacting with MAdCAM-1 and CCR9 and CCR10 interacting with CCL25 and CCL28, respectively. However, the homing marker most consistently shown to regulate migration of IgA plasmablasts to the mammary gland in mice is CCR10 interacting with CCL28. When CCL28 is blocked in mice, the levels of IgA antibody in milk is reduced, resulting in a lack of ingested IgA by the nursing mouse (Wilson and Butcher, 2004). In addition, in a CCR10-deficient mouse model, IgA ASC accumulation in the mammary gland is abolished (Morteau et al., 2008). While these studies have not been replicated in swine, associated evidence exists. In mice and swine, CCL28 is most highly expressed in the mammary gland during lactation (Berri et al., 2008; Lazarus et al., 2003). The increase in CCL28 in the mammary gland of swine coincides with the number of accumulating IgA B cells over the course of gestation (Bourges et al., 2008; Meurens et al., 2006). Additionally, CCL28 protein can be detected in sow's milk, providing further evidence suggestive of the role of this chemokine in lactogenic immunity in the sow (Berri et al., 2008). In sows, MAdCAM-1 expression on blood vessel endothelial cells in the mammary gland is increased at the end of gestation and during early lactation (Bourges et al., 2008).

While the binding of $\alpha_4\beta_7$ to MAdCAM-1 has been implicated in the recruitment of IgA plasmablasts to the mammary gland in both swine and mice, contrary evidence exists for both species (Postigo et al., 1993; Tanneau et al., 1999). In mice, a functional blockade of $\alpha_4\beta_7$ to MAdCAM-1 did not diminish the accumulation of IgA in milk (Low et al., 2010). Furthermore, in swine, only a small fraction of the total IgA B cells in the mammary gland express $\alpha_4\beta_7$ after migration into the mammary gland (Bourges et al., 2008). These results suggest that while the $\alpha_4\beta_7$ /MAdCAM-1 interaction may be important in the gut-mammary-sIgA axis, other mechanisms of action may exist. The integrin $\alpha_4\beta_1$ and its respective addressin VCAM-1 are, however, associated with the migration of lymphocytes to the mammary gland in mice. In swine, $\alpha_4\beta_1$ -expressing IgA B cells can be detected in the mammary gland during lactation and are higher in number than $\alpha_4\beta_7$ -expressing IgA B cells. Additionally, VCAM-1 is expressed in the mammary gland prior to farrowing and during lactation (Bourges et al., 2008). When mice are treated with anti-VCAM-1 serum, IgA ASC accumulation in the lactating mammary gland is inhibited (Low et al., 2010). These data suggest that cell trafficking in mice may also be dependent on $\alpha_4\beta_1$ /VCAM-1 interaction. It is also important to consider the possible species differences in lymphoid structure between mice and swine that may contribute to dissimilarities reported in the literature (Rothkottter, 2009). The lack of research in swine necessitates further investigation to corroborate the results obtained from mouse studies. The trafficking of immunocytes to the mammary gland can be monitored by analyzing B and T cells and chemotactic factors in mammary gland tissue and secretions. Over the course of gestation, there are dynamic changes in B/T immunocyte compo-

sition and accumulation in the mammary gland. This is paralleled by a change in CCR10 and CCL28 mRNA expression as well. For instance, in the first trimester, IgA B cell and T cell numbers are low and there is little CCR10/CCL28 expression in the mammary gland. In the second trimester, T cells increase in the mammary gland while IgA B cell numbers remain similar to that seen in the first trimester. In the third trimester, T cell accumulation in the mammary gland peaks, while a moderate increase is seen in IgA B cell numbers and CCR10/CCL28 expression. By the time the animal farrows, IgA B cell numbers have reached their peak and CCR10/CCL28 expression continues to increase during lactation (Bourges et al., 2008; Chabaudie et al., 1993; Meurens et al., 2006). This exemplifies how important trafficking of B cells is to the immunology of the mammary gland and subsequent antibody secretion into colostrum and milk. Understanding the kinetics of immunocyte migration to the mammary gland during late gestation is imperative for regulating lactogenic immunity. While it is likely that initiating the gut-mammary-sIgA axis by means of natural infection or oral vaccination in naïve gilts/sows is required for optimal trafficking of pathogen-specific B and T cells, other variables, including the parity, gestational stage, and dose required for initiation remain unknown. We will discuss several of those variables in the following sections.

2.3. Parity

In a recent case study, a 6000 sow farm in the US became positive for PEDV in 2013 and the farm was given feedback exposure (Oral Live Virus Exposure [OLVE]) for the purpose of inducing lactogenic immunity in sows. However, nearly one year after OLVE initiation, PEDV was detected at the same facilities and diagnosed to be the same strain as that in the 2013 breakout. At this time, the entire farm was given a second round of OLVE. Nine weeks after the second OLVE, first parity gilts had higher mortality of their piglets (62%) compared to second (37%), third (24%), fourth (20%), fifth (22%), sixth (17%), seventh (20%), eighth (21%) and ninth (5%) parity gilts (Ackerman, 2016). The decrease in piglet mortality in higher parity sows could have been attributed to higher levels of immune factors in serum and lactogenic secretions. For instance, sow IgG and IgA immunoglobulin levels in serum, colostrum and milk, and piglet IgA immunoglobulin levels in serum are higher in multiparous compared to first parity sows or their piglets, respectively (Cabrera et al., 2012; Klobasa et al., 1986; van de Ligt et al., 2002). Additionally, parity impacts rotavirus vaccine efficacy in ruminants where multiparous cows had higher antibody titers in lactogenic secretions and required fewer doses of vaccine when compared to primiparous cows (Saif and Fernandez, 1996). However, other explanations may exist for the PEDV results, including the age at first PEDV exposure. For instance, the gilts farrowing in 2014 would have first been exposed to PEDV as neonatal piglets. The sows that farrowed in 2014 would have first been exposed to PEDV as older animals. It is possible that older animals were able to generate a larger pool of PEDV-specific memory B cells resulting in a greater anamnestic response during the second OLVE exposure in 2014. Additionally, an increase in the amount of mammary tissue in multiparous sows may coincide with an increase in number of resident memory B cells in the gland. More research is needed to determine if parity itself, age at PEDV exposure or a combination of the two impact lactogenic immunity and subsequent neonatal protection.

2.4. Gestational stage

As mentioned earlier, gestational stage of the animal influences the kinetics of lymphocyte and lymphoblast accumulation in the mammary gland. For instance, at day 80 of gestation in sows, T cell numbers peaked in the mammary gland at 5 cells per unit

(50–0.03 mm² microscopic fields) and decreased to 3 cells unit (30–0.03 mm² microscopic fields) into lactation. However, IgA B cell numbers did not peak until day 8 post-partum at approximately 14 cells per unit (30–0.03 mm² microscopic fields) and remained at approximately 11 cells per unit (30–0.26 mm² microscopic fields) during lactation (Bourges et al., 2008; Chabaudie et al., 1993). Additionally, an increase of mammary gland MAdCAM-1 expression at the end of gestation and during lactation coincides with an increase of $\alpha_{4\beta 7}$ B cells in sows (Bourges et al., 2008). In mice, sIgA lymphoblasts preferentially migrate to the mammary gland from the mesenteric lymph node rather than from the peripheral lymph node toward the end of gestation and during lactation (Roux et al., 1977). These results suggest that gestational stage influences lymphocyte trafficking to the mammary gland. There is a dearth of information on the impact of gestational stage on immunity to enteric viruses like PEDV and TGEV. However, we can draw comparisons from research done on the altered state of the immune response in pregnant women. For instance, there is a decreased ratio of type 1 T helper (Th1) to Th2 CD4T cells as pregnancy progresses in women (Yamaguchi et al., 2009). A change in cytokine profiles also corresponds to a change in immune responses and likely, lymphocyte trafficking. Even more convincing of the changed immunology state during pregnancy, pregnant women in their second trimester have higher natural killer and CD4T cell IFN- γ responses to an H1N1 vaccine when compared to women in their third trimester (Kay et al., 2014). It is conceivable that the differences in gestational stage immune responses are related to the drastic changes in hormone concentrations that pregnant women (and animals) experience.

2.5. Pregnancy-associated and mammogenic hormones

In an early report, administration of estrogen, progesterone, and prolactin in ovariectomized mice enhanced mesenteric lymph node-derived IgA plasmablast recruitment to the mammary gland. This was the first evidence to show that pregnancy-associated/mammogenic hormones influence lymphocyte trafficking from the intestine to the mammary gland (Weisz-Carrington et al., 1978). While this study has not been replicated in swine or other species, associative evidence exists between hormone levels and lymphocyte recruitment to the mammary gland. For instance, the expression of MAdCAM-1 is predominantly at the end of gestation and during lactation and there is an increase of the $\alpha 4$ integrin, CCL28 and VCAM-1 expression during lactation (Bourges et al., 2008). Additionally, in swine, there is a correlation between increased density of prolactin receptors on mammary epithelial cells and the rate of lymphocyte accumulation in the mammary gland (Salmon, 1987). Prolactin has also been reported to upregulate the polymeric immunoglobulin receptor (pIgR) in the mammary glands of rabbits and sheep. It is known that pIgR facilitates the selective migration of sIgA across the mucosal epithelium (Kaetzel, 2005). In swine, pIgR mediates the secretion of IgA and IgM in lactogenic secretions and the free secretory component can be detected in colostrum and milk (Kumura et al., 2000; Le Jan, 1993). Little research has been done in this area of hormone-controlled lymphocyte trafficking since the 1970's and there are many unanswered questions. A pregnant gilt/sow experiences dynamic changes in circulating plasma hormone levels. Unlike humans, whose estrogen and progesterone levels consistently increase throughout pregnancy, until plummeting at parturition, swine progesterone dominates in the first trimester and estrogen and prolactin dominate in the third trimester and into lactation, respectively (Foisnet et al., 2011; Kraeling et al., 1992; Robertson and King, 1974). The dramatic increase in estrogen and prolactin concentrations in the third trimester is followed by a subsequent

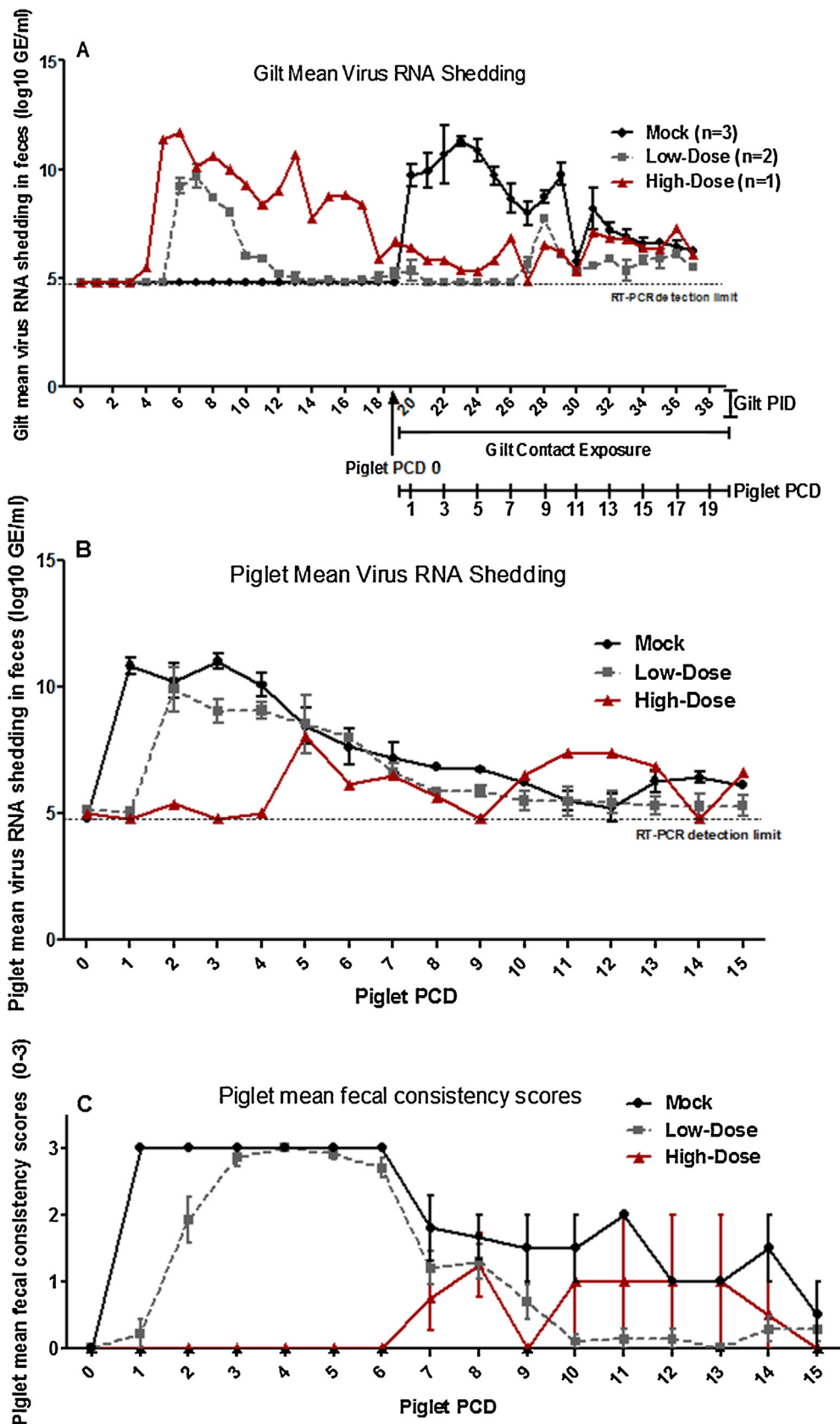


Fig. 2. A–C. Gilt and piglet fecal PEDV RNA shedding post-PEDV exposure and piglet diarrhea scores post-challenge. (A) PEDV RNA fecal titers were highest post-inoculation in the high-dose PEDV-inoculated gilt. PEDV RNA fecal titers were increased in contact exposed gilts after piglet PEDV challenge, but the low-dose PEDV and mock-inoculated gilts had the highest viral titers suggesting immune protection in the previously high-dose PEDV-inoculated gilt. (B) PEDV RNA fecal titers increased in piglets after PEDV challenge, but the piglets born to the low-dose PEDV and mock-inoculated gilts had higher viral titers suggesting lower immune protection.

increase in CCR10 and CCL28 expression and number of IgA cells in the mammary gland (Bourges et al., 2008; Meurens et al., 2006). While evidence of estrogen and prolactin impacting IgA plasmablast homing exists for mice, there is no research demonstrating this directly in swine. Also, it is unclear how these hormones affect the gut-mammary-sIgA axis in swine during an infection. By deducing the mechanism by which these hormones influence homing ligand and receptor expression and the gut-mammary-sIgA axis, we can target when the animal is most immunologically responsive to enteric infections or vaccines during pregnancy.

2.6. Exposure dose

Current studies in our lab have demonstrated that exposure dose of PEDV during gestation may influence lactogenic immune protection in PEDV-challenged neonatal piglets. Six gilts at 96 days of gestation (third trimester) were orally inoculated with an original highly virulent US PEDV PC22A strain at either: (1) $13 \log_{10}$ GE [(genomic equivalents), $5.7 \log_{10}$ plaque forming units (PFU)] ($n=1$, high dose); (2) $10 \log_{10}$ GE ($2.7 \log_{10}$ PFU) ($n=2$, low dose) of gnotobiotic pig-passaged virulent strain (PC22A) PEDV; or (3) mock inoculated ($n=3$, negative control). At 3–5 days of age, piglets were orally challenged with $10 \log_{10}$ GE ($2.7 \log_{10}$ PFU) of PEDV of the homologous strain. Virus shedding in rectal swabs was quantified by real-time quantitative reverse-transcription polymerase chain reaction (RT-qPCR) and diarrhea scores were recorded daily as described previously (Jung et al., 2015; Oka et al., 2014). Virus neutralization antibodies were detected in serum and whey using a plaque reduction assay modified from previously published protocols for TGEV (Bohl et al., 1972; Lanza et al., 1995) and PEDV (Lin et al., 2015). Vero cell culture adapted original virulent PEDV strain PC22A was used (Oka et al., 2014). Plaques were counted and virus neutralization titers were expressed as the reciprocal of the highest dilution of serum or whey showing an 80% reduction in the number of plaques. The high dose PEDV-inoculated gilt shed higher viral RNA titers ($11.7_{\log} 10$ GE/ml) in fecal samples that were detectable one day earlier and persisted longer (post-gilt inoculation) than in those of the low dose PEDV-inoculated gilts (Fig. 2A). Additionally, both high and low dose PEDV-inoculated gilts had lower and delayed peak fecal viral RNA titers post-piglet challenge when compared to mock-inoculated gilts contact exposed to their PEDV-inoculated piglets (Fig. 2A). The litter born to the high dose PEDV-inoculated gilt had delayed and lower PEDV RNA fecal titers throughout post-piglet challenge day (PCD) 8 and mean diarrhea scores <2 (defined as no watery diarrhea) throughout when compared with the low dose PEDV and mock-inoculated litters (Fig. 2B,C) demonstrating greater passive protection against both disease and infection. The low dose and mock gilt litters shed higher PEDV RNA levels in feces earlier and for a prolonged period (mainly in the mock litters). Interestingly, the PEDV high dose – inoculated pregnant gilt, and the lactating mock-inoculated gilts exposed to their PEDV challenged piglets had higher peak PEDV RNA titers in feces ($11.7_{\log} 10$ GE/ml and mean of $11.29_{\log} 10$ GE/ml, respectively) than the mean peak PEDV RNA titers ($11.02_{\log} 10$ GE/ml) of the challenged piglets of the mock-inoculated gilts. This data suggests that not only are PEDV-challenged seronegative piglets (of the mock gilt litters) likely to shed high fecal RNA levels, but also PEDV seronegative pregnant or lactating gilts may shed high titers of PEDV RNA in feces, further contributing to viral contamination and transmission on farms. The high dose PEDV-inoculated gilt

provided greater protection (0% mortality) to her PEDV-challenged piglets compared with the low dose (37% mortality) and mock-inoculated (90% mortality) gilts. This corresponded to increased mean PEDV neutralizing antibody titers in the serum at gilt mean post inoculation days (PID) 17 and 23 and in colostrum and milk through gilt mean post-partum day (PPD) 14 of the high dose gilt (Fig. 3A and B). For instance, in the high dose PEDV-inoculated gilt, PEDV neutralizing antibody titers in serum were detectable at gilt PID 17/piglet PCD –2. However, PEDV neutralizing antibody titers were delayed in serum in the low dose PEDV-inoculated gilts and were not detectable until gilt PID 23/piglet PCD 3. In milk, the high dose PEDV-inoculated gilt had higher PEDV neutralizing antibody titers in colostrum and milk throughout lactation than the low dose and mock-inoculated gilts, except at gilt PID 39/piglet PCD 17. Similarly, as expected for a primary immune response to PEDV, there was a delayed response in mean PEDV neutralizing antibody titers in serum (gilt PID 39/piglet PCD 17) but an earlier response in milk of the mock-inoculated gilts (gilt PID 30/piglet PCD 10). Milk PEDV neutralizing antibody titers in the low dose PEDV-inoculated gilts remained lower throughout lactation than in the high dose PEDV-inoculated gilt until reaching approximately 2-fold higher titers at gilt PID 39/piglet PCD 17. These results suggest that increased viral replication (higher viral RNA shedding in feces) in the high dose PEDV-inoculated gilt resulted in earlier and increased virus neutralizing antibody titers in serum and milk and greater piglet protection. These studies will be repeated in additional gilts or sows to confirm our preliminary findings, as well as to complete additional immunological assays. We hypothesize that the increase in PEDV replication in the gut reflected by viral RNA shedding in feces leads to an increase in IgA plasmablast and memory B cell generation. These gut IgA immunocytes then traffic to the mammary gland to provide passive lactogenic immunity to neonatal suckling piglets.

3. Correlates of lactogenic immunity in gilts/sows

3.1. IgA antibodies

Identifying the correlates of immunity after infection or vaccination is pertinent to assess the susceptibility of individual animals or herds and to understand the relationship between infection/vaccination, the host immune response, clinical signs and subsequent protection. Regarding lactogenic immunity, the role that antibodies play in host defense is best exemplified by the protection provided to neonates by maternal antibodies (Saif, 1999; Zinkernagel, 2001). It is well documented that swine give birth to agammaglobulinic offspring due to an impermeable placenta (Kim et al., 1966). Maternal antibodies provide the necessary lactogenic immunity needed for neonatal piglet immune defense until endogenous antibodies can be produced in sufficient amounts. Correlates of immunity have previously been identified for other enteric viruses like TGEV and rotavirus. As mentioned earlier, an increased rate of protection against TGEV in neonatal piglets was associated with high titers of sIgA antibody in colostrum and milk (Bohl et al., 1972; Bohl and Saif, 1975; Saif et al., 1972). Additionally, our lab was the first to identify IgA ASC trafficking in blood and intestine as an immunological marker of protective active gut immunity in an animal model of human-rotavirus-induced disease. In this study, rotavirus-specific IgA ASC, but not IgG ASC, in blood correlated with IgA ASC in the intestine and subsequent

(C) The litter born to the high-dose PEDV inoculated gilt had delayed and lower PEDV RNA fecal titers throughout post-piglet challenge day (PCD) 8 and mean diarrhea scores <2 (defined as no watery diarrhea) throughout when compared with the low-dose PEDV and mock inoculated litters. After PEDV inoculation, piglets were monitored for clinical signs 2–3 times daily until necropsy. Diarrhea was assessed by scoring fecal consistency as follows: 0 = solid; 1 = pasty; 2 = semi-liquid; 3 = liquid. Scores of 2 or more are considered diarrheic. (A–B) PEDV RNA titers in fecal samples were determined by qRT-PCR. The detection limit of qRT-PCR was $4.8 \log_{10}$ GE/ml. (A–C) Each bar represents the mean \pm SEM. Abbreviations: Post-inoculation day (PID), Piglet Challenge Day (PCD).

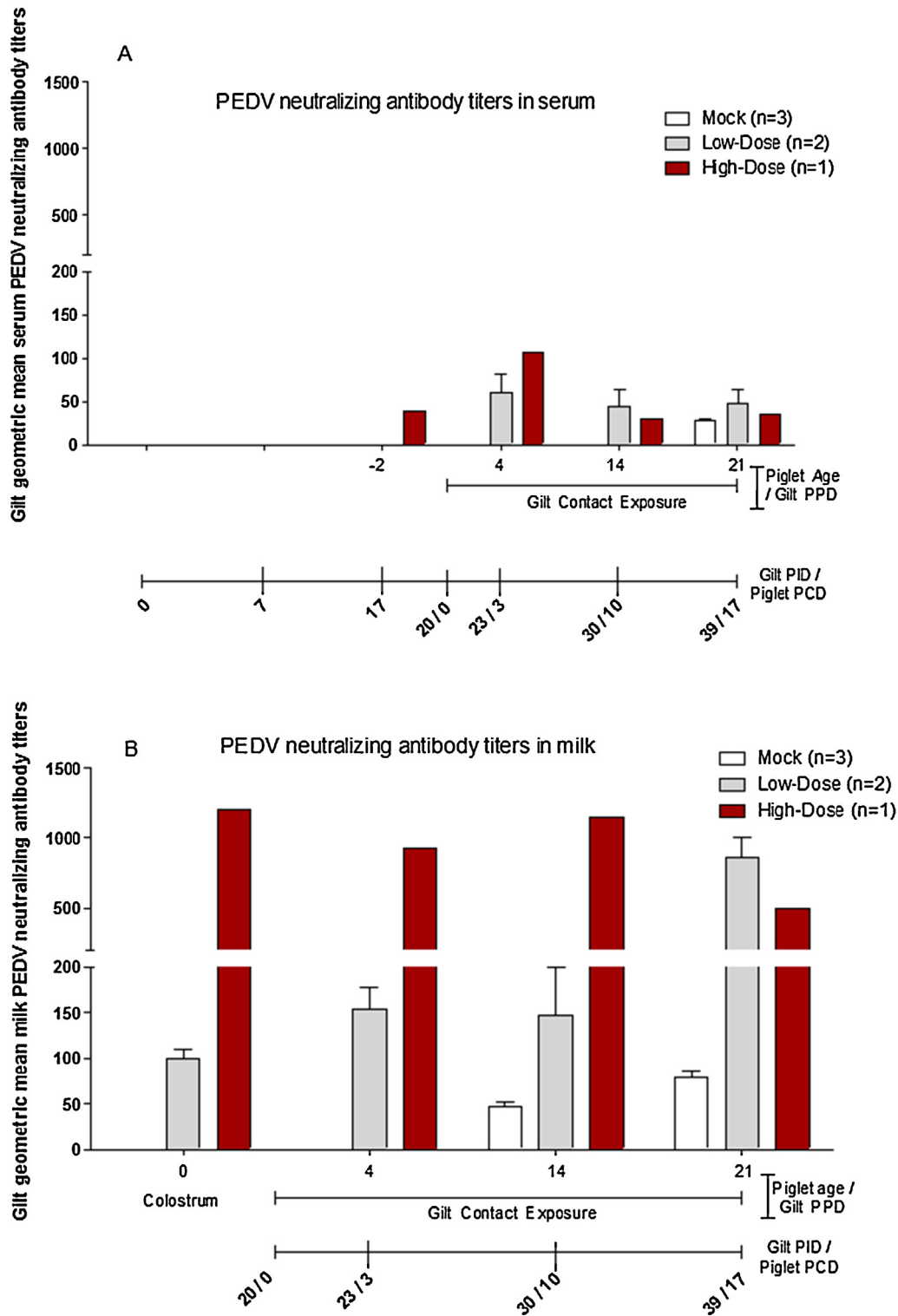


Fig. 3. A–B. Gilt geometric mean serum PEDV neutralization antibody titers.

(A) Serum PEDV neutralization antibody titers were first detected in the high dose PEDV-inoculated gilt at mean gilt post-inoculation day (PID) 17 ± 2 [mean piglet challenge day (PCD) 2 ± 2] while low dose PEDV-inoculated gilt neutralizing antibody titers were delayed, being detectable at gilt mean PID 23 ± 2 (piglet PCD 3 ± 2). PEDV neutralizing antibody titers were not detected in the mock-inoculated gilt until gilt mean PID 39 ± 3 (piglet PCD 17 ± 2). (B) Milk PEDV neutralizing antibody titers were highest in colostrum and milk of the high dose PEDV-inoculated gilt. Serum and milk PEDV neutralizing antibody titers were delayed in the mock-inoculated gilts until mean PID 39 ± 3 (piglet PCD 17 ± 2) and mean PID 30 ± 2 (piglet PCD 10 ± 2), respectively. (Mean gilt post-partum day, PPD). See Fig. 2 for PID and PCD.

protection against disease upon challenge with homologous virus (Yuan et al., 1996). This relationship was also demonstrated in human pediatric patients, where circulating rotavirus-specific IgA ASC were correlated with rotavirus-specific IgA ASC in the small

intestinal lamina propria (Brown et al., 2000). The specific time frame of cellular trafficking from the gut to other mucosal tissues is further highlighted when assessing lymphoproliferative responses of intestinal (mesenteric lymph node and ileal lamina propria),

circulating (blood) and systemic (spleen) mononuclear cells after rotavirus challenge in gnotobiotic pigs. Rotavirus-specific lymphoproliferative responses in blood correlated to rotavirus-specific lymphoproliferative responses in the intestine early after rotavirus inoculation or challenge. Furthermore, the magnitude of lymphoproliferative responses positively correlated with the number of rotavirus-specific ASC, supporting the hypothesis that lymphoproliferation quantifies the function of trafficking T-helper-cells which subsequently help to stimulate antibody production by B cells (Ward et al., 1996a; Yuan et al., 1996). These studies highlight the importance of identifying the window of time when immunocytes and ASC traffic in blood from the gut to the mammary gland post-PEDV challenge in gilts and sows.

This previous work demonstrates that immune factors associated with intestinal mucosal immunity, like sIgA, are important correlates of immunity. In regard to PEDV, in a recent report, researchers determined that following inoculation with PEDV, virus shedding was significantly reduced in nursing piglets that had received passive lactogenic immunity. A linear relationship was detected between fecal viral RNA shedding in the piglets and IgA antibody levels in the milk. Piglets receiving milk with higher IgA antibody titers had significantly lower PEDV RNA shedding in feces, adding further evidence that IgA antibody plays a role in lactogenic immunity and PEDV passive protection in neonatal piglets. Recent work suggests that systemic antibodies also contribute to protection against PEDV (Poonsuk et al., 2016a,b). In this study, neonatal piglets were intraperitoneally administered with varying concentrations of anti-PEDV serum antibodies between 2 and 5 days of age followed by PEDV inoculation 24 h later. Piglets that were positive for circulating anti-PEDV antibody returned to normal body temperature faster and experienced less mortality post-PEDV inoculation compared to piglets negative for PEDV antibody. However, piglet's growth rates, PEDV fecal shedding and humoral immune responses were not improved by intraperitoneal administration of anti-PEDV serum antibodies. While systemic antibodies may play a role in PEDV clearance, it is likely that protection of neonatal piglets is mostly attributed to maternal secretory IgA in milk.

A recent case study evaluating responses to oral immunization of sows in production units by feeding back intestines from euthanized moribund neonates, and a parenteral immunization with a commercial PEDV vaccine, further supports this hypothesis. In the first production unit (unit A), non-PEDV infected gilts and sows received the Harrisvaccines, Inc. first generation PEDV vaccine (iPED) intramuscularly at 6 and 3 weeks pre-farrowing. The iPED vaccine was formulated by alphavirus-based expression vector technology by using a recombinant Venezuelan equine encephalitis virus (VEE) strain TC-83 replicon to express the PEDV spike (S) glycoprotein. The recombinant VEE virus particles with encapsulated PEDV S RNA were harvested from infected Vero cells and the VEE replicon RNA expressing the S gene was recovered for use in the iPED vaccine (Kim et al., 2016). The vaccinated gilts and sows from production unit A generated low or negligible serum PEDV IgG and milk PEDV neutralizing antibody titers. In the second production unit (unit B), gilts and sows were given oral immunization by feedback at 4 months pre-farrowing at the time of an initial PEDV outbreak. These animals had high PEDV neutralizing antibody titers in milk, but no PEDV IgG antibodies were detected in serum. In the third production unit (unit C), the sows were given oral immunization by feedback on 3 successive days after an initial PEDV outbreak on the farm after which PEDV neutralizing and IgG antibodies were detected in milk and serum, respectively. This suggests that successful generation of milk PEDV neutralizing antibody titers is dependent on oral exposure or oral immunization of sows, as seen with TGEV. Additionally, as described for TGEV, serum IgG antibody titers may not be a correlate of immunity for PEDV (Bohl et al., 1972; Saif and Bohl, 1979; Saif et al., 1972). Scherba and col-

leagues also vaccinated gilts and sows from a fourth production unit (unit D) with the parenteral iPED vaccine at 9 and 2 weeks pre-farrowing in addition to oral immunization by feedback at 8 and 5 weeks pre-farrowing during two PEDV outbreaks. Production unit D also experienced a third PEDV outbreak shortly after farrowing. While serum PEDV IgG antibody levels waned over 4 weeks pre-farrowing in the gilts and sows from unit D, these levels did not coincide with the PEDV neutralizing antibody titers in milk, supporting the lack of a relationship between serum IgG antibody and milk virus neutralizing antibody titers to PEDV (Scherba et al., 2016). The authors included a control group of three gilts housed in an isolated research facility that were neither infected nor vaccinated and were sampled at the same time points as those in production units. An omission in this study was the lack of analysis of the levels of PEDV IgA antibodies in serum and milk and lack of reporting on piglet mortality and protection rates in subsequent outbreaks. Because these studies were conducted in production swine facilities, PEDV piglet challenge studies were not possible. It is important to note that because production units B–D experienced PEDV outbreaks in addition to oral immunization by feedback, animals from each production unit would have received varying doses of PEDV depending on production unit management practices and environmental PEDV load. Lastly, while the authors state that production unit A was PEDV naïve, no serological data was presented to confirm that serum was free from PEDV antibodies.

3.2. Neutralizing antibodies, PEDV vaccines and passive protection of piglets

After viral infection, a subset of antibodies generated has the ability to neutralize the viral pathogen. To do this, the antigen binding sites (paratopes) on the variable domain of the antibody must bind to epitopes on antigens that are associated with virus neutralization. Mechanisms of antibody neutralization include inhibition of many parts of the virus's infection cycle, including cellular surface binding, fusion, entry, endocytosis, and/or viral replication within the cell (Klasse and Sattentau, 2002). For many licensed vaccines, neutralizing antibodies have provided a quality correlate of vaccine efficacy (Zinkernagel, 2001). PEDV is an alphacoronavirus with at least seven open reading frames (ORF) encoding four major structural proteins, spike (S), envelope (E), membrane (M), nucleocapsid (N) and the accessory protein-encoding ORF3 gene (Huang et al., 2013). The S protein forms the characteristic protruding spikes that are situated on the outside of the nucleocapsid and consists of two domains, S1 and S2. The S1 domain is associated with binding to host cellular receptors while the S2 mediates viral fusion and entry into the cell (Bosch et al., 2003; Sturman et al., 1985; Wicht et al., 2014). For TGEV, the S1 domain is a potent inducer of virus neutralizing antibodies, which are important for piglet protection. Previous work with the PEDV S1 protein demonstrated that the region designated S1D (aa 636–789) reacted with PEDV antiserum and elicited PEDV neutralizing antibody titers in mice (Sun et al., 2007). In addition, passive immunization of neonatal piglets by oral administration of egg yolk antibodies (IgY) specific for the S1 domain provided protection post-PEDV challenge (Kweon et al., 2000). However, due to the lack of a negative control (PEDV-challenge with non-specific IgY treatment of piglets), it is unknown if non immune IgY could also mediate passive protection. The ability of S1 to induce neutralizing antibodies is conserved across many other coronaviruses, including severe acute respiratory syndrome (SARS)-associated coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV), members of the betacoronavirus genera (Sui et al., 2004; Yu et al., 2015). The ability of S1 to stimulate neutralizing antibodies may make it an attractive booster vaccine after initial PEDV priming in the gut (Park et al., 1998). Interference of sIgA with binding of the

spike ectodomain (receptor-binding subunit, S1, and membrane fusion subunit, S2) of PEDV would inhibit cell entry and subsequent viral infection. Therefore, sufficient amounts of sIgA in the lumen of neonatal piglets are important to prevent infection and PEDV-related deaths.

Sows given the South Korean vaccine strain (attenuated DR13 strain) orally provided greater passive protection to piglets (13% mortality) compared to mothers given the vaccine intramuscularly (60% mortality) (Song et al., 2007). The results of this study are in agreement with previous literature on oral vs intramuscular routes and induction of the gut-mammary-sIgA axis. This coincided with increased serum IgA PEDV antibodies at farrowing in pregnant sows infected via the oral route compared to those in inoculated intramuscularly. However, neutralizing antibody titers did not differ in the serum of the sows given oral or intramuscular vaccines. However, the orally inoculated sows had increased colostrum PEDV neutralizing antibody titers compared to the intramuscularly inoculated sows. The increase in colostrum PEDV neutralizing antibody titers in the oral sows was associated with increased serum IgA PEDV antibody. Also neutralizing antibody titers were increased in piglets' sera at 3 days of age pre-challenge born to sows infected via oral compared with intramuscular routes. Again, these results suggest that colostrum/milk neutralizing antibody titers induced by oral inoculation of sows are a better reflection of piglet protection for the attenuated DR13 PEDV vaccine. Colostrum/milk antibody correlates of protective immunity may be a more consistent diagnostic tool for determining vaccine efficacy than systemic antibody responses.

3.3. Antibody secreting cells

As described earlier, gut derived IgA ASC trafficking transiently in serum can be a correlate of immunity to enteric infections. In conventional piglets given either a virulent or an attenuated PEDV strain, only the piglets given the virulent strain induced specific ASC in the blood, intestine and spleen post-inoculation. After PEDV-challenge, piglets previously inoculated with the virulent strain had increased IgA and IgG ASC in the intestines when compared to the piglets given the attenuated strain. There was a strong positive correlation between protection and the ASC responses detected in the gut lymphoid tissues and blood (de Arriba et al., 2002). In gestating sows that were exposed to PEDV in the field, IgA and IgG PEDV-specific ASC were detected in the intestine of sows at 1 month post-PEDV exposure. The numbers of ASC waned but were still detectable in the sow intestines at 6 months post-PEDV exposure (Ouyang et al., 2015). However, this latter study did not investigate PEDV-specific ASC trafficking in blood. Additionally, because animals were PEDV-infected in the field and maintained at their respective farms, they may have been re-exposed to varying doses of PEDV between the time of initial infection and slaughter. This would confound the conclusions related to duration of immunity, by assuming a single time point exposure in the sows. More research is needed in controlled environments where animals are experimentally infected and piglets can be PEDV challenged so lactogenic protection and neonatal mortality can be measured. In addition, blood analysis of PEDV-specific ASC is needed to determine if blood ASC are a correlate for lactogenic protection as observed for rotavirus and TGEV.

4. Vaccine strategies to prevent epizootic TGEV and PEDV

Maternal vaccination strategies to induce lactogenic immunity against TGEV have been reviewed (Saif, 1999; Saif et al., 2012). They should also be applicable to emerging enteric infections such as PEDV. To summarize prior research, only attenuated oral TGEV

vaccines administered in multiple doses to pregnant sows (usually 2 doses at 5–6 and 2–3 weeks pre-partum) induced sIgA antibodies in milk and variable rates of passive protection. However, milk sIgA antibody levels and piglet protection rates were consistently lower in sows given attenuated oral TGEV than those in sows inoculated with virulent TGEV strains. Reasons for the discrepancies may include lower viral doses in the vaccines and loss of gastrointestinal stability and subsequent viral replication in the gut of the sow after virus attenuation.

The latter is an important but sometimes overlooked variable. In TGEV challenge studies of vaccinated sow litters, if the vaccine failed to induce active immunity in the gut of the sow, she became ill, developed diarrhea or anorexia and ceased milk production. Then invariably mortality rates among the nursing piglets were high (Saif et al., 2012). Thus, active mucosal immunity to protect the gut of the sow is critical for successful TGEV or PEDV maternal vaccines. As mentioned previously, parenteral immunization of seronegative sows with inactivated or subunit TGEV vaccines did not induce mucosal immunity, but elicited mainly systemic IgG antibodies that are dominant in serum and colostrum, that decline rapidly in milk and provide little lactogenic immunity to piglets. The exception was if extremely high levels of IgG antibodies were induced in serum that were then transudated into colostrum and remained elevated in milk to protect neonates transiently through the first week of life.

In regards to whether a killed or live injectable intramuscular PEDV vaccine generates sufficient immunity to PEDV in the sow, Paudel et al. (2014) analyzed sows given one of four vaccine treatments: an unvaccinated negative control group, a killed virus vaccinated group with a killed virus booster (K/K), a live virus vaccinated group with a live virus booster (L/L) and a combination group given the live virus and a killed virus booster (L/K). All vaccinations including using live virus were given intramuscularly twice, at four and two weeks prior to farrowing. The sows were confirmed PEDV negative by antibody ELISA (serum) and RT-PCR using fecal samples and only PEDV-negative sows were used. These investigators demonstrated that the sows given the K/K treatment had higher IgG and IgA antibody titers and neutralizing activity in serum, colostrum and in piglet serum, with the lowest levels in the L/L group. Due to the intramuscular injection route for the live virus vaccine, the virus is unlikely to reach the gut and replicate in intestinal enterocytes in most sows. Therefore, while this study may provide information regarding vaccine immunogenicity, it does not reflect immune responses after live oral PEDV exposure. Additionally, PEDV challenge of piglets is necessary to determine if the K/K vaccine treatment also provides sufficient lactogenic immunity and piglet protection from disease (Paudel et al., 2014).

4.1. Practical aspects of targeted homing of IgA immunocytes from the intestine to the mammary gland related to maternal vaccine design and feedback approaches

There are many unanswered questions related to timing and mechanisms of IgA immunocyte homing from the intestine to the mammary gland and how this relates to vaccine approaches and herd immune status. Few studies have investigated the optimal gestational maternal vaccination times or intervals to generate sufficient lactogenic immunity. Similarly no detailed studies have been done to compare vaccine responses in gilts vs sows. For gilts and sows an important question is when the gut-mammary-sIgA axis is initiated during gestation and if it can be evoked in pre-pubescent gilts exposed to virus. In the first reported experimental sow study with a US PEDV isolate, 4 sows (average parity 5.4) from a herd naturally infected with a 'mild' SINDEL strain of PEDV were used. On the farm the sows also received a live virus exposure to the original field isolate. Then 7 months later they were moved to iso-

lation facilities and experimentally re-exposed to a more 'virulent' PEDV isolate at 109 days of gestation. At about 3 days-of-age, piglets also received an oral challenge with the virulent virus and their survival to 4-days-post challenge (to one week of age) was monitored. In the PEDV positive sow group, all piglets survived challenge compared to the naïve sow group, which experienced a 33% mortality rate (Goede et al., 2015). This study demonstrated that sows in the field could produce lactogenic immunity; however, piglet protection from infection was not complete and was variable among the 4 litters indicating there could be a number of factors influencing the outcome. In a recent report, researchers detected PEDV serum antibody titers for at least five months after field exposure of sows. However, PEDV challenge studies of the suckling piglets from gilts previously exposed to PEDV before sexual maturity or prior to conception are necessary to determine the induction of lactogenic immunity in gilts and sows and its association with piglet passive protection (Schelkopf et al., 2016).

Another important question is how the dose and extent of virus replication in the gilt/sow intestine impacts induction of IgA antibodies in milk and whether this is sufficient for piglet protection to PEDV. As we described previously for TGEV vaccine research, replication of PEDV in the sow's gut, or in the case of attenuated vaccines, the stability and immunogenicity of the PEDV vaccine antigens needed to induce PEDV neutralizing antibody titers should be considered when designing maternal vaccines and feedback approaches.

4.2. Herd PEDV status

Herds can be categorized into three general statuses based on presence of PEDV, antibody and clinical signs. PEDV-negative herds are herds that have never been infected, while PEDV-active herds have ongoing clinical disease with virus detected by RT-qPCR. PEDV-stable herds previously experienced an epidemic, but subsequently no clinical disease is recognized, the herd is negative for PEDV fecal shedding based on RT-qPCR testing and some or all sows are PEDV-antibody positive. At the beginning of the fourth year of PEDV in the US, PEDV-active herds are ones that were likely previously infected earlier in the epidemic. In such cases, the clinical disease is usually not as extensive as when PEDV first entered the naïve herd suggesting some, albeit insufficient, maternal immunity in sows (Goede and Morrison, 2016). Although the incidence of new PEDV cases in the US has declined dramatically since the winter of 2013–2014, there are still occasional cases of PEDV-negative herds becoming infected for the first time and experiencing dramatic losses as first reported in 2013 (https://www.aphis.usda.gov/aphis/ourfocus/animalhealth/animal-disease-information/swine-disease-information/ct_pedv_info).

The different PEDV statuses described earlier may provide insights into possible PEDV control strategies. However, it is difficult to generalize because in the US because most pigs are raised using multi-site production and they are derived from sow farms containing more than 1000 co-housed sows. In addition, some sites may have a complex of large sow barns located close together. In contrast, there are more traditional farrow-to-finish farms located all on one site that contain relatively small numbers of sows. This diversity in swine husbandry contributes to a complex epidemiology for most swine diseases, and this may be especially true for PEDV.

4.3. Feedback methods

Initial attempts to control PEDV involved "feedback" or controlled whole herd exposure which involved exposing the entire sow herd to infectious material (i.e. feces and gut tissues col-

lected from infected piglets) in an attempt to stimulate active PEDV immunity (<https://www.aasv.org/>). The goal was to develop PEDV immunity in the sow, which would be transferred to her piglets through colostrum/milk. This would protect young piglets when they were most vulnerable. In general, this strategy seems to have worked well since the incidence of clinical disease has declined dramatically, but feedback has not been 100% effective (Goede and Morrison, 2016). The apparent failure of feedback practices to consistently reduce or eliminate clinical disease has raised questions about the duration and quality of sow immunity following a controlled infection. In addition, there are questions about the difficulty of trying to expose a large group of sows to virus, the quality of the biologic material used for feedback, and how well the workers actually perform the task.

Modifications to the feedback strategy have involved multiple feedback attempts to expose all sows 2–3 times over a two week period with the goal of making sure that all sows had at least one exposure to infectious virus (<https://www.aasv.org/pedv/Conceptsforherdexposure121713.pdf>). This practice improved conditions on some farms, but there are still field reports of farms that would have a chronic low level of clinical disease, which suggests that some of the breeding herd was not immunized through the feedback program. Or, if the sows were actually immunized (infected with wild-type virus), but their piglets were still clinically affected, (1) then the duration or magnitude of sow immunity was considered inadequate, or (2) the live virus feedback program may be too aggressive in sows near the time of farrowing that may lead to sows shedding an overwhelming dose of challenge virus in the farrowing room. These field observations further highlight a gap in knowledge of what influences lactogenic immunity in females and passive protection to PEDV in suckling piglets. Feedback may fail for similar reasons that attenuated vaccines fail: (1) if the virus dose is too low; (2) if the virus is non-infectious due to inadequate sampling/storage conditions; (3) if swine already have pre-existing immunity that blocks viral replication in the gut; or (4) if gestational stage at time of feedback influences lactogenic immunity as described earlier (Ackerman, 2016).

4.4. Herd size influence on PEDV persistence

As the US epidemic enters its fourth year, field case reports are being published (Goede and Morrison, 2016; Hough, 2016; Thomas, 2016). A lesson from this growing body of work is that the feedback strategy did help many farms reduce losses during the acute phase of the outbreak, and it seems to have prevented future disease (i.e. PEDV-stable herd). However, there are other farms that used the feedback practice but had a difficult time controlling the disease (PEDV-active herds). The negative status in smaller herds may reflect less movement of incoming pigs with potentially fewer contaminated vehicles and clothing on workers. Also, on smaller farms compliance of workers is easier to monitor than on larger farms. Smaller farms also can be more geographically separated than larger systems that are located more closely together.

Currently, in the US there are swine herds that have never been infected with PEDV which may reflect a high level of adherence to very strict biosecurity practices in these herds. PEDV-stable herds demonstrate how a herd once infected can eliminate PEDV from the site, and then be maintained virus free. The PEDV-active herds generally are large herds on a single site for which it has been difficult to eliminate the virus from the farm. This endemic PEDV infection probably reflects a lack of immunity in the sow herd more than the presence of a unique environmentally stable virus that can re-infect sows. Presumably, all of the PEDV isolates are physically similar and should have the same sensitivity to disinfectants, desiccation, etc. Thus, differences between farms are related to farm-specific activities and feedback approaches and not novel virus strains. It

may be that PEDV-stable herds are typically smaller, and thus more likely to be thoroughly disinfected than larger sites. In addition, there is experimental evidence for infectious PEDV to be detected in fecal slurries (<http://www.pork.org/wp-content/uploads/2014/05/goyal-13-215-main.pdf>) and manure storage lagoons (Tun et al., 2016) for extended periods of time which could serve as a potential reservoir of infectious virus to re-infect a farm. Differences in how manure is handled on small and large farms might explain some of the differences between PEDV-stable and active sites. “All-in-all-out” management practices versus continuous farrowing operations that have a continual source of newborn piglets may also influence PEDV transmission and persistence within herds. Despite all of the efforts to control intra and inter-farm PEDV transmission, swine herds are still vulnerable to infection through the movement of contaminated feed. Recent field reports and experiments support the movement of contaminated feed as a likely source of infection in a number of once naïve farms (Bowman et al., 2015; Dee et al., 2014; Dee et al., 2016; Kochhar, 2014) and supports the working hypothesis of the Chinese origin of the PEDV epidemic (Huang et al., 2013).

5. Prime/booster maternal vaccination strategies to enhance piglet protection in seropositive herds (enzootic PEDV and TGEV)

Studies of enteric virus vaccines [TGEV, rotavirus in swine; poliovirus in children (Jafari et al., 2014; John et al. 2014)] suggested that after effective priming of the gut by natural infection or oral attenuated vaccines, parenteral subunit or inactivated booster vaccines could enhance and maintain mucosal or lactogenic immunity. The oral prime/parenteral booster approach may explain why such parenteral vaccines are effective in certain scenarios in sows recovered from TGEV or rotavirus infections or after use of oral attenuated vaccines (Saif, 1999; Saif et al., 2012). For example, a parenteral TGEV spike subunit vaccine (contains the virus neutralization epitopes) alone was not effective to induce milk sIgA antibodies and passive protection to TGE in seronegative pregnant sows (Shoup et al., 1997). However when used as a booster vaccine in orally primed sows, the spike subunit or an inactivated TGEV vaccine enhanced milk sIgA antibody titers and passive protection (Park et al., 1998). Likewise non-replicating rotavirus-like-particle vaccines failed to induce sIgA intestinal antibodies or active protection in piglets, but they were more effective than oral attenuated rotavirus booster vaccines in piglets that had been primed with an oral rotavirus vaccine (Azevedo et al., 2013). Further enhancement of lactogenic immunity and subsequent immune protection in piglets may also be accomplished by boosting orally primed animals with parenteral vaccines as described earlier. These demonstrate that viral infection with stimulation of the intestinal mucosa influences lactogenic immunity via the gut-mammary-sIgA axis. This model system also is applicable to PEDV, as similar maternal vaccination strategies may be needed for gut-mammary-sIgA axis initiation and piglet protection.

In a recent report, investigators compared the effects of intramuscular injection of an inactivated killed PEDV vaccine (Zoetis Inc.) given to pregnant sows at five and two weeks pre-farrowing that were either previously exposed in the field to PEDV or were PEDV naïve. Although negative control sows were categorized as PEDV uninfected by RT-PCR, no serological data was presented to confirm naïve sow status. All piglets were orally challenged at 5 days of age with feces from 1-day-old piglets inoculated with PEDV at birth and diluted to a PCR cycle-threshold (Ct) of 19. This report showed that while PEDV previously exposed sows had significantly increased PEDV IgA antibody levels in serum and milk compared with naïve sows, vaccination with the inactivated killed

vaccine did not provide a booster effect in either group. Piglet mortality was inversely related to the IgA PEDV antibody titers in colostrum and milk: no mortality occurred in litters of previously exposed sows that had detectable IgA antibodies in colostrum and milk. In contrast, all piglets from the naïve sows had higher mortality rates compared to the previously exposed groups regardless of vaccination status. While the inactivated killed vaccine stimulated statistically significant increases in serum PEDV IgG antibody titers, it did not significantly increase serum or milk PEDV IgA antibody titers in previously exposed or naïve sows. This report suggests that the inactivated killed vaccine, under the conditions and times of administration tested, was not an effective primary or booster vaccine for naïve or previously PEDV-exposed sows, respectively, to induce lactogenic immunity (Schwartz et al., 2016).

6. PEDV vaccines: past and present

Due to the marked decrease in PEDV outbreaks in Europe in the 1980's and 1990's, PEDV vaccine development was targeted to Asian countries experiencing increased outbreaks of virulent PEDV strains and where the economic impact of PEDV was greatest. Since 1999, an inactivated bivalent TGEV and PEDV vaccine has been used in China (Ma et al., 1995). From 2003–2006 an additional attenuated PEDV vaccine developed from an attenuated PEDV strain was used in the Chinese pig population. These vaccines, particularly the inactivated bivalent TGEV/PEDV vaccine, have been used extensively to try to control PEDV. However, with the reemergence of a more highly virulent PEDV in China in 2010, there was a demand for more efficacious vaccines to control the current outbreaks. The lack of vaccine-induced piglet protection seen in the 2010 Chinese outbreak may have been due to differences between the highly virulent re-emergent strains and the classical PEDV vaccine strains based on genetic analysis of the *S*, *M*, and *ORF3* genes, but this remains unproven and both classical and the virulent emerging strains induce cross-neutralizing antibodies (Lin et al., 2015; Song et al., 2015). To provide a more efficacious vaccine, a trivalent vaccine, consisting of TGEV, PEDV, and rotavirus was licensed by the Chinese Ministry of Agriculture in December 2014 (Song et al., 2015). It reportedly has demonstrated increased protection rates in swine on experimental farms, but detailed studies of its proposed efficacy or mechanisms of immunity have not been published.

South Korea has used oral vaccination with an attenuated PEDV vaccine since 2004. The South Korean strain 'DR13' was serially passaged in Vero cell cultures until passage 100 where there were nine nucleotide changes over the course of attenuation and sequence variation at the ORF 3 gene determined by restriction fragment length polymorphism analysis (Song and Park, 2012). In Japan, a commercial attenuated virus vaccine of cell culture adapted PEDV (P-5-V) has been administered to sows since 1997. These vaccines were considered efficacious, but it was reported that not all sows given the vaccine developed solid lactogenic immunity (Song et al., 2015). Several issues to be addressed when lactogenic immunity is low include the dominant immunogens in the vaccines and their stability, the dose, and the route of administration, as well as the other parameters discussed earlier.

Since the outbreak in 2013, the US has conditionally licensed two PEDV vaccines. The first vaccine was developed by Harrisvaccines™ and is based on alphavirus-based expression vector technology as described earlier (Vander Veen et al., 2012). The first generation vaccine (iPED) was released in 2013 on a limited use basis while the second generation (iPED+) vaccine was released in January 2014. The iPED+ vaccine incorporates a longer segment of the spike gene than the iPED vaccine. The Harrisvaccines, Inc. PEDV vaccine is labeled for 1 dose intramuscularly to sows or gilts pre-farrowing. Secondly, an inactivated killed vaccine for PEDV has

been developed by Zoetis, Inc (Florham Park, New Jersey, Study Report No. B826R-US-13-258, Zoetis Inc.). This vaccine is labeled for 2 doses given intramuscularly to sows or gilts pre-farrowing. While these two vaccines are now used in the US, there is a lack of published data on their field efficacy or the correlates of immunity. More research is needed to determine the efficacy of these vaccines in experimental trials and in the field and to identify the immune correlates of piglet protection in suckling neonates.

7. Conclusions

Generating sufficient lactogenic immunity is integral for passive lactogenic protection against PEDV in suckling neonates. While much was learned from maternal vaccination efforts against TGEV, the emergence of PRCV that induced cross-protection against TGEV did not allow further delineation of the detailed variables related to the mechanisms the gut-mammary-sIgA axis and lactogenic immunity. Current research suggests that colostrum and milk IgA and PEDV neutralizing antibody titers may be a correlate for protective immunity against PEDV. Additionally, research from our lab suggests that sufficient levels of IgA and PEDV neutralizing antibody titers in lactogenic secretions may be dependent on viral dose and extent of replication in the gut of the gilt. However, much is still unknown on the mechanisms to induce and maintain the gut-mammary-sIgA axis. There are numerous factors that may contribute to the waning immunity seen in the field, particularly even after aggressive feedback procedures. These field observations and lack of lactogenic immunity seen in vaccination trials in Asia further highlight the gap in knowledge of what influences induction of lactogenic immunity. These factors include dose at inoculation, immunogenicity of the viral strain and/or vaccine viral antigens used, the age of the gilt/sow, and the sexual maturity of the animal at the gestational stage at vaccination. It is imperative that we develop vaccine strategies that target pregnant or lactating swine when they are most responsive. Identifying factors that influence lactogenic immunity may lead to improved PEDV and other enteric pathogen vaccine regimens in gestating swine, improving overall herd immunity and industry productivity.

Conflict of interest

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of this paper.

Acknowledgements

We thank Dr. Juliette Hanson, Dennis Hartzler, Sara Tallmadge, Moyasar Abdulhameed, John Blankenship, Kathryn Anderson, Marcia Lee, Juliet Chepngeno and Hui Hu for technical assistance in the animal experiments reported. Salaries and research support were provided by state and federal funds appropriated to the Ohio Agricultural Research and Development Center, The Ohio State University. The research on PEDV dose effects in gilts reported in this paper was supported by a grant from the US Department of Agriculture (USDA) Agricultural Research Service (ARS) National Animal Disease Center (NADC) (Ames, IA) (Project Number: 5030-32000-108-38).

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