Antibody response to Rotavirus A and C in gilts and their piglets after prenatal Natural Planned Exposure

by

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AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Diagnostic Medicine and Pathobiology
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Abstract

Rotaviruses (RVs) are a significant cause of diarrheal disease in piglets globally. From birth, piglets rely on maternal antibodies in colostrum and milk to be passively protected from severe disease and mortality. Due to difficulty in propagating RVA and lack of cell culture systems for RVC, Natural planned exposure (NPE) remains one of the most common method in swine production to boost lactogenic immunity through pre-farrow exposure to live virus. However, the efficacy of NPE protocols in providing lactogenic immunity against RVA and RVC has not been investigated. There is also lack of serological tools to asses genotypic-antibody response to RVs. Most importantly, we do not know the factors driving high prevalence of RVC in neonatal piglets. The objectives of this dissertation are manifold. In chapter 2, a longitudinal study on a commercial farm assessed effect of different doses and timings of natural planned exposure (NPE) on generating passive lactogenic immunity in gilts/sows. We determined antibody response to different G and P-genotypes (G4, G5, P[7] and P[23]) of RVA using in-house optimized indirect ELISAs. We found that 3 doses of pre-farrow NPE administration in gilts results in significantly higher anti-RVA IgG and IgA levels in their colostrum and milk. Piglets born to group 1 gilts/sows had higher IgG titers at day 0 compared to other groups. Only two litters shed RVA prior to weaning ad carried a G11P[34] combination, which was different than the parent strains in the NPE material.

RVC infection are more prevalent in neonatal piglets particularly in neonatal piglets, reasons of which are not completely understood. In the 3rd chapter, we sought to determine antibody response to two most prevalent G and P-genotypes (G6, P[5]) of RVC using in-house optimized indirect ELISAs and compare antibody responses to RVC shedding in pre-weaning piglets. We found that group 1 had higher colostral IgG and IgA titers compared to other groups.

Interestingly, group 1 RVC antibody levels in day 0 piglet serum were either significantly (P[5]) or numerically (G6) higher than other groups. Higher group 1 colostrum and piglet serum levels suggest that 3 doses of NPE in gilts prior to farrowing was able to better stimulate maternal immunity than other treatment groups. However, none of the NPE doses were able to prevent RVC shedding by piglets in the farrowing room.

A major goal of this work was to understand the antibody response to RVA and RVC, and how NPE shapes the genetic makeup of the RV strains in the piglet population. All 4 groups had significantly or numerically lower RVC colostrum antibody titers than RVA, irrespective of G and P-type. Piglet serum RVC IgA titers at day 0 were significantly lower than RVA titers. Low levels of colostrum and piglet serum antibody levels against RVC explains its higher prevalence in the neonatal piglets. The outer capsid proteins VP7 and VP4 of RVs are targets for the humoral immune response and they independently elicit neutralizing and protective immunity. In both Chapters 2 and 3 we observed that RVA and RVC IgG and IgA GMTs against VP4* were manifold higher compared to VP7 titers. To conclude, this work generates important data about humoral immune response to RVA and RVC NPE and lays ground for future research in the field of porcine RV immunology.

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Dedication

This thesis is dedicated to my parents, and wife Anisha who have provided me loving and constructive environment to grow and learn. Sadly, my mother could not see this thesis completed. Somewhere in heaven she is smiling down on me. Words are not enough to express my love for my wife Anisha, who all these years supported me and took care of our kids, while I was working on my dissertation. If I am able to complete my PhD, it's because of you. My PhD would have not been possible without your love and support. To my dear kids, Aaradhya and Mihir, you made me stronger, better and more content than I could have ever imagined.

Chapter 1 - Rotavirus Infection in Swine: Immune Responses, Current Management Practices, and role of Gut Microbiome in Rotavirus Immunity

1.1 Rotavirus genome, classification and host range

Rotaviruses (RVs) are double-stranded RNA viruses belonging to the Rotavirus genus in the Reoviridae family. RV genome is approximately 18,522bp in size and consists of 11 segments of dsRNA encoding six structural proteins (VP1-VP4, VP6 and VP7) and 5 non-structural proteins (NSP 1- NSP5/6) (Estes & Kapikian, 2007). The VP1, VP2, and VP3 proteins form the inner capsid of the virion. The middle capsid layer is made up of the VP6 protein while the outer capsid is composed of the VP7 and VP4 proteins. VP7, a glycoprotein with a molecular weight of 37 kDa, constitutes 30% of the virus protein, and forms the smooth external surface of the outer shell. The minor component of the outer shell, VP4, is present as a series of spikes that project outward from the VP7 shell. VP4 is non-glycosylated, has a molecular weight of 88 kDa, and constitutes 1.5% of the virus protein (R. D. Shaw et al., 1986). Both VP7 and VP4 proteins independently induce neutralizing and protective antibodies (Ludert et al., 2002). The VP4 is proteolytically cleaved into VP5 and VP8. The VP8* forms the spike that is used for host attachment and infectivity (Ramani et al 2016). VP4 has been implicated in several important functions, including cell attachment and penetration, hemagglutination, neutralization, host range, and virulence (Nejmeddine et al., 2000). Rotaviruses are unique since the NSP4 produces an enterotoxin, which contributes to viral pathogenesis (Lorrot & Vasseur, 2007).

Historically, RVs were grouped based on banding patterns of the dsRNA in an electropherogram and cross-neutralization capabilities in plaque reduction assays (Estes & Greenberg, 2013). However, with the improvement in molecular tools, RV species are now classified based on sequencing of the VP6 gene (Matthijnssens et al., 2008, 2012). A binary classification system is used to address vast rotavirus diversity on the basis of sequencing of VP7 (G) and VP4 (P) genes, which are also targets of neutralizing antibodies. The dual (G/P) typing system has been extended to a complete genome classification system based on nucleotide sequencing of all 11 RV segments with nucleotide percent identity cut-off values set for each segment. In this system, VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6 RV genes are designated as Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx (Vlasova et al., 2017). Currently, ten RV species, A through J (RVA-RVJ) have been classified. However, only species A, B, C, E, and H have been reported from swine (Alekseev et al., 2018; Chasey et al., 1986; Homwong et al., 2016; Marthaler et al., 2014). Humans and swine are affected by species A, B, C and H RVs. Birds are affected by rotavirus D, F and G, and species E has been reported exclusively in swine. Rotavirus I and J have been reported from dogs (Mihalov-Kovács et al., 2015) and bats (Bányai et al., 2017), respectively. For RVA G types, there is no difference between a serotype and a genotype; however, for P types, there are more genotypes than P serotypes (Desselberger, 2014).

Rotaviruses are ubiquitous in nature, and mixed infections with multiple RV strains seem more common as pigs grow older, indicating a temporal distribution of RV infectivity over a pig's lifetime. Once infected, piglets may exhibit clinical or subclinical symptoms and eventual recovery in most cases. However, neonatal and suckling piglets, which lack an established adaptive immune system are worst affected. Rotavirus A is the most characterized genogroup among RVs due to its

wide host range, high prevalence and pathogenicity (Alekseev et al., 2018). Host range of RVA includes humans (Giri et al., 2019; Sadiq et al., 2019), cows (Basera et al., 2010), goats (Kaminjolo & Adesiyun, 1994; Legrottaglie et al., 1993), wild animals (Abe et al., 2010), ostriches (Silva et al., 2012), chicken (Pauly et al., 2017; Ter Veen et al., 2017), dogs (Ortega et al., 2017) and horses (Nemoto et al., 2017, 2019). Rotavirus B has been identified in pigs (Alekseev et al., 2018; Marthaler et al., 2012; Medici et al., 2011), cows (Barman et al., 2004; Chang et al., 1997), humans (Alam et al., 2013; Sanekata et al., 2003), goats, lambs (Theil et al., 1985) and rats. RVC has been detected from a variety of sources including pigs (Kattoor et al., 2017; Marthaler et al., 2014; Medici et al., 2011), humans (Bhat et al., 2018; Kumazaki & Usuku, 2014; Tiku et al., 2017), cows (Soma et al., 2013), ferrets (Wise et al., 2009), cats (Otto et al. 2015), and dogs (Marton et al., 2015)

1.2 Rotavirus entry and Replication

RV transmission is through fecal-oral route, and the piglets become infected with RV shed from sows and other piglets. The target sites of RV replication are the mature, non-dividing enterocytes in the small intestine, especially the jejunum and ileum. Enterocytes have enterokinase enzyme which is necessary for activating trypsin, which activates RVs and facilitates viral entry into the cells. The virus particularly affects the middle and the tip of the villi causing destruction and eventually resulting in villous atrophy (Crawford et al., 2017; D. P. Shaw et al., 1989). The extent of villous atrophy induced by RV is lesser compared to other enteric viral pathogens of pigs such as transmissible gastroenteritis (TGEv) or porcine epidemic diarrhea (PEDv). The normal villus-height/crypt-depth ratio of the intestinal villi is approximately 7:1. TGEv reduces this ratio to 1:1, while RV infection slightly changes it to 5:1 (Hooper & Haelterman, 1969). The severity of RV infection depends on the length of the villi and the percentage of enterocytes that are

affected. Eventually, the mature columnar epithelial cells on the villi are replaced by immature cuboidal enterocytes that are unable to produce digestive enzymes and have lost their absorptive capabilities (Dewey et al., 2003).

The VP8* subunit of the VP4 outer capsid protein bind to permissive enterocytes by interacting with sialic-acid (Haselhorst et al., 2009) or histo-blood group antigens (HBGAa) (Hu, Crawford, Czako, et al., 2012) on the cell surface, which is followed by interaction with other cell surface receptors such as integrins and heat shock cognate protein (Hsc70) (Hu, Crawford, Hyser, et al., 2012; Lopez & Arias, 2006). RV–HBGA interactions depend on the P-genotype of the RV, not on the species of origin (Ciarlet et al., 2002). Sialoglycan ganglioside GM3 and GM1 serve as receptors for porcine RV strain OSU and human strains KUN and MO (Martínez et al., 2013; Rolsma et al., 1998). Specific VP4-HBGA interactions probably explains host range restriction among RVs. The virus is internalized into the cells by clathrin-dependent or clathrin-independent and caveolin-independent endocytic pathways (Arias et al., 2015; Crawford et al., 2017). The low calcium ion levels inside the endosome causes removal of the outer layer of virus particle and results in the formation of transcriptionally active double-layered particles (DLPs) into the cytoplasm (Hu, Crawford, Hyser, et al., 2012) (Figure 1). Viral mRNA functions as a template for the production of viral proteins and genome replication. The replicated RNA assembles to form new DLPs in the form of viroplasms, which are specialized structures consisting of viral and cellular proteins. The freshly made DLPs then interact with NSP4, which facilitates entry of DLPs into the endoplasmic reticulum (ER). Moreover, NSP4 is also responsible for increased cytoplasmic calcium levels required for virus replication (Hyser et al., 2010). In the ER, outer capsid proteins VP7 and VP4 proteins are added to the enveloped virus particles which results in

the loss of transient envelop and formation of triple layered particles (TLPs). The TLPs are then release from enterocytes through cell lysis (Figure 1).

1.3 Distribution and genotypic diversity of porcine rotaviruses

RVA has been reported from swine population globally. In the US, RVA detection is most common in pigs between 21 and 55 days old, with neonatal infection occurring slightly less commonly (Homwong et al. 2016). However, RVA is still a major cause of neonatal diarrhea in piglets worldwide (Halaihel et al., 2010; Tuanthap et al., 2019). Prevalence rates of 9.4% (Amimo et al., 2013a), 63.6% (Marthaler et al., 2012), 62% (Marthaler et al., 2014), 67.8% (Janke et al., 1990) and 81.1% (Homwong et al., 2016) are reported from swine populations in the US. There are 27 G genotypes and 35 P genotypes within RVAs (Matthijnssens et al., 2011). Of which, 12 G genotypes (G1-G6, G8-G12, and G26) and 16 P genotypes (P[1],P[5], P[6], P[7], P[8], P[11], P[13], P[14], P[19], P[23], P[26], P[27], P[32] and P[34]) have been reported from swine populations (Collins et al., 2010; Martella et al., 2007; Papp et al., 2013). Papp and co-workers (2013) reported G5 (71.43%) as the most common RVA genotype prevalent in the US followed by G4 (8.19%), G3 (3.57%), G9 (2.31%), G11 (1.89%), G10 (1.26%) and G1 (1.05%). Prevalence of other genotypes (G2, G6, and G10) was less than 1%. Among P genotypes, P[7] was the most common genotype (77.22%) followed by P[6] (12.07%), while other P-types individually constituted less than 1% of the reported RVA genotypes. Another study from the US reported G9P[13] as the most prevalent (60.9%) G and P-type combination followed by G9P[7] (8.7%), G4P[13] (8.7%), G11P[13] (4.3%), and G11P[7] (4.3%) (Amimo et al., 2013a).

Traditionally, RVA was considered the most prevalent and pathogenic in swine, but RVC has been emerging as a significant cause of enteritis in neonatal piglets (Theuns et al., 2016). Porcine RVC was first identified in 1980 and considered as an enteric pathogen with a moderate

prevalence rate of between 4 and 31% (Saif et al., 1980). In the US, RVC is a major cause of diarrhea in neonatal pigs, particularly in piglets younger than 3 days old (Marthaler et al., 2013). In the US, 51.1% porcine intestinal samples collected during 2009-2011 were positive for RVC (Homwong et al., 2016). Amimo et al., 2013b reported an overall RVC prevalence of 19.5% in diarrheic and non-diarrheic piglets collected from swine farms located in Ohio, US. The prevalence of RVC was 23.5% among nursing piglets compared to only 8.5% in weaned piglets. Another study from the US detected RVC in 46% samples of porcine origin (feces, fecal swabs, intestinal or lung tissues) collected during 2009-2011 in the US and Canada. Of these, 16% RVC was detected in very young pigs (<3 days old) and 21% in young pigs (4–20 days old). Interestingly, 34% of RVC positive samples were negative for RVA/RVB, and the highest percentage of single RVC infections was in very young (78%, <3 days) and young pigs (65%, 4–22 days) piglets compared to 6-39% in older age groups (Marthaler et al., 2013). Interestingly, single infections of RVC are more common in 0-3 days old piglets, with co-infection with other RV species being more prevalent post-weaning (Homwong et al., 2016; Marthaler et al., 2014)

In swine, 15 G genotypes (G12, G13, G8, G6, G5, G14, G9, G1, G17, G15, G7, G10, G3, G18, G16), and 16 P genotypes (P[1], P[5]-P[9], and P[12]-P[21]) of RVC have been identified (Niira et al., 2016; Suzuki & Inoue, 2018). The G6 genotype (70%) was the dominant RVC genotype followed by G5 (17%), G1 (12%), and G9 (1%). Chepngeno et al., 2019 reported a higher fecal prevalence (76.1%) of RVC from healthy and diarrheic piglets in the US. A recent study reported presence of RVC in piglets less than 1-week-old in Australian swine herds (Roczo-Farkas et al., 2021). Importantly, single RVC genotypes (either G5 or G6) were detected from neonatal piglets, however, older piglets (5-11 weeks) harbored multiple genotypes of RVC (G1 and G3). It is evident that RVC infections are more prevalent among neonatal piglets than weaned piglets, but

the reason(s) are not completely understood. Likely reasons include lack of RVC vaccine for use in swine, insufficient maternal RVC antibodies in colostrum or low minimum infectious dose of RVC required for infecting piglets compared to other swine enteric viruses (Chepngeno et al., 2019).

Unlike RVA and RVC, rotavirus B is more prevalent in older pigs and generally not considered an immediate cause of piglet mortality. Few studies have reported RVB prevalence from the United States (Homwong et al., 2016; Marthaler et al., 2012). A total of 31.8% diarrhea samples from pigs of North American origin were found positive for RVB (Homwong et al., 2016). Marthaler et al., 2012 reported 46.8% prevalence of RVB in pigs of all ages. Most of the RVB positive intestinal samples (70/81) in this study also tested positive for RVA and RVC. The highest prevalence (72.7%) of RVB positive samples was observed in pigs more than 55 days of age compared to only 12.9% RVB positive samples below 21 days of age. Kuga and colleagues (Kuga et al. 2009) reported 25.9% prevalence of RVB in pigs. Age distribution revealed 71.9% RVB positivity in diarrheal fecal samples from weaned pigs compared to 18.7% in diarrheal feces from suckling piglets. RVA and RVC were detected in 36.4% and 21.2% fecal samples, respectively. Despite having high detection rates in swine, pathogenesis of RVB has been scarcely researched (Alekseev et al., 2018; Theil et al., 1985). A recent study successfully reproduced clinical illness in 10-days old piglet experimentally inoculated with fecal suspension collected from RVB positive diarrheic piglets (Alekseev et al., 2018). The fecal samples were negative for common swine viral pathogens including RVA, and the presence of RVB was confirmed by next generation sequencing (NGS). Inoculated piglet developed diarrhea within 12 h of inoculation, and NGS of intestinal homogenate identified RVB. Of the 26 G genotypes and 5 P RVB genotypes known in all host

species, 21 G genotypes (G4 and G6-G26) and 2 P genotypes (P[4]-P[5]) have been identified in pigs (Shepherd et al., 2017).

There is only a single report of Rotavirus E in swine identified in 1980s, and the sample is no longer available (Chasey et al., 1986). There have been no reports of RVE since then to accurately analyze its host specificities and epidemiology. Rotavirus H was recently proposed and included three human strains (ADRV-N, J19 from China and B219 from Bangladesh) and a porcine RVH strain SKA-1 isolated from a pig with diarrhea in Japan (Alam et al., 2007; S. Jiang et al., 2008; Nagashima et al., 2008; Wakuda et al., 2011; H. Yang et al., 2004). In 2012, three more porcine RVH strains BR63, BR60, and BR59 were reported from Brazil (Molinari et al., 2014). In the same year, 15% of porcine intestinal samples comprising different age groups were positive for RVH in the US (Marthaler et al., 2014). Of the RVH positive samples, 18% were detected in 21-55 days old pigs, however, no RVH was detected in 1–3-day-old piglets. Phylogenetic analysis revealed that the RVH had been circulating in US swine herds at least since 2002 and had remained underdiagnosed. Coinfections with other rotavirus species are very common and should be investigated prior to conclusively diagnosing RVH as the causative agent of disease. Recently, 12 porcine RVH strains from Japan were sequenced and genotype constellations allotted (Suzuki & Inoue, 2018). Phylogenetic analysis classified porcine RVHs into multiple genotypes. A total of 10G, 6P, 6I, 3R, 4C, 7M, 6A, 2N, 4T, 6E and 3H representing VP7, VP4, VP6, VP1, VP2, VP3, NSP1, NSP2, NSP3 genes were identified. Most common G and P genotypes were G5 and P1, respectively.

1.4 Immune responses to rotavirus infection

The innate and adaptive immune response plays a key role in containing RV infection in infected hosts (Hakim et al., 2018). T-lymphocytes, mediating cellular immunity, along with B

lymphocytes, mediating humoral immunity, provide the adaptive immune response, which works in close association with the innate immune system.

1.4.1 Innate immune response

1.4.1.1 Role of RIG1 like receptors

The innate immune response is the combination of the host's non-specific defense mechanisms critical for early pathogen recognition and inhibition (Brisse & Ly, 2019). Different effectors of the innate immune response include macrophages, dendritic cells (DCs), natural killer cells (NKs), chemokines and various cytokines such as interleukins, and interferons (Marshall et al., 2018; Turvey & Broide, 2010). The initiation of the immune response against an invading microorganism like virus require that the host senses the organism and its constituents. The initial response is carried out primarily by pattern recognition receptors (PRRs), which are expressed by intestinal epithelial cells and recognize the conserved molecular footprint of pathogens called pathogen- or damage-associated molecular patterns (PAMPs and DAMPs) (Amarante-Mendes et al., 2018; Mogensen, 2009).

The presence of viral sensing PRRs in multiple cellular compartments allows innate cells to recognize and quickly respond to a broad range of viruses. RV dsRNA trigger cytoplasmic PRRs such as RIG-I-like receptors RIG-I (Retinoic acid-inducible gene I), MDA5 (Melanoma Differentiation-Associated protein 5), LGP2 (laboratory of genetics and physiology 2) and endosomal membrane associated PRRs like toll-like receptor (TLR) 3 expressed within intestinal epithelial cells (IECs) and DCs (Broquet et al., 2011; Sen et al., 2011). TLR2, TLR5 and TLR7 have also been implicated in the innate immune signaling of RVs (Wen et al., 2009; Zhang et al., 2014). Both RIG-I and MDA5 recognize different sections of the same viral genome due to their preferential binding to RNA, which illustrates their ability to work independently and

synergistically (Brisse & Ly, 2019; Sanchez David et al., 2016). This is particularly true in viral infections such as RV, in which both of these receptors are required to induce the necessary levels of IFN-β signaling to control infection (Brisse & Ly, 2019; Broquet et al., 2011).

Upon binding to dsRNA, the activated RIG-I and MDA5 interact with the mitochondrial antiviral signaling proteins (MAVS) and forms a multilayered protein complex containing several different proteins (Brisse & Ly, 2019; P. Tan et al., 2017). Infection of porcine intestinal epithelial cells (IECs) by RVs trigger TLR3, RIG-I and MDA-5 which then activates IRF3 and nuclear factor-κB (NF-κB), and also induce expression of IFN stimulated genes (Ishizuka et al., 2016). This early antiviral phase is characterized by the transcription of antiviral genes including ISG15, ISG54, ISG56, and IRF7. The induced IFNs and ISGs then function to restrict RV replication and virus-induced cell injury. Interferons secreted from infected cells amplifies the antiviral response and assist in the transcription of a wide range of antiviral genes (Ciarlet et al., 2002). A study using siRNA silencing in human intestinal epithelial cell lines (IECs) suggested that RIG-I and MDA-5 are more important for virus recognition and signaling for IFN production compared to TLR3 and dsRNA-activated protein kinase (PKR) 0/0/00 0:00:00 AM. Silencing RIG-I or MDA5, or the mitochondrial antiviral signaling protein (MAVS), significantly decreased IFN-β production and increased RV titers in infected IECs. RV-infected mice lacking TLR3 or PKR did not change the levels of IFN-β and amount of RV in intestinal epithelium and feces. A study in suckling mice showed that both type I and type III IFNs are required to protect the gastrointestinal tract against the heterologous simian RV infection. Moreover, both IFN types were demonstrated to independently contribute to innate antiviral defenses within the intestinal mucosa and cooperate to restrict extra-intestinal RV replication in other tissues (Lin et al., 2016).

Another member of the of RIG like receptors family, LGP2, appears to support RV replication, unlike RIG-I and MDA5 (Broquet et al., 2011). LGP2 receptors lack CARD domains found in RIG-I and MDA5 and hence cannot utilize MAVS signaling pathway (Li et al., 2021; Reikine et al., 2014). LGP2 has a dual role of a negative and positive regulator of RIG-I/MDA5 signaling. It negatively regulates RIG-I/MDA5 signaling by competing with these receptors for binding with RV RNA. Overexpression of LGP2 has been linked with decreased IFN-β production, decreases ISRE activation and increased RV titers in RV infected intestinal epithelial cells (IECs) (Broquet et al., 2011).

1.4.1.2 Role of Toll-like receptors

Among Toll-like receptors, TLR3 is the most extensively researched receptor in RV infection. All TLRs, except TLR10, have been detected in primary intestinal epithelial cells (IECs), however, their role in protection against RV infection is controversial (Otte et al., 2004). RV dsRNA and its synthetic analog polyinosinic-polycytidylic acid poly(I:C), induce severe mucosal damage via TLR3-dependent manner (Zhou et al., 2007). RV dsRNA, upon interacting with TLR3 within intestinal epithelial cells (IECs), stimulates the secretion of IL-15 which further increases the production of CD3+/NK1.1+ intestinal intraepithelial lymphocytes (IELs), a cell-type vital in maintaining the integrity of mucosal immune responses (Konkel et al., 2011). The enhanced cytotoxicity of IELs results in disrupted epithelial homeostasis and acute RV gastroenteritis indicating that that TLR3 pathways have a role in rotaviral pathogenesis (Zhou et al., 2007).

Another study showed that RV dsRNA induces severe apoptosis and regression of wound repair in intestinal epithelial cells (IECs) through a TLR3 dependent manner (Sato et al., 2006). The induction of apoptosis and reduction in wound repair in IECs were suppressed by the anti-TLR3 antibody. The ability of TLR3 to recognize RV and the outcome of such infection, has been

linked with the age dependent expression of TLR3 in the intestine (Pott et al., 2012). TLR3 expression was reported to be very low in the epithelium of the suckling mice but strongly increased during the post-natal period. Increased postnatal TLR3 expression positively correlated with decreased RV susceptibility, viral shedding and histological damage. The age dependent TLR3 upregulation was also found in human small intestinal biopsies (Pott et al., 2012). Differences in TLR3 expression perhaps explains the high severity of RV infection in infants/young children (low TL3 expression) and better protection in adults (high TLR3 expression).

The role of other TLRs in RV clearance has also been explored. A study found that the absence of MyD88 signaling protein results in higher RV infectivity indicated by high RV shedding in feces, intestinal lysates and high levels of virus in blood (Uchiyama et al., 2015). Loss of MyD88 also affected the humoral immune response evidenced by low RV-specific IgA and RV-specific IgG2c/IgG1 ratios. Since, MyD88, one of the most vital molecules of innate immunity, mediates signaling for all TLRs, except TLR3, it is apparent that TLRs other than TLR3 also play a pivotal role in development of both innate and adaptive immune responses to RVs. Bacterial flagellin has been reported to prevent and cure RV infection in mice via a TLR5 and NOD-like receptor C4 (NLRC4) based mechanism (Zhang et al., 2014). Both TLR5 and NLRC4 are receptors for bacterial flagellin. Flagellin-induced activation of TLR5 and NLRC4 resulted in the production of the IL-22 and IL-18, respectively. Interestingly, administration of IL-22 and IL-18 to mice fully recapitulated the capacity of flagellin to prevent or eliminate RV infection. Absence of both TLR5 and NLRC4 or MyD88, which is required for signaling by TLR5 and inflammasome associated cytokines, eliminated flagellin's protection against RV infection (Zhang et al., 2014).

Recently, a novel inflammasome sensor NLRP9b was recognized to have a role in RV dsRNA sensing (Zhu et al., 2017). Targeted deletion of NLRP9b, a NOD-like receptor in IECs of suckling mice resulted in increased diarrhea and RV shedding in feces compared to wild-type mice illustrating a vital role of NLRP9b in RV infection. Intestinal organoids lacking NLRP9b also illustrated defective pyroptosis and decreased IL-18 production (Zhu et al., 2017).

1.4.1.3 Other mediators of innate immune response

Other innate immune cells involved in controlling RV infection include macrophages and DCs. DCs are considered the link between innate and the adaptive immune responses (Iwasaki, 2007). DCs are the most efficient antigen presenting cells and play a vital role in the initiation of innate immune response against viral infections (Lopez-Guerrero et al., 2010). RV present in the intestinal lumen are transported to the Peyer's patches (PP) by M cells (Fasciano & Mecsas, 2020). Viral antigen is then captured by DCs which results in upregulation of CD40, CD80, and CD86 surface activation markers (Lopez-Guerrero et al., 2010). During RV infection, DCs not only trap virus particles abut also effectively presents viral antigens to T-cells. A study analyzed the initial response of DCs present in Peyers patches to the RV infection in a mouse model (Lopez-Guerrero et al., 2010). A two-fold increase in the absolute numbers of DCs and the upregulation of surface activation markers CD40, CD80, and CD86 was observed in the infected mice compared to the mice inoculated with UV-inactivated RV.

In vitro studies have shown that macrophages use mitochondrial antiviral-signaling proteins (MAVS) to produce IFN-β and IL-6 in response to RV infection (Di Fiore et al., 2015). Knocking out upstream signaling by MDA-5 and RIG-I showed that only RIG-I seems to be important for anti-RV signaling in macrophages. In intestinal lymphoid tissues, DCs seemed to be responsible for the higher levels of observed IFN-α production. Increasing the dosage of RV

inoculum in these gnotobiotic pigs did not change the levels of IFN-α produced, suggesting that host cells are able to inhibit IFN production above a certain concentration to limit the amount of intestinal damage caused by inflammation.

Tumor necrosis factor alpha (TNF-α) is a multifunctional cytokine that has a potent antiviral role against (Matikainen et al., 2006; Seo & Webster, 2002), hepatitis C (W. Wang et al., 2016), African swine fever virus (Gómez del Moral et al., 1999) and RV (Hakim et al., 2018). Anti-RV effects of TNF-α are independent of interferon production and JAK-STAT signaling pathways (Hakim et al., 2018). Instead, TNF-α was reported to signal through NF-κB (classical NF-κB pathway) to inhibit RV infection (Hakim et al., 2018). Use of TNF-α inhibitors such as infliximab, which bind specifically to TNF-α and blocks its interaction with TNF receptors, completely blocked the inhibitory effects of TNF-α. Significant increase in levels of TNF-α has been reported in RV infected children with fever and more episodes of diarrhea than those without fever and with fewer episodes of diarrhea. Although, the mechanism behind the increase in levels of TNF-α is not completely understood, the authors posited that TNF-α induces increased levels of chloride ion secretion in intestinal epithelial cells (B. Jiang et al., 2003).

1.4.2 Adaptive immune response

Although the innate immune response against RVs is important, adaptive immune responses ensure efficient viral clearance and protection from re-infection. Several studies using mice and gnotobiotic piglets deficient in different arms of the immune system have been crucial to understanding the role of innate and adaptive immunity in clearance and resistance development to RV infections. Mice without T or B cells develop chronic infections of RV, and the lack of B cells greatly affects their ability to develop resistance in the face of repeated exposure (Franco & Greenberg, 1995). However, mice without B cells are still able to eventually clear RV infection,

although infection occurs earlier after exposure and lasts several days longer (Franco & Greenberg, 1995; McNeal et al., 1995). Not surprisingly, this illustrates a multi-level and coordinated approach of all arms of the immune system to clear RV infection. In the absence of humoral immunity, cytotoxic T lymphocytes can clear infection, but these populations of T cells are usually short-lived and cannot confer long-term immunity (McNeal et al., 1995).

During RV infection, antibodies are produced against VP7, VP4, VP6, NSP3, and NSP4 (Caddy et al., 2020; Chang et al., 2001; Lappalainen et al., 2014; Nair et al., 2017; Qiao et al., 2009; Vizzi et al., 2005). However, the strength and type of immune responses against each of these proteins vary, and only VP7 and VP4 stimulate neutralizing antibody responses. Although, the number of intestinal IgA-specific antibody secreting cells (ASCs) have been considered a strong indicator of protective immunity, routine quantification of ASCs is not feasible and hence serum IgA titers are considered a fairly good indicator of mucosal IgA levels (Azevedo et al., 2004; Chang et al., 2001; Velázquez et al., 2000). Studies in mice (Blutt et al., 2002; Franco & Greenberg, 1995) and gnotobiotic piglets (Twitchell et al., 2016; Wen et al., 2016) have looked into the relative roles of B and T cells in active immunity against RVs. Wen and coworkers established a gnotobiotic pig model to study human rotavirus (HRV) infection. Specifically, B cell-deficient, CD8 T cell-depleted gnotobiotic pigs and wild-type pigs were vaccinated with an attenuated HRV vaccine and challenged with virulent HRV. Significantly longer duration of virus shedding was observed in vaccinated B cell-deficient than in WT pigs, highlighting the importance of B cells in vaccine-induced protective immunity. Vaccinated B cell- and CD8 T cell deficient pigs shed significantly higher number of infectious virus than WT pigs and CD8 T cell sufficient/B cell deficient pigs, indicating the importance of CD8 T cells in controlling virus replication. Therefore, both B cells and CD8 T cells play an important role in the protection against RV

infection. However, upon challenge it appears that CD4 T cells are able to compensate for this depletion and confer protection similar to CD8-sufficient animals. Unsurprisingly, long-term immunity is not present in these animals, showing the importance of B cell responses in developing immune memory.

There is no cross-protection between different RV species. Cross-protection against multiple genotypes of the same RV species (heterotypic protection) is an important component of the protective immune response against RVs in humans (Angel et al., 2012). Human studies suggest that immunization with a single strain of RV provides substantial protection from severe infection caused by other RV strains (De Vos et al., 2009; Ruiz-Palacios et al., 2006). Monovalent human RV vaccine containing G1P[8] induces significant protection against severe RV disease caused by multiple G and P types not included in the vaccine, which confirms at least some level of heterotypic protection from other G and P types. Although, the mechanisms and the antigenic determinants underlying the heterotypic protection are not well understood, the presence of antibodies against non-neutralizing cross-reactive VP7 and VP4 epitopes, or VP6 have been suggested (Clarke & Desselberger, 2015). First natural or vaccine induced RV infection results in mainly homotypic immunity mediated by antibodies against VP7 and VP4, whereas, previously exposed or adult animals produce homotypic as well as antibodies to a wide range of heterotypic RVs (Green et al., 1990). There are no enough studies to confirm heterotypic protection against RVs in swine. However, few recent studies provide mixed evidence of heterotypic protection against RV genotypes in swine (Hoshino et al., 2004; Shao et al., 2016). In a recent study, a porcine RVA G9P[13] genotype provided complete short-term cross protection in pigs against porcine G9P[13] or human Wa G1P[8] induced infection and diarrhea. However, the levels of cross-neutralizing antibody titers in hyperimmune serum against human Wa G1P[8], porcine OSU

G5P[7], and porcine Gottfried G4P[6]) were low, suggesting that heterologous protection against human Wa G1P[8] was not dependent on the heterotypic serum virus neutralization titers, and other factors such as upregulated innate, mucosal or cellular immune response might be responsible for heterotypic protection. It is important to note that piglets were not challenged with porcine OSU G5P[7] and Gottfried G4P[6]) strains to asses heterotypic protection against these porcine genotypes (Shao et al., 2016).

Another study reported that antiserum to porcine RVA A2 strain with a G9P[9] genotype (previously identified as a G4P[7] strain) significantly neutralizes different human G9 strains including 116E, R44, R143, US1205, INL1 and BD524 originating from different countries (Hoshino et al., 2004). Also, antiserum generated against each of these human G9 strains neutralized porcine A2 strain significantly. It was also reported that VP7 of the porcine A2 strain is similar to that of phylogenetic lineage 3 of human RVA G9 strains and also share amino acid substitutions with lineage 3 human G9 strains. Similarity in VP7 sequences among porcine and human G9 strains possibly explains the heterotypic immunity observed.

A study reported that infection-induced heterotypic immunoglobulins (Igs) are primarily directed to VP5*, the stalk region of the RV attachment protein. Although, heterotypic protective Igs against VP7, and VP8* (the cell-binding region of VP4), are also generated after infection; however, homotypic anti-VP7 and non-neutralizing VP8* responses occur more frequently (Nair et al., 2017). These results specifically outline the importance of the VP5* region in mediating broad-based protection against serotypically distinct RV strains. Interestingly, the authors found that all VP8* specific monoclonal antibodies were inactive in traditional neutralization assay using MA104 cells and did not prevent RV associated diarrhea in mice, which was unusual. In a recent publication from the same group, the authors reexamined the ability of monoclonal antibodies

(n=32) to neutralize RVs in human intestinal epithelial cells, including ileal enteroids and HT-29 cells (Feng et al., 2019). Most (18 of 20) of the "non-neutralizing" VP8* mAbs efficiently neutralized human RV in HT-29 cells or enteroids. Serum RV neutralization titers in adults and infants were significantly higher in HT-29 than MA104 cells. VP8* monoclonal antibodies also protected suckling mice from diarrhea in an in vivo challenge model. Authors concluded that since MA104 cells are the most commonly used cell line to detect anti-RV neutralization activity, previous studies might have underestimated the contribution of VP8* antibodies to the neutralization titer (Feng et al., 2019).

One promising avenue of understanding RV immunity involves using VP6 as a vaccine candidate. Anti-VP6 IgA antibody was delivered in a mouse "backpack tumor model," which resulted in prevention from RV infection (Burns et al., 1996). This same effect was not seen when the IgA antibodies were injected directly into the lumen, suggesting that the main mechanism of protection involves the transcytosis of anti-VP6 IgA. A full, triple-layered RV particle is not transcriptionally active due to a conformational change during VP7 and VP6 interaction which decreases the activity of the VP1 polymerase. Feng et al., 2019 used this knowledge to discover that anti-VP6 monoclonal antibodies would interact with VP6 in a similar manner to the VP7 protein to stop viral transcription and replication. The anti-VP6 monoclonal antibody 7D9 was able to neutralize RV and lower the amount of viral shedding in mice. The immune system response in a mouse model after immunization with VP6 required the presence of αβ CD4 T cells, rather than γδ T cells or B cells (McNeal et al., 2002).

1.5 Maternal immunity in sows and protection of piglets

Since there is no in-utero transfer of Igs in swine due to their epitheliochorial placenta, the newborn piglet survival depends critically on the intake of maternal derived antibodies in

colostrum and milk (Langel et al., 2020). Passive immune protection occurs in the form of high IgG antibodies in colostrum and high secretory IgA (sIgA) antibodies in colostrum and milk. In particular, sIgA antibodies play a major role in preventing RV infection at the gut mucosal level (Langel et al. 2020). There are several approaches to stimulate maternal immunity. A commercial modified live RVA vaccine (ProSystem Rota, Merck Animal Health) is available: it contains G4, G5, G9, P[6], and P[7] genotypes. The RVA strains in the ProSystem Rota vaccine do not closely match the RVA circulating in the swine population, which possibly explains the high RVA prevalence in weaned/nursery piglets, despite the use of vaccine. Another vaccine known as ProSystem RCE contains the same G5 and G9 serotypes of RVA along with C. perfringens type C toxoid and four major Escherichia coli pilus antigens - K88, K99, F41 and 987P. This vaccine is intramuscularly administered to pregnant gilts or sows at 5 and 2 weeks before farrowing to speed up the development of high persisting levels of RV antibodies in milk. However, diversity of RVA strains other than the vaccine strains co-circulating in swine farms may assist RVAs to escape immunity from the vaccine. Despite being the most common cause of rotaviral diahhrea in piglets less than 1 weeks of age, no vaccine is available for RVC due to inability of RVC to adapt to cell culture. However, recently a vectored vaccine platform known as Sequivity has been introduced by Merck animal health for use in pre-farrow gilts/sows against RVs. Sequivity is an RNA particle (RP) vaccine based on farm-specific VP7 sequences of RVA and RVC. Early trials of the Sequivity RP vaccine for RVs showed that RP vaccines yielded lower mortality and higher weight gain than NPE (Boyd et al., 2022), but more research is required to understand its effectiveness in providing protection against RVC in swine.

Passive, antibody-based immunity from sows is essential to protect piglets from RV infections, since piglets are born agammaglobulinemic (Hammerberg et al., 1989). The best

current approach to protect piglets from RV infection is to immunize sows before farrowing to boost their antibody levels, which can be passively transferred to the piglets through colostrum and milk. Since piglets get infected with RV at birth, it is impossible to expose the piglets to the RV field strains. Hence, boosting the lactogenic immunity appears to be the most efficient way of providing RV immunity to the piglets until the piglets reach an age at which they are less susceptible to rotaviral infections. IgG and IgA produced in the sow traffics to the mammary glands and is transferred through colostrum and milk to piglets, where RVs are locally neutralized in the gut (Bianchi et al., 1999; Chepngeno et al., 2019; Hodgins et al., 1999). Lactogenic IgA is effective within the intestinal tract of the neonatal pig because it has high affinity and is resistant to proteolysis (Song et al., 2015). IgA is the longest-lasting immunoglobulin present in lactating sows, but antibodies are typically strongly protective for only two weeks after farrowing (Fu et al., 1990). Levels of IgG and IgM in piglets also wane over time, following the trend in the sow, until active immunity in the piglets is induced and levels of anti-RV neutralizing antibodies increase (Tzipori et al., 1980). Early weaning and lack of colostrum leads to severe RV diarrhea in piglets, demonstrating the importance of maternal antibodies in protecting piglet health (Lecce & King, 1978). IgA levels in milk plays a vital role in lactogenic immunity and RV passive protection in suckling piglets. Studies from other swine enteric viruses have also identified IgA as an important correlate of passive immunity to piglets. An increased rate of protection against TGEv in neonatal piglets was associated with high sIgA levels in colostrum and milk (Bohl et al., 1972).

Lack of updated strains in the current RVA vaccine and absence of modified live virus (MLV) vaccines against RVC have prompted swine producers to mimic natural RV infection in gilts/sows in the form of “feedback” or “natural planned exposure (NPE)”, which contains RV-infected material. Gilts/sows ingest NPE to stimulate maternal immunity and to provide lactogenic

immunity to piglets. NPE during pregnancy is the most widely used method of stimulating lactogenic immunity, in the US. Using NPE precludes the need to generate RVB and RVC in vitro and hence is currently the only method available to prevent RVB and RVC infections in swine farms. However, stimulating high levels of passive immunity without introducing RV particles into the farrowing room is difficult since sows can have subclinical RV infections yet shed high amounts of virus into the environment. An optimal NPE dosing strategy has not been determined or standardized across the industry, making it difficult to know what strategies may be most effective in production settings. Moreover, it is extremely difficult to create NPE material with high RVC viral load. NPE method involves feeding live farm-specific RV strains mixed with feed a few weeks prior to farrowing. Attempts have been made to determine the best time for exposing sows to the feedback material. Natural planned exposure at 5, 4, and 3 weeks prior to farrowing is considered most successful against RVA. This method induced the highest level of RVA and RVC shedding in gilts following NPE and led to the least shedding and best performance in piglets (Shepherd, 2020b). However, a major concern with this method was that the sows were still shedding RVA and RVC when they entered the farrowing house, potentially exposing their piglets to higher levels of RV.

1.6 Gut microbiome and Rotavirus Immunity

1.6.1 Composition of swine gut microbiome

The gut microbiota help maintains normal functioning of the intestinal mucosal barrier and stimulate host immune response. Recent studies suggest that gut microbiota also play a crucial role in the regulation, elimination and potentiation of infectious diseases. In pigs, Bacteroidetes and Firmicutes are predominant phyla of gut microbiota regardless of age and breed. Normal gut microbiota of 4 to 21-day-old piglets includes Firmicutes (44%), Bacteroidetes (21%),

Verrucomicrobia (20%) Proteobacteria (10%), and Fusobacteria (5%) (Liu et al., 2015). Microbiome-virus interactions have been well characterized for Porcine Epidemic Diarrhea virus (PEDv), another important enteric viral pathogen of pigs (A. Huang et al., 2019; M.-Z. Huang et al., 2018; Liu et al., 2015; Song et al., 2017; Z. Tan et al., 2019a, 2019b). However, such information is completely lacking for RVs in pigs. Moreover, association between gut microbiome changes in pigs and immune response to RVs has not been explored yet. In pigs, Bacteroidetes and Firmicutes are predominant phyla regardless of age and breed. For example, normal gut microbiota of 4-21 days non-PEDv infected piglets included Firmicutes (44%), Bacteroidetes (21%), Verrucomicrobia (20%) Proteobacteria (10%), and Fusobacteria (5%) (Liu et al., 2015). However, PEDv infection greatly changed this equilibrium, in particular the abundance of Fusobacteria increased in piglets infected with PEDv (36%) compared to non-infected piglets (5%). A recent study tracking the pig microbiome from day zero until the market age also found Firmicutes to be the most abundant phylum followed by Bacteroidetes across each stage. These two phyla accounted for 70% of the total sequences (X. Wang et al., 2019).

1.6.2 Evidence from human rotavirus studies

Recently, changes in gut microbiome composition have been correlated with improved protection against viral diseases such as porcine reproductive and respiratory syndrome virus (Constance et al. 2021), porcine epidemic diarrhea virus (PEDv) (A. Huang et al., 2019), human RV (Harris 2018b; Shi et al. 2019) and porcine circovirus (Niederwerder et al., 2018). Much of our current understanding of association between RV immune response and gut microbiome, stems from studies carried out in human infants within last 5-6 years (V. Harris et al., 2018; V. C. Harris et al., 2017, 2018; Parker et al., 2018). Human RV studies show that changes in gut microbiome composition are associated with improved immune response to RV vaccines (V. C. Harris et al.,

2017, 2018). In-fact, a gut segmented filamentous bacteria (SFB) was found to prevent and cure RV infection in immunodeficient mice (Shi et al., 2019). However, such information is completely lacking for RVs in swine. In humans, RV vaccine immunogenicity correlated with an increased abundance of specific Proteobacteria (Escherichia coli and Serratia) in Pakistan and an increased abundance of Streptococcus bovis and decreased abundance of Bacteroidetes in Ghana (V. Harris et al., 2018; V. C. Harris et al., 2017). In both the studies, pre-vaccination intestinal microbiome of infants differed significantly between RV vaccine responders (post-vaccination serum IgA titer >20 IU/ml) and non-responders (post-vaccination IgA titer <20 IU/ml). Interestingly, microbiome composition of vaccine responders was more similar to age-matched healthy Dutch infants, which further strengthens the important role of gut-microbiome in shaping immune response to RVs. Proteobacteria in particular stimulates the specific immune responses through their expression of flagella or toxigenic LPS. In fact, report exist that bacterial flagella can prevent and cure RV infection in mice via a TLR5 and NOD-like receptor C4 (NLRC4) based recognition and subsequent production of IL-22 and IL-18 (Zhang et al. 2014). A study from India reported no significant differences in microbiome diversity, stability and taxon abundance between RV vaccine responders and non-responders (Parker et al., 2018). The poor seroconversion (31%) in this study was presumed to be due to the presence of a specific bacterial community inhibitory to RV replication.

1.6.3 Gut microbiome modulation and response to rotavirus infection

Microbiome modulation using probiotics have been used to improve immune response to RV vaccines in humans with varied success (Parker et al., 2018). Bifidobacterium and Lactobacillus spp. were found to significantly reduce the duration of RV induced diarrhea in infants (Park et al. 2017). Both probiotics also appeared to ease the duration

of fever, frequency of diarrhea, and vomiting; however, the association was not statistically significant. Rice bran, a prebiotic, provided complete protection against human RV induced diarrhea in *Lactobacillus rhamnosus* GG (LGG) and *Escherichia coli* Nissle (EcN) colonized gnotobiotic pigs (X. Yang et al., 2015). Also, rice bran significantly enhanced the growth and colonization of both LGG and EcN in the intestine of pigs, promoted body weight gain, protected against damage to intestinal epithelium and significantly enhanced intestinal IFN-γ and IgA levels compared to the non-rice bran group. A combination treatment including LCG and anti-RV antibodies significantly reduced RV induced diarrhea, prevented histopathological changes and reduced the viral load in the intestines in mice (Pant et al., 2007). In contrast to the studies demonstrating positive effects of probiotics, zinc and probiotic supplementation did not significantly improve the low immunogenicity of RV vaccine given to infants in a poor urban community in India (Lazarus et al., 2018). In another study, dietary intake of *Bifidobacterium lactis* and *Streptococcus thermophilus* failed to decrease the duration of RV induced diarrhea in infants (Mao et al., 2008). The therapeutic ability of probiotic compounds is mainly attributed to their ability to reinforce the intestinal mucosal barrier, production of antimicrobial compounds and stimulation of gut-specific immune response (Pant et al., 2007). However, the probiotic based studies have failed to address the key question of whether there is a causal association between gut microbiome and RV vaccine immune response.

Recently, malnutrition was suggested to reduce the protective efficacy of oral live attenuated human RV vaccine (attHRV) in human infant fecal microbiota (HIFM) gnotobiotic piglet challenge model (Michael et al., 2020). Four groups of gnotobiotic piglets were fed either sufficient (with and without HIFM) or deficient diets (with and without HIFM). Pigs in deficient HIFM and sufficient HIFM groups were orally inoculated with 2 ml of diluted HIFM stock at 4

days of age. All pigs were given oral attenuated HRV vaccine twice after fecal transplantation at PTD 7 and 17, subsequently challenged with virulent HRV and euthanized at PTD31. Piglets fed deficient diets had reduced HRV-specific IgG and IgA antibody secreting cells (ASCs) in blood or intestinal tissues following AttHRV vaccination and before VirHRV challenge. Few studies have reported effect of nutritional supplements in enhancing immune response to swine viral pathogens (Langel et al., 2019; Zijlstra et al., 1999). Oral vitamin A supplementation was found to enhance lactogenic immune response and protection of piglets from porcine epidemic diarrhea virus (PEDV) (Langel et al., 2019). Piglets born to vitamin A (VA) supplemented gilts had a better survival rate (74.2%) compared to non-VA supplemented gilt litters (55.9%), when challenged with PEDV at 3-5 days of age. Also, VA supplemented gilts had increased PEDV IgA antibody secreting cells and PEDV IgA antibodies in serum pre-partum and IgA+β7+ gut homing cells in milk post piglet challenge compared with non-VA PEDV gilts (Langel et al., 2019). In another study, malnutrition resulted in delay in subsidence of RV induced intestinal damage and diarrhea in two-day old piglets. RV induced diahhrea subsided in 9 days in well-nourished piglets and persisted through 16 days post-infection in malnourished piglets (Zijlstra et al., 1999).

Gut microbiome modulation using narrow spectrum antibiotics has been reported to influence the response to oral RV vaccine in humans (Harris et al. 2018b). In this randomized-controlled trial, healthy adults were randomized and administered broad-spectrum (oral vancomycin, ciprofloxacin, metronidazole), narrow-spectrum (vancomycin), or no antibiotics and then vaccinated with oral RV vaccine. Although no difference was observed in anti-RV IgA levels 28 days post-vaccination, the group administered vancomycin revealed slight increase in anti-RV IgA titers 7 days after vaccination. In addition, groups given antibiotics had increased fecal shedding of RV compared to no antibiotic treatment group, which suggest RV replication within

the intestine (V. C. Harris et al., 2018). The study provides the first evidence that the gut microbiome has a role to play in RV vaccine immunity in humans. Recently, gut segmented filamentous bacteria (SFB) was found to prevent and cure RV infection in immunodeficient mice (Shi et al. 2019). Authors accidentally identified a mouse breeding colony that was highly resistant to RV infection and found that resistant mice carried distinct microbiota (SFB). Co-housing and fecal microbiota transplant resulted in the transfer of RV resistance to the new hosts. It was further revealed that SFB reduces RV infectivity and provide protection by shedding of epithelial cells and replacement with new cells. The results of this study clearly suggest a role of specific gut microbiome in combating RV infections.

Human data apparently supports a link between microbiome composition and oral RV immunogenicity. However, the association between gut microbiome and porcine enteric viruses has not been studied. Gnotobiotic piglets, because of their close anatomical and physiological resemblance to human infants, have been utilized as models to investigate the effects of nutritional deficiencies, RV infection and vaccine efficacy in humans(Kumar et al., 2018; Miyazaki et al., 2018; Twitchell et al., 2016; Vlasova et al., 2016). We do not know how RV infection in pigs affects the structure and functionality of gut-microbiome. Also, the association between gut-microbiome changes in pigs due to RV infection immune response to RVs remains unexplored. Given the lack of porcine RVC vaccine and lack of protection against RVC by commercial porcine RVA vaccine, it would be interesting to study the effects of NPE on gut microbiome composition and any association between NPE-induced microbiome changes and RV immune response in gilts and piglets. More effective on-farm management of RV can be achieved by understanding whether there is an association between gut microbiota composition and RV immunogenicity in pigs, and

by identifying which commensal organisms are associated with enhanced immune response to RVs in pigs.

1.7 Conclusion

Rotaviruses are ubiquitous in nature and all swine herds most likely have a history of RV infection and circulation. RVA and RVC are the most common species among all RV species reported in swine. Although, RVA is considered most prevalent in swine populations, recent data suggest that RVC is emerging as a significant cause of enteritis in neonatal piglets. Studies detailing immune response to porcine RVs are lacking and a better understanding of porcine RV immune response to RV is needed. Presently, NPE is the only method available to provide protection against porcine RVCs due to difficulties in growing RVA in the laboratory and complete lack of cell culture system to grow RVC. There is an urgent need to identify better NPE protocols (time and dosage) to enhance maternal immune response to RVs in gilts and lactogenic protection of the piglets, until an effective porcine RVC vaccine is developed. NPE however carries risk of introducing other viral and bacterial pathogens into the herd and also it is extremely difficult to prepare high NPE material with high RVC load. More research is required to better characterize emerging RV strains in swine herds to identify novel variants that can evade herd immunity. Alternative approaches to develop a porcine RVC vaccine such as viral vector-based vaccines (replication competent and incompetent) and recombinant protein-based vaccines needs to be explored. There is complete lack of gut microbiome and rotavirus immune response data in swine. A better understanding of such an interaction might result in more effective management of RVs in commercial swine farms.

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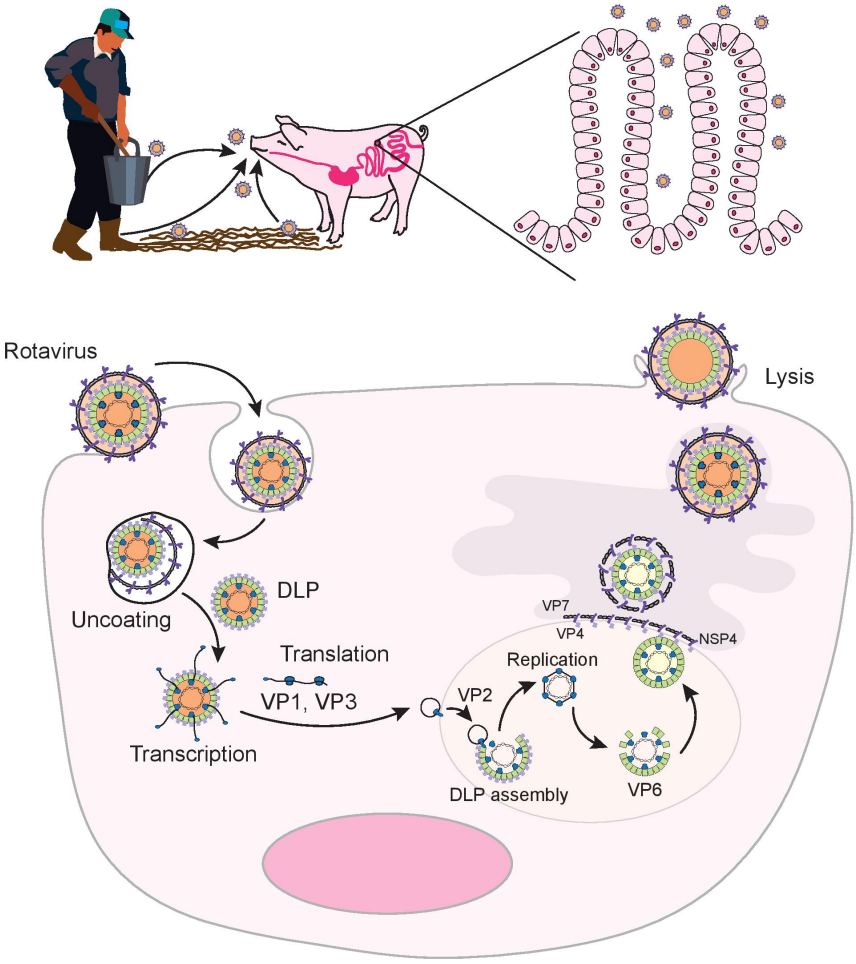
Figure 1.1.

Rotavirus Replication Cycle



Note. The rotavirus replicates in the cytoplasm of the enterocytes. Rotavirus enter the host cells by receptor-mediated endocytosis. The low calcium levels inside the endosome trigger the removal of outer capsid layer, which releases the transcriptionally active double layered particle (DLP) in to the cytoplasm. Viral mRNA is transcribed to form the structural proteins of the capsid. The RNA genome is replicated and packaged into newly made DLPs in viroplasms. DLP binds with NSP4, which serves as an intracellular receptor followed by budding of DLPs into the endoplasmic reticulum (ER). In the ER, VP4 and VP7 proteins are added onto the DLPs thus forming a triple layered particle (TLP). The matured virion are releases from cells through cell lysis.

Chapter 2 - Role of Pre-farrow Natural Planned Exposure to Gilts in Shaping the Passive Immune Response to Rotavirus A in Piglets

2.1. Introduction

Rotaviruses (RVs) are double-stranded RNA viruses belonging to the Rotavirus genus in the Reoviridae family. The RV genome is approximately 18,522bp in size and consists of 11 segments of dsRNA encoding six structural proteins (VP1-VP4, VP6 and VP7) and 5/6 non-structural proteins (NSP 1- NSP5/6) (Estes and Kapikian 2007). RV species are classified based on sequencing of the VP6 gene (Matthijnssens et al. 2008a; Matthijnssens et al. 2012), and ten RV species, A through J (RVA-RVJ) have been classified (Banyai et al. 2016). A binary classification system of G (VP7) and P types (VP4) is used to address vast rotavirus diversity with a species. Also, the VP7 and VP4 proteins independently induce neutralizing and protective antibodies (Ludert et al 2002). A complete genome classification system was developed based on nucleotide sequencing of all 11 RV segments with nucleotide percent identity cut-off values set for each segment where the VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6 RV genes are designated as Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx (Matthijnssens et al. 2008b; Vlasova et al. 2017).

While five RV species (RVA, RVB, RVC, RVE, and RVH) have been identified in swine, RVA strains have been considered the most pathogenic and epidemiologically diverse of all RV groups infecting swine, with detection most common in post-weaning piglets (Homwong et al. 2016). Prevalence rates ranging from 9.4% to 81.1% have been reported from the US swine population (Amimo et al. 2013, Marthaler et al. 2012, Marthaler et al. 2014, Janke et al. 1990, Homwong et al. 2016). The genotypes G5 (71.43%) and P[7] (77.22%) constitute the most

prevalent RVA genotypes circulating in swine herds in the US given the limited sequencing data from the US (Papp et al. 2013).

Passive, antibody-based immunity from gilt or sow colostrum and milk is essential to protect piglets from RV infections since in-utero transfer of immunoglobulins (Igs) does not occur in swine due to epitheliochorial placenta. IgG and IgA produced in the sow traffics to the mammary glands and is transferred through colostrum and milk to piglets, where RVs are locally neutralized in the gut (Shepherd et al. 2020; Chepngeno et al. 2019; Ward et al 1996; Gellberg et al. 1991). In particular, secretory IgA (sIgA) antibodies play a major role in preventing RV infection at the gut mucosal level to neutralize RV infections (Langel et al. 2020). The IgA plasmablasts from the sow gut and IgG from serum into the mammary gland secrete these immunoglobulins in colostrum and milk (Chepngeno et al. 2019; Bohl et al., 1972). The outer capsid proteins VP7 and VP4 of RVs are targets for the humoral immune response and independently elicit neutralizing and protective antibody response (Nair et al. 2017; Estes and Greenberg, 2013). Upon the presence of trypsin in the gut, VP4 is cleaved into VP8 and VP5 (Settembre et al. 2011). Both the VP8 and VP5 stimulate neutralizing antibodies, and most of the recognized neutralizing epitopes have been mapped to VP8 and antigen domain of VP5 (Trask et al. 2012; Li et al. 2018).

Although a modified live RVA vaccine (ProSystem RCE, Merck Animal Health) is available which contains G5, G9, P[6], and P[7] genotypes, NPE prior and during pregnancy is the most widely used method of stimulating lactogenic immunity against RVs in the US (Pittman, 2016, Anderson et al 2022). NPE method includes collection of RV-positive material from the farm, which is fed to pregnant gilts and sows to boost antibody production against specific RVs circulating on the farm. However, the efficacy of NPE protocols in providing lactogenic immunity

to piglets and shaping the genetic changes in RV strains in piglet population has not been previously investigated.

In view of above knowledge gaps, a longitudinal study in a commercial swine farm was designed to test different NPE dosing strategies to gilts on providing lactogenic immunity to their piglets.

2.2 Materials and Methods

2.2.1 Study design, NPE material and sample collection

The study was conducted on an 1,800-head commercial, breed-to-wean gilt farm in the United States. Pregnant gilts were randomly allocated into 4 groups. Group 1 received 3 doses of NPE at 5, 4, and 3 weeks pre-farrow (WPF), group 2 received 2 doses of NPE at 5 and 3 WPF, group 3 received one dose of NPE at 5 WPF, and group 4 received no NPE (control group) (Figure 2.1). Each treatment group initially contained 12 gilts resulting in 12 piglet litters for each group. Post farrowing, 2 litters were excluded due to savaging and agalactia. Forty-six litters (Group 1 = 12, Group 2 = 12, Group 3 = 11, Group 4 = 11) were evaluated for rotaviral fecal shedding and antibody titers. Gilts were housed by treatment group, and all movement between groups was restricted by slatted fencing. Depending on the gilt farrow date, piglets were weaned between 19-25 days of age (after the week 3 sampling timepoint) and moved to a separate nursery barn prior to the week 4 sampling timepoint. The piglets continued to be separated by group in the nursery barn. NPE material was created using the master seed method (Pittman 2016). Five piglets from each litter were selected for serum sample collection throughout the study. Blood samples from gilts were collected at weeks -5, -3, 0 (farrowing) and 3. To assess the lactogenic immunity, colostrum was collected at birth and milk was collected 1-3 weeks post farrowing. Blood samples

from 5 piglets per litter were collected at weeks 0 (farrowing), 1, 2, 3, 4, 5, and 6 for a total of 7 blood samples per piglet (Table 1).

2.2.2 Generation of rotavirus A VP7 and VP4* expression constructs

NGS identified G (G4 and G5 VP7) and P (P[7] and P[23] VP4) genotypes in the NPE material. G4 and G5 VP7 constructs were prepared for expression in mammalian Expi293™ Expression System (Gibco). Truncated VP4* protein constructs for P[7] and P[23] genotypes were generated for bacterial expression. Full length VP7 sequences of G4 and G5 genotypes were modified to add in-frame 8-His tag and Streptavidin tags at N and C terminals respectively to track protein expression and affinity purification of recombinant proteins. Gene sequences were codon optimized for mammalian expression. A kozak sequence was also added at N terminal to facilitate enhanced protein expression. Linker sequences were added just preceding each affinity tag. CD5 secretory signal was fused at N-terminal for efficient secretion of the recombinant protein into the culture media. The dual tagged synthetic rotavirus VP7 genes were subcloned into pcDNA3.1+ mammalian expression vector (InvitrogenTM). Truncated VP4* (aa26-476) of P[7] and P[23] genotypes were cloned in to pET-24a(+) vector with a linker followed by a 8-his tag at C-terminal. Codon optimization, gene synthesis, cloning into pcDNA3.1 (+) and pET-24a(+) vectors, and gene sequence validation was outsourced to Genscript.

2.2.3 Recombinant protein expression

G4 and G5 VP7 pcDNA3.1 (+) plasmid constructs were transformed into DH5α competent cells. Positive clones for each construct were identified by PCR screening and used for recombinant protein expression in the mammalian Expi293TM Expression System (Gibco) as per manufacturer's protocol and as previously described (Sangewar et al. 2020). Briefly, Expi293 cell

suspension cultures were transfected with pcDNA3.1+ constructs expressing the G4 or G5 VP7. To check the efficiency of protein expression 300µl transfected Expi293 cells were plated in a 12 well plate and incubated at 37C for 2-3h. Fixed cells were used for immunocytometric analysis using anti-his monoclonal antibody (1:2000) and anti-mouse AP-conjugated secondary antibody (1:5000). To determine whether the protein was secreted or in the cell cytosol, culture media (100µl) and a small cell pellet were collected for both proteins and an ELISA was . was performed.

For bacterial expression, pET-24a(+) vector carrying DNA of P[7] and P[23] VP4* was individually transformed into Rosetta cells (Thermo Fisher Scientific) and grown overnight on LB agar plates with 30µg/ml kanamycin at 37℃. Individual colonies were amplified overnight in 20 mL of LB broth with kanamycin at 37℃with shaking overnight. The overnight culture was added to 1 L of LB broth with kanamycin (30 µg/ml), grown at 37℃ with shaking until reaching an OD600 approximately 1. Cultures were induced with IPTG added to a final concentration of 0.5 mM for 16 hours at 16℃ with shaking. Various time/temperature and IPTG combinations were tested to optimize the production of soluble protein. After expression, bacterial cultures were centrifuged and the resulting cell pellets were used for protein purification.

2.2.4 Protein purification and validation

Recombinant proteins were purified by immobilized metal affinity chromatography (IMAC) using TALON Cobalt resin (Takara Bio) following a hybrid batch/gravity procedure as per manufacturer with modifications. Expi293 cell culture supernatant was used to purify G5, Expi293 cell lysate was used to purify G4, and bacterial cell lysates were used for purification of P[7] and P[23]. Filtered culture supernatant of G5 was directly added to TALON resin. Cell pellets of G4, P[7] and P[23] were resuspended in lysis buffer (Sodium phosphate 50mM, NaCl 300mM, Imidazole 10mM, Glycerol 10%, pH7.0), homogenized using ultra sonication (30% amplitude, 10

sec on and 30 sec off, 10 cycles on ice), and centrifuged at 15000g for 20 minutes at 4℃. Protease inhibitor was added to to prevent protein degradation. Supernatant containing soluble protein was filetered (0.45µ added to cobalt resin, and rotated at 4C on a rocking platform for 1.5 hrs to allow protein binding. Suspension was centrifuged (700g for 5 min) and the supernatant was discarded. Resin pellet was washed twice with wash buffer (Sodium phosphate 50mM, NaCl 300mM, Imidazole 20mM, Glycerol 5%, pH7.4). Washed resin was transferred to a gravity-flow column and again washed on column. His-tagged proteins were eluted using an elution buffer (Sodium phosphate 50mM, NaCl 300mM, Imidazole 150mM, Glycerol 5%, pH7.4) in multiple 1.5 ml fractions. The affinity purified proteins were quality control validated by SDS-PAGE and Western blotting. Pure protein fractions were pooled and concentrated using 10K protein concentrators. Concentrated proteins were quantified using BCA assay and stored in -80 until further use. Expressed proteins were resolved in NuPAGE® Bis-Tris gel (InvitrogenTM, NP0322) by denaturing electrophoresis. The gel was then stained with AcquaStain (Bulldog-Bio) for visualization of the protein bands. The proteins were resolved on a gel as above and transferred to Immun-Blot PVDF membrane (BioRad) by electrophoresis for Western blotting. After transfer, the blot was incubated in blocking buffer (5% non-fat dry milk in 1X PBST) at 4°C for 1h, and then probed for 1 h with anti-His monoclonal antibody (1:2000) at room temperature. Following 3 washes with 1X PBST, the blot was incubated with anti-mouse peroxidase-conjugated secondary antibody diluted 1:5000 in blocking buffer. Pierce DAB substrate (Thermo Scientific, catalog #34002) was used for chromogenic detection of protein bands.

2.2.5 Development of recombinant protein ELISAs to quantitate RVA antibodies

Indirect ELISAs were individually optimized to detect genotype-specific RVA IgG and IgA antibodies in porcine serum and colostrum/milk. A checkerboard titration method was used

to determine optimal coating protein concentration for each protein and secondary antibody concentrations. Different concentrations (25ng, 50ng, 100ng, 150ng, 200ng, 300ng and 400ng) of individual proteins were diluted in ELISA carbonate buffer (0.05 M carbonate–bicarbonate, pH 9.6) and coated on immunoassay plates (2HB plates, Life Technologies). Plates were incubated overnight at 4°C and washed 4x using 1X PBST containing 0.05% Tween-20 using an automatic plate washer. Plates were blocked using 5% non-fat dry milk (NFDM) prepared in 1xPBST containing 0.05% Tween-20 at room temperature for 1h and subsequently washed 4 times. Five serum samples were randomly selected from the sample inventory and diluted 1:200 in 5% NFDM. Diluted serum was added (100 µl) in duplicate to the wells of washed immunoassay plates containing different concentration of coated protein. The plates were incubated at 37 °C for 1 h and washed 4x using wash buffer. Anti-porcine IgG (1:10,000 in 5% NFDM, 100 µL) conjugated to horseradish peroxidase (Abcam) was added to each well and incubated at 37 °C for 1 h. Plates were again washed 4x with wash buffer and 100µl of ABTS substrate was added to each well. Plates were covered and incubated at room temperature for 20 minutes. The reaction was stopped with 1x ABTS peroxidase stop solution (100µl). The plates were read using an ELISA microplate reader (Epoch) at 410 nm. The ELISA antibody titer was expressed as the reciprocal of the highest dilution that had a A410 value greater than twice the mean of negative control wells.

2.2.6 Anti-RVA IgG and IgA endpoint titer determination

Blood samples were centrifuged at 2000g for 15 min to obtain serum and stored at −80 °C until use. Colostrum and milk samples were centrifuged at 5000g overnight at 4℃ to separate fat, debris and whey. Fat layer was carefully separated using sterile pipette tips and clear fluid (whey) was collected in sterile 2 ml Eppendorf tubes. Whey was stored in -80℃ until further use. ELISA protocol detailed in section 2.5 was used to quantify antibodies against RVA in porcine serum and

colostrum/milk samples. To determine endpoint titer of RVA IgG and IgA antibodies, serum and colostrum/milk samples were serially diluted (1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400, 1:12800 and 1:25600) in 5% NFDM prepared in 1x PBST and added (100 µl) in duplicates to the wells of overnight protein coated, blocked and washed immunoassay plates. Care was taken to invert original sample tubes 2-3 times before preparing the dilutions. Washing, time and temperature incubation, and substrate conditions were same as detailed in section 2.5 above. Anti-porcine IgA was used in a concentration of 1:3,000 diluted in 5% NFDM 1X PBST, 100µl). The end point titer was expressed as the reciprocal of the highest dilution that had a A410 value greater than twice the mean of negative control wells. Each ELISA plate had a serially diluted positive and negative control to control plate to plate variation. Since, true positive controls (antiserum against each protein) were not available, few high titer serum samples were pooled and used as positive control throughout the ELISA testing to maintain uniformity.

2.2.7 Next generation sequencing of RV strains in NPE material and piglet feces

Whole genome sequencing (WGS) of RV strains in the NPE material was conducted at Molecular NGS laboratory at Kansas State Veterinary Diagnostic Laboratory (KSVDL), Kansas State University. Piglet fecal samples were chosen for sequencing from weeks 0-3 samples to assess viruses shed in the presence of lactogenic immunity (Table 2). Litters from which RVA was detected for multiple weeks in a row with Ct values less than 26 were selected for sequencing. Four more piglet fecal samples from week 4 (nursery) were also included for sequencing to determine RVA genotypes circulating outside the lactogenic immune pressure (Table 2). WGS of piglet fecal samples was conducted at Centers for Disease Control and Prevention (CDC), Atlanta, Georgia.

2.2.8 Statistical analysis

The significance of the differences between the treatment and the control groups was determined by two-way Analysis of Variance (ANOVA). All Statistical analysis was performed using GraphPad Prism 7 (Version 7.04, GraphPad Software, Inc. La Jolla, CA) and a significance level of $p < 0.05$ was used for all analyses.

2.3 Results

2.3.1 Expression of recombinant proteins and optimization of ELISAs

G5 protein was efficiently secreted into the Expi293 culture media, however, G4 protein was localized inside the cell pellet with minimal secretion into the media. An estimated 37-kDa and 55-kDa bands corresponding to the expected molecular weight of recombinant VP7 and truncated VP4* proteins, respectively were detected on SDS-PAGE and Western Blot using anti-His monoclonal antibodies (Figure 2.2). Immunocytometric analysis of HEK-293A cells transfected with pcDNA3.1 (+) plasmid encoding G4 and G5 VP7 genes, probed with anti-his monoclonal antibodies also confirmed protein expression (Figure 1 and 2). Protein concentrations of 50ng (G4 VP7), 100ng (G5 VP7 and P[7] VP4*) and 150ng (P[23] VP4*) resulted in optimal OD value readouts. Blocking the immunoassay plates with 5% NFDM prepared in 1x PBST with 0.05% Tween-20 and four washings after each incubation step resulted in minimal background. The optimal incubation temperature and time combination for samples (serum/colostrum/milk) and secondary antibodies was at 37℃ for 1h. Respective concentrations of 1:10,000 and 1:3,000 for peroxidase conjugated IgG and IgA were found to produce best OD readouts.

2.3.2 Antibody response to RVA NPE

2.3.2.1 Gilt serum

Since RVA is prevalent in swine herds, the study gilts had likely experienced RVA infection prior to their enrollment in the study, which resulted in varied levels of IgG and IgA antibodies before the administration of 1st NPE dose at 5-weeks pre-farrow (WPF) (Figure 2.3). Geometric mean titers (GMT) IgG levels at 5WPF were G5 (GMT 1667-1932), G4 (GMT 1243.53-1940.94), P[7] (GMT 5079.68-10159.37) and P[23] (GMT 4381.12-6816.26). GMT IgA levels at 5WPF were G5 (GMT 400-1029.33), G4 (GMT 548-852.03), P[7] (GMT 1704.07-2539.84) and P[23] (GMT 1243.53-2334.17). Control group IgG and IgA levels were always higher compared to treatment groups at 5WPF except for P[7] IgG levels, where group 3 IgG levels were higher than control group. Two doses of NPE in group 1 (5 and 4 WPF) and one dose each in group 2 and 3 (5WPF) resulted in increased IgG levels at 3WPF compared to the control group, which decreased at 3WPF in the absence of NPE. Gilt serum IgG levels dropped sharply in all treatment groups at farrowing (F). Serum IgG levels gradually increased after farrowing until 3-weeks post-farrowing (weaning).

Serum IgA levels for treatment groups 1, 2 and 3 increased at 3WPF after administration of respective NPE doses (Figure 2.3). Control group serum IgA levels decreased at 3WPF for G4 and G5 in the absence of 1st NPE dose (Figure 2.3 B and D). Interestingly, an increase in serum IgA levels of control group was observed for P[7] and P[23] at 3WPF (Figure 2.3 F and H). Similar to IgG levels, IgA levels also increased post-farrowing until weaning.

2.3.2.2 Colostrum and milk

Colostrum and milk samples were collected at farrowing (day 0) and then at weekly interval until weaning (day 7, 14 and 21). At day 0, treatment group 1 (3 NPE) had significantly

higher G5 and G4 IgG Ab titers compared to group 2 (2 NPE), group 3 (1NPE) and the control group (Figure 2.4 A and C). The P[7] and P[23] IgG Ab titers at day 0 were significantly higher in treatment group 1 compared to the control and group 3 (Figure 2.4 E and G). Overall at day 0, treatment group 1 had significantly higher colostrum IgG titers for all antigens compared to the control group, and either significantly or numerically higher IgG titers than group 2 and 3. In addition, the colostrum IgG levels for all antigens were highest on day 0, which rapidly declined and reached the baseline at day 7 in milk, and remained so during the subsequent samplings (Figure 2.4 A, C, E and G).

The G5 IgA Ab titers were significantly higher in group 1 (3 NPE) compared to group 2 and the control group, while the G4 IgA Ab titers were not significantly different for any treatment groups (Figure 2.4 B and D). The P[7] and P[23] VP4* IgA Ab titers were significantly higher in the treatment group 1 compared to other treatment and control groups (Figure 2.4 F and H). Overall at day 0, colostrum IgA levels for all antigens (except G4) were significantly higher in treatment group 1 than control group, and either significantly or numerically higher compared to group 2 and 3 (Figure 2.4 B, D, F and H). G5 VP7 IgA titers declined at day 7 and gradually increased until eventually reaching the same Ab titers at day 0 (Figure 4B). However, treatment group 1 did not reach the same G5 IgA Ab titer as on day 1. The P[7] and P23] IgA titers generally increased until day 21 but never reached the same values as day 0. Lastly, the VP4*-specific IgG and IgA titers were at least 5 times higher than VP7-specific IgG and IgA titers (Figure 2.4 A-H).

2.3.2.3 Piglet serum

Piglet serum samples were collected at birth and then at weekly interval until 6 weeks of age (day 42). At birth (day 0), piglets born to treatment group 1 gilts had significantly high IgG Ab titers against G5, P[7] and P[23] compared to group 2, group 3 and the control group piglets

(Figure 2.5 A, E and G). Group 1 piglets had significantly higher G4 IgG titers compared to the control group at day 0, and numerically high IgG titers than group 2 and 3 (Figure 2.5 C). G5 and G4 IgG titers for all four groups declined at day 7 but increased slightly at day 14 followed by a gradual decrease until day 42 (Figure 2.5 A and C). P[7] IgG titers for all groups declined post-birth reaching the baseline at day 42 (Figure 2.5E). P[23] IgG Ab titers for treatment groups 1, 2 and 3 declined at day 7 with group 2 and 3 showing slight increase at day 14 and 21. P[23] VP4* IgG titers increased slightly at day 42 (Figure 2.5G). Interestingly, P7 IgG levels in all three treatment groups at day 7 did not decrease as sharply as for other proteins (Figure 2.5E).

Group 3 serum samples had higher IgA Ab titers at day 0 for all proteins compared to group 1, 2 and the control group, however the levels were not significantly different (Figure 2.5 B, D, F and H). At day 0, group 1 IgA titers were significantly higher than group 2 and the control group for all four antigens. Overall, for all antigens, serum IgA levels of all four groups were highest at day 0, which rapidly declined at day 7 and reached the baseline on subsequent sample collection time points.

2.3.3 RVA fecal shedding and association with antibody levels

Realtime PCR of feedback (NPE) material revealed RVA ct-values of 24.43, 22.46 and 24.15 for feedback 1 (5WPF), 2 (4WPF) and 3 (3 WPF), respectively. Gilt and piglet RVA fecal shedding results have been described elsewhere and also summarized in the supplementary table 1 and 2 (Anderson et al. 2022). All piglets' fecal swabs collected within 24 hours of farrowing were negative for RVA by qRT-PCR. A single litter in treatment groups 1 (litter 41049) and 3 (litter 40996) shed RVA for multiple weeks prior to weaning. These pre-weaning samples and 4 samples at week 4 were selected for NGS to investigate genetic changes in response to immunity (Table 2.2). A complete RVA genome could only be recovered from treatment group 3, week 2

while the four samples at week 4 yielded complete RVA genomes. An association between Ab levels and RVA shedding in the piglets could not be established when compared to the Ab levels of the other piglets regardless of the treatment group. Antibody levels of the two piglets shedding RVA pre-weaning along with colostrum IgA levels of respective gilts are summarized in table 2.3. Piglet belonging to the litter 40996 had higher serum IgA levels for P[23] and G5 at week 1 of age compared to piglet of litter 41049 (ct-value 16.09). Sequencing revealed a RVA genome constellation of G11-P[34]-I5-R1-C1-M1-A8-N1-T7-E9-H1 from week 2 sample of litter 40996. Week 4 sample of litter 40996 along with other week 4 samples yielded genome constellation of G9-P[23]-I5-R1-C1-M1-A8-N1-T7-E1-H1. G and P-type combination (G11P[34]) detected in pre-weaning sample of litter 40996 was different from genotypes present (G4, G5, P[7] and P[23]) in the original NPE material fed to the gilts (Table 2.4).

2.3.4 Sequence analysis and antigenic variation among the RVA strains

We found that G11 VP7 sequence from piglets in the farrowing room shared 82.77% nucleotide and 89.57% amino acid percent identity with the G5 VP7 sequence of the NPE. However, nucleotide and amino acid percent identity with the G4 VP7 NPE sequence were 72.58% and 75.46%, respectively. G9 VP7 sequences from nursery shared low nucleotide (75.23-78.29%) and amino acid (78.22-83.13%) percent identity to parent G4 and G5 sequences. To determine the sequence variation, the neutralizing epitopes of the VP7 of five RVA sequences recovered from piglet feces were compared to the G4 and G5 sequences of the original NPE material. Out of 32 residues in the neutralizing epitopes, 7 residues were conserved (D95, S103, K143, S190, T192, T209 and T210) (Table 2.5). The G11 strain expressed the highest number of differences to the G4 strain (n=20) compared to the G5 strain (n=10) of the NPE. Four G9 sequences completely differed from G4 and G5 sequences at 10 amino acid positions (90, 94, 100, 122, 147, 189, 208,

212, 213 and 221). A series of common B-cell epitopes for RVA VP7 has been proposed recently (Shepherd et al. 2020). These include residue positions 87, 90-92, 94-97, 99, 122, 147 and 210-213. A higher amino acid variability was observed at these positions with 5 piglet RVA VP7 sequences differing either to the G4 or G5 NPE sequences at multiple residues. For example, piglet VP7 sequence completely varied at positions 90, 94, 122, 147, 212 and 213 (Table 2.5).

For VP4, out of 34 residues spread across VP8* and VP5* regions, 9 residues completely matched P[7] and P[23] sequences of the NPE (D100, Q125, N132, G150, N193, Y194, Y385, G392, and R425). The P[34] genotype completely differed from P[7] and P[23] sequences at 17 residues (87-89, 113, 116, 133, 146, 148, 173, 188, 192, 195-196, 393, 433, and 458). However, P[23] sequence from piglets only differed at residue 188 (Y188T) with the parent NPE P[23] strain (Table 2.6).

2.4 Discussion

This study was designed to investigate the dynamics of antibody response to different doses of NPE to gilts and transfer to their piglets. To evaluate the effectiveness of RV NPE protocols and differences in antibody response to different proteins, indirect ELISAs were developed to investigate antibody response to RVA G4, G5, P[7] and P[23] genotypes in gilt serum, colostrum/milk and piglet serum samples. We also investigated association between antibody levels to RVA genotypes and fecal RV shedding in piglets. Sequence analysis was performed to determine genetic changes in RV genotypes recovered from piglets in the presence of NPE.

Two doses of NPE in group 1 and one dose of NPE in treatment groups 2 and 3 resulted in increased IgG and IgA levels at 3WPF, reflecting the stimulation of active immunity against RVA in gilts. As expected, gilt serum IgG levels in all treatment groups dropped sharply at farrowing due to transudation of serum immunoglobulins into the colostrum swine (Bourne & Curtis, 1973).

However, serum IgA levels at farrowing did not drop as distinctly as IgG, which could be either due to increased numbers of IgA-producing cells at sub-mucosal sites which raise serum IgA levels by uptake from the lymphatics, release of IgA locally produced in the mammary gland into gilt serum, or a reduced transportation of serum IgA into exocrine fluid (Klobasa et al.1985). Similar to our results, Klobasa and coworkers also found elevated sow serum IgA levels during last weeks of gestation in contrast to serum IgG levels, which dropped sharply at farrowing.

Our results indicated group 1 gilts with 3 doses of NPE had significantly higher colostrum anti-RV IgG titers for all antigens compared to the control group, and either significantly or numerically higher IgG titers than group 2 and 3. For all groups, colostrum IgG levels declined sharply and reached baseline (dilution 1:200) at day 7. Rapid decline in colostrum IgG levels occurred in parallel with rapid rise in sow serum IgG titers post-farrowing until weaning. Similar to our results, high RV-specific colostrum antibody titers (8-32 fold) have been reported compared to milk collected at 18 days post-farrowing (Fu et al. 1990). Overall, 3 doses of NPE in group 1 resulted in higher colostrum IgG levels at day 0 compared to other groups for all antigens. We observed that three doses of NPE in group 1 also resulted in higher anti-RV IgA levels in colostrum (day 0) compared to the control group for all antigens. In a study comparing efficacy of maternally derived anti-RV antibodies on piglet protection against RVA, significantly higher RV-specific IgG levels and anti-RV virus neutralization titers (1600 versus 340) were reported for immune colostrum (collected from sows immunized with RVA) compared to the conventional colostrum (non-immunized) (Ward et al. 1996). We also found that colostrum IgA levels were highest at day 0 followed by a decline at day 7 and then steady increase at day 14 until day 21, which reflects the increased number of RVA-specific IgA plasmablasts in the mammary gland tissue and continuous supply of secretory-IgA in the colostrum and milk throughout the lactation. High pathogen-specific

IgA levels in milk have been associated with lower incidence of enteric disease including RVs in swine (Ward et al. 1996; Bohl et al. 1972). In addition, RVA IgA and IgG levels in colostrum and milk of RVA exposed field sows decreased gradually over time and provided protection against RVA infection in piglets for the first 1-2 weeks in a virus challenge model (Fu et al 1990). Overall, group 1 gilts had higher colostrum IgA levels (day 0) for all antigens (except G4) compared to other study groups. High IgA levels in milk from birth until weaning substantiate the role of IgA in providing protection against RV infection prior to weaning.

Maternally derived IgG and IgA levels in piglet serum were highest on day 0 and then declined thereafter which could be attributed to the cessation of absorption of intact immunoglobulins (gut closure) around 36 hours post-birth. The antibodies provided in colostrum and milk play a crucial role in the protection of the piglets against rotaviral infection (Ward et al. 1996). The ingested maternal Igs remain intact within the digestive tract probably because of the low proteolytic activity of piglets' digestive tract (Sangild et al. 1991). As expected, IgA levels declined more sharply across sampling points because of their shorter half-life of approximately 6 days as compared to 24 days for IgG (Mankarious et al. 1988; Challacombe et al. 1979).

For all antigens, piglet serum IgG levels reached the low levels at day 28 (week after weaning). Compared to other proteins, P[23] IgG titers increased at day 42 probably due to the active immunity to the P[23] genotype present in the nursery. In contrast, no increase in day 42 piglet serum IgG titers for G4, G5 and P[7] was detected, which was supported by no detection of these genotypes by sequencing. However, the absence of G4, G5 and P[7] cannot be confirmed due to the limited number of samples sequenced.

At weaning, the piglet serum IgA levels were very low and ranged 200-350 for all antigens while serum IgG levels were higher and ranged between 1156 to 2645 for P-types and 266 to 672

for G-types. Although serum IgA is considered a good indicator of intestinal IgA levels (Velázquez et al. 2000; To et al. 1998), studies suggest that serum IgG also provide protection against RV infection (Westerman et al. 2005, O'Ryan et al. 1994). Piglet fecal RVA shedding data suggest that maternal antibodies were able to resist natural RV infection in the farrowing room. Although, two litters shed RVA prior to weaning, belonging to a G and P-type combination (G11P[34]) different than NPE material administered to the gilts.

Similar to our findings, low levels of anti-RVA antibodies has been reported in piglet serum at 3 weeks of age (Fu et al. 1990). Authors were also able to determine protective levels of anti-RVA antibody titers and reported that piglets shed RVA when antibody titers in serum fell below 1/1600 at day 21 of piglets age. In our study, a positive correlation was not observed between the levels of anti-RV antibody and protection in individual litters as the two piglets shedding RVA in the farrowing room had serum IgA levels of 1/200-1/400 (G-types) and 1/1600 (P-types) at the time of shedding (week 1 of age). It is important to note, we determined protein-based (VP7 and VP4*) end point titers in contrast to the whole virus antibody titers by Fu and coworkers, and hence any comparison of antibody titers between two studies might not be correct.

The outer capsid proteins VP7 and VP4 of RVs are targets for the humoral immune response and independently elicit neutralizing and protective antibody response (Nair et al. 2017; Estes and Greenberg, 2013; Hoshino et al. 1985). The full length VP7 and truncated version of VP4* (aa26-476) proteins were generated as expression constructs as described in previous studies (Li et al. 2018; Wen et al 2012). Our IgG and IgA GMTs against VP4* were at least 5 times higher compared to VP7 protein, which is consistent with the reports published earlier. Ishida et al 1996 reported 9-27 times higher serum IgG titers for VP4 (titer = 1350) compared to VP7 (titers 50 and 150) against recombinant baculovirus-expressed EHP VP4 and RRV VP7 in mice. Similarly, Yuan

et al (2004) found that the magnitude of homotypic IgA antibody responses (fold GMT increase) in infant serum to VP4 was higher than VP7 in all study groups. This difference in the magnitude of antibody response against VP4 and VP7 could be due to less neutralizing epitopes (NEs) on VP7 (n=4) compared to 9 NEs on VP4. However, difference in endpoint antibody titers does not necessarily reflect the differences in the ability of these proteins to stimulate neutralizing antibody response.

Only two litters shed RVA in the farrowing room, suggesting protective homotypic passive immunity was stimulated against RVAs by the lower RVA Ct values in the NPE material (Table 2.7). This assumption was further strengthened by the detection of P[23] genotype (similar to NPE) in the piglet feces from treatment groups collected outside the window of lactogenic immunity (post-weaning samples). No association between genotype-specific serum IgA levels (G4, G5, P[7] and P[23]) of both piglets at birth and fecal RV shedding could be observed, which is logical since the RV genotypes shed in piglet feces (G11P[34]) prior to weaning were different than RV genotypes present in NPE. Even though G11 strain shared 89.57% amino acid percent identity with the parent G5 strain, a set of point-mutations at 10 key amino acid sites were observed. Of these, 7 sites (91, 96, 99, 122, 211-213) had been earlier predicted as shared B-cell epitopes for RVA (Shepherd et al. 2020). These predicted B-cell epitopes shared across multiple RVA genotypes have been proposed to be the common structural targets of RV antibodies regardless of genotype (Shepherd et al. 2020). Importantly, higher amino acid diversity at these positions could result in the development of virus immune escape mutants. We hypothesize that multiple point mutations at shared amino acid sites might have resulted in the development of immune escape mutants, which was confirmed by the detection of G11P34] genotype combination in the farrowing room. Our results emphasize the need of routine surveillance and genotypic analysis of RV

genotypes circulating in swine farms including environmental samples because we do not know if RV strains detected from piglet feces before weaning were already present in the farrowing room environment or resulted due to immune escape mutations.

In conclusion, the goal of this study was to investigate the dynamics of antibody response to different doses of NPE to gilts and transfer of lactogenic immunity to their piglets. Treatment group one with 3 doses of pre-farrow NPE resulted in significantly higher IgG and IgA levels in the colostrum and milk. RVA NPE stimulated antibodies in all groups regardless of treatment doses and prevented G4, G5, P[7] and P[23] RVA shedding prior to weaning. RVA is more prevalent and pathogenic compared to other porcine RVs, and every sow experience multiple RVA infections within their lifetime. Therefore, it is highly likely that sows harbor more RVA-specific memory B cells than RVC-specific memory B cells which upon repeated exposure to RVA antigens, proliferate and differentiate into RVA-specific antibody producing plasma cells. Control group gilts despite having no viral stimulation in the form of "NPE", prevented RVA fecal shedding in piglets in the farrowing room. Piglets born to group 3 gilts had higher serum IgA levels compared to other groups. Hence, we recommend using one dose of "RVA only NPE" at 5-weeks prior to farrowing. Results from this study expand our understating of the antibody response to RVA in swine and the role of NPE in providing lactogenic immunity to naïve piglets.

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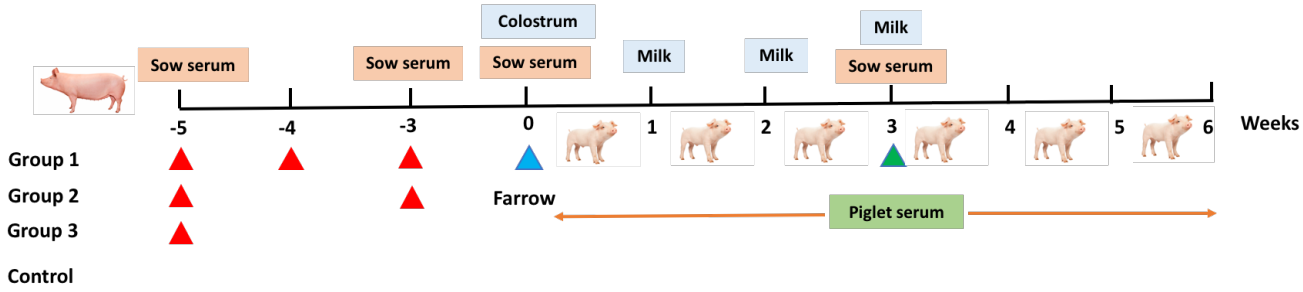
Figure 2.1.

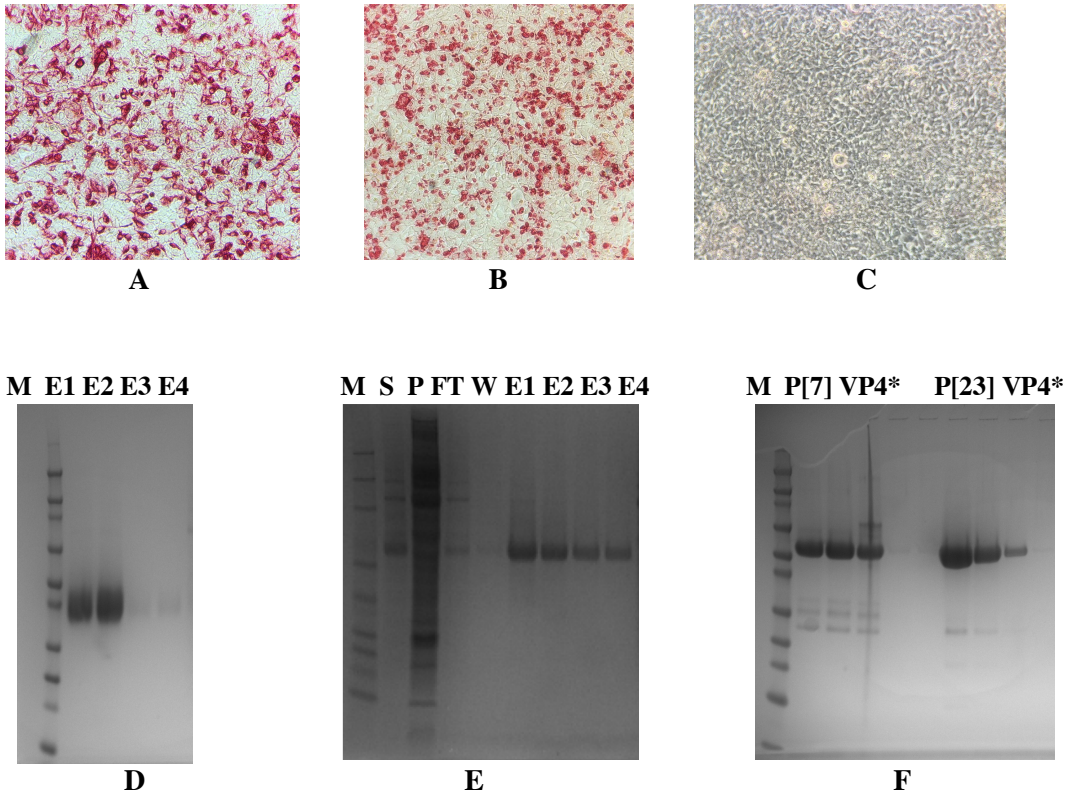
Feedback Administration and Blood Sample Collection Schedule



Note. Gilts (N=12 per group) and five piglets per gilt/litter were sampled individually.

Figure 2.2.

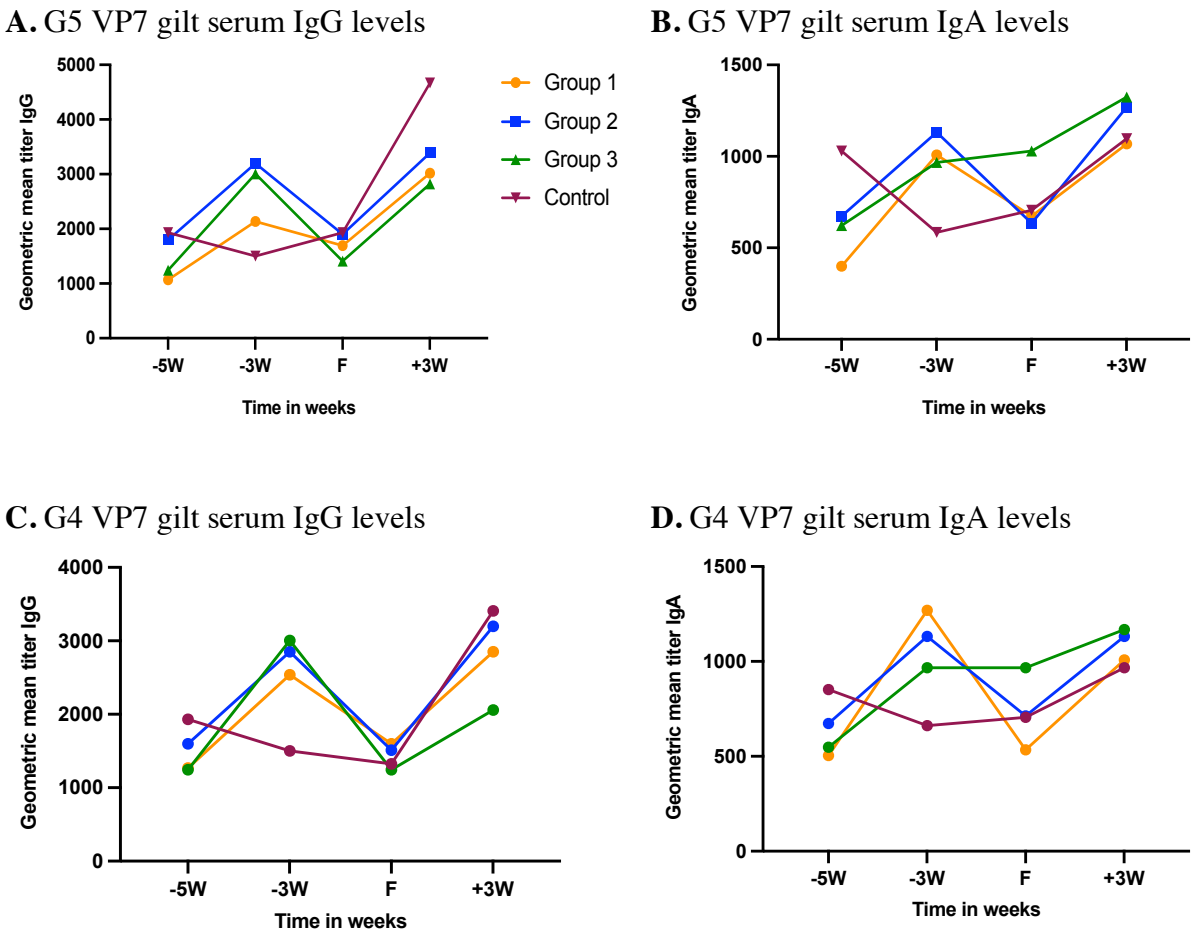
Expression and confirmation of Rotavirus A antigens



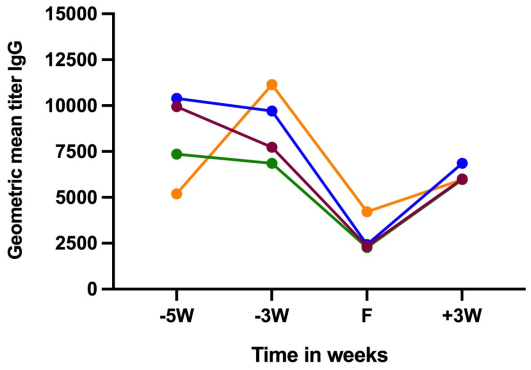
Note. Protein expression by the constructs encoding rotavirus A antigens was evaluated by immunocytometric analysis of HEK 293A cells. **(A).** Cells transfected with pcDNA3 constructs encoding G5 VP7 protein of rotavirus A. **(B).** Cells transfected with pcDNA3 constructs encoding G4 VP7 protein of rotavirus A. Transfected cells were probed with anti-his monoclonal antibody. **(C).** Negative control. **(D).** Purified G5 RVA VP7 (37kd), **(E).** Purified G4 RVA VP7 (37kd), M – Protein marker, S – culture supernatant, P – cell pellet, FT – flow through, E1 – E4 – protein elutes, **(F).** Purified P[7] and P[23] VP4* (55kd) protein elutes

Figure 2.3.

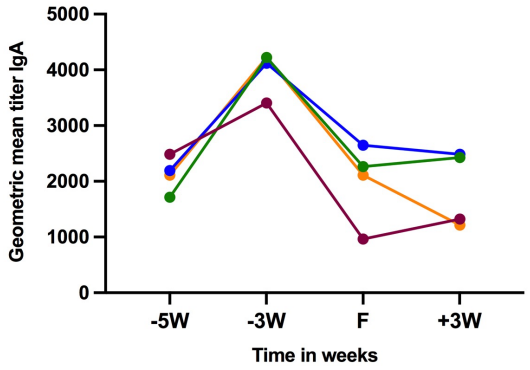
Longitudinal Gilt Serum Antibody Response to RVA NPE



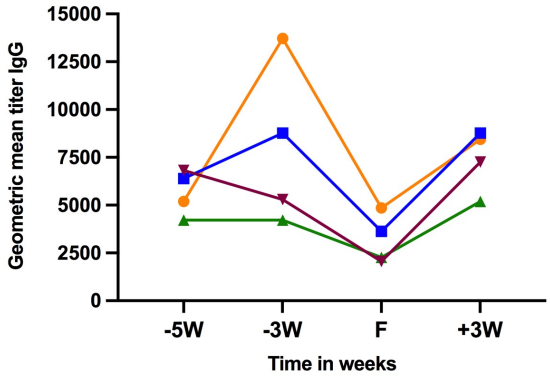
E. P[7] VP4* gilt serum IgG levels



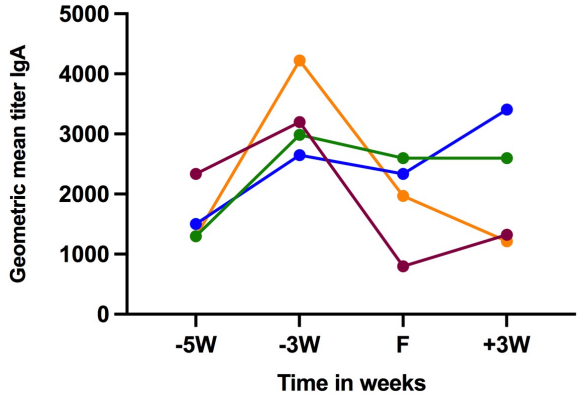
F. P[7] VP4* gilt serum IgA levels



G. P[23] VP4* gilt serum IgG levels



H. P[23] VP4* gilt serum IgA levels

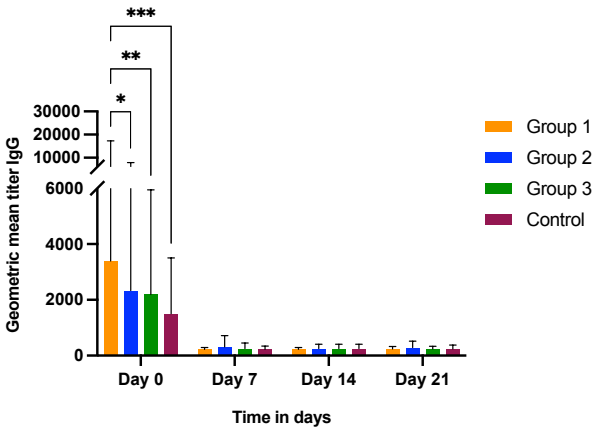


Note. Progression of RVA IgG and IgA levels over time in gilts receiving three (group 1), two (group 2), one (group 3) or no (group 4) doses of natural planned exposure. Gilts farrowed at week 0. Horizontal axis represents multiple sample collection time-points (-5W = 5 weeks pre-farrow; -3W = 3 weeks pre-farrow; F = at farrowing; +3W = 3 weeks post-farrow or at weaning). Vertical axis represents geometric mean antibody titers.

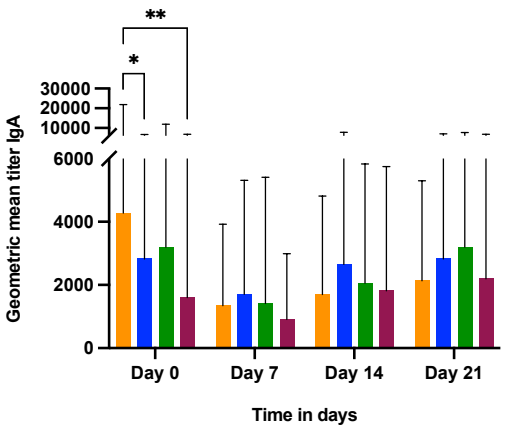
Figure 2.4.

Longitudinal Gilt/Sow Colostrum/Milk Antibody Levels against RVA

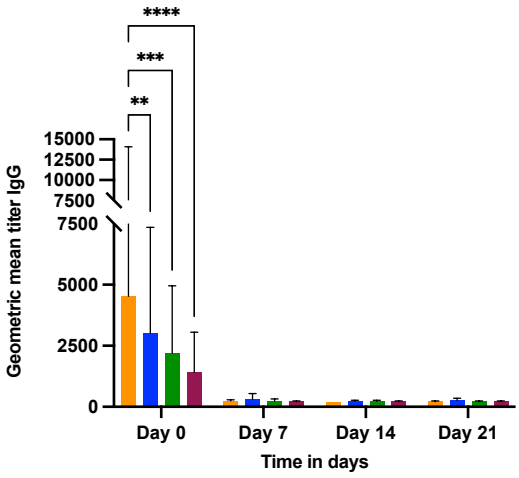
A. G5 VP7 colostrum/milk IgG levels



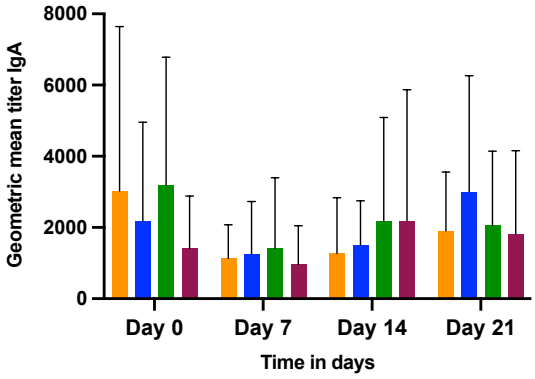
B. G5 VP7 colostrum/milk IgA levels



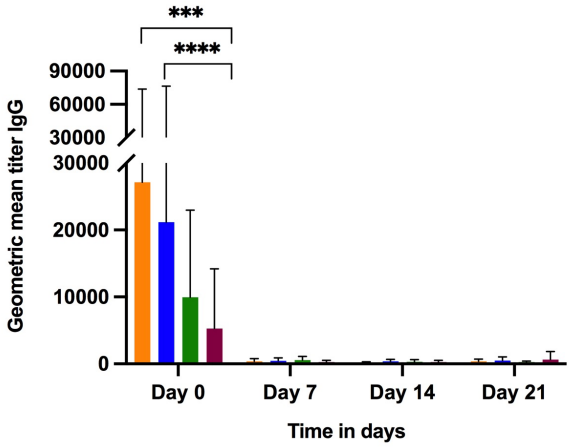
C. G4 VP7 colostrum/milk IgG levels



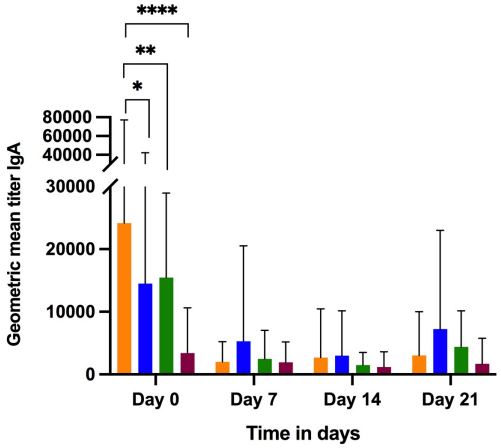
D. G4 VP7 colostrum/milk IgA level



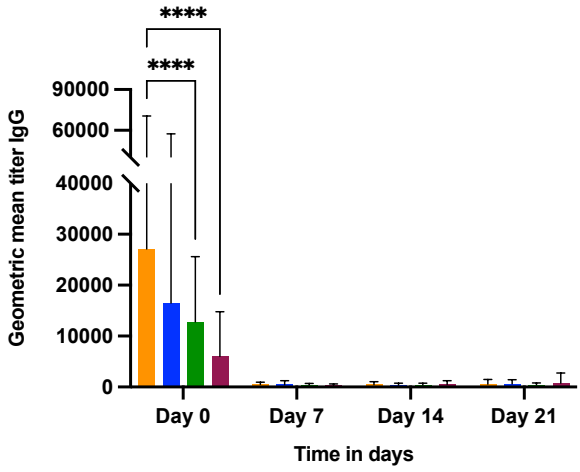
E. P[7] VP4* colostrum/milk IgG levels



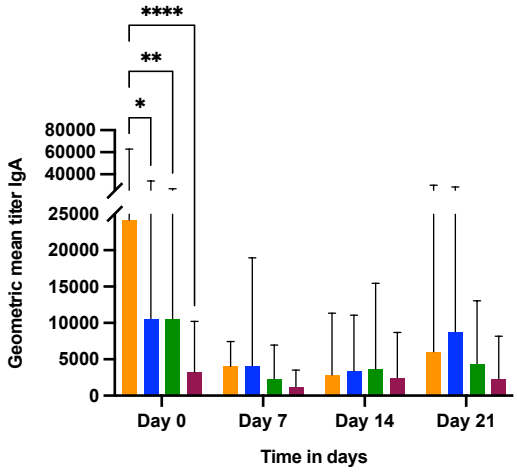
F. P[7] VP4* colostrum/milk IgA levels



G. P[23] VP4* colostrum/milk IgG levels



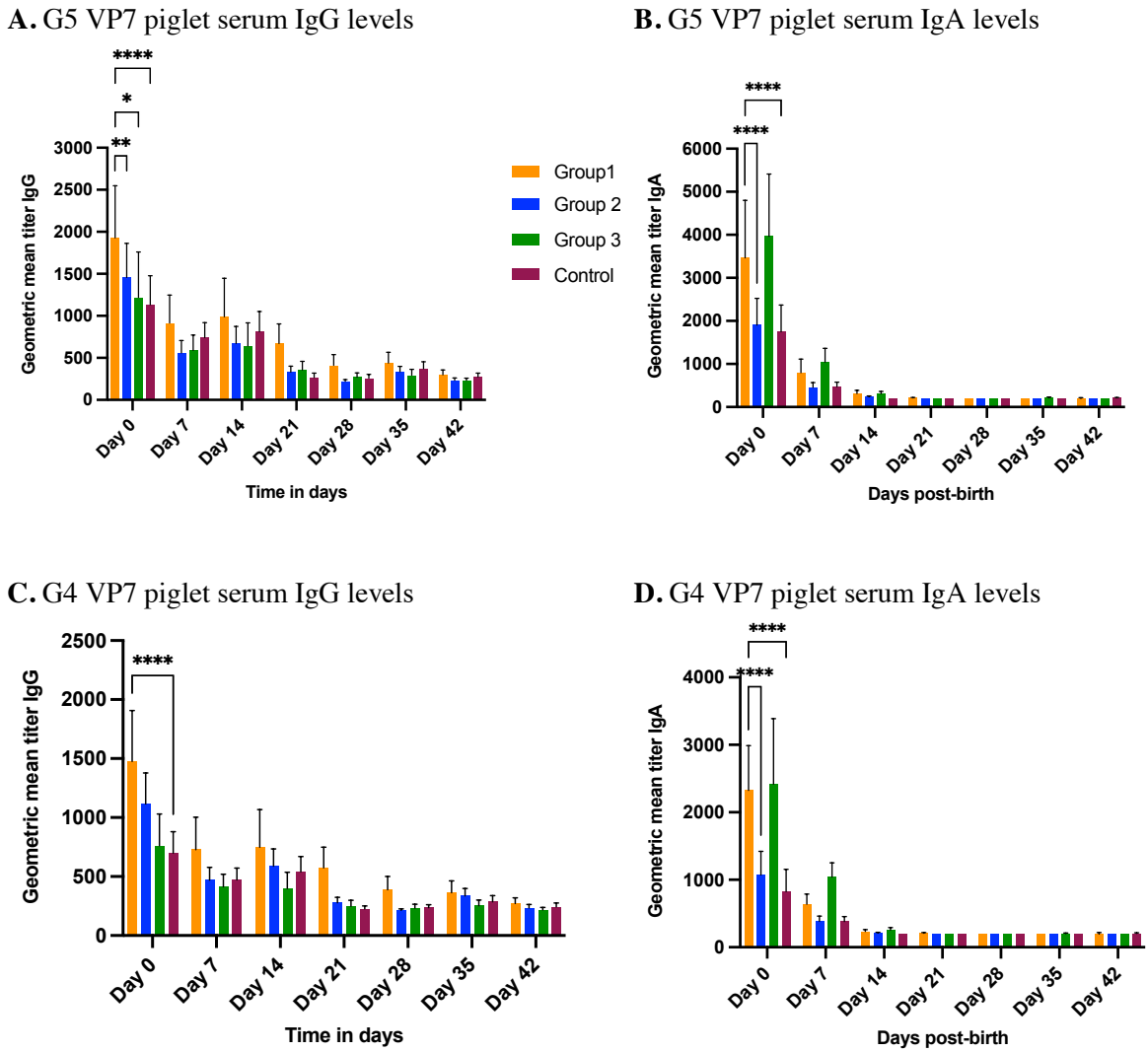
H. P[23] VP4* colostrum/milk IgA levels



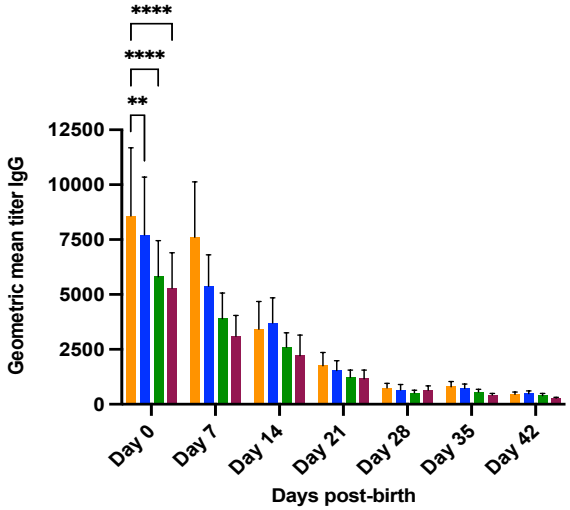
Note. Progression of RVA IgG and IgA levels over time in gilts colostrum/milk receiving three (group 1), two (group 2), one (group 3) or no (group 4) doses of natural planned exposure. Horizontal axis represents multiple sample collection time-points. Vertical axis represents geometric mean antibody titers.

Figure 2.5.

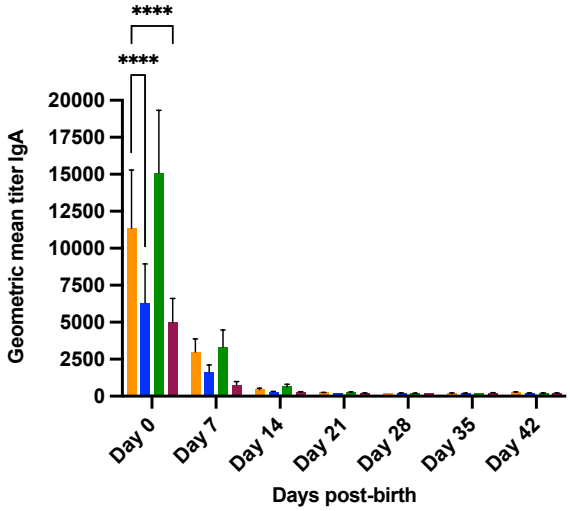
Longitudinal Piglet Serum Antibody Response to RVA NPE



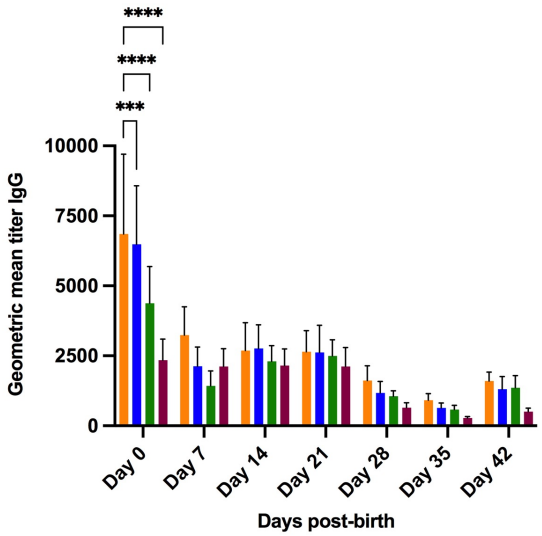
E. P[7] VP4* piglet serum IgG levels



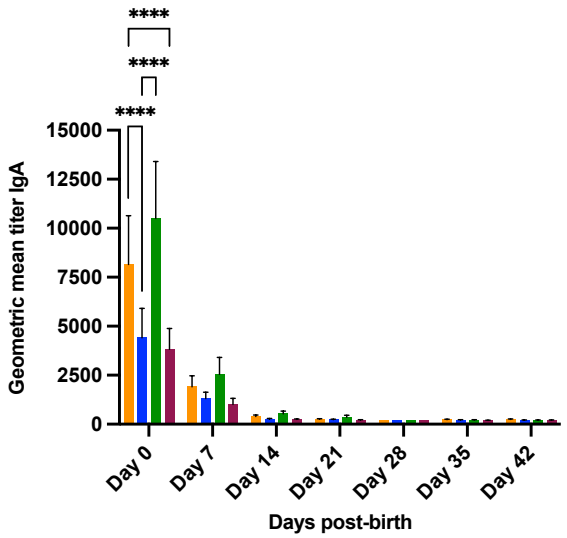
F. P[7] VP4* piglet serum IgA levels



G. P[23] VP4* piglet serum IgG levels



H. P[23] VP4* piglet serum IgA levels



Note. Progression of RVA IgG and IgA levels over time in piglet serum born to gilts receiving three (group 1), two (group 2), one (group 3) or no (group 4) doses of natural planned exposure. Horizontal axis represents multiple sample collection time-points. Vertical axis represents geometric mean antibody titers for respective study groups.

Table 2.1.

Piglet Fecal Samples Sequenced for RVA Variant Detection

Litter Id.	Group	Week	RVA Ct
41049	1	1	15.42
		2	18.62
40996	3	2	18.48
		3	17.28
41071	1	4	13.07
41045	2	4	11.29
40996	3	4	12.62
41053	4	4	12.15

Table 2.2

Antibody Levels of Two Piglets Shedding RVA in the Farrowing Room. Piglet Samples were Collected at Day 0 (week 0) and Then at Weekly Interval Until Week 6

	Colostrum IgA			**Piglet serum IgA levels at multiple time points**						
Litter	**Protein**	**Week 0**	**Week 0 group GMT**	**Week 0**	**Week 1**	**Week 2**	**Week 3**	**Week 4**	**Week 5**	**Week 6**
41049 (Group 1)	P[7]	12800	11353	22400	1600	200	200	200	1600	3200
	P[23]	25600	8145	12800	1600	400	200	200	1600	1600
	G5	800	3466	3200	200	200	200	200	200	200
Fecal RVA shedding (ct)				-	16.09	20.96	27.13	12.80	26.96	25.13
40996 (Group 3)	P[7]	25600	15097	12800	1600	1600	800	200	200	200
	P[23]	25600	10500	25600	3200	800	1600	200	200	200
	G5	800	3966	800	400	200	200	200	200	200
Fecal RVA shedding (ct)				-	28.09	20.09	15.5	14	22.62	18.07

Table 2.3.

Genome Constellation of RVA Strains Detected in Piglet Feces

Litter ID	Group	Week	RVA Ct	Genome constellation
40996	3	2	18.49	G11-P[34]-I5-R1-C1-M1-A8-N1-T7-E9-H1
		4	12.62	G9-P[23]-I5-R1-C1-M1-A8-N1-T7-E1-H1
41071	1	4	13.07	G9-P[23]-I5-R1-C1-M1-A8-N1-T7-E1-H1
41045	2	4	11.29	G9-P[23]-I5-R1-C1-M1-A8-N1-T7-E1-H1
41053	4	4	12.15	G9-P[23]-I5-R1-C1-M1-A8-N1-T7-E1-H1

Table 2.4.

Antigenic Variation in the VP7 Protein among the RVA Strains Recovered from Piglet Feces and Parent NPE Strains

	NE		NE		NE		NE	NE																						NE	NE
	87	90	91	92	94	95	96	97	99	100	103	119	122	123	124	126	143	144	147	152	189	190	192	208	209	210	211	212	213	214	216	221	223	242
G4-NPE	T	R	T	O	N	D	N	E	K	D	S	N	S	N	V	E	K	F	G	I	S	S	T	Q	T	T	N	A	N	T	E	S	K	X
G5-NPE	N	A	T	E	A	D	T	K	T	E	S	K	A	D	I	S	K	Y	N	M	T	S	T	S	T	T	D	I	N	S	E	A	K	X
G11-Piglet	N	A	R	E	A	.	D	K	K	D	.	K	T	D	I	S	.	Y	N	M	T	.	.	L	.	.	N	S	A	T	.	A	.	.

Table 2.5.

Antigenic Variation in the VP4 Protein among the RVA Strains Recovered from Piglet Feces and Parent NPE Strains

	VP8*																												VP5*						
	87	88	89	100	113	114	115	116	125	131	132	133	135	146	148	150	173	180	183	188	190	192	193	194	195	196	217	385	388	392	393	425	433	458
P[7]-NPE	T	V	E	D	Q	T	T	N	Q	E	N	T	Q	T	P	G	R	T	N	Y	S	T	N	Y	D	T	T	Y	A	G	A	R	G	Q
P[23]-NPE	S	N	A	D	P	S	E	S	Q	E	N	V	T	T	I	G	K	E	N	Y	T	T	N	Y	D	T	T	Y	R	G	A	R	E	G
P[34]-Piglet	K	N	D	.	I	S	T	T	.	S	.	M	T	Q	S	.	L	E	N	Q	T	S	.	.	S	E	T	.	R	.	K	.	L	D

Table 2.6.

Longitudinal RVA detection levels in piglets' feces

Study groups	Sow ID	Day 0 colostrum IgA		Day 0 piglet serum IgA		RVA ct values at different time points						
		P[7] RVA	GMT	Litter mean	GMT	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
Group 1	40399	3200	24163	6400	11353					13.04	34.81	15.24
	38868	51200		14080						15.43	29.13	26.74
	41031	6400		6800						12.19	26.67	22.36
	41071	25600		21760						13.30	24.55	24.90
	41049	12800		22400			16.09	20.96	27.13	12.80	26.96	25.13
	41267	51200		5440						13.65	27.78	22.65
	40973	25600		21333						14.18	24.94	25.30
	40652	204800		51200						13.49	24.23	22.76
	40956	51200		25600						12.89	28.50	19.85
	41262	25600		8200						12.09	31.52	26.47
	41144	6400		11200						14.45	26.20	22.38
	41181	51200		6720						12.46	24.16	17.09
Group 2	41289	51200	15221	25600	6315					13.55	28.64	23.47
	41030	25600		7200						13.08	27.36	15.29
	41052	3200		333						14.64	21.15	24.51
	40960	6400		3120						11.87	28.23	17.46
	41011	6400		12800						15.22	22.52	22.07
	41010	3200		7680						12.85	24.88	21.92
	40984	12800		4800						12.87	25.43	25.93
	41069	51200		20480						13.12	23.03	19.57
	41046	25600		3200						13.05	24.01	18.87
	40954	51200		8800						13.68	23.72	17.61

	41045	12800		16640					11.64	29.08	20.20
Group 3	41009	25600	14132	22400	15097				12.52	29.28	16.36
	41027	6400		1600					12.91	27.58	16.18
	40975	12800		25600					11.61	25.14	18.60
	40995	25600		12800					11.40	29.42	18.82
	41004	25600		38400					12.76	30.99	21.15
	40994	12800		21333					14.66	27.66	25.29
	41062	6400		21333					13.25	27.23	14.29
	41249	25600		18133					14.30	28.59	22.57
	41285	6400		11520					12.45	24.04	16.47
	40996	25600		23040		28.09	20.09	15.50	14.00	22.62	18.07
	41219	25600		8320					14.28	31.01	16.63
Group 4	40969	1600	3408	6400	4981				12.46	35.25	27.84
	41112	3200		2000					14.52	27.77	24.55
	41025	800		3840					14.70	23.80	16.80
	41014	12800		4800					14.02	21.91	17.15
	41053	3200		19200					13.39	18.58	24.17
	41070	3200		22400					14.12	27.94	19.68
	41174	3200		10560					14.03	28.47	20.10
	40964	1600		3680					13.89	29.02	20.49
	40979	800		4800					16.59	28.95	18.47
	41026	12800		6400						27.74	16.37
	41057	25600		2880					14.96	28.50	18.19

Note. Progression of levels of RVA detected over time in piglets' feces based on RT-PCR of fecal samples pooled by litter. Week 0 is farrowing, and piglets were moved to the nursery after the week 3 sample was collected. Colostrum P[7] RVA IgA levels, day 0 piglet litter mean serum P[7] IgA levels and group GMTs are also indicated.

Chapter 3 - Natural Planned Exposure Result in Low Genotype-specific Rotavirus C Antibodies in Gilt/Sow Colostrum and Piglet Serum

3.1 Introduction

Rotaviruses (RVs) are triple-layered, icosahedral, non-enveloped viruses belonging to the Rotavirus genus in the Reoviridae family. RV genome is approximately 18kb in size and consists of 11 segments of double-stranded RNA encoding six structural proteins (VP1-VP4, VP6 and VP7) and 5 non-structural proteins (NSP 1- NSP5/6) (Desselberger, 2017; Lestari et al., 2020) . The VP1, VP2, and VP3 proteins form the inner capsid of the virion. The middle capsid layer is made up of the VP6 protein while the outer capsid is composed of the VP7 and VP4 proteins (Ludert et al., 2002). VP7, a glycoprotein with a molecular weight of 37 kDa, constitutes 30% of the virus protein, and forms the smooth external surface of the outer shell. The minor component of the outer shell, VP4, is present as a series of spikes that project outward from the VP7 shell. VP4 is non-glycosylated, has a molecular weight of 88 kDa, and constitutes 1.5% of the virus protein ((Shaw et al., 1986)). Both VP7 and VP4 proteins independently induce neutralizing and protective antibodies (Ludert et al 2002). The VP4 is proteolytically cleaved into VP5 and VP8. The VP8* forms the spike that is used for host attachment and infectivity (Clarke & Desselberger, 2015; Ramani et al., 2016)). Both VP7 and VP4 proteins independently induce neutralizing and protective antibodies (Angel et al., 2007; Ludert et al., 2002) .

Currently, ten RV species, A through J (RVA-RVJ) have been classified on the basis of sequencing of the VP6 gene (Matthijnssens et al., 2008, 2012; Bányai et al., 2017). A binary classification system is used to address vast rotavirus diversity on the basis of sequencing of G

(VP7) and P types (VP4), which are also targets of neutralizing antibodies. The dual (G/P) tying system has been extended to a complete genome classification system based on nucleotide sequencing of all 11 RV segments with nucleotide percent identity cut-off values set for each segment. In this system, VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6 RV genes are designated as Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx (Matthijnssens, Ciarlet, Rahman, et al., 2008a). Out of 10 RV species, only species A, B, C, E, and H have been reported from swine ((Alekseev et al., 2018; Chasey et al., 1986; Homwong et al., 2016; Marthaler et al., 2014). RVC has been detected from a variety of sources including swine (Kattoor et al., 2017; Marthaler et al., 2014; Medici et al., 2018), humans (Kumazaki & Usuku, 2014; Tiku et al., 2017), cows (Soma et al., 2013), ferrets (Wise et al., 2009), cats (Otto et al., 2015), and dogs (Marton et al., 2015). Porcine RVC was first identified in 1980 and considered as an enteric pathogen with a moderate prevalence rate of between 4 and 31% (Saif et al., 1980). Previously, RVA was considered the most prevalent and pathogenic in swine, however recent data suggest that RVC is a major cause of diarrhea in neonatal pigs, particularly in piglets younger than 3 days old (Marthaler et al., 2013; Theuns et al., 2016). The G6 genotype (70%) is the dominant RVC genotype followed by G5 (17%), G1 (12%), and G9 (1%). The prevalence as high as 76.1% has been reported from the piglet population in the US (Chepngeno et al., 2019).

There is no in-utero transfer of antibodies in swine due to epitheliochorial placenta. Piglets are agammaglobulinemic at birth and their adaptive immune system is immature (Chepngeno et al. 2019). Hence, they are completely dependent on colostrum the intake of maternal derived antibodies in colostrum and milk (Langel et al., 2020). IgG and IgA produced in the sow traffics to the mammary glands and is transferred through colostrum and milk to piglets, where RVs are locally neutralized in the gut (Chepngeno et al., 2019; Gelberg et al., 1991; F. K. Shepherd et al.,

2020; Ward et al., 1996). IgG is the most prevalent immunoglobulin in colostrum and protects against systemic infections, while secretory IgA (sIgA) is prevalent in milk and is associated with the mucosal protection (Langel et al., 2020; Mantis et al., 2011).

Epidemiological data suggest that RVC infections are more prevalent among neonatal piglets than weaned piglets, however the reasons are not completely understood (Chepngeno et al., 2019; Marthaler et al., 2013). Likely reasons include lack of RVC vaccine for use in swine, insufficient maternal RVC antibodies in colostrum or low minimum infectious dose of RVC required for infecting piglets compared to other swine enteric viruses (Chepngeno et al., 2019). Despite being the most common cause of RV diahhrea in piglets less than 1 weeks of age, no vaccine is available for RVC due to inability of RVC to adapt to the cell culture. Recently a vectored virus vaccine platform known as Sequivity has been introduced by Merck animal health for use in pre-farrow gilts/sows against RVs. However, its field efficacy data is not available to assess the protection conferred to the swine against RVCs. Hence, natural planned exposure (NPE) to gilts prior and during pregnancy is the most widely used method of stimulating lactogenic immunity against RVs in the US (Pittman, 2016; F. K. Shepherd et al., 2020). RV-positive material from feces or intestines of sick piglets is fed to pregnant gilts and sows to boost antibody production against RVs. Studies investigating the efficacy of NPE protocols against RVC infection in swine are lacking. This study was carried out to investigate the maternal immunity induced by pre-farrow RV NPE to gilts/sows, passive lactogenic immunity to piglets and role of NPE in shaping the genetic makeup of RVC strains in piglet population.

3.2 Materials and Methods

3.2.1 Study design and sampling

Study design and sampling details have been described in Chapter 2 section 2.2.1. Briefly, pregnant gilts were randomly allocated into 4 study groups. Group 1 received 3 doses of NPE at 5, 4, and 3 weeks pre-farrow (WPF), group 2 received 2 doses of NPE at 5 and 3 WPF, group 3 received one dose of NPE at 5 WPF, and group 4 received no NPE (control group) (Figure 2.1). Forty-six litters (Group 1 = 12, Group 2 = 12, Group 3 = 11, Group 4 = 11) were evaluated for rotaviral fecal shedding and antibody titers. NPE material was created using the master seed method (Pittman 2016). Five piglets from each litter were selected for serum sample collection throughout the study. Blood samples from gilts were collected at weeks -5, -3, 0 (farrowing) and 3. Colostrum was collected at birth and milk was collected 1-3 weeks post farrowing. Blood samples from 5 piglets per litter were collected at weeks 0 (farrowing), 1, 2, 3, 4, 5, and 6 for a total of 7 blood samples per piglet.

3.2.2 Generation of protein expression constructs

NGS identified G6 and P[5] RVC genotypes in the NPE material. G6 VP7 was expressed using mammalian Expi293^{TM} Expression System (Gibco) and P[5] VP4* was expressed using bacterial expression system. Full length VP7 sequences of G6 genotype was modified to add in-frame 8-his tag and streptavidin tags at N and C terminals respectively to track protein expression and affinity purification of recombinant proteins. Gene sequences were codon optimized for mammalian expression. A kozak sequence was also added at N terminal to facilitate enhanced protein expression. Linker sequences were added just preceding each affinity tag. CD5 secretory signal was fused at N-terminal for efficient secretion of the recombinant protein into the culture

media. The synthetic rotavirus VP7 genes were subcloned into pcDNA3.1+ mammalian expression vector (InvitrogenTM). Truncated VP4* (aa26-476) of P[5] RVC was cloned in to pET-24a(+) vector with a linker followed by a 8-his tag at C-terminal. Codon optimization, gene synthesis, cloning into pcDNA3.1 (+) and pET-24a(+) vectors, and gene sequence validation was outsourced to Genscript.

3.2.3 Protein expression, purification and validation

Mammalian (Expi293) and bacterial expression of G6 and P[5] was carried as described in Chapter 2 section 2.2.3 with minor modifications. Recombinant proteins were purified using immobilized metal affinity chromatography (IMAC) using TALON Cobalt resin (Takara Bio) following a hybrid batch/gravity procedure as described in section 2.2.4 of Chapter 2 with modifications. The affinity purified proteins were quality control validated by Western blotting and pure protein fractions were pooled and concentrated using 10K protein concentrators as detailed in section 2.2.4 of Chapter 2. Contentrated proteins were quantified using BCA assay and stored in -80℃ until further use.

3.2.4 Development of recombinant protein ELISAs to quantitate RVC antibodies

Indirect ELISAs were individually optimized to detect genotype-specific RVC IgG and IgA antibodies in porcine serum and colostrum/milk. A checkerboard titration method was used to determine optimal coating protein concentration for each protein and secondary antibody concentrations. Various ELISA parameters such as coating antigen concentration, blocking condition, secondary antibody concentration (anti-IgG and anti-IgA), ELISA plate incubation time and temperature, and plate washing steps were optimized as detailed in as described in section

2.2.5 of Chapter 2. The ELISA antibody titer was expressed as the reciprocal of the highest dilution that had a A410 value greater than twice the mean of negative control wells.

3.2.5 Screening serum and milk of gilts/sows and piglet serum for RVC antibodies using genotype-specific ELISA

Blood samples were centrifuged at 2000g for 15 min to obtain serum and stored at −80 °C until use. Colostrum and milk samples were centrifuged at 5000g overnight at 4C to separate fat, debris and whey. Fat layer was carefully separated using sterile pipette tips and clear fluid (whey) was collected in sterile 2 ml Eppendorf tubes. Whey was stored in minus 80 until further use. To determine endpoint titer of RVC IgG and IgA antibodies, serum and colostrum/milk samples were serially diluted (1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400, 1:12800 and 1:25600) in 5% NFDM prepared in 1X PBST and added (100 µl) in duplicates to the wells of overnight protein coated, blocked and washed immunoassay plates. Washing, incubation time and temperature, and substrate conditions were same as detailed in the section 2.2.6 of chapter 2. Anti-porcine IgG (1:10,000 in 5% NFDM, 100 µL) and IgA (1:3,000 diluted in 5% NFDM 1X PBST, 100µl) conjugated to horseradish peroxidase (Abcam) was added to each well and incubated at 37 °C for 1hour. The end point titer was expressed as the reciprocal of the highest dilution that had a A410 value greater than twice the mean of negative control wells. If any sample had antibody titer more than the higher end of dilution range (>1:25,600), that sample was retested with more dilutions (1:25,600 -1:102,400). Each ELISA plate had a serially diluted positive and negative control to control plate to plate variation. Since, true positive controls (antiserum against each protein) were not available, few high titer serum samples were pooled and used as positive control throughout the ELISA testing to maintain uniformity.

3.2.6 Next generation sequencing of RVC strains in NPE material and piglet feces

Whole genome sequencing (WGS) of RVC strains in the NPE material was conducted at Molecular NGS laboratory at Kansas State Veterinary Diagnostic Laboratory (KSVDL), Kansas State University. Piglet fecal samples were chosen for sequencing from weeks 0-4 samples to assess viruses shed in the presence of lactogenic immunity (Table 2). Litters from which RVC was detected for multiple weeks in a row with Ct values less than 26 were selected for sequencing. A total of 30 piglet fecal samples from all 4 groups were submitted for sequencing. WGS of piglet fecal samples was conducted at Centers for Disease Control and Prevention (CDC), Atlanta, Georgia.

3.2.7 Statistical Analysis

The significance of the differences between the treatment and the control groups was determined by two-way Analysis of Variance (ANOVA). Statistical analysis was performed using GraphPad Prism 7 (Version 7.04, GraphPad Software, Inc. La Jolla, CA) and a significance level of $p < 0.05$ was used for all analyses.

3.3 Results

3.3.1 Recombinant protein expression and ELISA optimization

G6 protein was efficiently secreted into the Expi293 culture media. An estimated 37-kDa and 55-kDa bands corresponding to the expected molecular weight of recombinant VP7 and truncated VP4* proteins were detected on SDS-PAGE (Figure 3.1 A and B). Protein size and specificity was confirmed by a Western Blot using anti-His monoclonal antibodies (Figure 3.1 C and D). Immunocytometric analysis of HEK-293A cells transfected with pcDNA3.1 (+) plasmid carrying G6 gene and probed with anti-his monoclonal antibodies confirmed protein expression

(Figure 3.2). Protein concentrations of 50ng (G6 VP7) and 100ng (P[5]) resulted in optimal ELISA OD value readouts. Blocking the ELISA plates with 5% NFDM prepared in 1x PBST with 0.05% Tween-20 and four washings after each incubation step resulted in minimal background. The optimal incubation temperature and time combination for samples (serum/colostrum/milk) and secondary antibodies was at 37℃ for 1hr. Concentrations of 1:10,000 and 1:3,000 for peroxidase conjugated IgG and IgA were found optimal.

3.3.2 Antibody response to RVC NPE

3.3.2.1 Gilt serum

All gilts irrespective of the study group had some levels of anti-RVC antibodies before the administration of 1st NPE dose at 5-weeks pre-farrow (WPF) (Figure 3.3 A-D). Geometric mean titers (GMT) IgG levels at 5WPF for G6 and P[5] genotypes were in the range of 514.67- 1198.55 and 2262.74 – 5079.68, respectively (Figure 3.3 A and C). Two doses of NPE in group 1 (5 and 4 WPF) and one dose each in group 2 and 3 (5WPF) resulted in elevated IgG levels at 3WPF for both G6 and P[5]. Control group IgG levels at 3WPF showed a minimal increase in the absence of NPE. Serum IgG levels dropped sharply in all treatment groups at farrowing (F) followed by a quick rebound until 3-weeks post-farrowing (weaning) for both proteins. P[5] IgG GMTs at 3WPF in all treatment groups were at least 5-fold higher than G6 IgG levels (Figure 3.3 A and C).

GMT IgA levels at 5WPF were G6 (GMT 237.44 – 514.67) and P[5] (GMT 236.77 – 503.97) (Figure 3.3 B and D). Gilt serum IgA levels for both proteins in treatment groups increased at 3WPF after respective NPE doses. Control group gilt serum IgA levels for G6 decreased at 3WPF and showed a slight increase for P[5] at 3WPF (Figure 3.3 B and D). Similar to IgG levels, IgA levels also increased sharply post-farrowing until weaning.

3.3.2.2 Colostrum and milk

Colostrum and milk samples were collected at farrowing (day 0) and then at weekly interval until weaning (days 7, 14 and 21). At day 0, treatment group 1 and 2 had significantly higher G6 IgG Ab titers compared to group 3 (1 NPE) and numerically higher IgG levels than the control group (Figure 3.4 A). Group 1 P[5] IgG Ab titers at day 0 were significantly higher compared to groups 2, 3 and the control group (Figure 3.4 C). Group 2 and 3 also had significantly higher IgG GMT levels than the control group at day 0 (Figure 4C). Overall at day 0, group 1 had significantly higher colostrum IgG titers for both antigens compared to the treatment groups, and either significantly or numerically higher IgG titers than the control group (Figure 3.4 A and C). As expected, the colostrum IgG levels for both antigens were highest at day 0, which rapidly declined and reached the baseline at day 7, and remained so during the subsequent sampling points (Figure 3.4 A and C).

Day 0 G6 IgA GMTs were not significantly different for any of the study groups (Figure 3.4 B). However, group 1 had numerically higher IgA GMTs compared to other groups at day 0 (Figure 3.4 B). In contrast, colostrum P[5] IgA titers were significantly higher for group 1 compared to group 3 and control group and numerically higher than group 2 (Figure 3.4 D). Overall, IgA titers declined at day 7 and then gradually increased until weaning (Figure 3.4 B and D). Group 2, 3 and control IgA titers for both proteins at weaning matched or exceeded their titers in colostrum at day 0 (Figure 3.4 B and D). However, treatment group 1 IgA titers for both antigens did not reach the colostrum IgA levels at day 0 (Figure 3.4 B and D). Lastly, the IgG and IgA VP4* (P[5]) titers were manifold higher than IgG and IgA VP7 (G6) titers (Figure 3.4 A-D).

3.3.2.3 Piglet serum

Piglet serum samples were collected at birth and then at weekly interval until 6 weeks of age (day 42). At birth (day 0), none of the study groups had significantly different G6 IgG levels, which ranged from 729.38 (lowest) for control group to 810.76 (highest) for group 1 (Figure 3.5 A). However, day 0 group 1 P[5] IgG levels were significantly higher (GMT 4177.68) than group 2 (GMT 2914.92), 3 (GMT 3307.38) and control (GMT 2527.28) (Figure 3.5 C). P[5] IgG GMTs of group 1 remained significantly higher than other groups at day 7 too (Figure 5 C). G6 IgG levels decreased at day 7 followed by a gradual decrease until day 28 (Figure 3.5A). An increase in G6 IgG titers for all study groups was observed days 35 and 42 of piglets' age (Figure 3.5 A). P[5] IgG titers for all groups declined post-birth reaching the baseline (serum dilution 1:200) at day 28 (Figure 3.5 C). Similar to G6 IgG levels, an increase in P[5] IgG levels was observed at days 35 and 42 of sample collection (Figure 3.5 C).

At day 0, group 1 G6 IgA piglet serum levels were higher than all other groups although levels were not significantly different (Figure 3.5 B). However, group 2 G6 IgA levels were significantly different compared to group 3 and group 3 IgA levels were significantly different than control group at day 0. (Figure 3.5 B). Overall, serum G6 IgA levels of all four groups were highest at day 0, which rapidly declined at day 7 and subsequently reached the baseline (1:200). Group 1 P[5] IgA levels were significantly higher (GMT 2235.38) than group 2 (GMT 1665.28), 3 (GMT 1766.54) and control group at day 0 (GMT 1065.19) (Figure 3.5 D). Both group 2 and 3 P[5] IgA levels were also significantly higher than control group at day 0. Interestingly, group 1 P[5] IgA levels remained significantly higher than other study groups at day 7 (Figure 3.5 D). Overall, G6 and P[5] serum IgA levels showed minimal increase post-weaning compared to serum IgG levels (Figure 3.5 A-D).

3.3.3 Levels of antibodies against RVC were lower than RVA in colostrum

RVA antibody levels for sow colostrum/milk have been discussed in detail in Chapter 2. In this section, RVA and RVC antibody levels in colostrum (day 0) for all study groups are compared. For all 4 study groups, colostrum RVC IgG and IgA titers were lower than RVA titers irrespective of G and P-type (Figure 3.6 A-D). G4/G5 RVA IgG and IgA levels for all 4 groups were numerically higher than G6 RVC levels (Figure 3.6 A-D). Specifically, for group 1, P[7] and P[23] RVA antibody levels were either significantly (IgA) or numerically (IgG) higher than P[5] RVC antibody levels (Figure 3.6 A). In group 2, P[7] and P[23] RVA had significantly higher IgG and numerically higher IgA levels than P[5] RVC levels (Figure 3.6 B). Similar to group 1, group 3 also had significantly higher P[7]/P[23] RVA IgA and numerically higher IgG levels than P[5] RVC (Figure 3.6 C). Control group P-type RVA IgG and IgA levels were numerically higher than P[5] RVC (Figure 3.6 D). VP4* (P-specific) IgG and IgA titers were manifold higher than VP7 (G-specific) antibody titers for all 4 groups (Figure 3.6 A-D).

3.3.4 Piglet serum at birth has lower antibodies against RVC than RVA

RVA antibody levels in piglet serum have been discussed in detail in Chapter 2. For all 4 study groups, day 0 piglet serum RVC IgG and IgA titers were lower than RVA titers irrespective of G and P-type (Figure 3.7 A-C). For group 1 and 2, day 0 P[7] and P[23] RVA IgG and IgA levels were significantly higher compared to P[5] RVC antibody levels (Figure 3.7 A and B). G4 and G5 RVA antibody levels were either significantly or numerically higher than P[5] RVC antibody levels (Figure 3.7 A and B). Importantly, control group P[7] and P[23] RVA IgA levels were significantly higher compared to P[5] RVC IgA levels (Figure 3.7 C). P[7] RVA IgG levels were also significantly higher than P[5] RVC IgA levels. No significant difference was observed between P[23] RVA and P[5] RVC IgG titers (Figure 3.7 C).

3.3.5 RVC fecal shedding in piglets and association with antibody levels

Realtime PCR of feedback (NPE) material revealed RVC ct-values of 32.55, 29.32 and 30.30 for feedback 1 (5WPF), 2 (4WPF) and 3 (3 WPF), respectively. Gilt and piglet RVC fecal shedding results have been described in detail earlier and also summarized in the supplementary table 1 (Shepherd, 2020b). As expected, piglets' fecal swabs collected within 24 hours of farrowing were negative for RVC by qRT-PCR. RVC was first shed at week 1 in all 4 groups, but high viral load of RVC (low ct-values) were only observed in the control group piglets (Table 3.3). Multiple litters in all 4 groups shed RVC before weaning. At week 1, when RVC was first detected, the piglet pools from control gilts contained the most positive litters (58%), while 17%, 42% and 9% of litters in groups 1, 2, and 3 were positive, respectively. Higher number of RVC positive litters and high viral load at week 1 correlated with low colostrum IgA G6 and P[5] IgA colostrum IgA levels. Although, G6 piglet serum IgA levels at day 0 did not differ significantly between the groups, control group had lowest P[5] IA titers in piglet serum compared to the 3 treatment groups. Analyzing the day 7 piglet serum revealed that litter P[5] IgA titers lower than 800 significantly correlated with litter being RVC positive.

3.3.6 Sequence analysis of RVC from NPE and piglet feces

A total of 30 pre-weaning piglet fecal samples representing pre-weaning RVC shedding by all 4 groups were sequenced to investigate genetic changes in response to lactogenic immunity. Complete RVC genome could only be recovered from 11 fecal samples representing treatment group 2, 3 and control group piglets (Table 3.1). Sequencing revealed a RVC G and P-type combination of G6P[5] from all 11 samples regardless of the shedding week (1, 2 or 3), which was similar to the RVC genotypes present (G6, P[5]) in the original NPE material fed to the gilts. Sequence analysis revealed very high nucleotide (98.62-99.90%) and amino acid (98.22-100%)

percent identity between 11 G6 VP7 sequences from piglet feces and parent G6 NPE strain. Six G6 sequences completely matched the G6 NPE strain. To determine the sequence variation, the neutralizing epitopes of the 11 G6 sequence recovered from piglet feces were compared to the parent G6 strain. Five G6 sequences illustrated deletions at 245-247 amino acid positions and also differed with the parent G6 strain at one amino acid position 248 (L248I) (Table 3.2). Other 6 G6 sequences from piglets completely matched the parent G6 strain. Similarly, P[5] VP4 sequences from piglet feces shared very high nucleotide (99.09-100%) and amino acid (99.11%) percent identity with parent P[5] strain in the NPE material. VP4 sequences from piglets only carried 4-point mutations at amino acid positions 41 (T41I), 203 (I203L), 262 (R262W) and 350 (G350D).

3.4. Discussion

RVC infections are a major cause of diarrhea in suckling neonatal piglets, particularly in 0-3 days old piglets (Homwong et al., 2016; Marthaler et al., 2013), however, the reasons are not completely understood (Chepngeno et al., 2019). Possible reasons include insufficient maternal RVC antibodies in colostrum, lack of RVC vaccine for use in swine, distinct pathogenic mechanism compared to RVA, or low minimum infectious dose of RVC compared to other swine enteric viruses (Chepngeno et al., 2019). RVC is extremely difficult to adapt to cell culture and very few reports exist of its successful propagation in-vitro (Saif et al., 1980). Since a modified live or live attenuated vaccine is lacking against RVC in swine, NPE is the only cost-effective method of stimulating passive lactogenic immunity to protect piglets against RVs. However, due to lack of serological tools to detect RVC antibodies, it is impossible to truly assess the effectiveness of NPE protocols. There is only one report of RVC virus like particle (VLP) based ELISA to detect genotype-specific RVC antibodies in gilt/sow serum and lacteal secretions (Chepngeno et al., 2019). To fill this knowledge gap, we optimized VP7 (G6) and truncated VP4*

(P[5]) specific indirect ELISAs and investigated antibody responses against RVC in gilts after NPE and passive immunity in their piglets. To our knowledge, this is the first longitudinal study to investigate antibody levels against RVC in gilts/sows (pre and post-farrow serum), colostrum/milk, and their piglets at multiple time-points.

In gilt serum, 2 doses of NPE in group 1 and 1 dose of NPE in groups 2 and 3 resulted in increased IgG and IgA levels at 3WPF reflecting the development of active immunity against RVC in gilts. Antibody levels dropped at farrowing (F) suggesting transport of RVC-specific antibodies into the colostrum. Drop in gilt serum IgG levels was more distinct compare to serum IgA levels (Figure 2 A-D). Similar trend was observed for RVA antibody levels at farrowing and has been explained in section 2.4 of chapter 2. Possible reasons for this difference could be the release of IgA synthesized in mammary parenchyma into the gilt/sow serum or reduced transportation of serum IgA into exocrine fluid (Klobasa et al.1985). A study found increased sow serum IgA levels against RVA during last weeks of gestation in contrast to serum IgG levels, which dropped sharply at farrowing (Klobasa et al. 1985). However, the difference between serum IgG and IgA levels at farrowing may not truly indicate their respective levels in colostrum, as only 24-54% of IgA in colostrum comes from serum whereas all colostral IgG is derived from serum in swine (Bourne & Curtis, 1973).

Since no intra-uterine passage of immunoglobulins occur in swine during gestation, piglets are born agammaglobulinemic and uptake of pathogen-specific colostrum/milk immunoglobulins is critical for their survival during first few days of life (Chepngeno et al., 2019; Matías et al., 2017; Moffett & Loke, 2006). We found that group 1 has significantly or numerically higher colostral (day 0) IgG and IgA titers compared to other groups for both antigens, suggesting that 3 doses of NPE administered to group 1 gilts prior to farrowing was able to better stimulate maternal

immunity compared to other NPE doses. In contrast, control group gilts (no NPE) had lowest colostral IgG and IgA levels resulting in highest RVC fecal shedding (58%) in piglets at day 7 of age compared to 17%, 42% and 9% in groups 1, 2 and 3, respectively. Rapid drop in the colostrum IgG levels in day 7 milk occurred in parallel with rapid rise in sow serum IgG titers post-farrowing until weaning. On the contrary, IgA levels in milk increased steadily post day 7 until day 21, suggesting the increased local production of RVC-specific IgA in mammary glands and subsequent secretion in the milk. Similar trends of colostrum/milk IgG and IgA were observed against multiple genotypes of RVA post-farrowing as reported in Chapter 2. For example, group 1 gilts with 3 doses of NPE had higher IgG and IgA levels for both RVC and RVA compared to other treatment groups. Interestingly, G6 and P[5] RVC milk IgA levels for all groups at day 21 mirrored the gilt/sow serum antibody levels at day 21 (Figure 3 and 4). For instance, group 2 and 4 IgA levels were higher than group 1 and 3 in day 21 sow serum, which was reflected in higher milk IgA levels for group 2 and 4 at day 21.

Data regarding lactogenic protection against porcine RVC is very rare and most of the swine RV lactogenic immunity studies have been done for RVA (Fu et al. 1990; Ward et al. 1996). Recently, RVC antibody titers in gilt/sow milk and serum samples were reported using genotype-specific and cocktail of genotype-specific virus like particles (VLPs) based indirect ELISAs (Chepngeno et al., 2019). Authors reported no difference in levels of IgG and IgA against RVC G6 genotype in milk collected after 2-11 days of farrowing. Similar to Chepngeno study, we also observed that control group gilts (no NPE) had similar G6 RVC-specific IgG and IgA levels in day 0 colostrum samples. Comparison of antibody titers against RVA and RVC in colostrum (day 0) revealed that for all 4 study groups, anti-RVC antibody titers were lower (significantly or numerically) compared to RVA titers irrespective of the G and P-type. Variation in antibody levels

to RVA and RVC in colostrum could be due to differences in respective virus replication in gilts as evidence by RV gilt fecal shedding results before farrowing (Anderson et al. 2022). For RVA, 1st dose of NPE in treatment groups resulted in 71.4% (25/35) gilts shedding RVA at 4.5 weeks pre-farrow in comparison to only 20% (7/35) gilts shedding RVC after 1st of NPE. (Frances et al 2020). Overall, NPE administration in treatment groups resulted in higher levels of RVA shedding in gilts compared to RVC.

Significantly higher group 1 P[5] antibody levels in day 0 and 7 piglet serum mirrored higher P[5] IgA levels of group 1 in colostrum suggesting that 3 dose of NPE stimulated slightly better antibody response against RVC than other NPE regimens. However, G6 IgG levels in day 0 piglet serum were not significantly different for any group and G6 IgA levels were in a narrow range for all groups. Significant differences in antibodies against RVA and RVC levels in day 0 piglet serum were also observed. Importantly, serum RVC IgA titers in day 0 piglet serum were significantly lower than RVA titers irrespective of G and P-type (Figure 7 A-C). We hypothesize that higher ct-value of RVC (ct-values 32.55, 29.32 and 30.30) compared to RVA (ct 24.43, 22.46 and 24.15) in the NPE material failed to induce sufficient immunity and resulted in low colostrum RVC antibody titers and failed to passively protect piglets from natural RV infection in the farrowing room. RV fecal shedding data from piglets also support this assumption. Only two litters (5.8%, 2/34) shed RVA prior to weaning (Anderson et al. 2022) compared to 8 litters (23.5%, 8/34) at day 7 and 18 litters (53%, 18/34) each at day 14 and 21 for RVC. Very less RVA shedding in the farrowing room suggest that the better passive immunity was induced against RVA, which had the lower ct-values in the NPE compared to RVC.

Low RVC antibody titers generated in gilts are known to be associated with higher rates of clinical disease in piglets (Chepngeno et al., 2019). We also found that litter P[5] IgA GMTs less

than 800 were positively correlated with litter being tested RVC positive. Interestingly, we observed that day 0 piglet serum P[7] and P[23] RVA IgA levels in the control group were significantly higher compared to P[5] RVC IgA levels (Figure 7C). This finding is significant because control group gilts did not receive pre-farrow NPE and higher anti-RVA IgA levels in piglets at birth suggest higher passive immunity to RVA than RVC even in the absence of NPE administration to gilts.

NGS detected G and P-type combination of G6P[5] in pre-weaning piglet fecal samples which was similar to the RVC genotypes present (G6, P[5]) in the original NPE material fed to the gilts. Moreover, high nucleotide and amino acid percent identities and very few point mutations among RVC strains from piglets reiterate that lactogenic immunity stimulated by the RVC NPE was not sufficient to prevent piglets from RVC infections in the farrowing room. In contrast, we earlier identified a G and P-type RVA combination (G11P[34]) in two pre-weaning samples shedding RVA, which was different from the genotypes present (G4, G5, P[7] and P[23]) in the original NPE material fed to the gilts (Chapter 2 section 2.3.3). The VP7 and VP4 proteins of RVs independently elicit neutralizing and protective antibody response (Nair et al. 2017; Estes and Greenberg, 2013). We observed that RVC IgG and IgA levels induced by VP4* were higher compared to VP7 protein, which is possibly due to more neutralizing epitopes on VP4 compared to VP7. We have earlier reported similarly high levels of antibodies against VP4* than VP7 for RVA (Chapter 2 section 2.4).

In summary, treatment group one with 3 doses of pre-farrow NPE resulted in significantly higher anti-RVC antibody levels in colostrum. Although 3 doses of NPE appear better in stimulating lactogenic immunity, none of the NPE doses were able to prevent RVC shedding by piglets in the farrowing room, reflecting higher ct values of RVC in the NPE material. Finally, our

results suggest that gilt/sow colostrum and piglet serum contain significantly lower levels of antibodies to RVC than RVA, which possibly explains higher prevalence of RVC in neonatal piglets. Since RVA is more prevalent in swine farms and gilts/sows normally carry higher levels of antibodies against RVA than RVC, it is proposed to administer only RVC NPE to gilts at 5,4, and 3 weeks before farrowing. Also, more research is required to find ways to increase RVC load in the NPE material. Results of this study expand our understating of the antibody response to RVC in swine and the role of NPE in providing lactogenic immunity to naïve piglets.

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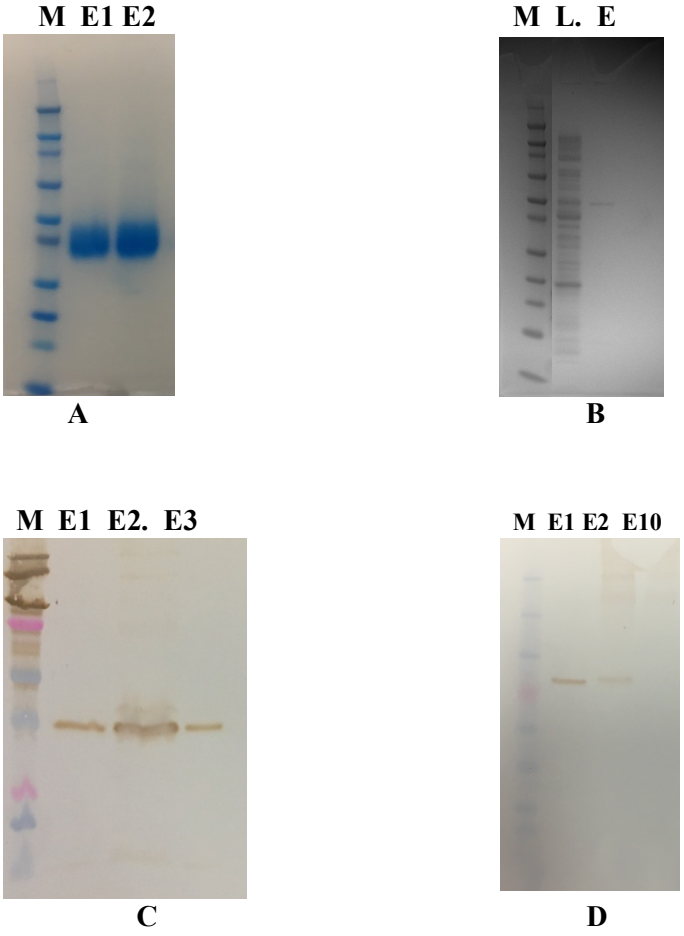
Figure 3.1.

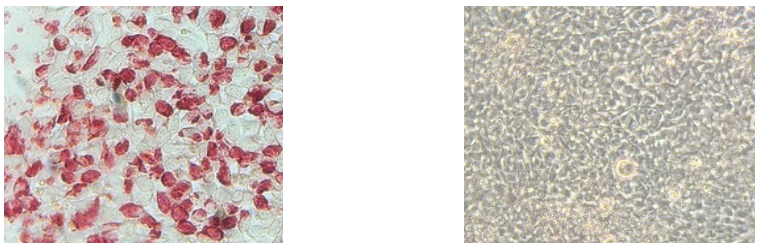
SDS PAGE and Western Blot Confirmation of Affinity Purified Proteins



Note. **(A)** SDS-PAGE of purified G6 VP7 (37kd), **(B).** SDS-PAGE of purified P[5] VP4* (55kd), (C) Western blot confirmation of G6 VP7, (D) Western blot confirmation of P[5] VP4* M – Protein marker, E1, E2, E3 and E10 – protein elutes, L - lysate

Figure 3.2.

Protein Expression by RVC Construct



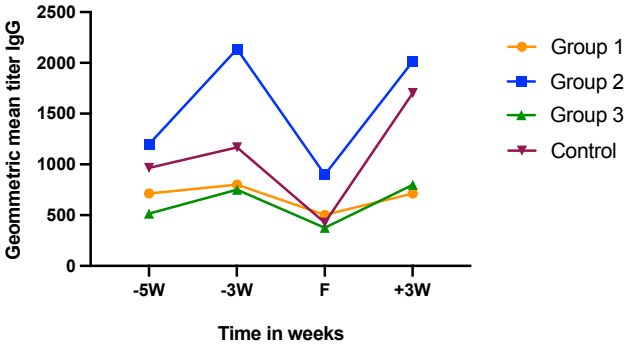
A **B**

Note. Protein expression by the constructs encoding RVC VP7 was evaluated by immunocytometric analysis of HEK 293A cells. **(A).** Cells transfected with pcDNA3 constructs encoding G6 VP7 protein of rotavirus A. Transfected cells were probed with anti-his mnoclonal antibody. **(B).** Negative control

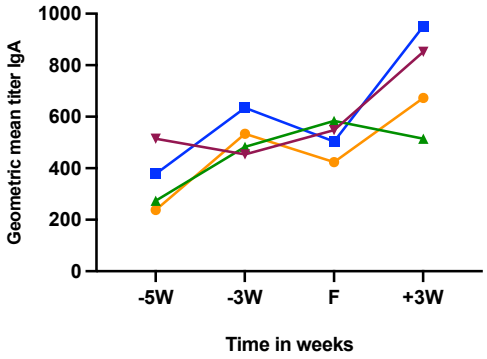
Figure 3.3.

Kinetics of Longitudinal Gilt Serum Antibody Response to RVC NPE

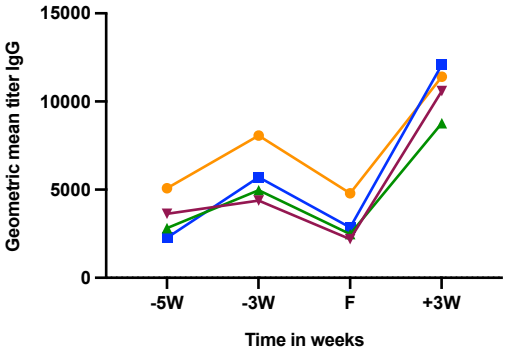
A. G6 VP7 gilt serum IgG levels



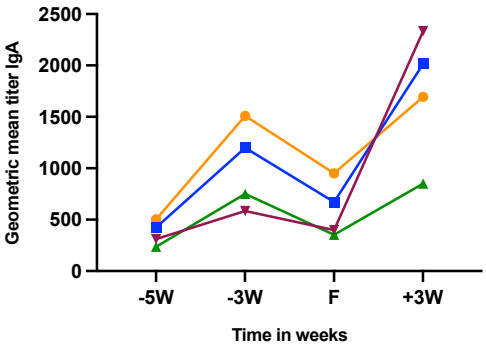
B. G6 VP7 gilt serum IgA levels



C. P[5] VP4* gilt serum IgG levels



D. P[5] VP4* gilt serum IgA levels



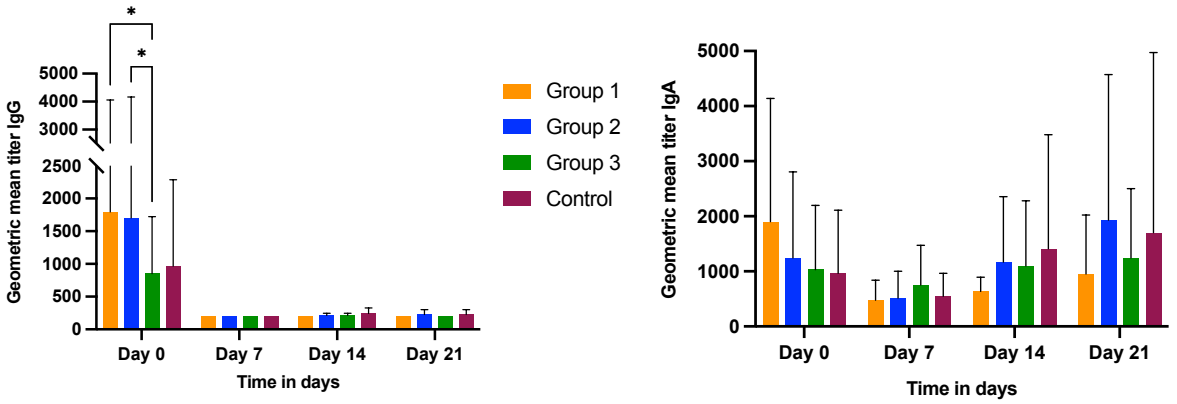
Note. RVC IgG and IgA levels over time in gilts receiving three (group 1), two (group 2), one (group 3) or no (group 4) doses of natural planned exposure. Gilts farrowed at week 0. Horizontal axis represents multiple sample collection time-points (-5W = 5 weeks pre-farrow; -3W = 3 weeks pre-farrow; F = at farrowing; +3W = 3 weeks post-farrow or at weaning). Vertical axis represents geometric mean antibody titers.

Figure 3.4.

Kinetics of Gilt/Sow Colostrum/Milk Antibody Levels against RVC

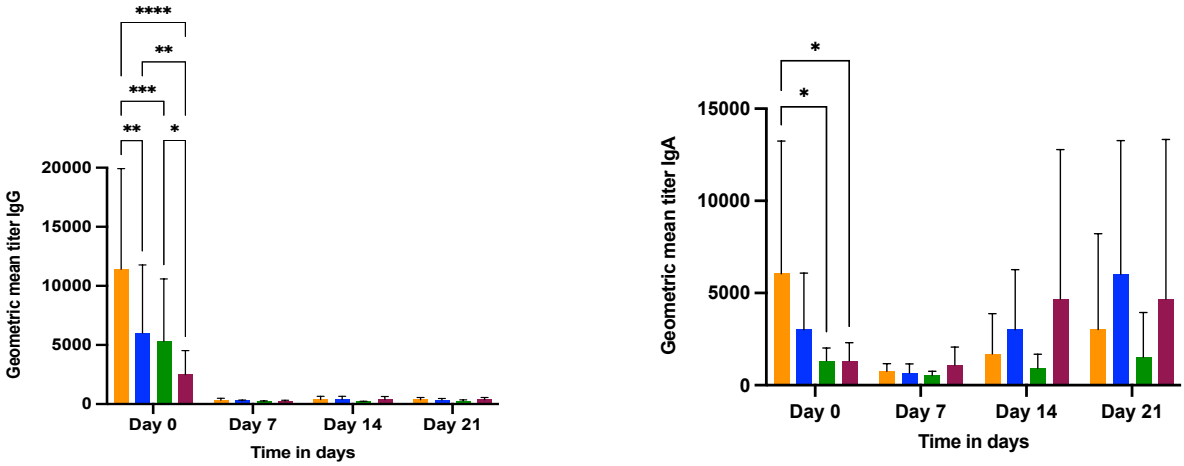
A. G6 VP7 colostrum/milk IgG levels

B. G6 VP7 colostrum/milk IgA levels



C. P[5] VP4* colostrum/milk IgG levels

D. P[5] VP4* colostrum/milk IgA levels



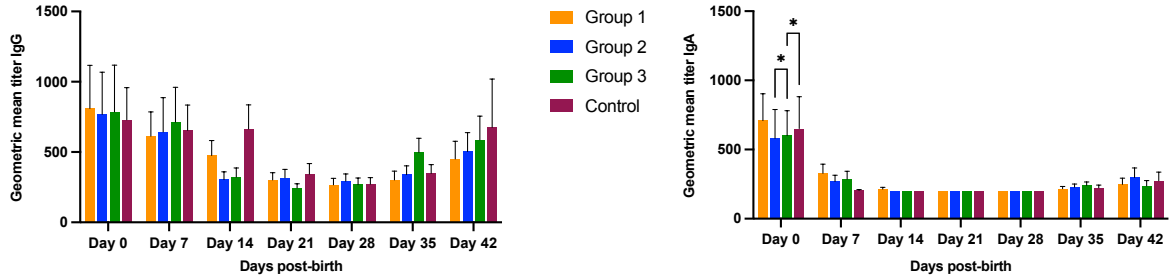
Note. Progression of RVC IgG and IgA levels over time in gilts colostrum/milk receiving three (group 1), two (group 2), one (group 3) or no (group 4) doses of natural planned exposure. Horizontal axis represents multiple sample collection time-points. Vertical axis represents geometric mean antibody titers for respective study groups.

Figure 3.5.

Kinetics of Piglet Serum Antibody Response to RVC NPE at Multiple Time-Points

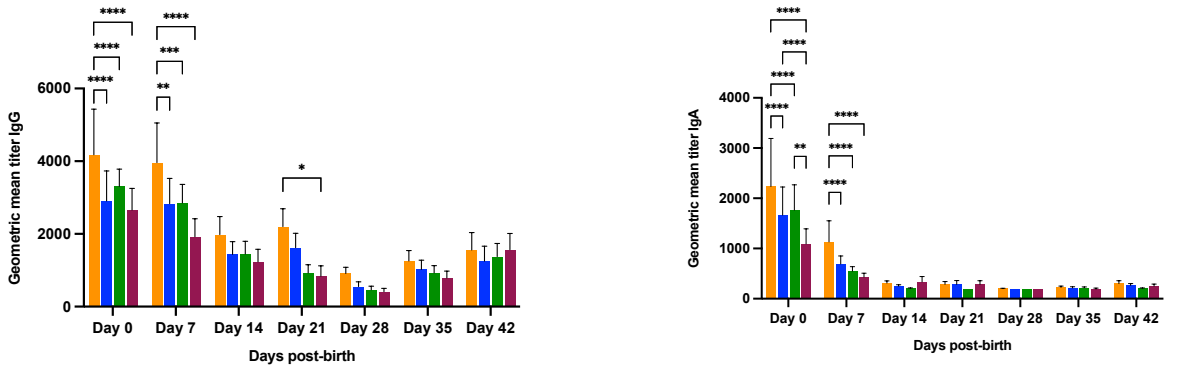
A. G6 VP7 piglet serum IgG levels

B. G6 VP7 piglet serum IgA levels



C. P[5] VP4* piglet serum IgG levels

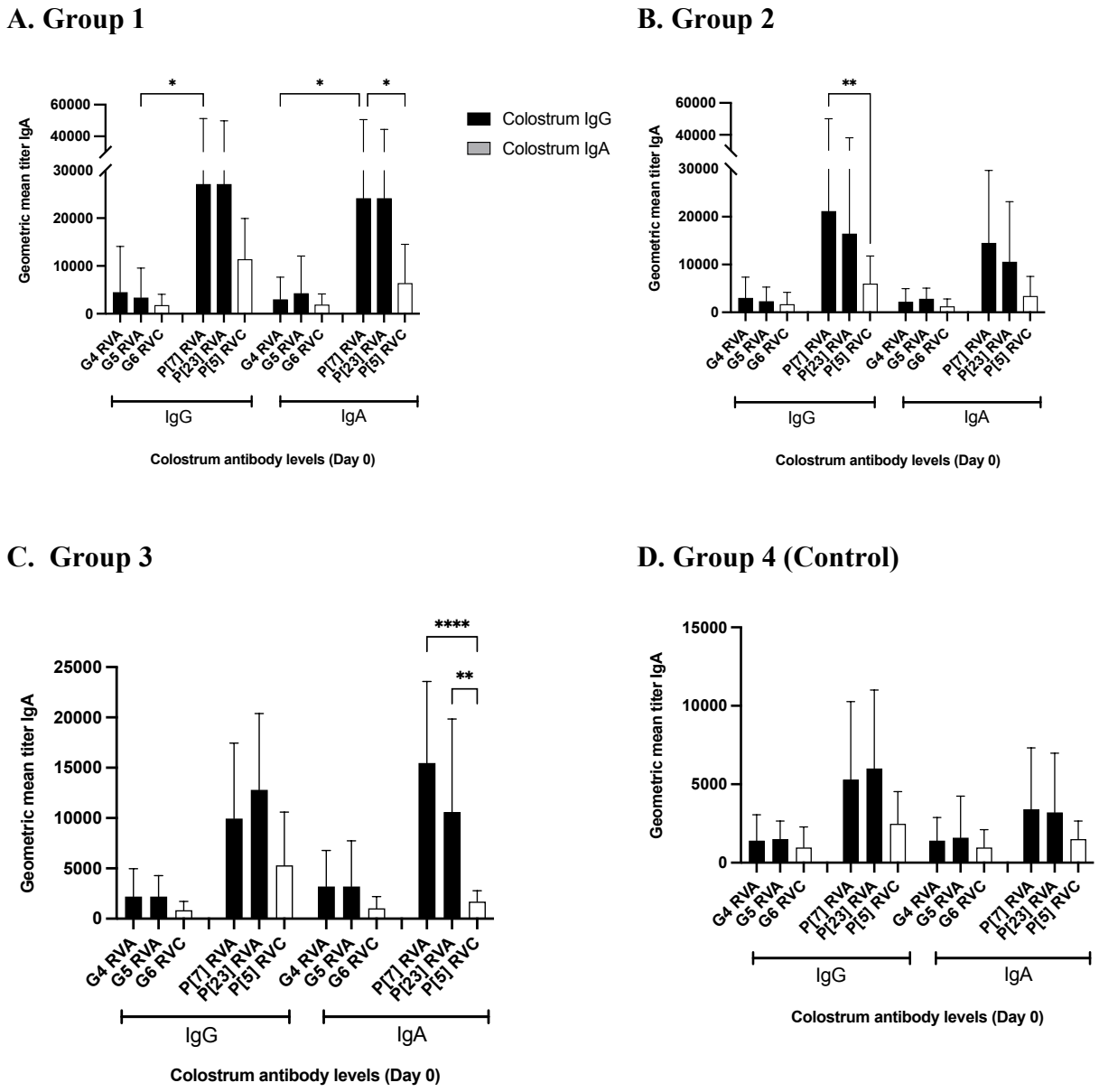
D. P[5] VP4* piglet serum IgA levels



Note. RVC IgG and IgA levels over time in piglet serum born to gilts receiving three (group 1), two (group 2), one (group 3) or no (group 4) doses of natural planned exposure. Horizontal axis represents multiple sample collection time-points. Vertical axis represents geometric mean antibody titers for respective study groups.

Figure 3.6.

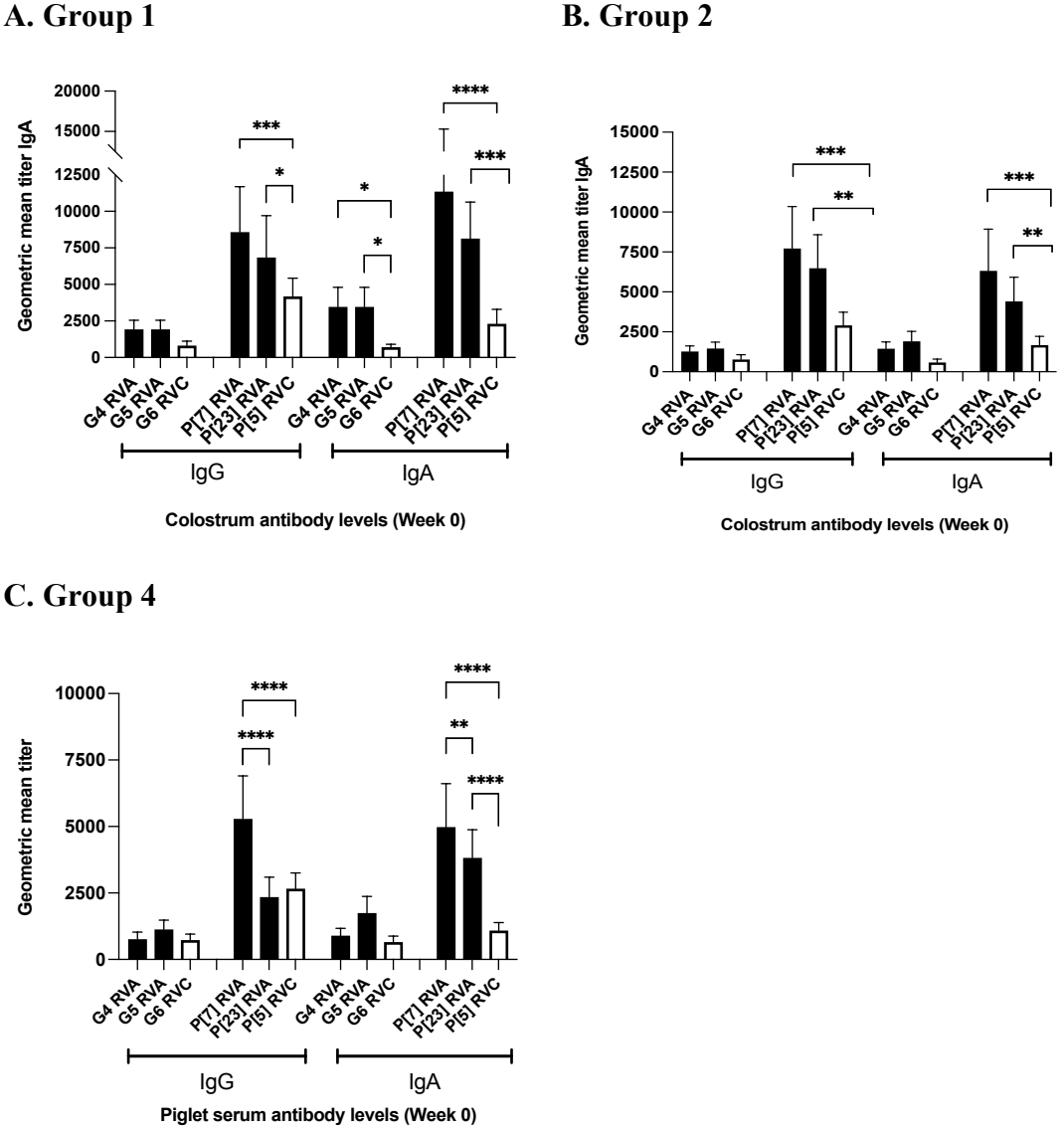
Comparison of Antibody Levels against RVA and RVC in Sow Colostrum (Day 0)



Note. Genotype-specific RVA and RVC antibody levels in gilts colostrum/milk in different study groups. **(A).** Group 1. **(B).** Group 2, **(C).** Group 3, **(D).** Control group. Horizontal axis represents different RVA and RVC G and P-genotypes. Vertical axis represents geometric mean antibody IgA levels.

Figure 3.7.

Comparison of Antibody Levels against RVA and RVC in Day 0 Piglet Serum



Note. Genotype-specific RVA and RVC antibody levels in day 0 serum samples from piglets born to different study groups. **(A).** Group 1. **(B).** Group 2, **(C).** Control group. Horizontal axis represents different RVA and RVC G and P-genotypes. Vertical axis represents geometric mean antibody IgA levels.

Table 3.1.

Details of Piglets Fecal Samples selected for Sequencing and Genome Constellation of RVC Strains

Litter ID	Group	Week	RVC Ct	Genome constellation
40960	2	1	17.66	G6-P[5]-I5-R1-C1-M1-A1-N6-Tu-Eu-H1
		2	18.39	G6-P[5]-I5-R1-C1-M1-A1- N6-Tu-Eu-H1
41045	2	2	22.57	G6-P[5]-I5-R1-C1-M1-A1- N6-Tu-Eu-H1
		3	21.7	G6-P[5]-I5-R1-C1-M1-A1- N6-Tu-Eu-H1
41009	3	2	17.48	G6-P[5]-I5-R1-C1-M1-A1- N6-Tu-Eu-H1
		3	19.68	G6-P[5]-I5-R1-C1-M1-A1- N6-Tu-Eu-H1
41285	3	2	19.64	G6-P[5]-I5-R1-C1-M1-A1- N6-Tu-Eu-H1
		3	22.49	G6-P[5]-I5-R1-C1-M1-A1- N6-Tu-Eu-H1
41014	4	1	17.94	G6-P[5]-I5-R1-C1-M1-A1- N6-Tu-Eu-H1
41174	4	1	17	G6-P[5]-I5-R1-C1-M1-A1- N6-Tu-Eu-H1
41025	4	2	21.13	G6-P[5]-I5-R1-C1-M1-A1- N6-Tu-Eu-H1

Table 3.2.

Antigenic Variation in the VP7 Protein among the RVC Strains Recovered from Piglet Feces and NPE Material

	84	88	89	90	91	92	150	151	152	153	154	155	156	194	195	197	226	245	246	247	248	249	250	251	252
G6-NPE	**A**	**S**	**P**	**G**	**P**	**G**	**E**	**P**	**K**	**N**	**S**	**E**	**A**	**E**	**D**	**D**	**D**	**S**	**S**	**S**	**L**	**N**	**Q**	**L**	**Q**
RVC/PIG/USA/S8/2017/G6P[5]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
RVC/PIG/USA/S18/2017/G6P[5]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
RVC/PIG/USA/S20/2017/G6P[5]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	--	--	--	I	.	.	.	.
RVC/PIG/USA/S31/2017/G6P[5]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	--	--	--	I	.	.	.	.
RVC/PIG/USA/S21/2017/G6P[5]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
RVC/PIG/USA/S32/2017/G6P[5]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	--	--	--	I	.	.	.	.
RVC/PIG/USA/S22/2017/G6P[5]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
RVC/PIG/USA/S33/2017/G6P[5]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
RVC/PIG/USA/S12/2017/G6P[5]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	--	--	--	I	.	.	.	.
RVC/PIG/USA/S13/2017/G6P[5]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
RVC/PIG/USA/S24/2017/G6P[5]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Table 3.3.

Longitudinal RVC detection levels in piglets' feces

Study group	Sow ID	Day 0 colostrum IgA				Day 0 piglet serum IgA		RVC ct values at different time points						
		G6	Group GMT	P[5]	Group GMT	Litter mean	GMT	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
Group 1	40399	1600	1902	6400	6040	709.56	2235.38					24.14	20.86	27.56
	38868	6400		1600								25.27	20.28	26.19
	41031	200		1600					24.29	20.43	25.59	27.50	19.37	26.00
	41071	3200		800					25.78	25.18	25.23	19.15	19.65	22.98
	41049	3200		6400						31.06		17.29	18.36	27.75
	41267	6400		6400						25.05	35.44	24.63	19.61	25.15
	40973	800		12800						28.77		32.01	18.20	26.73
	40652	6400		25600							29.86	24.27	18.35	22.11
	40956	1600		25600								29.02	17.37	19.37
	41262	400		25600						25.43	30.59	20.27	18.31	23.25
	41144	800		1600								20.01	17.73	17.02
	41181	3200		12800								27.05	18.03	24.98
Group 2	41289	6400	1243	6400	3004	580.97	1665.28					23.19	17.51	17.10
	41030	800		3200					33.79	23.75	27.22	33.83	19.23	20.90
	41052	400		800							27.10	28.58	29.18	26.35
	40960	400		800					23.71	20.96	39.43	27.29	18.55	25.11
	41011	200		800						23.96	27.45	20.81	24.97	24.43
	41010	1600		1600					28.99	23.43	29.14	33.01	17.35	25.37
	40984	6400		6400					22.64	25.16	23.09	30.85	15.72	22.16
	41069	1600		3200								28.43	19.13	26.00
	41046	3200		12800								20.83	17.11	25.07
	40954	3200		12800								22.85	17.53	18.47
	41045	400		3200					23.38	24.82	25.22	26.01	20.09	23.23

Group 3	41009	800	1029	1600	1324	600.17	1766.54		20.23	23.09	25.01	18.19	26.85
	41027	200		1600					22.72	24.43	20.06	19.18	26.51
	40975	800		1600					20.53	26.01	26.07	23.45	26.26
	40995	400		800					22.32	26.43	20.05	19.92	22.30
	41004	1600		400					21.05	24.05	29.13	20.20	24.16
	40994	1600		800							24.89	17.78	18.70
	41062	800		3200							23.16	19.20	21.53
	41249	3200		1600							23.41	15.91	24.98
	41285	3200		800				22.23	21.95	24.04	26.69	26.51	26.88
	40996	200		1600							24.10	22.41	23.20
	41219	6400		3200							24.40	20.45	23.07
Group 4	40969	1600	966	800	1324	650.17	1090.42		34.91		23.45	18.55	29.13
	41112	1600		1600							24.43	20.81	21.38
	41025	800		1600					21.48	28.71	28.38	18.73	24.57
	41014	3200		1600				19.48	18.75	28.88	31.44	30.51	21.57
	41053	400		400				28.06	23.33	23.63	28.62	20.23	20.83
	41070	3200		3200				25.84	34.54		24.47	23.07	19.12
	41174	400		400				17.19	29.79	29.80	30.27	21.81	28.31
	40964	200		800				19.34	29.19	35.01	33.39	22.35	27.36
	40979	200		1600				20.88	26.62	30.11	28.17	18.84	32.10
	41026	800		1600								19.15	24.84
	41057	6400		6400				19.86	28.67	34.14	27.87	18.18	24.31

Note. Progression of levels of RVC detected over time in piglets' feces based on RT-PCR of fecal samples pooled by litter. Week 0 is farrowing, and piglets were moved to the nursery after the week 3 sample was collected. Colostrum G6 and P[5] RVC IgA levels, day 0 piglet litter mean serum IgA levels and group GMTs are also indicated.

Chapter 4 - Conclusion

Rotaviruses (RVs) are one of the significant causes of piglet diarrhea in swine herds globally. RVA strains have been considered the most pathogenic and epidemiologically diverse of all RV groups infecting swine, with detection most common in post-weaning piglets (Homwong et al., 2016). However, recent data suggest that RVC is emerging as a major cause of diarrhea in neonatal pigs, particularly in piglets younger than 3 days old (Marthaler et al., 2013; Theuns et al., 2016). Lack of updated strains in the current porcine RVA vaccine and absence of modified live virus (MLV) vaccines against RVC have prompted swine producers to mimic natural RV infection in gilts/sows in the form of "natural planned exposure (NPE)" to provide lactogenic immunity to piglets (Pittman, 2016). Since no intra-uterine passage of immunoglobulins occur during gestation in swine, piglets are born agammaglobulinemic and uptake of pathogen-specific colostrum/milk immunoglobulins becomes critical for their survival during first few days of life (Chepngeno et al., 2019; Matías et al., 2017). Due to lack of serological tools to asses antibody response to RVs in swine, efficacy of NPE protocols cannot be examined. Molecular methods such as quantitative real-time PCR are used to asses viral fecal shedding in gilts and piglets to indirectly measure RV-specific antibody response. There are some major gaps in knowledge in the field of porcine RV immunology: 1). Lack of serological tools to asses genotypic-antibody response to RVs; 2). Lack of understanding of timing and doses of pre-farrow NPE administration to gilts/sows in generating passive immunity; and 3). Paucity of data on reasons of high RVC prevalence in neonatal piglets and absence of comparative data on antibody response to RVA and RVC in swine.

Hence, our aim in the first study was to determine antibody response to different G and P-genotypes (G4, G5, P[7] and P[23]) of RVA using in-house optimized indirect ELISAs. We found that 3 doses of pre-farrow NPE administration in gilts results in significantly higher anti-RVA IgG

and IgA levels in their colostrum and milk. Piglets born to group 1 gilts/sows had higher IgG titers at day 0 compared to other groups. Although, group 3 (1dose of NPE) piglet serum samples had higher IgA titers than other groups, antibody levels were not significantly different than group 1 piglets. Only two litters shed RVA prior to weaning and hence no clear association between the piglet serum IgA levels of both piglets and fecal RVA shedding could be observed. NGS results revealed that RVA shed in piglet feces prior to weaning carried a G and P-type combination of G11P[34], which was different than the parent strains in the NPE material. We hypothesize that better homotypic passive immunity was stimulated against RVAs, which prevented G4, G5, P[7] and P[23] RVA shedding in the farrowing room in all study groups including control. This finding of no RVA shedding in control group piglets prior to weaning suggest that piglets received sufficient antibodies though colostrum/milk to prevent natural RVA infection in the farrowing room. Our results of RVA antibody and fecal shedding levels in piglets are very important to the producers. Although all study groups including control were able to prevent pre-weaning natural RVA shedding, we recommend to use 1 dose of RVA NPE 5 weeks before farrowing owing to the higher serum IgA levels detected in piglets born to group 3 gilts. Serum IgA titers are considered a good indicator of intestinal IgA levels against RVA (To et al., 1998; Velázquez et al., 2000). Results of Chapter 2 expand our understanding of the antibody response to RVA in swine and the role of NPE in providing lactogenic immunity to naïve piglets.

RVC infection are more prevalent in neonatal piglets particularly in 0-3 days old piglets (Homwong et al., 2016), reasons of which are not completely understood (Chepngeno et al., 2019). Some of the possible reasons include insufficient maternal RVC antibody levels in the colostrum, lack of RVC vaccine, distinct pathogenic mechanism compared to RVA, and low minimum infectious dose of RVC (Chepngeno et al., 2019). In the absence of RVC vaccine, NPE to

gilts/sows is the only available methods to prevent piglets from RVC infections. However, studies investigating the efficacy of NPE protocols in generating immunity against RVC in swine are completely lacking. Hence, in Chapter 3 we sought to determine antibody response to two most prevalent G and P-genotypes (G6, P[5]) of RVC using in-house optimized indirect ELISAs and compare antibody responses to RVC shedding in pre-weaning piglets. We found that group 1 with 3 doses of NPE had higher (significantly or numerically) colostral (day 0) IgG and IgA titers compared to other groups for both antigens. Interestingly, group 1 RVC antibody levels in day 0 piglet serum were either significantly (P[5]) or numerically (G6) higher than other groups. Higher group 1 colostrum and piglet serum levels suggest that 3 doses of NPE in gilts prior to farrowing was able to better stimulate maternal immunity in gilts compared to other NPE doses. Although 3 doses of NPE resulted in high antibody levels to RVC, none of the treatment groups were able to prevent RVC shedding by piglets in the farrowing room. There is very limited information on lactogenic protection against porcine RVC and most of the swine RV lactogenic immunity studies have been done for RVA.

The primary goal of this work was to understand the differences in dynamics of antibody response to RVA and RVC in swine and to investigate effects of NPE administration to gilts in shaping the genetic makeup of the RV strains in the piglet population. To the best of our knowledge, this is the first study comparing antibody responses to RVA and RVC in gilts and their piglets, and investigating role of NPE in providing genotype-specific lactogenic protection to piglets against RVA and RVCs. All 4 groups had significantly or numerically lower RVC colostrum antibody titers than RVA titers, irrespective of G and P-type. Piglet serum RVC IgA titers at day 0 were significantly lower than RVA titers. RVA and RVC fecal shedding data from piglets correlated with the antibody levels in the colostrum. For example, only 2 litters (5.8%,

2/34) shed RVA prior to weaning compared to 8 litters (23.5%, 8/34) at day 7, and 18 litters (53%, 18/34) each at day 14 and 21 for RVC (Shepherd, 2020b). It is evident that low RVC levels in the NPE material failed to induce sufficient immunity, resulted in low colostrum RVC antibody titers, and failed to passively protect piglets from natural RVC infection in the farrowing room. Low colostrum and piglet serum antibody levels against RVC explains their higher prevalence in the neonatal piglets. Based on these results we recommend to use 3 doses of RVC only NPE in gilts/sows at 5,4, and 3 weeks before farrowing.

This dissertation research has answered many unresolved questions. First, now we know that 3 doses of NPE administration results in high anti-RV antibody levels in colostrum. Second and most importantly, we now know that colostrum and piglet serum at birth contain low levels of anti-RVC antibodies compared to anti-RVA antibodies. Low anti-RVC antibody titers in colostrum compared to RVA are perhaps linked to low RVC levels in the NPE material. These findings are very important for the swine producers who rely on RV NPE protocols to generate maternal immunity against RVs particularly against RVCs. There is a need to explore methods to increase the RVC concentration in the NPE material at the farm level, until a cell culture system is established for RVCs. One such intervention could be administration of immunosuppressive medication to the piglets used for generating the NPE material prior to RV inoculation. Use of immunosuppressive agents will suppress piglets' innate immune response to RV inoculation and hence result in increased virus replication inside the gut. Another possible way to improve RVC levels in the NPE could be to optimize the best time of harvesting the intestinal contents from RV inoculated piglets which yields higher RVC levels.

The lack of reproducible culture system for RVCs has remained a major barrier in developing vaccines for use in swine. RVC is emerging as a major pathogen of concern in neonatal

piglets and hence establishing an in-vitro cell culture system is critical to develop effective RVC vaccines. Intestinal enteroids are a recent development for studying human rotavirus infections and can overcome many limitations of using transformed cell lines (Saxena et al., 2015). Recently, intestinal enteroids using pig intestinal epithelial cells have been successfully used to propagate and study RVA and PEDv (Guo et al. 2021; Li et al. 2019). Porcine intestinal enteroids may overcome the difficulties of adapting porcine RVs to non-intestinal cell lines such as MA104. Additionally, porcine small intestinal epithelial cell line (IPEC-J2) should also be investigated as a possible cell culture system for RVCs. IPEC-J2 is a non-transformed, non-tumorigenic intestinal epithelial cell line, which maintains differentiated characteristics and exhibits strong similarities to intestinal epithelial cells (Schierack et al. 2006). Since RVs replicate inside intestinal epithelial cells, IPEC-J2 cells might support the RVC replication in-vitro. IPEC-J2 cell line has been successfully used as an infection model to study RVA (Liu et al. 2010). An established cell culture system for RVCs will help in determining virus neutralization titers and also expand our knowledge of protein-specific neutralization titers. For example, unique VP4 sequences could be reassorted onto a common backbone to generate VP4-specific neutralization titers. Such reassortants will help to determine relative contributions of VP7 and VP4 to overall neutralization titers.

The outer capsid proteins VP7 and VP4 of RVs independently elicit neutralizing and protective immunity (Hoshino et al., 1985). In both Chapters 2 and 3 we observed that RVA and RVC IgG and IgA GMTs against VP4* were higher compared to VP7 titers. Although we could not determine serum neutralization titers due to difficulty in propagating RVA and RVC in cell culture, magnitude of anti VP4* titers emphasize its inclusion in any futures vaccine formulations against RVs. Moreover, previous research suggests the existence of heterotypic antibodies to

human RVA, in particular against the VP5* region of the VP4 protein (Nair et al., 2017a). Nair et al. further reported that homotypic anti-VP7 and non-neutralizing VP8* responses occur more frequently (Nair et al., 2017a). Hence, including VP5* region of VP4 will be important, as heterotypic immunity seems to be more often generated to the stalk of the VP4 rather than the head (Nair et al., 2017a). In chapter 2, NGS identified G11 and P[34] genotypes in pre-weaning piglet fecal samples. These genotypes were different from the genotypes (G4, G5, P[23] and P[23]) present in the NPE, which suggests that RVA NPE was able to prevent homotypic natural RVA infection in the farrowing room. Although heterotypic immunity is not typically observed in field conditions involving passive immunity, existence of heterotypic antibodies to human RVA has been reported earlier (Nair et al., 2017b). If similar broad acting antibodies against RVA could be identified in swine, scaled up and administered to the piglets at birth, a heterotypic protection against multiple genotyped could be achieved. This area of research remains unexplored and requires more investigation.

There is no data on protective antibody titers against RVA and RVC in swine. We do not know what levels of antibodies are sufficient to provide protection against RVs in swine. Next logical step of this dissertation will be to assess neutralization titers against RVA and use a RV challenge model to know levels of antibodies induced by NPE sufficient to protect from RV infection. Original NPE material can be used as a viral challenge, and anti-RV antibody levels in colostrum/milk and piglet serum can be determined. Multiple cell lines could be tried to grow RVA and RVC genotypes to identify the best cell line that support their growth.

Lastly, we recommend producers to use 1 dose of "RVA only NPE" at 5-weeks pre-farrow and 3-doses of "RVC only NPE" at 5, 4- and 3-weeks pre-farrow to generate sufficient lactogenic immunity to protect RV infection in neonatal piglets. It is known that RVA is more prevalent and

pathogenic compared to RVC, and every sow experience multiple RVA infections within their lifetime. Therefore, it is highly likely that sows harbor more RVA-specific memory B cells than RVC-specific memory B cells which upon repeated exposure to RVA antigens, proliferate and differentiate into RVA-specific antibody producing plasma cells. Data from Chapter 2 also support this assumption. For example, control group gilts despite having no viral stimulation in the form of "NPE", prevented RVA fecal shedding in piglets in the farrowing room. Hence, we believe that one dose of "RVA only NPE" at 5-weeks prior to farrowing will result in sufficient lactogenic immunity to protect piglets from RVA infection until weaning. Our recommendation of administering 3 doses of "RVC only NPE" to gilts prior to farrowing is based on the fact that group 1 (3 doses) had higher colostrum and piglet serum antibody levels compared to other study groups resulting in lowest RVC shedding in group 1 piglets before weaning. Three doses of high RVC titer NPE prior to farrowing will result in increased RVC replication in gilts and enhanced lactogenic immunity. Moreover, our previous research (unpublished) on RV fecal shedding in piglets suggest that RVA and RVC fecal shedding occurs in a cyclical pattern (higher RVA shedding followed by high RVC shedding in a weekly pattern) suggesting a competitive gut colonization pattern of RVA and RVC in swine.

Since RVC is very difficult to prevent in neonatal piglets, methods other than stimulating maternal immunity or developing a vaccine for piglets, needs to be explored. Once such method could be feeding probiotics to the piglets immediately after birth until weaning. Different bacteria (*Lactobacillus* and *Bifidobacterium* spp.) living in mammalian guts as commensals have been proven to improve immune responses to rotavirus vaccines in gnotobiotic piglets (Kumar et al. 2018; Huang et al. 2018). We believe that probiotics will colonize the piglets' gut and reduce the availability of intestinal epithelial cells for RVC replication, thus minimizing RVC infection.

Another method could be to feed colostrum from multiparous sows to the piglets born to the gilts. Recently it was reported that multiparous sows contain significantly higher IgA and IgG antibody titers in milk compared to gilts due to better local secretion of IgA in milk or trafficking of IgA plasmablasts from the gut to the mammary glands in multiparous sows (Chepngeno et al. 2018). We believe that supplementing piglets with colostrum and milk from multiparous sows will provide antibodies required to prevent RVC infection at an early age.

Finally, results of this dissertation research have advanced our understanding of the dynamics of antibody response to RVA and RVC in gilts and their piglets. To the best of our knowledge this is the first time that a comparative study has been carried out to investigate the differences in antibody response to RVA and RVC in swine. In particular, RVC data from this dissertation assumes more significance since there is very limited reports on immunity against porcine RVCs. We believe that these results will help in formulating better immunization and management strategies to prevent RVs in swine herds.

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