Investigation of Novel Prophylactics Against Human Rotavirus Using Gnotobiotic Pig Models

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Academic Abstract

Human rotavirus (HRV) is a major causative agent of acute gastroenteritis (AGE), which causes severe dehydrating diarrhea in children under the age of five and results in up to 215,000 deaths worldwide each year. There are two live oral attenuated vaccines licensed for use in the United States that are highly effective in high-income countries but much less so in low-and middle-income countries (LMICs). Several factors contributing to decreased efficacy in these areas include chronic malnutrition, gut dysbiosis, and concurrent viral infection. Along with this, currently used vaccines require constant cold-chain storage to maintain vaccine stability, and those resources can be scarce in LMICs. These areas continue to maintain a high burden of HRV morbidity and mortality, and more efficacious vaccines are needed. The gnotobiotic (Gn) pig model of HRV infection and diarrhea has long been used in the evaluation of novel HRV vaccines due to Gn pigs' susceptibility to HRV infection, development of clinical signs, histopathological changes in the intestine, and the infection kinetics that mimic those seen in human infants. The first project in this dissertation used the Gn pig model to evaluate a thermostable live oral attenuated vaccine administered as a dissolvable film. Two doses of the tetravalent dissolvable film vaccine conferred significant protection from virus shedding by delaying its onset and reducing peak titers in feces. It also significantly delayed the onset of diarrhea and reduced the duration and area under the curve (AUC) of diarrhea. The dissolvable film was highly

immunogenic, inducing high titers of serum virus neutralizing (VN) antibodies specific to each of the four G-types included in the vaccine formulation, HRV-specific serum IgA and IgG, and intestinal IgA. These data confirm the thermostable platform as a useful alternative to liquid vaccines that require cold-chain. The second project evaluated three mRNA-based nonreplicating vaccine candidates in the Gn pig model. All three mRNA candidates encoded a universal CD4+ T cell epitope, P2, derived from tetanus toxoid, fused with the encoded VP8* from P[4], P[6], and P[8] HRVs. Two candidates also encoded for a lumazine synthase (LS) domain fused with the P2-VP8*. A dose response study of the LS-P2-VP8* candidates was conducted simultaneously. Significant protection against virus shedding was induced by all three candidates, with LS-P2-VP8* candidates inducing significantly higher VP8*-specific serum IgG. All three candidates induced significantly higher numbers of P[8]-VP8*-specific IgG antibody-secreting cells (ASCs) and IFN-γ-producing T cells in the ileum, spleen and blood. These data provide guidance for further development of the relatively new mRNA-based technology for use in HRV vaccine development. In the final study of this dissertation, we used the Gn pig model of both P[8] and P[6] HRV infection to evaluate a cocktail nanoparticle-based HRV vaccine. This vaccine was made up of an S_{60} nanoparticle, self-assembled from the S domain of the human norovirus capsid protein. The exposed C-termini on the S_{60} nanoparticle were utilized as an antigen display platform, where VP8* from P[4], P[6] and P[8] HRVs was fused. This vaccine was tested as both a two-dose intramuscular (IM) regimen, or as an IM booster preceded by an oral priming immunization with commercial monovalent Rotarix®. Pigs were challenged with either P[6] or P[8] HRV to evaluate cross-protection of the nanoparticle vaccine. Both regimens were highly immunogenic, inducing high titers of serum VN, IgG and IgA antibodies. Furthermore, the prime-boost regimen conferred significant protection against virus shedding in P[8] HRV-challenged

pigs as evidenced by the shortened duration of fecal virus shedding. There was also significant protection in P[6] HRV-challenged pigs vaccinated with the prime-boost regimen, as evidenced by the shortened duration, reduced mean peak titer and AUC of virus shedding. Prime-boost-vaccinated pigs challenged with P[8] HRV had significantly higher P[8]-specific IgG ASCs in the spleen post-challenge. Prime-boost-vaccinated pigs challenged with P[6] HRV had significantly higher numbers of P[6] and P[8]-specific IgG ASCs in the ileum, as well as significantly higher numbers of P[8]-specific IgA ASCs in the spleen post-challenge. Oral priming followed by parenteral boosting appears to be a promising vaccination strategy for HRV and these data warrant further investigation into this regimen. Through these studies, we improved our understanding of the effect of different vaccination routes and formulations in the effectiveness of conferring protection against an enteric virus. The knowledge will facilitate the development of more effective vaccination strategies against HRV, the leading cause of infantile diarrhea in LMICs, as well as other enteric viruses.

Investigation of Novel Prophylactics Against Human Rotavirus Using Gnotobiotic Pig Models

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General Audience Abstract

Human rotavirus (HRV) is a major causative agent of acute gastroenteritis (AGE) in children under the age of five. Acute gastroenteritis is characterized by nausea, vomiting, and potentially deadly dehydrating diarrhea. There are two highly effective vaccines licensed for use in the United States; however, these vaccines are much less effective in low- and middle-income countries (LMICs), where HRV disease burden is the highest. There are several reasons thought to be responsible for the decrease in effectiveness seen in these areas, including chronic malnutrition and gut dysbiosis. Non-biological reasons for decreased efficacy may include the breakdown of cold-chain storage for these vaccines, which require constant low temperature storage that is often unavailable in LMICs. Thermostable vaccines are necessary for increasing vaccine distribution and efficacy in these areas. Because many of the biologic factors thought to interfere with the effectiveness of these vaccines appear to be confined to the gastrointestinal tract, development of next generation HRV vaccines has focused on the parenteral route of administration. The gnotobiotic (Gn) pig model is a highly relevant animal model that has been used for decades to evaluate novel HRV vaccine efficacy. Our first study evaluated a thermostable, dissolvable live oral vaccine administered as a dissolvable film in our Gn pig model. Two doses of this vaccine significantly reduced the severity of diarrhea and virus shedding in the stool. Our second study evaluated three mRNA-based intramuscular (IM) vaccines in the Gn pig model. Three doses of all mRNA

candidates provided significant protection from virus shedding in the stool, as well as inducing the production of strong HRV-specific antibodies in the serum and high numbers of virus-specific T cells in the tissues. In our final study, we evaluated a nanoparticle-based vaccine as a two-dose IM regimen or as an IM booster preceded by an oral immunization using the commercially available Rotarix® vaccine. The prime-boost regimen significantly shortened the duration and severity of virus shedding in the stool. We also detected more cross-strain HRV-specific antibody-secreting cells in the tissues. All three vaccines evaluated in this dissertation offer differing novelty in the field of HRV vaccine development, and the Gn pig model has been instrumental in the evaluation of these vaccines.

To my husband Jared, there is nothing I am prouder of than being your wife. Thank you for loving me through this journey and beyond. To my three babies, Isaiah, Isaac, and Malachi, no accomplishment could ever top being your mama.

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Table of Contents

List of Figures

Chapter 2

Chapter 3

Chapter 4

List of Tables

Chapter 2

Chapter 3

Chapter 4

Chapter 1

Literature Review

Human Rotavirus Infection, Disease and Vaccine Development

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1. Molecular Virology of Human Rotavirus

1.1 Genome and Virion Structure

Human rotaviruses (HRVs) are a genus within the family *Reoviridae*, and possess a double-stranded segmented RNA genome encoding six structural (VP1-4, and VP6-7) and six nonstructural proteins (NSP1-6) (Matthijnssens, Ciarlet et al. 2011). The mature virion is a triple-layered particle (TLP) around 100nm in diameter (Pesavento, Crawford et al. 2006, Knipe and Howley 2013). The innermost layer, which surrounds the genome, is made up of 120 copies (60 dimers) of VP2, 12 copies of VP1, the RNA-dependent RNA polymerase (RdRp) and 12 copies of VP3 (Pesavento, Crawford et al. 2006). The middle layer involves 260 trimers of VP6, and the outer later is comprised of 260 trimers of VP7, with 60 dimers of VP4 protruding from the surface as spikes (Pesavento, Crawford et al. 2006). Upon contact with host proteases in the digestive tract, VP4 is cleaved into the proximal VP5* and distal VP8*; both of which play a vital role in the attachment and entry of the virion into target cells, as well as immune recognition (Zarate, Espinosa et al. 2000, Zarate, Cuadras et al. 2003, Knipe and Howley 2013).

1.2 Classification

Rotaviruses (RVs) as a genus is divided into nine serologic groups designated A-D and F-J, with group A RVs (RVAs) causing the majority of HRV infections (Pesavento, Crawford et al. 2006, Knipe and Howley 2013). Recent evidence has implicated groups B and C in human infection, as well (Marthaler, Homwong et al. 2014, Joshi, Walimbe et al. 2019). Group A RVs are further divided into serotypes based on antibody reactivity in neutralization assays and genotypes based on sequence homology for the outer capsid proteins, VP7 and VP4 (Knipe and Howley 2013). From these assays, RVAs can be

classified into G and P serotypes/genotypes, respectively, and there are currently 41 G-types and 57 P-types known (RCWG 2021). The ability of RVs to reassort results in independent variation of G and P types, hence the binomial typing system used, G[x]P[y]. Despite the diversity and reassortment combinations of RVAs, there are only a few G/P type HRVs that circulate the globe consistently, including G1-4, G9 and G12, and P[4], P[6], and P[8] (Zhou, Zhou et al. 2020). In 2008, the Rotavirus Classification Working Group established a nucleotide sequence-based genome classification system for further classifying RVs according to their previously established nucleotide cut off values (Matthijnssens, Ciarlet et al. 2011). The genotypes are described as Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx and correspond to the genes of VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6, respectively (Matthijnssens, Ciarlet et al. 2011).

1.3 Binding/Entry

Upon contact with host proteases present in the digestive tract, RV's spike protein, VP4, is cleaved into proximal VP5* and distal VP8*. Intact VP4 and its cleavage product, VP8*, are widely considered to be the proteins primarily responsible for interacting and binding with host cell receptors and co-receptors, though VP8* is required for entry into the cell (Knipe and Howley 2013). Human rotaviruses preferentially target mature enterocytes in the villi of the small intestine, and though specific receptors have not yet been definitively identified, evidence has shown RVs are capable of entering target cells via glycan receptors and co-receptors which include histo-blood group antigens (HBGAs), heat shock protein 70 (hsc70), gangliosides and integrins (Yolken, Willoughby et al. 1987, Guo, Nakagomi et al. 1999, Hewish, Takada et al. 2000, Londrigan, Graham et al. 2003, Zarate, Cuadras et al. 2003, Isa, Realpe et al. 2004, Lopez and Arias 2004, Hu, Crawford et al. 2012, Liu,

Geng et al. 2020). Early evidence suggests that HRV utilizes terminal sialic acid (SA) residues for anchoring to host cells, but further research has concluded the majority of HRVs are, in fact, SA-independent. (Guerrero, Bouyssounade et al. 2002, Liu, Huang et al. 2012). Despite this dichotomy, there are studies that suggest some HRVs, including the Wa strain, have specific protein conformations, defined via NMR spectroscopy and molecular modeling tools, within their VP8* which may accommodate the binding of internal sialic acid moieties found in host cell surface glycans (Haselhorst, Blanchard et al. 2007).

VP5* and VP7 have also been implicated in the binding of RVs to host cells though the use of integrins. Previous studies showed antibodies specific to integrins or synthetic peptides mimicking integrin-ligand motifs found in VP5* and VP7 can reduce RV infectivity *in vitro* (Hewish, Takada et al. 2000). VP5*- and VP7-specific neutralizing antibodies will also inhibit virus binding to integrins (Fleming, Graham et al. 2007). VP5* may also play a major role in the attachment and entry of RVs via hsc70, as studies have shown hsc70-specific antibodies can block RV infection (Guerrero, Bouyssounade et al. 2002, Zarate, Cuadras et al. 2003).

Histo-blood group antigens as receptors for RVs are an area of active research because they are thought to play a large role in the ability of human norovirus, another enteric virus, as well as human coronaviruses, to bind target cells (Hutson, Atmar et al. 2002, Guillon, Clement et al. 2008). These glycan structures are expressed on the surface of many human cells including red blood cells and intestinal epithelial cells (Clausen and Hakomori 1989). Furthermore, they are expressed into bodily fluids such as breastmilk and saliva in

individuals who have a functional FUT2 gene (secretors), potentially increasing the susceptibility to enteric viral infection (Marionneau, Cailleau-Thomas et al. 2001).

Once bound to the host cell, a series of events facilitated by conformational changes in the viral capsid and spike proteins results in entry into the cell. There has been debate over the mechanism by which RV enters the cell. Previous research suggested entry of RVs was through endocytic pathways; however, there is a plethora of evidence that has shown direct penetration to be the more likely scenario (Ruiz, Leon et al. 2009). Such evidence includes studies that showed inhibition and deacidification of intracellular trafficking of endosomal vesicles did not decrease RV infectivity in several *in vitro* experiments (Kaljot, Shaw et al. 1988, Bass, Baylor et al. 1995, Cuadras, Arias et al. 1997). These data together suggest endocytosis is not a major mechanism of entry into the cell after binding of activated RV, and rather direct penetration into the cytoplasm is likely the mechanism which subsequently results in more robust infection.

1.4 Replication

Following the complex events of penetration and uncoating of VP4 and VP7 from the TLP in the host cell, the now double-layered particle (DLP) begins the replication process within the cytoplasm. The VP1 (RdRp) of RV is responsible for transcription of the dsRNA, followed by capping through the guanyltransferase and methyltransferase activity of VP3 (Ruiz, Leon et al. 2009, Knipe and Howley 2013). Instead of a polyA tail, RV genome ends consist of a consensus sequence bound with NSP3, effectively functioning as a polyA tail (Ruiz, Leon et al. 2009). mRNA exits the DLP and is translated in polysomes, while other mRNA is used to produce viral genome for packaging, which occurs within the DLP (Ruiz, Leon et al. 2009, Knipe and Howley 2013). After transcription and translation have

occurred, and an accumulation of viral protein and mRNAs has been produced, an electron-dense inclusion within the cytoplasm called a viroplasm develops via mechanisms directed by NSP2 and NSP5 (Ruiz, Leon et al. 2009, Knipe and Howley 2013). In the viroplasm, new DLPs are produced. Interestingly, RV DLPs must translocate to the endoplasmic reticulum (ER) for maturation using NSP4 as a surrogate intracellular receptor (Ruiz, Leon et al. 2009, Knipe and Howley 2013). Through this process, virions become transiently enveloped, and after the outer VP4 and VP7 are assembled within the ER, the envelope disappears (Ruiz, Leon et al. 2009, Knipe and Howley 2013). Fully mature TLPs then bud from the ER and exit the cell via either vesicular transport or cell lysis (Ruiz, Leon et al. 2009, Knipe and Howley 2013).

2. Pathobiology

2.1 Transmission and Epidemiology

Human rotavirus is spread via the fecal-oral route through contaminated surfaces, food and water. It is highly contagious in susceptible hosts, with a dose of fewer than 100 particles being required for infection (Knipe and Howley 2013). This makes person-to-person spread inevitable in close contact settings like daycare centers, especially since virus is shed in the stool at extremely high titers. Fecal virus shedding can persist after the cessation of symptoms, which further contributes to the ease of transmission of the virus. Furthermore, the nonenveloped virus is very hardy in the environment and is resistant to many traditional household cleaners and hand sanitizer solutions.

Though G1-4 and P[8], P[6] and P[4] HRVs have been the predominant genotypes circulating worldwide in humans, a combination of variables including geographical location, vaccination rates and efficacy, and interspecies transmission, have led to the

emergence of other uncommon strain combinations such as G9P[11] and G2P[4] (Mukherjee and Chawla-Sarkar 2011). The zoonotic potential of RVs and its impact on transmission is an important factor to consider, as interspecies transmission and reassortment events between humans and several animal species have been reported (Luchs, Cilli et al. 2012, Ben Hadj Fredj, Heylen et al. 2013, Degiuseppe, Beltramino et al. 2013, Mukherjee, Mullick et al. 2013, Papp, Matthijnssens et al. 2013, Truong, Nguyen et al. 2022). Though several animal species have been identified as reservoirs for zoonotic RV, swine are associated with a large number of RVA genotypes known to also infect humans (Vlasova, Amimo et al. 2017). Rotavirus infection can be devastating in intensive farming operations, especially when outbreaks involve piglets. Like humans, older pigs may be infected by RVA subclinically, and combined with the high prevalence of the virus in pigs, persistence and severity of outbreaks in both swine operations and people who work closely with them is of concern (Brnic, Colic et al. 2022). The segmented structure of RV's genome allows for the generation of chimeric reassortant strains, which are able to infect multiple species. There are numerous reports of interspecies transmission of RVs between humans and swine, particularly in rural areas (Degiuseppe, Beltramino et al. 2013, Brnic, Colic et al. 2022). A report detailing the genomes of two G4P[6] RVAs detected in the stool sample of symptomatic children in Argentina found that the genomic constellations were nearly identical to the prototype Gottfried strain of swine, and both strains were Wa-like, but more closely related to swine RV strains (Degiuseppe, Beltramino et al. 2013). Both children infected with these strains lived in unsanitary housing severely affected by major flooding in the few years prior to detection (Degiuseppe, Beltramino et al. 2013). This G4P[6] strain was successfully passaged in Gn pigs

and the infectious dose was determined for the preclinical evaluation of HRV vaccines (Nyblade, Hensley et al. 2022). Despite not living in direct/close contact with swine, contact with contaminated flood waters has been a major source of zoonotic disease transmission for other pathogens (Okaka and Odhiambo 2018). Though not considered a major source of human infection currently, the immense genetic diversity of RVs coupled with the emergence of more unusual RV strains in humans necessitates more investigation into the impact of interspecies transmission on the perpetuation of HRV outbreaks and generation of novel strains.

3. Immunity

3.1 B Cells

The role of cell-mediated immunity in the resolution and prevention of HRV infection has been difficult to fully understand, due to issues with timely sample acquisition from human infants. It is known that strong local intestinal IgA response is vital to prevention of HRV infection, hence the focus of vaccine development efforts on live attenuated vaccines (Sinha, Kanungo et al. 2018). In the gnotobiotic (Gn) pig model of HRV infection and diarrhea, intestinal IgA-secreting B cells have been historically associated with significant protection from reinfection and diarrhea development after subsequent exposure to virulent HRV (Yuan, Ward et al. 1996). In a study involving B cell-deficient Gn pigs (HCKO), it was shown that when these pigs were vaccinated with an attenuated HRV (AttHRV) vaccine, they were not as well protected after virulent HRV challenge as wild-type (WT) pigs were (Wen, Bui et al. 2016). They were, however, still partially protected, as HCKO pigs who received a mock vaccination had significantly higher fecal virus shedding and more severe clinical signs of disease than AttHRV-vaccinated HCKO pigs (Wen, Bui et al.

2016). This suggests intact B cell responses are critical for decreasing severity of HRV infection and clinical signs, but other types of effector cells also contribute to protective immunity. B cells appear to be much more important in complete RV clearance in mice. In one study, athymic mice were able to clear RV infection completely with a small delay, as well as resisting subsequent infection upon a second challenge (Franco and Greenberg 1997). In this same study, mice devoid of T cells were able to mount an RV-specific IgA response in the intestine that correlated with timing of viral clearance, and they were also resistant to reinfection (Franco and Greenberg 1997). In humans, B cell-deficient children can be chronically infected with HRV, and serum and intestinal antibodies are associated with protection in children (Coulson, Grimwood et al. 1992, Gilger, Matson et al. 1992, Velazquez, Matson et al. 2000).

3.2 T Cells

Unlike mice, in the Gn pig model, T cells appear to be required for the protection from and resolution of HRV infection and diarrhea. In the previously mentioned study using HCKO pigs, researchers also depleted T cells in the model. Attenuated HRV-vaccinated pigs who were devoid of CD8+ T cells in addition to B cells (HCKO/CD8-), had significantly more severe clinical signs of disease after challenge as compared to AttHRV-vaccinated pigs who were B cell-deficient only (Wen, Bui et al. 2016). The duration and cumulative scores of diarrhea of HCKO/CD8- pigs were actually more comparable to the HCKO pigs who were vaccinated with a sham vaccine (Wen, Bui et al. 2016). Virus shedding burden was also significantly higher in AttHRV vaccinated HCKO/CD8- pigs (Wen, Bui et al. 2016). These data suggest CD8+ T cells are essential for protection against HRV infection, as well as being important effectors after vaccination. In Gn pigs specifically, HRV-specific

interferon gamma (IFN-γ)-producing CD4+ and CD8+ T cells are important correlates of protection against HRV infection and diarrhea after both virulent HRV exposure and AttHRV vaccination (Yuan, Wen et al. 2008).

3.3 Correlates of Protection

Correlates of protection from HRV infection in humans remain incompletely understood but have been well studied in animal models. In Gn pigs, virus-specific IFN-γ T cells and IgA antibody-secreting cells (ASCs) in the intestine are strongly correlated with protection from virulent HRV challenge (Yuan, Ward et al. 1996, Yuan, Wen et al. 2008). Interestingly, transient detection of IgA ASCs in the blood was associated with IgA ASCs temporal presence in the intestine, indicating serum IgA ASCs as a potential correlate of intestinal protection (Yuan, Ward et al. 1996). In humans, it has been difficult to elucidate cellular immune correlates of protection due to sampling difficulty in infants. Intestinal IgA is thought to be a major contributor to protection from rotavirus infection in humans, and, similar to Gn pigs, serum IgA is thought to be an indicator of intestinal IgA (Angel, Franco et al. 2012). Thus, serum IgA has been historically used as an indicator of vaccine take (Franco, Angel et al. 2006). Because infants are unable to mount significant antibody responses to natural HRV infection early on in life, this correlate of protection is flawed at best, especially when evaluating vaccine responses (Franco, Angel et al. 2006, Ward, Kirkwood et al. 2006).

4. Rotavirus Vaccine Development

4.1 History of HRV Vaccines

4.1.1 Live Attenuated Vaccines

Due to the induction of protective immune responses by natural HRV infection, the focus of vaccine development has been on live attenuated vaccines, with the first, Wyeth Laboratories' RotaShield®, being licensed by the Food and Drug Administration (FDA) in August 1998. This vaccine was a live attenuated tetravalent rhesus RV (RRV-TV) vaccine containing an RRV strain with human G3's VP7, as well as human-rhesus reassortant strains that contain G1, G2, and G4 HRV VP7 genes (Delage 2000). This vaccine was shown to be safe and highly effective at preventing severe disease in infants who received the two-dose series in clinical trials (Delage 2000). By October 1999, however, 101 cases of intussusception in infants who had received at least one dose of the vaccine had been reported to the Vaccine Adverse Event Reporting System (Delage 2000). The manufacturer voluntarily withdrew the vaccine from further distribution by mid-October 1999. After post-withdrawal investigation, it was determined there was enough evidence to establish a causal relationship between intussusception and RotaShield® administration; however, it occurred disproportionately in infants who received their first dose after three months of age (Delage 2000, Bines 2005). A following phase II clinical trial with infants in Africa receiving the first dose during the neonatal period (0-27 days of age) and the second dose before 60 days of age, demonstrated its safety, immunogenicity and efficacy of 63.1% against rotavirus gastroenteritis of any severity associated with any of the four serotypes in

the vaccine (Armah, Kapikian et al. 2013). The vaccine is now slated for commercialization again as a cost-effective alternative to Rotarix® and RotaTeq® for Gavi-eligible countries. Nearly seven years after the initial withdrawal of RotaShield®, two new live attenuated vaccines were licensed by the FDA. Rotarix® (GlaxoSmithKline), a monovalent vaccine comprised of the attenuated 89-12 strain (G1P[8]), and Rotateq® (Merck), a pentavalent, bovine RV-based vaccine containing G1-4P[8] went through large safety and efficacy trials (Glass, Tate et al. 2021). They were both shown to be highly efficacious (>90%) against severe disease and they were implemented into national immunization programs shortly thereafter (Glass, Tate et al. 2021). Despite their high efficacy in high-income areas like the United States, these vaccines have proven to be much less effective in low- and middle-income countries (LMICs). The reason for this is multifactorial. Malnutrition, gut dysbiosis, and concurrent use of other oral vaccines (poliovirus) are all thought to be some of the contributing factors to the decreased efficacy in these areas, and the need for more efficacious vaccines is urgent (Patel, Glass et al. 2012, Gilmartin and Petri 2015, Lamberti, Ashraf et al. 2016). Though Rotarix® and RotaTeq® are most widely distributed and studied, some countries have licensed other live attenuated vaccines for use, including the monovalent Lanzhou lamb (LLR) (G10P[12]) in China, RV3-BB (G3P[6]) in Indonesia, Rotavac (G9P[11]) in India, and Rotavin-M1 (G1P[8]) in Vietnam (Dang, Nguyen et al. 2012, Li, Zhang et al. 2019, Kanungo, Chatterjee et al. 2022, Witte, Handley et al. 2022). There are multivalent vaccines being implemented as well, including Rotasiil, a pentavalent human-bovine reassortant containing G1-4,

9 and LLR3, a trivalent vaccine using LLR as a backbone with VP7 encoding human G2-4 (Xia, Du et al. 2020, Kanungo, Chatterjee et al. 2022).

4.1.2 Next Generation of Rotavirus Vaccines

Due to issues with currently used oral vaccines for HRV, mostly associated with the gastrointestinal tract, much research has focused on the development of parenteral nonreplicating vaccines. A trivalent P2-VP8* subunit vaccine adjuvanted with Alhydrogel™ recently made it to phase III clinical trials, though the efficacy was disappointing in comparison to Rotarix®, effectively ending the trial earlier than expected (Chen, Grow et al. 2022). Though mRNA as a therapeutic has been used for decades, the COVID-19 mRNA vaccines are the first to be licensed by the FDA (Schlake, Thess et al. 2012). Since the COVID-19 pandemic, mRNA platforms for vaccine development have gained attention as a potential solution for other infectious diseases. An inactivated vaccine candidate developed at the CDC utilizing CDC-9 (G1P[8]) HRV has shown promise in both mice and Gn pig models, particularly in the presence of concomitant poliovirus vaccine administration (Jiang, Wang et al. 2008, Wang, Azevedo et al. 2010, Jiang, Wang et al. 2013). Nanoparticle vaccines have also shown promise in mice and Gn pigs (Tan, Huang et al. 2011, Ramesh, Mao et al. 2019). A P24-VP8* vaccine, comprised of 24 copies of a norovirus capsid P domain displaying 24 copies of HRV VP8*, was highly immunogenic and conferred significant protection from virus shedding in Gn pigs (Ramesh, Mao et al. 2019).

5. Animal Models for Evaluation of HRV Vaccine Immunogenicity and Protective Efficacy

5.1 Mice

Mice are perhaps the most extensively used species for biomedical research. They require little space, have short gestation times, and a short lifespan (Bryda 2013). They share many anatomic and physiologic characteristics with humans, including sharing a large portion of their genome (Bryda 2013). Furthermore, with the completion of their full genome being sequenced in 2002, they are easily manipulated for the creation of transgenic models for use in an almost limitless capacity (Bryda 2013). Mice are naturally infected with murine RV, also referred to as epizootic diarrhea of infant mice (EDIM). Mice are only susceptible to the development of clinical signs after infection before 15 days of age, so adult mice are typically used as an infection only model (Franco and Greenberg 1999, Franco, Angel et al. 2006, Knipping, McNeal et al. 2011). In one of the first studies using mice as an RV infection model, infant mice inoculated with EDIM at one day of age exhibited severe diarrhea that lasted up to ten days, with infectious virus being detected in intestinal homogenates as late as 21 days post inoculation (Sheridan, Eydelloth et al. 1983). Naïve mice infected at seven days of age exhibited much less severe disease as compared to the younger mice, as well as a 50% reduction in duration of diarrhea (Sheridan, Eydelloth et al. 1983). Naïve mice inoculated at 21 days of age exhibited no signs of disease, though viral antigen was detected in the intestine (Sheridan, Eydelloth et al. 1983). Because of this apparent host-age restriction of the induction of disease coupled with the fact that HRV is unable to infect mice, the adult mouse model of RV infection has mostly been used to evaluate potential immune correlates of protection from infection that may be translatable

to humans (Franco and Greenberg 1999, Franco, Angel et al. 2006, Knipping, McNeal et al. 2011). Many researchers have utilized gene knockout mice to evaluate the necessity of cellular versus humoral immunity for the elimination of infection in mice, often with conflicting results. Though mice are cheap, easy to manipulate and naturally infected with murine RV, they are less suitable than other models for evaluating HRV pathogenesis and vaccine efficacy.

5.2 Nonhuman Primates

Currently, nonhuman primates (NHPs) make up a very small number of animals used in biomedical research, but their similarities to humans have made them invaluable in scientific discoveries that have resulted in the saving of millions of human lives (Friedman, Ator et al. 2017). Nonhuman primates are very closely related to humans genetically, and display similar mechanisms of infection and disease pathogenesis for a variety of agents. Their physiology and immunologic development and responses are also comparable to humans, making them the gold standard for translational biomedical research. Unfortunately, they require costly facilities and extensively trained personnel for handling, so they are often prohibitively expensive. Like mice, NHPs are naturally infected with RV. The first report of RV infection in an NHP occurred in 1963 in South Africa, where the prototype SA11 strain was isolated from a vervet monkey. This strain has been used extensively in studies examining RV growth and reproduction kinetics. In 1980, another RV strain was isolated from a rhesus monkey (RRV). This strain was used as the basis for the aforementioned rhesus-human reassortant live attenuated vaccine, Rotashield®. A recent report of a neonatal rhesus monkey model of SA11 RV infection and diarrhea showed that neonatal monkeys who received 10^8 FFU orally shed viral antigen in their stool

for 7 days, along with developing severe diarrhea that resolved itself gradually (Yin, Yang et al. 2018). Furthermore, viral antigen and histopathological changes such as edema, vacuolization, and destruction of mature villi were detected in the small intestinal epithelium of infected monkeys (Yin, Yang et al. 2018). These findings concur with what is seen in human infants, making this model a potentially promising tool for the future evaluation of HRV vaccine candidates, though the cost concerns for this model remain (Barnes and Townley 1973, Davidson and Barnes 1979).

5.3 Gnotobiotic Pigs

Pigs have long been used in biomedical research because of their similarities to human physiology, anatomy and genetics (Walters and Prather 2013). Additionally, pigs produce HBGA antigens that share high homology with human HBGAs (Guo, Candelero-Rueda et al. 2021). As such, pigs are naturally infected with several strains of RVs and are susceptible to experimental infection with HRV (Ward, Rosen et al. 1996, Nyblade, Hensley et al. 2022). Because conventional pigs are naturally infected with RV, seroconversion occurs early in life, thus making them unusable for HRV vaccine efficacy studies (Hodgins, Kang et al. 1999). Gnotobiotic pigs have been successfully and reliably infected with HRVs experimentally (Ward, Rosen et al. 1996, Nyblade, Hensley et al. 2022). The G1P[8] Wa HRV Gn pig model is a well-established model that has been used in HRV vaccine evaluation for more than two decades (Ward, Rosen et al. 1996, Yuan 2022). A new Gn pig model of G4P[6] Arg HRV was recently described by our laboratory and exhibits many of the same features as the Gn pig model of Wa infection (Nyblade, Hensley et al. 2022). It is especially useful as P[6] strains of HRV are becoming increasingly prevalent, and this new model allows for the evaluation of cross P-type

protection induced by HRV vaccine candidates (Nyblade, Hensley et al. 2022). The Gn pig is the only laboratory animal that develops HRV infection, and the development of intestinal pathology closely mimics that seen in humans infected with HRV (Ward, Rosen et al. 1996, Nyblade, Hensley et al. 2022, Yuan 2022). This model consistently develops clinical signs of disease (diarrhea) post-oral challenge with virulent HRV (Ward, Rosen et al. 1996, Nyblade, Hensley et al. 2022, Yuan 2022). Along with this, the Gn pig mirrors human immunity to HRV and vaccines, with serum IgA, IgG, intestinal IgA, and HRV-specific IFN-γ producing T cells correlating to protection from HRV infection and diarrhea (Yuan, Kang et al. 1998, Desselberger and Huppertz 2011, Wen, Cao et al. 2015, Ramesh, Mao et al. 2019, Yuan 2022). Furthermore, the Gn pig lacks any microbiota and the porcine placenta prevents the passive transfer of maternal antibodies (Yuan 2022). Therefore, Gn piglets are immunologically naïve but immunocompetent at birth (Yuan 2022). This prevents immune interference from commensal bacteria and maternal antibodies but allows for normal immunological development in response to preclinical vaccines (Yuan 2022). This is especially preferable in the preclinical evaluation of HRV vaccine candidates.

6. Concluding Remarks

Human rotavirus remains a major cause of AGE in LMICs. Disease burden is concentrated in these areas, and the need for more efficacious vaccines is apparent. Live attenuate vaccines have proven to be highly effective in high-income countries, but difficult-to-solve biological and logistic problems have hindered the improvement of their efficacy in LMICs. New technologies such as mRNA and nanoparticle antigen displaying platforms are promising tools for advancing HRV vaccines in these areas. The Gn pig model of HRV infection and diarrhea is an ideal model for preclinical vaccine efficacy studies due to its similarities to human anatomy/physiology and

immune response to both experimental HRV infection and vaccination. Their role in preclinical evaluation of next generation HRV vaccines is crucial.

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Chapter 2

Thermostable, Dissolvable Buccal Film Rotavirus Vaccine is Highly Effective in Neonatal Gnotobiotic Pig Challenge Model

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Abstract

Difficulties related to storage and transport of currently available live oral rotavirus vaccines can have detrimental consequences on the efficacy of the vaccines. Thus, there is a great need for thermostable vaccines that can eliminate the necessity for cold chain storage or reconstitution before administration. In this study, we developed a dissolvable oral polymeric film comprised of a live attenuated thermostable tetravalent rhesus-human reassortant rotavirus vaccine (RRV-TV) powder and antacid ($CaCO_3$). Immunogenicity and protective efficacy of the vaccine after buccal delivery was evaluated in the gnotobiotic pig model of human rotavirus (HRV) infection and diarrhea. Two doses of the vaccine were highly immunogenic and conferred strong protection against virus shedding and diarrhea upon challenge with a high dose of a virulent G1 HRV in gnotobiotic pigs. Those pigs vaccinated with the preserved film vaccine had significantly delayed onset of diarrhea; reduced duration and area under the curve of diarrhea; delayed onset of fecal virus shedding; and reduced duration and peak of fecal virus shedding titers compared to pigs in both the placebo and the reconstituted liquid oral RRV-TV vaccine groups. Associated with the strong protection, high titers of serum virus neutralization antibodies against each of the four RRV-TV mono-reassortants and G1 HRV-specific serum IgA and IgG antibodies, as well as intestinal IgA antibodies, were induced by the preserved film vaccine. These results demonstrated the effectiveness of our thermostable buccal film rotavirus vaccine and warrant further investigation into the promise of the novel technology in addressing drawbacks of the current live oral HRV vaccines.

Keywords: thermostable; oral film vaccine; rotavirus; diarrhea; gnotobiotic pigs

1. Introduction

Rotavirus is the leading cause of severe infantile diarrhea worldwide. Even with live oral vaccines widely available since 2006, rotavirus infections are still responsible for the majority of cases of diarrheal disease in children globally, accounting for up to 50% of these cases (Knipe and Howley 2013, CDC 2019). Of the many children under five years of age infected with rotaviruses, up to 215,000 die each year from this vaccine-preventable disease, and 90% of these deaths occur in low- and middle-income areas of Asia and Africa(Knipe and Howley 2013, Collaborators 2017, WHO 2018).

Rotavirus vaccine development has been most successful in the area of live attenuated, orally delivered vaccines. There are currently two licensed human rotavirus (HRV) vaccines available on the markets in the United States and many other countries, though there are others in various stages of preclinical and clinical trials (Bines and Kirkwood 2015). RotaTeq® (Merck, Kenilworth, NJ, USA) and Rotarix® (GSK, Brentford, UK) are both prequalified for worldwide use by the World Health Organization (WHO), and as of 2009, WHO has recommended that HRV vaccines be incorporated into all national immunization programs (WHO 2009). These vaccines are highly efficacious (~90%) at preventing severe rotaviral diarrhea in properly vaccinated children in the United States and other high-income countries, but their efficacies are much lower in low- to middle-income countries (LMIC) (39–70%) and the cause is multi-faceted (Vesikari, Karvonen et al. 2007, Armah, Sow et al. 2010, Boom, Tate et al. 2010, Patel, Glass et al. 2012, Gilmartin and Petri 2015, Lamberti, Ashraf et al. 2016, CDC 2019, Ramesh, Mao et al. 2019).

Despite their high efficacy in developed countries, RotaTeq® and Rotarix® lack some important characteristics for maintaining their efficacy in developing countries and increasing their distribution worldwide. First, both vaccines require consistent and stable temperatures for storage

and transport (2 °C to 8 °C) and are sensitive to extreme conditions. Fluctuations in temperature can lead to degradation of the vaccine components, resulting in inadequate dosing. Along with this, both vaccines are in either a liquid form (RotaTeq®) or freeze-dried powder requiring reconstitution with a liquid diluent (Rotarix®). This complicates the administration of vaccines to infants. According to the Vaccine Adverse Events Report System, the most common issues with both vaccines are related to the infant spitting up the liquid, diluent mix-ups, accidental injection, and accidental splashing into administrator's eyes (Hibbs, Miller et al. 2014). All of these scenarios present not only a health risk to healthcare workers, but also the risk of inadequate dosing and subsequent impaired protective efficacy for the infants receiving the vaccine. Furthermore, HRV vaccines are prohibitively expensive in LMIC, even in those receiving Gavi funding and subsidization (Pecenka, Debellut et al. 2018). A large portion of the costs for vaccines is their distribution that requires an intact cold chain. Based on an article published at The Conversation, WHO estimated that up to 50% of vaccines get wasted globally every year because of temperature control, logistics, and shipment-related issues (Thomas 2018). The appeal of inexpensive, thermostable, and easily administered vaccines is clear.

The first objective of this study was to develop a thermostable rotavirus vaccine in a mucoadhesive dissolvable polymeric film for reconstitution-free administration to the buccal or sublingual surfaces in order to provide simpler, more accurate dosing to neonates and to allow storage, shipment, and delivery of the vaccine at ambient temperatures. The second objective was to evaluate the immunogenicity and protective efficacy of the buccally administered thermostable tetravalent human-rhesus rotavirus reassortant (RRV-TV) film vaccine in a neonatal gnotobiotic (Gn) pig model of HRV infection and disease.

RRV-TV was selected because it is a proven vaccine: it was first licensed in 1998 but withdrawn in 1999 due to a rare association with intussusception, which occurred disproportionately in infants receiving their first dose at more than three months of age (Bines 2005). A phase II clinical trial with infants receiving the first dose during the neonatal period (0–27 days) and the second dose before 60 days of age demonstrated its safety, immunogenicity and efficacy of 63.1% against rotavirus gastroenteritis of any severity associated with any of the four serotypes in the vaccine in Africa (Armah, Kapikian et al. 2013). The vaccine is slated for commercialization again as a cost-effective alternative to Rotarix® and RotaTeq® for Gavi-eligible countries.

The Gn pig model is very well established, and is routinely used in HRV vaccine pre-clinical evaluation (Yuan 2017). It is the only available model large enough for buccal administration of the full-size film designed for human infants. This model is not only ideal due to its size, but for its physiologic, anatomic, and immunologic similarities to human infants. Their similar development of antibodies, lymphoid organs and cell-mediated immunity make them a practical choice for translational vaccine research (Yang 2014, Yuan 2017). In this study, the duration and severity of diarrhea and virus shedding in Gn pigs were monitored to assess the protection conferred by the vaccine against challenge with a virulent HRV strain. The intestinal and systemic virus-specific IgA and IgG antibody and serum virus-neutralizing antibody responses to G1-G4 rotaviruses after vaccination and challenge were measured to assess the immunogenicity of the preserved vaccine.

Polymeric films comprising vaccines for oral delivery have been formulated before (H. Oprins 2011, Bajrovic, Schafer et al. 2020). In both of these formulations, polymeric film was produced by solvent casting from aqueous solutions, which limits both the vaccine stability that can be

achieved at high ambient temperatures and incorporation of the amount of antacid necessary to protect from low gastric pH during intestinal delivery. Universal Stabilization Technologies, Inc. (UST) has introduced an alternative approach for production of thermostable vaccines encapsulated in water-dissolvable polymeric films, which comprises two steps: (1) Formulation of thermostable vaccine powders using Preservation by Vaporization (PBV) and subsequent micronization; (2) Anhydrous encapsulation of vaccine powder in polymeric films using polymers soluble in both acetone and water (Bronshtein 2015, Bronshtein 2016). This allows anhydrous production of films from an acetone-based polymer solution comprising suspensions of dry vaccine powders and other dry excipients/buffers (i.e., antacids) if needed.

PBV is a state-of-the-art foam-drying process that has been successfully used to produce various thermostable biologics, including live vaccines (Smith, Siirin et al. 2015, Universal Stabilization Technologies 2020, Luczo, Bousse et al. 2021). This technology is efficient, inexpensive, and drastically increases the shelf-life of its products without compromising the potency of the components (Bronshtein 2005, Bronshtein 2019, Universal Stabilization Technologies 2020). PBV-preserved live attenuated rabies virus (RABV) did not lose viral titers or antigenic content when stored at ambient temperatures and was effective in inducing neutralizing antibodies and protection in mice from peripheral RVBV challenge (Smith, Siirin et al. 2015). A live attenuated influenza vaccine (LAIV) intranasal powder was also developed from PBV LAIV and shown to be thermostable, immunogenic, and protective in ferrets against homologous challenge (Luczo, Bousse et al. 2021).

Using UST's approach, we formulated a thermostable PBV rotavirus vaccine and demonstrated stability of the vaccine encapsulated in polymeric films at ambient temperatures.

This dissolvable oral film containing thermostable RRV-TV was designed for buccal delivery, thus mitigating the previously described disadvantages of currently used oral HRV vaccines.

2. Materials and Methods

2.1. Vaccine Formulation

In this study, we used an RRV-TV vaccine containing three human-rhesus mono-reassortant rotaviruses carrying G1, G2, and G4 VP7 of HRV respectively, on the RRV MMU18006 strain backbone and the G3 RRV (P5B[3]G3) (Kapikian, Hoshino et al. 1996). Bulk live attenuated rotavirus vaccines were sourced from BravoVax Co. Ltd. in Wuhan, China (Bines 2005, Armah, Kapikian et al. 2013).

2.1.1. Preservation by Vaporization

Rotavirus G1, G2, G3, G4 serotype vaccines were preserved individually using PBV (Bronshtein 2016). Before drying, vaccines were mixed 1:1 with preservation solution comprising 35% sucrose and 5% mannitol. The vaccine-preservation solution mixture was distributed into 0.5 mL portions in 5 mL borosilicate glass serum vials, and dried using the PBV foam drying process in a conventional Virtis Genesis freeze-dryer (Virtis, Gardiner, NY, USA). PBV protocol consisted of less than 2 h of primary drying starting after the application of vacuum, after which the material became a stable foam, followed by secondary drying, which included 20 h at 45 °C under vacuum. The vials were then sealed under vacuum. Vials of PBV-dried rotavirus vaccine foams were stored in bags with Drierite desiccant at room temperature (RT; 22 °C ± 3 °C) and in incubators (37 °C) for use in film fabrication or subsequent long-term stability evaluation.

2.1.2. Micronization

To produce thermostable vaccine powder, PBV-dried rotavirus vaccine foams that had been stored at RT for 12 months were micronized in a humidity-controlled dry room at 13–15% relative humidity (RH). Micronization into powder was achieved using a Laarmann Labwizz lab-scale ball mill and sieved through a 63 µm mesh.

2.1.3. Film Fabrication

Dissolvable vaccine films were produced at UST using a conventional solvent casting approach. Films were designed for buccal administration to human infants. Each one square-inch film contained 60 mg of micronized PBV RRV-TV (4×10^5 focus forming units (FFU)/dose) and 60 mg calcium carbonate ($CaCO_3$) as antacid to protect viruses from low gastric pH during oral delivery. The dose of 60 mg of $CaCO_3$ was selected based on similar antacid dosing contained in Rotarix® during administration (GlaxoSmithKline).

Micronized rotavirus vaccine and antacid ($CaCO_3$) powders were mixed into an acetone solution comprising 8.5% *w*/*w* hydroxypropyl cellulose (HPC; M_w 80,000; Sigma Aldrich, St. Louis, MO, USA) and 0.75% *w*/*w* triacetin (plasticizer) to produce the polymeric vaccine mixture. HPC is soluble both in water and acetone and is a GRAS-listed ingredient. The polymeric vaccine mixture was poured onto a substrate sheet on an Elcometer 4340 automatic film applicator machine (Elcometer, Manchester, UK) and spread using a doctor blade at 3.0 mm thickness. The film sheet was air dried inside a humidity-controlled dry room (13–15% RH) for three hours, then was additionally dried under vacuum for one hour. The dry film sheet was removed from the substrate, weighed, and cut into dose-appropriate pieces. Film pieces were distributed into 10-mL serum

vials and dried under vacuum to remove remaining acetone, and subsequently sealed under vacuum for use in stability and challenge studies.

2.2. Rotaviruses for Challenge and Immunoassays

The challenge virus inoculum used for this study was derived from a pool of intestinal contents from the 29th passage of the virulent Wa strain (G1P[8]) HRV (VirHRV) in Gn pigs. The median infectious dose (ID_{50}) and median diarrhea dose (DD_{50}) of the VirHRV in Gn pigs were previously determined to be approximately 1 FFU (Ward, Rosen et al. 1996). The lysates of Wa HRV-infected African green monkey kidney MA104 cells (ATCC CRL-2378.1™) were partially purified by centrifugation through 40% (wt/vol) sucrose cushion and used as detector antigen in the ELISA for the detection of serum and intestinal antibody responses in Gn pigs as described previously (Twitchell, Tin et al. 2016).The human-rhesus mono-reassortant rotavirus G1, G2, G4 strains and G3 RRV strain were used in the virus neutralization (VN) assays for the detection of G type specific serum VN titers in the Gn pigs.

2.3. Vaccine Inoculation and Virus Challenge of Gn Pigs

The vaccine films and PBV RRV-TV powders were vacuum sealed in serum vials and transported in one shipment on ice by overnight FedEx t from Universal Stabilization Technologies, San Diego, CA to Virginia Tech, where they were stored at 4 °C upon arrival. They were stored for 42 days before being placed in the Gn pig isolators for the first vaccination of Gn pigs. The vaccines stayed in the isolator port for one hour under a controlled room temperature of ~30–34 °C (first dose at 34 °C on post-inoculation day [PID] 0 and second dose at 30 °C on PID 10) during the sterilization procedure.

Gnotobiotic pigs used in this study were surgically derived and maintained as previously described (Yuan 2017). Gn pigs (large white crossbreed) were fed ultra-high temperature treated (UHT) sterile whole cow's milk (The Hershey Company, Hershey, PA, USA) until PID 21 and were then transitioned to Similac® baby formula (Abbott Laboratories, Chicago, IL, USA) until the end of the study. The pigs remained bacteria-free throughout the experiment confirmed by weekly rectal swabs tested on blood agar plates and in thioglycolate broth.

A total of 17 Gn pigs (both male and female) were assigned randomly to three groups: (1) Preserved film (n = 5); (2) Placebo film (n = 6); and (3) Liquid vaccine (n = 6). Pigs received the first dose of vaccine or control at five days of age (PID 0) and the second dose at 15 days of age (PID 10).

The preserved film vaccine and placebo film were administered buccally and the liquid vaccine was administered orally. The films in two 1 × 1-inch pieces were placed in the left and right cheek pouch of the pigs. Contact time was longer than anticipated, with time to dissolve taking up to 20 min. A PBV thermostabilized powder RRV-TV containing the 4×10^5 FFU/dose was reconstituted in 5 mL of Diluent #5 [minimum essential media (MEM, ThermoFisher Scientific, Inc. Waltham, MA, USA); 100 IU of penicillin per mL, 0.1 mg of dihydrostreptomycin per mL; and 1% HEPES] immediately before use and a total volume of 5 mL was administered orally to Gn pigs for comparison of immunogenicity and protective efficacy with the preserved film vaccine. The liquid vaccine was fed through a needleless syringe and immediately swallowed by the pigs, with very brief contact to the buccal mucosa.

Serum samples were collected at PID 0, PID 10, PID 17, PID 28 and PID 35 for the detection of Wa HRV-specific IgA and IgG antibody responses by ELISA and G1, G2, G3, and G4 reassortant rotavirus strain-specific VN antibody titers by microplate VN assays. Pigs were orally

challenged with 6×10^5 FFU of VirHRV Wa strain at PID 28. Twenty minutes prior to vaccination and oral challenge with VirHRV, pigs were fed 4 mL of 200 mM sodium bicarbonate ($NaHCO_3$) to neutralize stomach acidity. Clinical signs were monitored daily and rectal swabs were taken for the detection of virus shedding from post-challenge day (PCD) 0 to PCD 7.

All pigs were euthanized on PCD 7. At euthanasia, small and large intestinal contents (SIC and LIC) were collected aseptically and processed as previously described, for the detection of intestinal antibody responses and antigen presence by ELISAs, as well as infectious virion counting by cell culture immunofluorescence (CCIF) (Parre, Hodgins et al. 1999).

2.4. Assessment of Diarrhea and Detection of Fecal HRV Shedding by Rotavirus Antigen ELISA and CCIF

For the assessment of severity of diarrhea post-challenge, fecal consistency scores were recorded daily as 0: normal, 1: pasty, 2: semi-liquid, and 3: liquid (Saif, Ward et al. 1996, Ward, Rosen et al. 1996). Pigs are considered diarrheic with scores of ≥2. Daily rectal swabs were collected and used for HRV antigen detection by ELISA and infectious virus particle detection by CCIF as we described previously (Twitchell, Tin et al. 2016). For using the capture ELISA to detect rotavirus antigen, a 96-well plate was coated with 100 µL/well goat anti-bovine rotavirus polyclonal unconjugated antibody (PA1-7241, ThermoFisher Scientific, Waltham, MA, USA; AB_561090) at a dilution of 1:250 in carbonate buffer (pH 9.6). The plate was incubated overnight at 4 °C, washed twice with PBST (PBS containing 0.05% Tween® 20 (Sigma Aldrich, Co. St. Louis, MO, USA), blocked with 300 µL/well of PBS (pH 7.4) containing 5% nonfat dry milk then incubated for 1 h at 37 °C. After washing three times with PBST), 100 µL/well of diluted sample (in Diluent #5) was added in duplicate. Semi-purified AttHRV antigen or swab samples from HRV-negative Gn pigs were used as a positive and negative control respectively. Plates were

incubated for 1 h at 37 °C then washed three times with PBST. Goat anti-bovine rotavirus polyclonal HRP-conjugated antibody (PA1-73015, ThermoFisher Scientific Waltham, MA, USA; AB_1018382) diluted 1:200 in PBS containing 1% BSA was then added to each well (100 µL/well) and incubated for 1 h at 37 °C, followed by three washes with PBST. The plate was developed with ABST peroxidase substrate solution for 15–30 min at room temperature before being stopped with ABST stop solution diluted 1:5. The optical density was measured at 405 nm using a microtiter plate reader (Twitchell, Tin et al. 2016).

2.5. Detection of Wa HRV-Specific Serum and Intestinal IgA and IgG Antibody by ELISA

To determine the Wa HRV-specific IgA and IgG antibody titers in serum, SIC and LIC samples, direct ELISA was performed as described previously, with modification (Twitchell, Tin et al. 2016). Briefly, 96-well microtiter plates were coated with semi-purified attenuated Wa HRV antigen at 68 µg/mL or mock-infected MA104 cell culture control lysate at 68 µg/mL in 0.05 M carbonate buffer (pH 9.6) for 1 h at 37 °C. The concentration of the semi-purified attenuated Wa HRV antigen was determined by NanoDrop™ One (Thermo Scientific, Waltham, MA, USA) and titrated in the range of 68 to 102 µg/mL, with 68 µg/mL determined as the optimal coating concentration. Following the incubation, plates were washed five times with TBST (Tris-buffered saline with 0.05% Tween® 20, pH 8.0) and blocked overnight at 4 °C with TBS-5% BSA. In separate deep 96-well plates, samples were diluted 4-fold serially with TBST-5% BSA, including one positive and two negative controls. One hundred µL/well of the serially diluted samples were then transferred to the coated plates and incubated overnight at 4 °C. The plates were washed five times with TBST and incubated in the dark at room temperature for 2 h with 100 µL/well horseradish peroxidase (HRP) conjugated goat anti-pig IgA antibody or HRP-conjugated goat anti-pig IgG-Fc antibody (Bethyl Laboratories, Inc. Montgomery, TX, USA) diluted 1:3000 in TBST-

1% BSA. Plates were washed five times with TBST and developed with 100 µL/well room temperature ABTS peroxidase substrate (1:1 ratio of KPL ABTS® peroxidase substrate solutions A and B from SeraCare Life Sciences, Inc. Milford, MA, USA) in the dark, for 15-30 min before ABTS peroxidase stop solution was added. Optical density was read at 405 nm.

2.6. Detection of G1, G2, G3 and G4 Rotavirus Strain-Specific Serum VN Antibody Responses

Rotavirus G1 to G4-type specific VN antibody titers in sera of all pigs collected on PID 28 and PCD 7 were determined by a microplate cell culture immunofluorescence staining assay as described previously (Twitchell, Tin et al. 2016). In brief, MA104 cells (passage 10 to 16) were grown in 96-well plates for three days to 100% confluence. Monolayers were washed once with Eagle's minimum essential medium (EMEM) (Corning, NY, USA), and the 96-well plates were incubated with serum-free EMEM at 37 °C for 2 h. In a separate 96-well plate, serum samples were diluted 4-fold in duplicate from 1:4 to 1:16384 and then were incubated with 4×10^3 FFU of mono-reassortant rotavirus G1, G2, G4 or RRV G3 strain for 1 h. The serum-free EMEM was discarded from the MA104 cell plates after a 2 h incubation. The plates were then inoculated in duplication with 50 µL/well of the mixtures of serum samples and G1 to G4 rotaviruses or EMEM and incubated at 37 °C for 1 h. Fifty µL of EMEM with trypsin (0.5 µg/mL) was added to each well and the plates were incubated at 37 °C with 5% CO_2 for additional 18–24 h. The plates were fixed with 80% cold acetone and stained with goat anti-bovine rotavirus polyclonal antibody (PA1-7241, ThermoFisher Scientific, Waltham, MA, USA) and FITC-conjugated rabbit anti-goat IgG antibody (F7367, Sigma, Inc. St. Louis, MO, USA). Fluorescing cells were observed by fluorescent microscopy. The virus-neutralizing antibody titer was defined as the reciprocal of the maximum dilution of sera that began to have any fluorescing cells.

2.7. Vaccine Stability Assessment by Focus Forming Assay

PBV foams of individual rotavirus serotype vaccines and RRV-TV vaccine films were stored with Drierite desiccant for stability assessment at room temperature (RT; 22 °C ± 3 °C) and in incubators (37 °C). Samples were removed from storage and tested at specific time points. PBV foams were tested at initial yield (0 month), three months, six months, and 12 months. PBV foam titers stored at RT or 37 °C were compared to liquid control vaccine (stored at –80°C). RRV-TV vaccine films were evaluated out to three months at varying storage temperatures, ranging from RT, 37 °C, 45 °C, and 50 °C.

Samples were tested using immunoperoxidase focus forming assay on MA104 cells on 96-well plates (Nest Scientific, Beltsville, MD, USA. TC-treated). PBV foams and vaccine films were reconstituted with serum-free Dulbecco's modified Eagle medium (DMEM; Corning) and trypsinized (final trypsin concentration 5 µg/mL) for 1 h at 37 °C. Trypsinized samples were serially diluted with serum-free DMEM to 1×10^{-8} dilution. MA104 monolayers were washed with serum-free DMEM, inoculated with 50 µL of activated sample from each dilution and incubated at 37 °C for 1 h. After adsorption, 150 µL of DMEM (serum-free, 1µg/mL trypsin) was added to each well, and incubated at 37 °C for 18 to 19 h. After incubation, cells were washed once with serum-free DMEM, then fixed in cold (−20°C) 80% acetone for 15 to 20 min at −20 °C, and air dried at room temperature. PBS + 0.5% Tween−20 was added to each well to wet the cell monolayer, then immediately aspirated. Cells were treated with goat anti-rotavirus antibody (Sigma Aldrich, St. Louis, MO, USA. AB1129; diluted 1:1000 in PBS + 1% BSA) and incubated at 37 °C for 1 h. Cells were washed three times with PBS + 0.5% Tween−20, then treated with HRP-conjugated rabbit anti-goat IgG antibody (Sigma Aldrich, St. Louis, MO, USA. AP106P; diluted 1:1000 in PBS + 1% BSA) and incubated at 37 °C for 1 h. Cells were washed three times

with PBS + 0.5% Tween−20, then developed using an AEC staining kit (Sigma Aldrich, St. Louis, MO, USA. AEC101) according to kit instructions. Sodium azide (0.05% in PBS) was added to each well following development. Focus forming units were counted under an inverted microscope at 100x magnification.

2.8. Statistical Analysis

Pigs were randomly assigned into treatment groups upon derivation by animal care technicians, regardless of gender and body weight. Kruskal-Wallis test followed by Dunn's test for multiple comparisons was used to evaluate cumulative diarrhea scores, days with diarrhea, area under the curve (AUC) of diarrhea, shedding onset day by ELISA and CCIF, and days with shedding by ELISA and CCIF. AUC of CCIF titers, mean peak titers and mean duration of diarrhea were analyzed by ordinary one-way analysis of variance (ANOVA). CCIF titers, antigen ELISA optical density (OD) values, and HRV-specific IgA, IgG, and VN antibody titers among the treatment groups were analyzed by two-way ANOVA with repeated measures and followed by Tukey's test for multiple comparisons. Diarrhea scores of individual pigs were analyzed by Friedman's test. All analyses were carried out using GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA). Data of vaccine stability after storage was analyzed by two-way ANOVA using SigmaPlot 14.5 (Systat Software Inc., San Jose, CA, USA). For all analyses, a p value lower than 0.05 was accepted as statistically significant.

3. Results

3.1. Preserved Thermostable Film Vaccine Conferred Strong Protection against Diarrhea and Virus Shedding upon Challenge with VirHRV

Vaccinated and control Gn pigs were challenged with VirHRV at PID 28 and were monitored daily for clinical signs of infection (diarrhea) and virus shedding from PCD 1 to PCD 7. For diarrhea, although the preserved film vaccine did not totally prevent diarrhea (four pigs had mild diarrhea for 1–2 days), pigs that received the preserved film vaccine had a significantly delayed onset of diarrhea (4.4 days), compared to the liquid vaccine (1.7 days) and the placebo film (1.8 days) vaccinated groups, as well as a significantly reduced mean diarrhea score on PCDs 3 and 4 (**Table 1** and **Figure 1A**). Preserved film-vaccinated pigs had a significantly reduced mean duration of diarrhea (1.8 days) compared to the liquid vaccine (4.5 days) and placebo film vaccine (4.3 days) pig groups (**Table 1**). The preserved film vaccine significantly reduced the AUC of diarrhea compared to the liquid vaccine and placebo groups (6.8, 9.7, and 10.6, respectively) (**Table 1**). There were no significant differences in any parameters assessing the severity of diarrhea between liquid vaccine and placebo groups (**Table 1** and **Figure 1B**).

For virus shedding, although rotavirus was detected by CCIF in the fecal samples of all the VirHRV-challenged pigs, the preserved film-vaccinated pigs only shed virus on the first two days and had significantly lower virus shedding titers by CCIF on PCDs 3, 6, and 7, whereas the liquid vaccine did not reduce virus shedding when compared to the placebo film controls (**Figure 1C,D**). Preserved film-vaccinated pigs showed significantly reduced duration of virus shedding (2.2, 5.7, and 6.8 days, respectively), mean peak titers (~ 4-fold lower) and AUC (~ 6- to 8-fold lower) of virus shedding compared to both placebo and liquid vaccine groups (**Table 1**). The amount of rotavirus antigen in fecal samples measured by ELISA OD values were substantially lower in both

preserved film vaccine and the liquid vaccine groups compared to the placebo control from PCDs 2–6, but there were no significant differences on any time point (**Figure 1E,F**).

3.2. Strong Serum IgG and IgA and Interstinal IgA Antibody Responses were Induced by Both the Preserved Film Vaccine and the Liquid Vaccine

Serum was collected from Gn pigs at PID 0, PID 10, PID 17, PID 28 (PCD 0), and PCD 7, as well as SIC and LIC on PCD 7 to evaluate antibody responses induced by the vaccination. Pigs vaccinated with both preserved film vaccine and liquid vaccine showed significantly higher serum IgA and IgG titers than placebo film-vaccinated pigs from PID 17 onward (**Figure 2A**). They both also had significantly higher IgA titers in SIC and LIC on PCD 7 compared to pigs in the placebo film group. Notably, however, IgA titers in the LIC of preserved vaccine group were significantly higher than the liquid vaccine group, and this is associated with the significantly higher protection conferred by the preserved vaccine (**Figure 2B**). Intestinal IgG titers were low and there was no difference in both SIC and LIC of any groups (**Figure 2B**).

3.3. Significantly Higher Titers of G1, G3, and G4 Rotavirus Serum VN Antibody Responses were Induced by the Preserved Film Vaccine than the Liquid Vaccine at PCD 7

G1 to G4 rotavirus-specific VN antibodies were measured in serum samples from pigs at PID 28 (PCD 0) and PCD 7. The preserved film-vaccinated pigs had similar VN GMTs to the liquid vaccinated pigs at PID 28, but higher or significantly higher G1, G2, G3, and G4 VN antibody levels compared to the liquid vaccinated pigs on PCD 7 (**Figure 3**). This coincided with the significantly higher IgA titers in the LIC at PCD 7 and is associated with the significantly higher protection against diarrhea and virus shedding in the preserved film vaccine group. In the preserved film vaccine group, the highest VN antibody GMT was against G3 at PID 28. The VN GMTs

increased substantially from PID 28 to PCD 7 with the highest fold increase against G1 (52-fold), reflecting the anamnestic response to the homologous G1 rotavirus challenge, followed by G3 (18-fold), G4 (12-fold), and G2 (9-fold) rotaviruses (**Table 2**). In the liquid vaccine group, the highest VN GMT at PID 28 was also against G3. However, from PID 28 to PCD 7, it only increased 13-fold against G1, the same fold increase to G1 as in the placebo film group. Post-challenge, the VN GMT to G2, G3, and G4 rotaviruses only increased slightly (2–4 fold) in the liquid vaccine group and placebo film group (3-fold to G3 and no increases to G2 and G4).

3.4. Safety of Preserved Film and Liquid Vaccine in Neonatal Gn Pigs

Overall health status of the Gn pigs was monitored daily throughout the experiment by certified animal care staff. There was no intussusception after vaccination or VirHRV challenge in any of the pigs. There were no other signs of adverse effects after vaccination except occasional incidences of diarrhea (fecal scores ≥ 2) were recorded in the preserved film vaccine group (10), liquid vaccine group (8), and the placebo film group (3) after PID 10 (**Supplemental Table S1**). We changed the pigs' diet from whole cow's milk on PID 21 to Similac® baby formula until the end of the study. Usually, the mild diarrhea caused by indigestion of cow's milk can be resolved by switching to baby formula which contains less fat. On PID 28/PCD 0, there were still 1–2 pigs in each group that had diarrhea. Those diarrhea scores before challenge were taken into consideration as the covariance in a Kruskal-Wallis test, which could reduce the impact of the pigs who had diarrhea before challenge and could be potentially prolonged into post-challenge period. The analysis results showed there was no difference among treatment before challenge, and the results also showed that the pre-challenge diarrhea scores only have a very little influence on the diarrhea scores after challenge.

3.5. Preservation by Vaporization Stabilizes Live Oral Rotavirus Vaccine at Ambient and High Temperatures

After storage at different temperatures, live rotavirus vaccine titer was measured via immunoperoxidase focus forming assay. In carbohydrate foam format, no significant loss (determined as 0.5 logs or more of loss as compared to liquid control vaccine stored at –80 °C) was observed in any of the rotavirus serotype vaccines even after a 12-month storage period at 37 °C (Figure 4). Highest observed titer loss was seen in rotavirus serotype G3 after 12 months at 37 °C, measuring 0.39 logs of loss compared to frozen liquid control (**Figure 4C**). Stability of RRV-TV vaccine films was also evaluated out to three months at varying storage temperatures, ranging from RT (22 ± 3 °C), 37 °C, 45 °C, and 50 °C (**Table 3**). Less than 0.5 logs of loss was measured at each storage time/temperature group. The PBV method confers excellent thermostability to live attenuated rotavirus vaccines in foam format and in films incorporating micronized PBV powder.

4. Discussion

Preservation by Vaporization is a very gentle and quick drying process. Primary drying only requires approximately 2 h, and less than 0.1 logs of titer loss occurs due to the drying process in all rotavirus serotypes with the exception of rotavirus G2, in which we observed 0.3 logs of loss (**Figure 4B**). From our long-term stability evaluation, we observed <0.5 logs of loss in PBV foams after drying and subsequent 12 months of storage at both RT and 37 °C in all serotypes (**Figure 4**). PBV-dried RRV-TV is also well-preserved during micronization and encapsulation in films, with <0.3 logs of loss observed. From the same film batch that was produced for use in Gn pigs, we observed no loss in titer during three months of storage at RT, and <0.2 logs loss at 37 °C (**Table 3**). We believe our dissolvable oral RRV-TV vaccine films to be thermostable and well-suited for cold chain-free storage, shipment, and delivery.

The immunogenicity and protective efficacy of the preserved thermostable dissolvable oral polymeric film RRV-TV vaccine were evaluated in neonatal Gn pigs in comparison with the reconstituted liquid RRV-TV vaccine. After two buccal doses of vaccine, the preserved film vaccine conferred significant protection against diarrhea and virus shedding upon challenge with the virulent Wa HRV. The preserved film vaccine significantly delayed the onset of diarrhea, shortened the duration of diarrhea, and reduced the AUC of diarrhea compared to the placebo film. The preserved film vaccine also significantly reduced virus shedding compared to the placebo film. Preserved film-vaccinated pigs had significantly reduced duration, mean peak titers, and AUC of virus shedding compared to pigs in the placebo film group. In contrast, the reconstituted liquid RRV-TV did not confer any protection. Among the various transmucosal vaccination routes, buccal mucosa is the best choice as it has excellent patient accessibility, can induce both humoral and cell-mediated protective immunity, especially strong mucosal immune responses, and the buccal area has direct access to the systemic circulation through the internal jugular vein (Kraan, Vrieling et al. 2014, Uddin, Allon et al. 2019). It avoids the first pass effect leading to high bioavailability of the vaccine and can have a dose sparing effect. This may be another reason to explain the discrepancy between the similar immune responses but different protection conferred by the buccal preserved film and oral liquid vaccines.

The preserved film vaccine demonstrated high immunogenicity in neonatal Gn pigs. Two doses of the film vaccines induced strong IgG and IgA antibody responses in serum, LIC and SIC, and also high levels of serum VN antibody responses pre-challenge to all four G types of the rotaviruses corresponding to the RRV-TV vaccine in this study. Post-challenge, the GMT of serum VN antibodies in preserved film vaccine group had the highest fold increases to all the four G types among the three treatment groups from pre-challenge (**Table 2**), suggesting the preserved

film vaccine primed for the strongest anamnestic responses against the VirHRV challenge. However, the preserved film vaccine did not totally prevent virus infection in any pigs. Virus shedding in the preserved film-vaccinated pigs was brief, mostly occurred at PCD 1–2. After PCD 2, there were only two pigs shedding low titers of rotavirus for one day at PCD 4 (CCIF titer 1600) and PCD 5 (400), respectively (**Figure 1C**). This type of protection is characteristic of T-cell mediated immunity. In previous studies of live oral HRV vaccines in Gn pigs, three doses of oral immunization with the Wa AttHRV vaccine prevented virus infection in 68% of the pigs upon challenge with the homologous Wa VirHRV, in which there was a significant correlation between intestinal and serum IgA antibody responses with the protection rate (Yuan, Ward et al. 1996). It is perceivable that humoral immune responses did not play major role in the protective immunity conferred by the preserved film vaccine. The hallmark of T-cell mediated protection is that it does not totally prevent infection but the memory T cells induced by the vaccine residing in the small intestinal lamina propria can be reactivated quickly to secret anti-viral cytokines, become cytotoxic cells or facilitate anamnestic antibody responses to clear the virus infection post-challenge (Mackay and von Andrian 2001). It is likely that the rotaviruses in the preserved film vaccine replicated significantly better in the pig intestine than the liquid vaccine and thus induced a much stronger memory T-cell response in these pigs, resulting in stronger protection against virus infection and diarrhea upon challenge with VirHRV. Unfortunately, T-cell responses were not measured in this study.

A possible reason for the different vaccine effectiveness between preserved film and liquid vaccine is the inclusion of antacid. The preserved film vaccine contains antacid in the film formulation, so it was simultaneously delivered to the pigs whereas liquid vaccine does not contain antacid. The sodium bicarbonate was fed to pigs 20 min prior to vaccine inoculation. It is possible

that while the stomach pH may have been neutralized at first, within the 20 min timeframe, additional gastric acid sufficient to lower the pH was produced, enough to reduce the virus viability and hence vaccine efficacy.

Although not protective, the liquid vaccine induced similar magnitude of antibody responses as the preserved film vaccine in Gn pigs pre-challenge. The serum IgG and IgA, and VN antibodies to all the four G type viruses, as well as IgA antibody responses in SIC induced by the liquid vaccine were comparable to the preserved film vaccine at PID 28. After the challenge, however, the VN GMTs in the liquid vaccine group was much lower than the preserved vaccine group because the fold increases were much smaller compared to the preserved film vaccine, indicating its weaker immunogenicity for memory responses.

In a large placebo-controlled efficacy trial of the RRV-TV in humans, no consistent relationship was found between the titers of any VN antibodies [Wa (G1), DS-1 (G2), P (G3), ST-3 (G4), RRV (G3)] or rotavirus-specific IgA following vaccination and protection against rotavirus, thus suggesting that serum antibody titers would not be useful markers of protection with the RRV-TV vaccine (Ward and Bernstein 1995). In our study, the Gn pigs vaccinated with the preserved film vaccine had significantly higher titers of IgA in LIC and significantly higher serum VN antibody titers to G1, G3, and G4 rotavirus on PCD 7 than the liquid vaccine, which may also indicate that the main immune effectors conferring the protection upon challenge were T cells. Stronger helper T-cell responses might have been primed by the preserved film vaccine, leading to stronger anamnestic antibody immune responses after infection caused by VirHRV challenge.

In addition to the stronger anamnestic intestinal and systemic immune responses induced by the preserved film vaccine, salivary IgA responses may have also played a role. Because the

preserved film vaccine was administered buccally (but was not quickly dissolved), the result was a prolonged exposure to the mucosal surfaces in the mouth. It is possible that a virus-specific salivary IgA antibody response may have been stimulated by the buccal film vaccine, resulting in stronger protection against infection from oral virus challenge. Buccal mucosa is rich in immune cells such as dendritic cells and Langerhans cells and these cells are able to uptake vaccines in the film (Uddin, Allon et al. 2019). Rotavirus-specific antibody-producing B cells can then develop and reside in salivary glands or homing to intestinal mucosal surfaces through the common mucosal immune system and produce IgA antibodies (Brandtzaeg 2013). Secondary IgA responses tend to be quicker, stronger and longer-lasting upon subsequent exposure to antigen than primary responses (Murphy 2005). Since pigs were vaccinated twice, both with prolonged mucosal exposure to preserved film vaccine, we would expect to see an increased salivary IgA response after the second vaccination on PID 10, lasting long enough to mitigate oral challenge with VirHRV at PID 28. After natural infection by rotavirus or oral inoculation with RRV-TV in infants, ~50% of infants had rotavirus-specific saliva antibody responses; however, the role of salivary IgA in rotavirus protective immunity was not studied(Aiyar, Bhan et al. 1990, Friedman, Segal et al. 1993). Regrettably, we did not collect salivary samples from the pigs in this study. It will be interesting to measure salivary IgA antibody responses in future studies of animals or humans vaccinated with buccal film vaccines to validate this hypothesis.

The mucoadhesive dissolvable polymeric film vaccines are designed to adhere quickly once applied to the buccal mucosal membrane in the mouth and exposed to moisture. The film dissolves over time and releases the antacid ingredients and vaccine for oral delivery. Two initial safety studies were outsourced to determine toxicity of film ingredients and irritation/sensitivity on oral mucosal membranes. A preliminary 14-day toxicity study was performed by Charles River

Laboratories using four groups of five male rats (20 in total), administered with either PBS solution, one sq. inch dose of film (full human dose), one-tenth sq. inch dose of film, or one sq. inch dose of film containing no PBV powder. Films used in the toxicity study contained no antacid; the $CaCO_3$ antacid used is FDA-approved and fully characterized, and the excessive dose for rat size would have conflated any adverse clinical effects caused by high dose antacid with those caused by films. Doses were administered twice via oral gavage, on days 1 and 7, and all groups were terminated on day 14. Study results showed no clinical observations of toxicity, effects on body weight or food consumption, changes in hematology or clinical chemistry parameters, or effects on organ weight, and no macroscopic or microscopic findings at any dose level (unpublished data).

A preliminary oral mucosal irritation/sensitivity study in hamsters was performed by NAMSA. The right cheek pouch of 10 hamsters was implanted with full human dose film (containing both vaccine PBV powder and antacid). The left cheek pouch of all animals was implanted with polymethylmethacrylate (PMMA) disks which served as control article. Both articles were held in place with fitted collars. Good adhesion to hamster mucosal membrane was also observed after administration. Animals were euthanized after 24 h of exposure and pouch mucosa were subsequently removed for evaluation. The results showed no macroscopic or microscopic reactions of the RRV-TV film to hamster cheek tissue (unpublished data).

Due to the relative newness of the technology used to develop the buccal film, it was apparent during animal experiments that the formulation needs further improvement to offer a usable oral vaccine alternative in human infants. As mentioned above, the film comprised a high percentage of powders, causing it to be brittle, thick, and difficult to manipulate. Plasticity of the films was evaluated by observation of film behavior after repeated bending and folding. It was determined

that film plasticity strongly decreases when the amount of total powders incorporated within a one sq. inch film increases above 60 mg. Due to dosing requirements, total mass of powder in our vaccine formulation was 120 mg, and the resulting films expressed brittle behavior, which made them difficult to manipulate and administer. To increase plasticity of the films, the mass of the vaccine powder per film can be substantially decreased by concentrating the viruses before drying. There is also significant room to reduce the mass of antacid per film without negatively affecting vaccine potency. A recent study has demonstrated that 10 mL of 200 mM buffer concentration is sufficient to neutralize 4 mL of 0.1 N HCl, which mimics infant stomach conditions (Kumar, Pullagurla et al. 2021). To eliminate the negative effect of low gastric pH on orally delivered rotavirus vaccines, a dose of 40 mg of $CaCO_3$ per film should be sufficient. Another study found no statistical difference in IgA seroconversion rate between Rotarix® RIX4414 vaccine reconstituted with liquid $CaCO_3$ buffer or with water (Kerdpanich, Chokephaibulkit et al. 2010). The mass of $CaCO_3$ used per film could be potentially lowered several times while still guaranteeing vaccine efficacy.

A formulation with a thinner, more flexible texture and quicker to dissolve film would be ideal as has been discussed in the review article by Uddin et al. (Uddin, Allon et al. 2019). Promisingly, this issue can be easily resolved by concentrating the starting bulk liquid vaccine, thus reducing the total mass of micronized vaccine powder that must be incorporated into the film. Future formulations will include more concentrated vaccine powder and lower amounts of antacid per film, resulting in thinner and more flexible films. Starting with bulk liquid rotavirus vaccine with 5–10 times higher concentration will allow production of adequately concentrated vaccine powders for this purpose.

Certainly, further investigations to identify the specific immune responses that mediated the strong protection by the preserved thermostable film vaccine are warranted. Investigating T-cell responses, including intestinal IFN-γ producing effector/memory T cells and T follicular helper cells which play a critical role in helping B cells to produce antibody against foreign pathogens, would generate the insight on the cell-mediated immunity conferred by the vaccine (Yuan, Wen et al. 2008, Holmgren, Parashar et al. 2017). Due to the novel route of administration, evaluating salivary antibody responses would further clarify mechanisms of protection, as well.

5. Conclusions

Our study demonstrated the safety, immunogenicity, and effectiveness of the thermostable, dissolvable oral film RRV-TV rotavirus vaccine using the neonatal Gn pig model of HRV infection and diarrhea. Two doses of the preserved film vaccine were highly immunogenic and conferred significant protection against virus shedding and diarrhea upon challenge with a high dose of a virulent G1 HRV. Associated with the strong protection, higher or significantly higher GMTs of anamnestic serum VN antibodies against each of the four serotype rotaviruses and significantly higher titers of G1 HRV-specific large intestinal IgA antibodies were induced by the preserved film vaccine compared to the non-protective liquid vaccine, implying strong memory immune responses (i.e., helper T-cell responses) were primed by the preserved film vaccine. The characteristics of the protection against virus infection suggest that T-cell mediated immunity played a more important role than humoral immunity in the preserved film-vaccinated pigs. Future studies of the vaccine should evaluate the correlation of effector/memory T-cell responses and protection. Our results provided solid evidence supporting the further film formulation optimization and evaluation of the thermostable, dissolvable polymeric oral film RRV-TV rotavirus vaccine in human clinical trials, which will lead to a licensed thermostable RRV-TV

rotavirus vaccine that is of low cost in manufacture, storage, and shipment and is highly immunogenic and protective for neonates in the developing world.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10 .3390/vaccines9050437/s1, Table S1: Diarrhea incidences in Gn pigs from PID 0 to PID 28/PCD 0 before challenge.

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Table 1. Diarrhea and virus shedding in preserved film-vaccinated and control pigs after challenge with virulent Wa HRV.

		Clinical Signs of Diarrhea					Virus Shedding				
Treatments	***n***	**% with Diarrhea**	**Mean Days to Onset**	**Mean Duration Days**	**Mean Cumulative Fecal Score**	**AUC of Diarrhea**	**% Shedding Virus**	**Mean Days to Onset**	**Mean Duration Days**	**Mean Peak Titer (FFU/g of feces)**	**AUC of Virus Shedding**
Preserved Film	5	80% (4/5)	4.4 (1.12) [A]	1.8 (0.49) [B]	9.0 (0.89)	6.8 (1.14) [B]	100% (5/5)	1.0 (0.25)	2.2 (0.45) [B]	3120 (1242) [B]	4080 (1418) [B]
Placebo Film	6	100% (6/6)	1.8 (0.40) [B]	4.5 (0.72) [A]	12.0 (1.15)	10.6 (0.31) [A]	100% (6/6)	1.0 (0.17)	6.8 (0.17) [A]	12600 (1217) [A]	33200 (3265) [A]
Liquid Vaccine	6	100% (6/6)	1.7 (0.33) [B]	4.3 (0.67) [A]	13.3 (1.69)	9.7 (1.99) [AB]	100% (6/6)	1.0 (0)	5.7 (0.61) [A]	12533 (3023) [A]	24767 (5819) [A]

Note: a. Pigs were immunized twice with preserved film, placebo film buccally, or liquid vaccine orally at 5 (post-inoculation day [PID] 0) and 15 days (PID 10) of age. On PID 28, all pigs were orally challenged with 6×10^5 FFU of virulent Wa HRV and monitored for diarrhea and virus shedding for 7 days post-challenge. b. Fecal consistency scores were used to assess diarrhea; scores are defined as 0: solid, 1: pasty, 2: semi-liquid, and 3: liquid. Scores of 2 or higher are considered diarrheic. Rotavirus shedding titers were determined by rotavirus antigen ELISA (detect viral antigen) and CCIF (determine the number of infectious viral particles). If there is no diarrhea or virus shedding, the mean days to onset were assigned as one day after the pigs were euthanized (8) for statistical analysis. c. Different letters indicate significant differences between groups ($n = 5$–6; $p < 0.05$), while shared letters or no letters indicate no significant difference. d. Numbers in parentheses are the standard error of the mean (SEM).

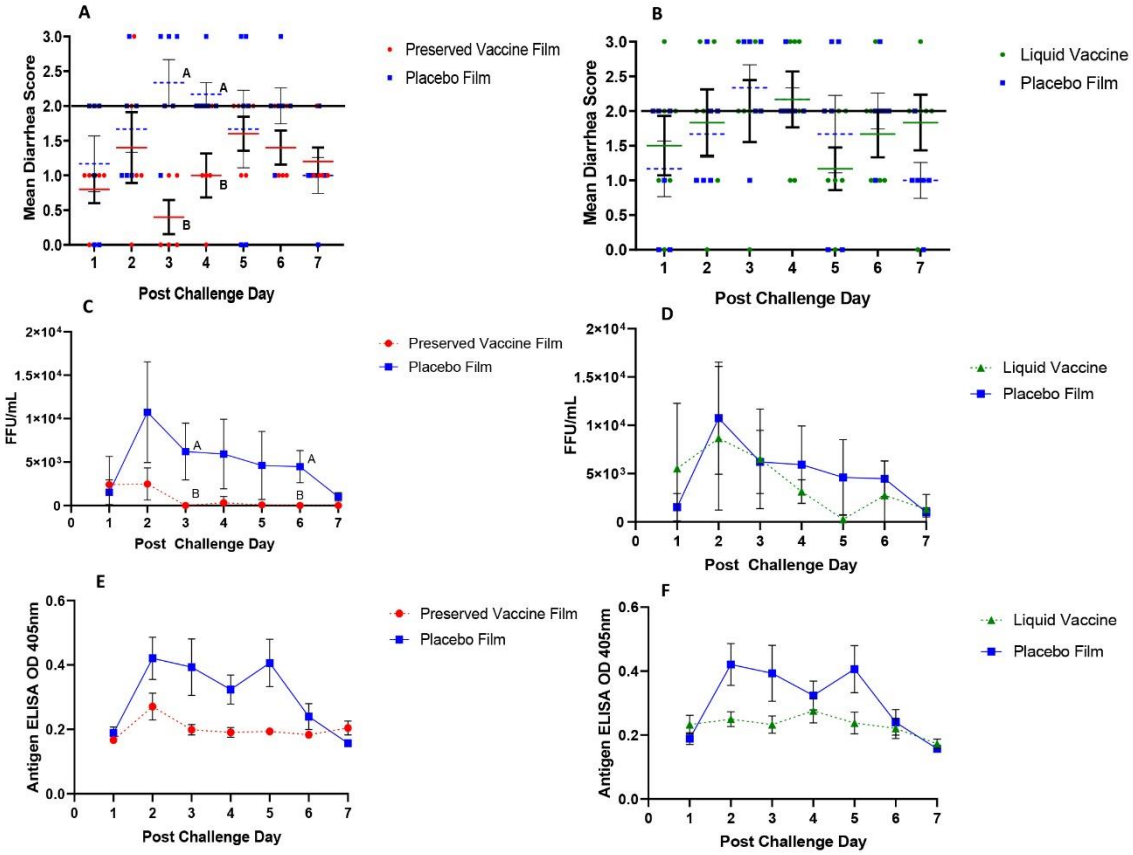

Figure 1. Diarrhea score and virus shedding post-challenge in Gn pigs vaccinated with preserved film vaccine (A,C,E) or liquid vaccine (B,D,F). The pigs vaccinated with preserved film vaccine had significantly reduced diarrhea on PCDs 3–4 compared to the placebo film group (**A**). Pigs in the preserved film vaccine group also showed a reduced overall virus shedding by CCIF (**C**) and antigen ELISA (**E**). The mean ± SEM bars of pig groups are indicated by solid horizontal line and vertical thicker lines, respectively (**A**–**B**). Fecal rotavirus antigen was measured by ELISA and results are expressed as OD units, and infectious virus particles were measured by CCIF and expressed as FFU/mL. Fecal samples from mock-infected pigs were used as negative controls in both antigen ELISA and CCIF. Different capital letters (**A**,**B**) indicate significant differences ($p < 0.05$) between groups at the same time point, while shared letters or no letters indicate no significant difference. ELISA, enzyme-linked immunosorbent assay; FFU, focus forming units; OD, optical density.

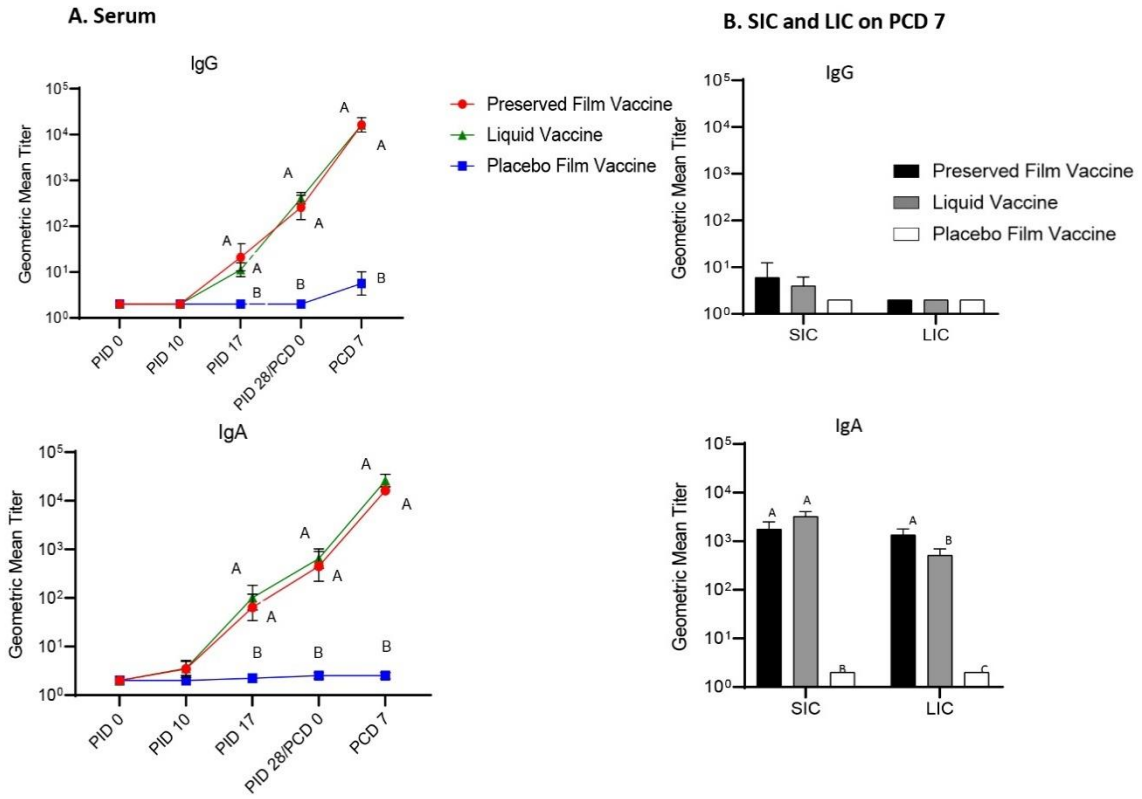

Figure 2. Geometric mean titers of Wa HRV-specific IgG and IgA antibodies in Gn pig serum samples collected at PID 0, PID 10, PID 17, PID 28, and PCD 7 (A), and in small intestinal contents (SIC) and large intestinal contents (LIC) collected on PCD 7 (B). Serum, SIC, and LIC samples were tested at a series of 4-fold dilutions, beginning at 1:4. All negative samples were given a titer of 2 to allow for data analysis and use in graphical depictions. Different capital letters (A, B, C) indicate significant differences ($p < 0.05$) between groups, while shared letters indicate no significant difference. Data points without capital letters indicate no significant difference between any of the groups at that time point.

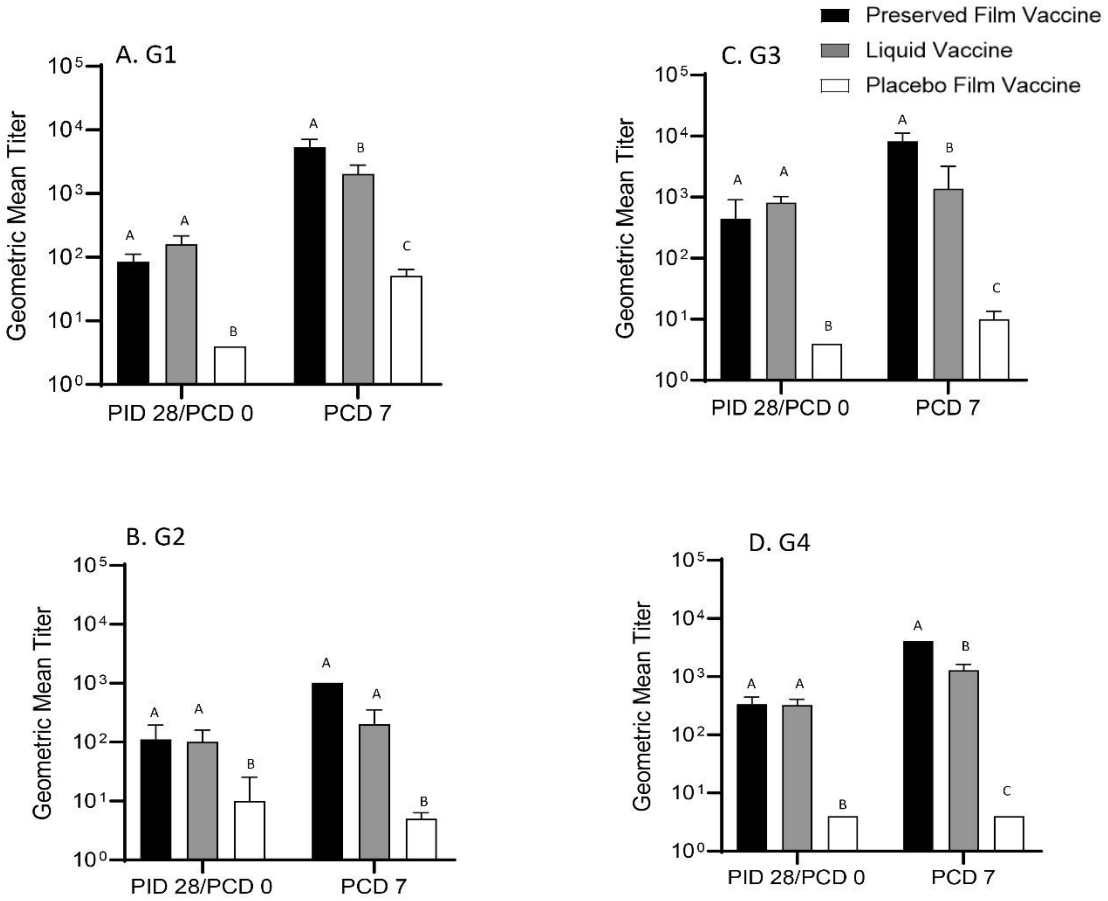

Figure 3. Geometric mean titers of virus-neutralizing (VN) antibodies in Gn pig serum samples at PID 28 and PCD 7. The sera were tested in a series of 4-fold dilutions, beginning at 1:4 in EMEM to 1:16384 for neutralization of 4×10^3 virions of G1 (**A**), G2 (**B**), G4 (**D**) human-rhesus reassortant rotaviruses and G3 (**C**) RRV. Different capital letters (A, B, C) on the top of the bars indicate a significant difference ($p < 0.05$) between groups, while shared letters indicate no significant difference.

Table 2. Serum VN antibody GMT increases from pre-challenge (PID 28) to post-challenge (PCD 7).

GMT	PID 28	PCD 7	Fold Increase	PID 28	PCD 7	Fold Increase	PID 28	PCD 7	Fold Increase	PID 28	PCD 7	Fold Increase
Group/type	G1			G2			G3			G4		
Preserved film vaccine	111	5793	52	111	1024	9	446	8192	18	338	4096	12
Liquid vaccine	161	2048	13	102	203	2	813	1380	2	323	1290	4
Placebo film vaccine	4	51	13	4	5	1	4	10	3	4	4	1

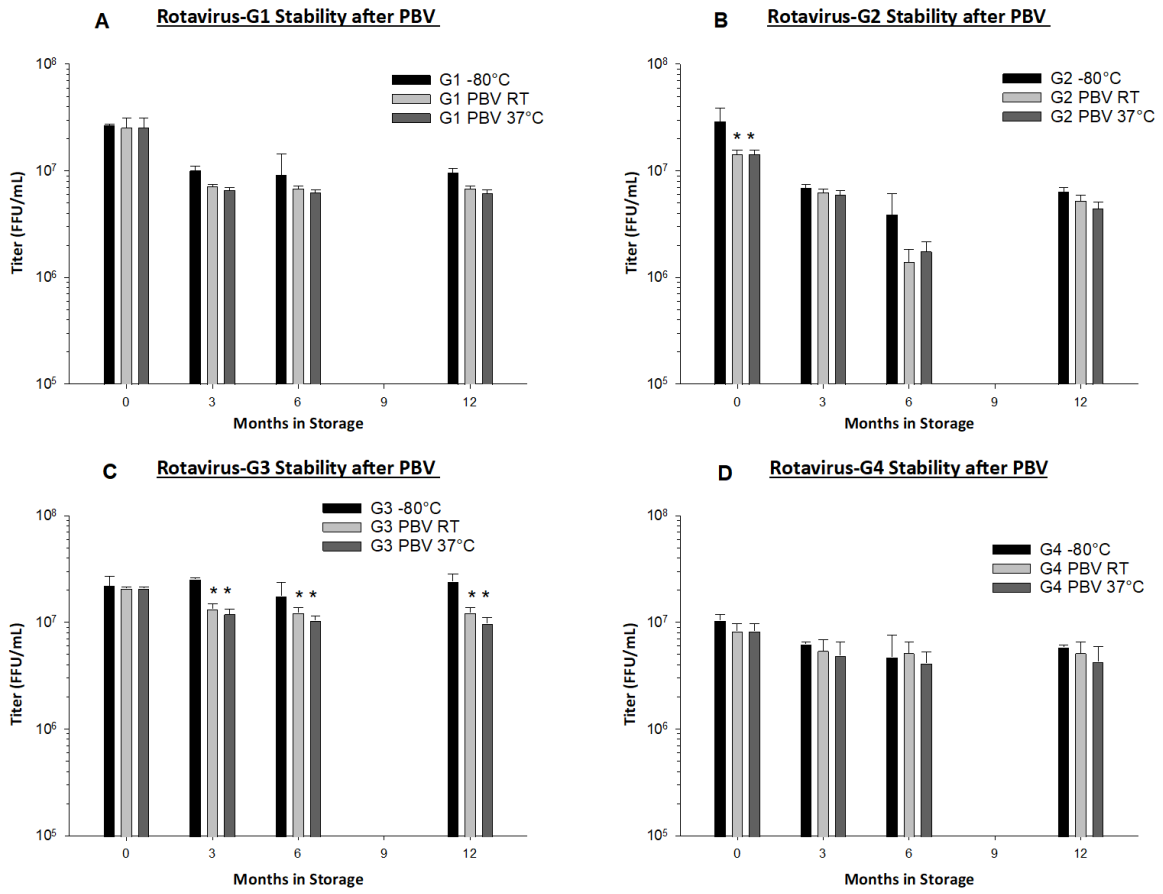

Figure 4. Long-term stability of preservation by vaporization (PBV) live rotavirus vaccines G1 (A), G2 (B), G3 (C) and G4 (D) at different temperatures was tested via immunoperoxidase focus-forming assay on MA104 cells. PBV provided high long-term stability and viral recovery for individual rotavirus vaccine strains after 1 year of storage at room temperature (RT) and 37 °C in the dry carbohydrate foam format. Samples of PBV vaccines stored at RT (22 ± 3 °C) and 37 °C were tested at initial yield (0 month), three months, six months, and 12 months in comparison to liquid control vaccine stored at –80 °C. Asterisk (*) indicates significance ($p < 0.05$) between test group and –80 °C control (two-way ANOVA). Less than 0.5 logs loss in measured titer was observed in all groups in comparison to –80 °C control vaccine, even groups where significance was noted.

Table 3. Stability of PBV RRV-TV dissolvable films.

Storage Time	Storage Temperature	Mean Film Titer (FFU)	stdev	Logs of Loss
0 month	--	5.80×10^5	$\pm 0.27 \times 10^5$	−0.24
2 weeks	50 °C	4.01×10^5	$\pm 0.43 \times 10^5$	−0.40
1 month	RT	5.73×10^5	$\pm 0.20 \times 10^5$	−0.24
	37 °C	4.89×10^5	$\pm 0.11 \times 10^5$	−0.31
2 months	RT	5.42×10^5	$\pm 0.09 \times 10^5$	−0.27
	45 °C	3.57×10^5	$\pm 0.12 \times 10^5$	−0.45
3 months	RT	5.54×10^5	$\pm 0.16 \times 10^5$	−0.26
	37 °C	4.01×10^5	$\pm 0.27 \times 10^5$	−0.40

Note: Logs of loss calculated from target titer of 1×10^6 FFU per film tested.

Chapter 3

mRNA-based vaccines are highly immunogenic and confer protection in the gnotobiotic pig model of human rotavirus diarrhea

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Abstract

An mRNA-based vaccination platform that uses sequence-optimized mRNA, has demonstrated promising results against a variety of viral pathogens in several animal models. In this study, we assessed the immunogenicity and protective efficacy of two mRNA-based human rotavirus (HRV) trivalent vaccine candidates (encoding genotypes P[8], P[6] and P[4] of VP8*) in the gnotobiotic (Gn) pig model of Wa (G1P[8]) HRV infection and diarrhea. Gn pigs were randomly assigned into five groups (n=8-13) and immunized intramuscularly three times at two-week intervals with one of two lipid nanoparticle (LNP)-formulated mRNA vaccines: P2-VP8* (30 µg), LS-P2-VP8* (30 µg) or LS-P2-VP8* (12 µg). A trivalent alum-adjuvanted P2-VP8* protein vaccine or an LNP-formulated irrelevant mRNA vaccine served as positive and negative control, respectively. All pigs were orally challenged seven days after the last immunization with virulent Wa HRV and monitored for seven days post-challenge. Significant protection from virus shedding was induced by all three candidates. Both LS-P2-VP8* doses induced significantly higher VP8*-specific IgG antibody titers in the serum after immunizations than the mock control. The P[8] VP8*-specific IgG antibody-secreting cells in ileum, spleen and blood increased in all three mRNA-vaccinated pig groups compared to the controls seven days post-challenge. VP8*-specific IFN-γ-producing T cell numbers also increased in the mRNA-vaccinated pigs, though not significantly so. The demonstrated strong humoral immune responses, priming for effector T cells, and partial protection in Gn pigs induced by the two mRNA vaccine candidates provide guidance for further development of mRNA-based rotavirus vaccines.

Keywords: Rotavirus, mRNA vaccine, gnotobiotic pigs, P2-VP8*, diarrhea

1. Introduction

Human rotavirus (HRV) is the leading cause of childhood viral gastroenteritis, leading to significant morbidity and mortality, primarily in low- and middle-income countries (LMICs) (Carcamo-Calvo, Munoz et al. 2021, Donato and Bines 2021). The inclusion of live-attenuated oral vaccines in national vaccination programs has been recommended by the World Health Organization (WHO) since 2006, but their efficacy in LMICs has remained low (Carcamo-Calvo, Munoz et al. 2021). Several factors are thought to be responsible, including circulating maternal antibodies, concurrent use of other oral vaccines, concurrent enteric viral infection, gut dysbiosis, and chronic malnutrition (Carcamo-Calvo, Munoz et al. 2021, Donato and Bines 2021). Along with these factors, oral HRV vaccines come with a history of increased risk of intussusception, a rare but potentially fatal bowel blockage (Carcamo-Calvo, Munoz et al. 2021). To circumvent these factors that are mainly associated with the gastrointestinal (GI) tract, other routes of administration have continued to be pursued (Carcamo-Calvo, Munoz et al. 2021). Intramuscular (IM) non-replicating rotavirus vaccines (NRRV) may offer a solution to the aforementioned problems. The non-replicating nature of these vaccines makes them a safer alternative to live-attenuated vaccines regarding HRV vaccine-specific intussusception and live-attenuated vaccination risks in general (Ramesh, Mao et al. 2019, Carcamo-Calvo, Munoz et al. 2021).

To address the obvious need for improved HRV vaccines in LMICs, CureVac, has developed an mRNA-based RNActive® NRRV for intramuscular administration. mRNA as a therapeutic molecule has been studied for decades, but until recently, there were no licensed mRNA-based vaccines available(Schlake, Thess et al. 2012). mRNA-based vaccines have made headlines of late as the saviors of the COVID-19 pandemic, and two are now FDA-licensed for use in the United States.

Vaccines employed here consist of chemically unmodified, sequence-engineered mRNA featuring optimized non-coding regions previously described in the context of SARS-CoV-2 vaccine development (Hoffmann, Corleis et al. 2021, Gebre, Rauch et al. 2022, Roth, Schon et al. 2022). mRNAs are encapsulated in lipid nanoparticles (LNPs) and encode for VP8*, a proteolytic cleavage product of the rotavirus spike protein VP4 that contains neutralizing epitopes and defines the P genotype (Wen, Cao et al. 2012, Wen, Wen et al. 2014, Crawford, Ramani et al. 2017). Both mRNA vaccine designs employed here are trivalent and encode for VP8* derived from P[4], P[6] and P[8] genotypes. Two different mRNA designs, described in more detail in Roier *et al*., were tested. Firstly, P2-VP8*, an mRNA that features the universal T cell epitope P2 and mirrors a clinically tested protein vaccine(Kovacs-Nolan and Mine 2006, Wen, Wen et al. 2014, Groome, Fairlie et al. 2020). Secondly LS-P2-VP8*, in which P2-VP8* is fused to a multimerization domain derived from *Aquifex aeolicus* lumazine synthase (LS) (Wei, Kumar et al. 2018). Expression of VP8* in this context leads to nanoparticle formation aimed to mimic the structure of a viral capsid. The inclusion of this molecule in other vaccines has shown to facilitate more efficient and natural antigen presentation and B cell activation (Jardine, Julien et al. 2013, Azuma, Edwardson et al. 2018, Ladenstein and Morgunova 2020, Geng, Tai et al. 2021)

The objective of this study was to evaluate the immunogenicity and protective efficacy of these mRNA vaccine candidates in the gnotobiotic (Gn) pig model of HRV infection and diarrhea using the trivalent alum-adjuvanted P2-VP8* protein vaccine, kindly provided by PATH, as a comparator. The G1P[8] Wa HRV Gn pig model is a well-established model that has been used in HRV vaccine evaluation for more than 27 years (Ward, Rosen et al. 1996, Yuan 2022). It is the only laboratory animal that develops HRV infection, including mimicking human intestinal pathology, and reliably shows clinical signs of disease (diarrhea) (Ward, Rosen et al. 1996, Yuan

2022). Along with this, the Gn pig mirrors human immunity to HRV and vaccines, with serum IgA, IgG, intestinal IgA, and HRV-specific IFN-γ producing T cells correlating to protection from HRV infection and diarrhea (Yuan, Kang et al. 1998, Desselberger and Huppertz 2011, Wen, Cao et al. 2015, Ramesh, Mao et al. 2019, Yuan 2022). In general, pigs are physiologically and immunologically similar to humans, making results using swine directly translatable to humans (Ward, Rosen et al. 1996, Yuan 2022). Furthermore, the Gn pig lacks any microbiota and the porcine placenta prevents the passive transfer of maternal antibodies. Therefore, Gn piglets are immunologically naïve but immunocompetent at birth (Yuan 2022). This prevents immune interference from commensal bacteria and maternal antibodies but allows for normal immunological development in response to preclinical vaccines (Yuan 2022).

2. Materials and Methods

2.1 Rotaviruses for Challenge and Immunoassays

The virulent Wa HRV (G1P[8]) inoculum consisted of a pool composed of small intestinal contents collected from the 28th or 29th passage of the virus in Gn pigs. A dose of 10^5 focus-forming units (FFU) diluted in 5 mL of Diluent #5 (minimal essential media [ThermoFisher Scientific]; 100 IU of penicillin per mL, 0.1 mg of dihydrostreptomycin per mL; and 1% HEPES) was used for the oral challenge of Gn pigs. The median infectious and diarrhea doses of Wa HRV were determined previously to be approximately 1 FFU (Ward, Rosen et al. 1996). The supernatant of Wa HRV-infected African green monkey kidney MA104 cells (ATCC CRL-2378.1™) were semi-purified by centrifugation through 40% (wt/vol) sucrose cushion and used as a positive control in antigen enzyme-linked immunosorbent assay (ELISA) and cell culture immunofluorescence (CCIF) (Twitchell, Tin et al. 2016). Recombinant P genotype-specific P2-VP8* proteins were used

as detector antigens in ELISA and ELISpot and as stimulating antigens in flow cytometry analysis of T cell responses.

2.2 mRNA and Protein Vaccines

The trivalent LNP-formulated mRNA vaccines P2-VP8* and LS-P2-VP8* were recently described by Roier *et al*. The mRNA vaccines are based on CureVac's RNActive® platform and do not contain chemically modified nucleosides. They comprise a 5′ cap1 structure, a 5′ untranslated region (UTR) from the human hydroxysteroid 17-beta dehydrogenase 4 gene (*HSD17B4*), a GC-enriched open reading frame, a 3′ UTR from the human proteasome 20S subunit beta 3 gene (*PSMB3*), a histone stem-loop, and a poly(A) tail. LNP encapsulation of mRNA was performed using LNP technology from Acuitas Therapeutics (Vancouver, Canada). LNPs are composed of an ionizable amino lipid, phospholipid, cholesterol, and a PEGylated lipid. The P2-VP8* mRNA vaccine design encodes a universal CD4+ T cell epitope (P2) derived from tetanus toxin (Kovacs-Nolan and Mine 2006) (aa residues 830-844; NCBI reference sequence: WP_011100836.1) N-terminally linked to a P genotype-specific rotavirus VP8* subunit protein (aa 65-223 of VP4) by a GSGSG linker. The LS-P2-VP8* mRNA vaccine design encodes full-length lumazine synthase (NCBI reference sequence: WP_010880027.1) from *Aquifex aeolicus* containing two point mutations (C37A to remove an unpaired cysteine and N102D to remove a glycosylation site (Jardine, Julien et al. 2013, Melo, Porter et al. 2019)) N-terminally linked via $(GGS)_4$-GGG to P2-VP8*, which is designed as described above, except that a longer version of VP8* (aa 41-223 of VP4) is encoded. The P genotype-specific VP8* sequences always derive from the following human rotavirus A strains: BE1128 for P[8] VP4 genotype (GenBank accession number: JN849135.1), BE1322/F01322 for P[6] VP4 genotype (GenBank accession number: JF460826.1), and BE1058 for P[4] VP4 genotype (GenBank accession number: JN849123.1). The trivalent

mRNA vaccines encoding genotypes P[8], P[6] and P[4] of VP8* were formulated by mixing the respective mRNAs in a 1:1:1 weight ratio prior to LNP encapsulation. An LNP-formulated mRNA encoding rabies virus glycoprotein (RABV-G) served as an irrelevant mRNA vaccine and thus as a negative control in the present study (Lutz, Lazzaro et al. 2017, Aldrich, Leroux-Roels et al. 2021).

The recombinant fusion proteins P2-VP8* P[8], P2-VP8* P[6], and P2-VP8* P[4] expressed in *Escherichia coli* were kindly provided by PATH (Seattle, WA, USA) and consist of tetanus toxin T cell epitope P2 linked to aa 65-223 (for P[8]) or aa 64-223 (for P[6] and P[4]) of VP4 from rotavirus strain Wa (P[8]), 1076 (P[6]), or DS-1 (P[4]), respectively, using a GSGSG linker as previously described (Wen, Cao et al. 2012, Wen, Wen et al. 2014, Fix, Harro et al. 2015, Groome, Fairlie et al. 2020, Lakatos, McAdams et al. 2020). The protein vaccines were alum-adjuvanted by adsorption of the recombinant fusion proteins to aluminum hydroxide (Alhydrogel®; Croda, Frederikssund, Denmark) essentially as previously described (Lakatos, McAdams et al. 2020, McAdams, Lakatos et al. 2021). Briefly, a two-fold working stock containing 720 µg/mL of trivalent P2-VP8* antigens (1:1:1 weight ratio; 240 µg/mL per antigen) in 0.5 mM PO_4 in 0.9% saline was combined with an equal volume of an Alhydrogel® suspension (4.48 mg Al/mL diluted in 0.5 mM PO_4 in 0.9% saline) using gentle mixing for 20-24 hours to achieve a formulation with 360 µg/mL total trivalent P2-VP8* antigen containing aluminum hydroxide at 2.24 mg Al/mL in 0.5 mM phosphate buffer in 0.9% saline (pH 6.9).

2.3 Vaccine Inoculation, Virus Challenge, and Sample Collection of Gn Pigs

Gnotobiotic pigs used in this study were derived via hysterectomy of near-term sows and maintained in germ-free isolators for the duration of the study (Yuan 2017). Sterility was verified

by weekly rectal swabs (RS) on blood agar and in thioglycolate broth. Pigs were fed ultra-high temperature (UHT) treated sterile whole cow's milk (The Hershey Company, Hershey, PA, USA) for the duration of the study. On each vaccination day, pigs were injected IM into *M. brachiocephalicus* (PID 0) or left (PID 14) or right (PID 28) *M. biceps femoris* with 30 µg of irrelevant mRNA vaccine (100 µL), with 90 µg (560 µg Al) of P2-VP8* protein vaccine (250 µL), with 30 µg of P2-VP8* mRNA vaccine (100 µL), or with 30 µg or 12 µg of LS-P2-VP8* mRNA vaccine (100 µL each). The first dose of the respective vaccine was inoculated on post-partum day (PPD) 5 (also referred to as post-inoculation day [PID] 0) followed by two more doses at 14-day intervals for a total of three vaccinations. Serum was collected 14 h after prime vaccination on PID 1, for evaluation of vaccine-induced IFN-α levels. Serum was also collected before each following vaccination at PID 14 and 28 and at euthanasia (post-challenge day [PCD] 7) to evaluate P2-VP8*-specific IgG and IgA by direct ELISA and virus neutralizing (VN) titers by virus neutralization assay. Whole blood was taken on PID 32 for extraction of mononuclear cells (MNCs) for detection of vaccine-induced P2-VP8*-specific IgG and IgA antibody-secreting cell (ASC) responses by enzyme-linked immunosorbent spot (ELISpot) assay and P2-VP8*-specific IFN-γ-producing CD4+ and CD8+ T cells by flow cytometry.

A total of 49 pigs were assigned randomly to five groups (**Table 1**). Pigs were orally challenged on PID 35 (PCD 0) with 1 x 10^5 FFU of virulent Wa HRV and monitored for diarrhea and virus shedding via daily RS from PCD 0-7; RS were processed as described previously (Yang, Twitchell et al. 2015). All pigs were fed 200 mM sodium bicarbonate 10 minutes prior to challenge to neutralize stomach acidity. Pigs were euthanized at PCD 7. At euthanasia, small intestinal contents (SIC) and large intestinal contents (LIC) were collected and processed as previously described, for the detection of intestinal antibody responses and antigen presence by direct and sandwich ELISA,

respectively, as well as infectious virus particle counting by CCIF (Parre, Hodgins et al. 1999). Spleen, ileum, and whole blood were collected for extraction of MNCs to be used in the detection of VP8*-specific IgG and IgA ASC responses by ELISpot and P2-VP8*-specific IFN-γ-producing CD4+ and CD8+ T cells by flow cytometry (Yuan, Ward et al. 1996, Yuan, Kang et al. 1998, Yuan, Wen et al. 2008).

2.4 Assessment of Diarrhea and Detection of Fecal Virus Shedding by Antigen ELISA and CCIF

To assess severity of diarrhea, fecal consistency was recorded, using the RS taken from PCD 0 to 7, as 0: solid, 1: pasty, 2: semi-liquid, and 3: liquid, with any score ≥2 being considered diarrhea (Saif, Ward et al. 1996, Ward, Rosen et al. 1996, Yuan and Saif 2002). Antigen ELISA was used for detection of HRV VP6 and CCIF for detection of infectious virus particles, as we previously described (Saif, Ward et al. 1996, Saif, Yuan et al. 1997, Yuan and Saif 2002, Vega, Garaicoechea et al. 2021).

2.5 Detection of Interferon-α Induction after Prime Immunization

Serum was taken from all pigs 14 h after the prime immunization (PID 1) and interferon-α (IFN-α) levels were evaluated using a commercial ELISA kit (Thermo Fisher cat#ES7RB) according to manufacturer's instructions. The IFN-α concentrations were calculated based on the standards in the kit and expressed as pg/mL.

2.6 Detection of P2-VP8*-specific Serum and Intestinal IgA and IgG Antibody by ELISA

Serotype-specific anti-P2-VP8* IgA and IgG antibody titers in serum (collected at PID 0, 14, 28 and PID 35/PCD 0 and PCD 7) and intestinal contents (collected at PCD 7) were measured by

using a serotype-specific antibody ELISA (To, Ward et al. 1998, Parre, Hodgins et al. 1999, Twitchell, Tin et al. 2016). Recombinant P[8], P[6], or P[4] P2-VP8* proteins were used as detector antigens with the coating concentration of 0.5 µg/mL. Negative control wells were coated with coating buffer only (0.05M carbonate buffer, pH9.6). Coated plates were incubated for 1 h at 37°C, washed five times with TBST, and blocked with TBS + 5% BSA overnight at 4°C. Plates are washed five times with TBST and incubated with four-fold serial dilutions of each serum sample (starting from 1:4 to 1:65536) overnight at 4°C. For detection, plates were incubated with goat anti-pig IgA antibody horseradish peroxidase (HRP)-conjugated and goat anti-pig IgG-Fc antibody HRP-conjugated (Bethyl Laboratories, Inc. USA), 1:3000 diluted in TBST with 5% BSA, and developed with ABTS peroxidase substrate (1:1 ratio of KPL ABTS® peroxidase substrate solution A and KPL peroxidase substrate solution B from Seracare Life Sciences, Inc. USA). The reaction was stopped with ABST peroxidase stop solution diluted 1:5 in ddH_2O. Optical density was read at 405 nm. IgA and IgG titers were expressed as the reciprocal of the highest sample dilution in which the mean A_{405} of P2-VP8*-coated wells was greater than the mean A_{405} of negative controls plus 3-fold SD. Titers of <4 were assigned a value of 2 for calculation of geometric mean titer (GMT).

2.7 Flow Cytometry for Detection of IFN-γ-producing CD3+CD4+ and CD3+CD8+ T Cells

Mononuclear cells isolated from peripheral blood (PCD 0 and 7) were diluted to a concentration of 1×10^6 cells/mL and seeded into 12-well plates (Yuan, Wen et al. 2008). Cells were re-stimulated for 17 h at 37°C in 5% CO_2 with one of seven antigens: (1) medium (negative control), (2) PHA (10 µg/mL; positive control), (3) P[4] P2-VP8* protein (12 µg/mL), (4) P[6] P2-VP8* protein (12 µg/mL), (5) P[8] P2-VP8* protein (12 µg/mL), and (6) semi-purified attenuated Wa HRV antigen (12 µg/mL). Anti-CD49d mAb (0.5 µg/mL) was added to all samples

before incubation for co-stimulation. After 12 h, Brefeldin A (5 µg/mL) was added for 5 h at 37°C in 5% CO_2. After the total 17 h incubation, cells were washed with 2 mL of commercial stain buffer and transferred to 5 mL Falcon round-bottom polypropylene tubes for 8 min centrifugation at 800 x g at 4°C, followed by discarding of the supernatant. Primary antibodies used for staining were mixed in a cocktail that included FITC-conjugated mouse (IgG2b) anti-pig CD4a, SPRD-conjugated mouse (IgG2a) anti-pig CD8a, and mouse (IgG1) anti-pig CD3ε in 100µL of stain buffer per sample. Samples were incubated with the cocktail for 15 mins at 4ºC and subsequently washed with 500 µL of wash buffer, followed by 8 min centrifugation at 800 x g at 4°C. Then, secondary antibody APC-conjugated rat (IgG1) anti-mouse for CD3ε diluted in 100 µL of stain buffer was added and incubated for another 15 mins at 4ºC. The washing and centrifugation steps were repeated, then, 100 µL of Permeabilizing/Fixation solution was added into each sample and incubated for 30 mins at 4ºC. Washing and centrifugation were then repeated. PE-conjugated mouse (IgG1) anti-pig IFN-γ diluted in 100 µL of stain buffer was added and incubated for another 30 mins at 4ºC. A last washing step with 2 mL of stain buffer was performed followed by centrifugation. Cells were resuspended in 250 µL of stain buffer and stored away from light at 4°C until delivery to Virginia Tech's flow cytometry core for acquisition on a BD FACSArial™ II flow cytometer.

2.8 Detection of P2-VP8*-specific Antibody-Secreting Cells by ELISpot Assay

Plates (96-well) were coated with 50µL P[8] P2-VP8* (1.41 mg/mL), P[6] P2-VP8* (1.16 mg/mL) or P[4] P2-VP8* (1.04 mg/mL) proteins diluted to the working concentration of 5 µg/mL in 50 mM carbonate coating buffer (pH 9.6). Plates were incubated at 37ºC for 30 min, then stored at 4ºC overnight. Plates were washed twice with PBST (pH 7.4, with 0.05% Tween 20), blocked with 100 µL/well 4% BSA in PBS (pH 7.4) and incubated at 37ºC for 1 h. Plates were washed three

times with ddH_20. Separately, extracted MNCs were diluted to a single-cell suspension at a concentration of 5 x 10^6 cells/mL with E-RPMI and 100 µL of each diluted cell suspension was added to duplicate wells. Plates were then centrifuged at 500 rpm for 5 min at RT, followed by incubation at 37ºC for 12 h. Plates were then washed five times with PBST, and 100 µL/well of biotinylated goat anti-porcine IgA diluted 1:20,000 (Bethyl A100-102B, 1 mg/mL) or IgG (Bethyl A100-104B, 1 mg/mL), diluted 1:20,000 in PBST was added to each well. Plats were incubated at RT for 2 h and washed five times with PBST. To ensure MNCs were removed completely, the plates were knocked vigorously on paper towels between each wash. HRP-conjugated streptavidin diluted 1:30,000 in PBS was added (100 µL/well) and the plates were incubated at RT for 1 h. Plates were washed five times with PBST, and 50 µL/well KPL TrueBlue HRP substrate (ready-to-use substrate, VWR catalog number 95095-168) was added to each well. Plates were then incubated for 1-2 h at RT until spots become dark blue. Spots were counted with ELISpot analyzer S5 and reported as IgA or IgG ASC per 5 x10^5 MNC.

2.9 Virus Neutralization Assay

The rotaviruses Wa (G1P[8]), 1076 (G2P[6]) and DS-1 (G2P[4]) were used in this assay. These viruses were originally obtained from the National Institutes of Health and a stock was grown for use in the assay. The rotavirus-neutralizing antibody assay was performed by titrating respective serum samples and incubating them with a constant amount of a specific strain of rotavirus to allow neutralization of the virus. The rotavirus/serum mix was then incubated on MA104 cells in 96-well plates for 1 hour to allow adsorption of the virus. The plates were washed to remove serum and then overlaid with media. Control wells on each 96-well plate received either diluted virus without sera (virus control) or no virus or sera (cell controls). After an overnight incubation allowing for rotavirus growth, the cells were frozen and thawed to lyse the cells to release the

viruses, and then the lysates were resuspended. The relative amount of non-neutralized rotavirus was determined using an antigen capture ELISA to detect rotavirus. The ELISA plates were coated with rabbit anti-rotavirus IgG antibodies. The resuspended lysates from the neutralization part of the assay were added and incubated. Following blocking with nonfat milk, guinea pig anti-rotavirus antiserum was added to the wells to detect captured rotavirus. Horseradish peroxidase (HRP) conjugated rabbit anti-guinea pig IgG (Jackson ImmunoResearch, West Grove, PA, USA) was added to detect the guinea pig anti-rotavirus antibody. The wells were developed with o-Phenylenediamine (Sigma-Aldrich), a peroxidase-sensitive colorimetric substrate. Optical density of each well was read as absorbance at 492 nm. The amount of rotavirus present in the resuspended lysate from each well is inversely related to the amount of neutralizing antibody present in the serum. Each serum dilution series was modeled using a logistic regression function. For each fitted curve the reciprocal serum dilution which corresponds to a 40% response (ED_{40}), compared to the virus controls, was determined and reported as the titer. The ED_{40} represents the titer of the serum against a given virus, which represents a 60% reduction in the amount of virus (Knowlton, Spector et al. 1991).

2.10 Statistical Analysis

Pigs were randomly assigned to treatment groups by animal care staff upon derivation, regardless of sex or size. Vaccine Immunology Statistical Center (VISC) performed statistical analyses on efficacy endpoints, including AUC of virus shedding, peak titer of virus shedding, AUC of diarrhea, cumulative diarrhea scores, and duration of diarrhea and virus shedding. Analyses for immunogenicity endpoints, including IFN-α levels, and P-type specific serum IgG and IgA responses were also performed by VISC. Litter variability for all VISC-evaluated endpoints was assessed and minimal evidence was found that supported litter variability being responsible for the

differences seen. Daily mean diarrhea scores were evaluated using two-way ANOVA followed by Holm-Šídák's multiple comparisons test and daily mean CCIF titers and ELISA ODs were evaluated using two-way ANOVA followed by Tukey's multiple comparisons test. Mixed-effects model followed by Tukey's multiple comparisons test was used for comparing intestinal antibody responses and serum VN titers. Total numbers and mean frequencies of T cell subsets were analyzed using ordinary one-way ANOVA followed by Dunnett's multiple comparisons test. Mean numbers of ASCs in post-mortem tissues were analyzed using the Kruskal-Wallis test followed by Dunn's multiple comparisons test. All non-VISC analyzed parameters were performed using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA). A p value lower than 0.05 was accepted as statistically significant.

3. Results

3.1 LS-P2-VP8*mRNA vaccine induce IFN-α in serum of vaccinated animals 14 h post immunization

Serum collected 14 h post-prime immunization was evaluated for systemic induction of IFN-α. As expected, the lowest levels of IFN-α were detected in animals vaccinated with P2-VP8* protein vaccine. All mRNA vaccines induced IFN-α with lowest levels (2-4 fold less) detectable for LS-P2-VP8* at a dose of 12 µg.LS-P2-VP8* (**Figure 1**).

3.2. Partial Protection against Diarrhea was Conferred by both mRNA Vaccine Candidates, and Significant Protection against Infectious Virus Shedding was Conferred by Both Doses of LS-P2-VP8*

Gn pigs were challenged with virulent Wa HRV at post-inoculation day (PID) 35/post-challenge day (PCD) 0 and clinical signs/virus shedding was monitored via daily RS. These data are summarized in **Table 2**. Pigs who received P2-VP8* and LS-P2-VP8* (30 µg) had a significantly shortened duration of diarrhea as compared to the control pigs (3.1 days and 2.5 days versus 4.6 days, respectively) (**Table 2**). All pigs who received mRNA vaccines had a significantly decreased area under the curve (AUC) of diarrhea that was 2.2-2.6 lower than control pigs (**Table 2**). Indeed, control pigs experienced a double peak of diarrhea at PCDs 3 and 6, while pigs vaccinated with LS-P2-VP8* (12 µg and 30µg**)** only experienced one peak at PCD 3 which then tapered off to consistently lower scores for the rest of the study (**Figure 2A**)LS-P2-VP8*(**Figure 2B**). The reduced severity of diarrhea is also reflected by significantly decreased cumulative fecal scores that were 2.1-2.4 lower in these groups as compared to the controls (**Table 2**). Protein P2-VP8*-vaccinated pigs experienced similar trends, as the LS-P2-VP8* groups; however, the mean diarrhea scores after the peak at PCD 3 trended slightly higher than both LS-P2-VP8* groups for the duration of the study (**Figure 2C, D**). An overall trend towards improved clinical signs elicited by LS-P2-VP8* compared to P2-VP8* mRNA was detected.

The mean duration days of virus shedding was significantly reduced (to 3.6-4.1 days) in all pigs who received mRNA vaccines as compared to the control group (5.4 days) (**Table 2**). The area under the curve of virus shedding was also significantly reduced in all pigs who received mRNA vaccines, ranging from 2.2-fold to 5.7-fold lower than the control (**Table 2**). Similar to the diarrhea data, when looking at mean daily titers, control and protein P2-VP8*-vaccinated pigs experienced

a classic double peak of daily mean CCIF titers at PCDs 2 and 5 (**Figure 3A**), though more dramatic in control pigs (Ward, Rosen et al. 1996, Azevedo, Yuan et al. 2005, Wen, Bui et al. 2016, Yuan 2022). LS-P2-VP8* (12 µg)-vaccinated pigs shed at significantly lower titers compared to control pigs on PCDs 2-6 (**Figure 3A**). When comparing LS-P2-VP8* (12 µg)-vaccinated pigs to protein-P2-VP8*-vaccinated pigs, this group shed significantly lower titers on PCDs 2 and 5 (**Figure 3A**). Results for LS-P2-VP8* (30 µg)-vaccinated pigs were not as pronounced; however, the group's mean titer around the time of the typical second shedding peak (PCD 5) was significantly lower than both control and protein-P2-VP8*-vaccinated pigs (**Figure 3B**). This is reflected by the previously mentioned significantly shortened duration of virus shedding in this group (**Table 2**). For peak viral titers, P2-VP8* and LS-P2-VP8* (12 µg)-vaccinated pigs exhibited significantly reduced (3.0-fold to 4.7-fold lower) titers as compared to control pigs. When evaluating mRNA-vaccinated pigs against the protein-P2-VP8* vaccine (control excluded in analysis), LS-P2-VP8* (12 µg) conferred significant protection with 2.3-fold lower AUC of virus shedding, 2.2-fold lower peak titer and 1.3 days less shedding. LS-P2-VP8*

3.3. Both Doses of LS-P2-VP8* Vaccine Induced Strong P-type-specific Serum IgG Antibody Responses Pre- and Post-Challenge and Primed for Stronger P[8] -specific Serum IgA Antibody Responses Post-Challenge

Serum samples were collected from all pigs before each vaccination (PID 0, 14, 28), challenge (PID 35/PCD 0), and at euthanasia (PCD 7). P-type-specific IgG and IgA antibody responses were detected in the serum at each time point via ELISA and results are summarized in **Figure 4**. Both doses of LS-P2-VP8* induced significantly stronger IgG responses to P[8], P[6] and P[4] in the

serum as compared to all other groups, beginning at PID 14, after only one immunization (**Figure 4A)**. This trend continued to PCD 7, at which point both dose groups still had significantly higher titers than all other groups for all three P-types.

P[8]- and P[6]-specific serum IgA titers were significantly higher in the LS-P2-VP8* (30 µg) group at PCD 0 as compared to control, protein P2-VP8* and P2-VP8*-vaccinated pigs (**Figure 4B**). Post-challenge, both doses of LS-P2-VP8* vaccine primed for significantly higher serum IgA against P[8] and P[6] VP8* as compared to control and protein P2-VP8*-vaccinated pigs. P[4]-specific IgA titers were significantly higher in LS-P2-VP8* (12 µg)-vaccinated pigs than the control pigs post-challenge (**Figure 4B**).

3.4. Both Doses of LS-P2-VP8* Vaccine Primed for Stronger P[8]-specific Intestinal IgA and IgG Antibody Responses Post-Challenge

Small and large intestinal contents (SIC and LIC, respectively) were collected at euthanasia (PCD 7) for quantification of local P-type specific IgG and IgA responses induced by the vaccine candidates using the same ELISA as was used for serum samples. In the SIC, LS-P2-VP8* vaccines at both doses primed for stronger P[8]-specific IgA and IgG responses post-challenge as compared to controls (**Figure 5A**). There were some detectable titers of P[4]-specific IgA and IgG in the SIC, but not to a significant degree (**Figure 5A**). This is likely explained by the relatively high sequence homology close relationship between P[8] (challenge strain) and P[4] genotypes (Matthijnssens, Ciarlet et al. 2008). There were no detectable IgG or IgA titers in the LIC at PCD 7, except for nominal P[8]-specific titers in the LS-P2-VP8* (12µg) group. (**Figure 5B**).

3.5 mRNA Vaccine Candidates Induced Strong HRV-specific T cell Responses in the Blood Pre-challenge and Primed for Stronger Responses Post-challenge

MNCs were extracted from peripheral blood pre-challenge (PCD 0) and post-challenge (PCD 7), and frequencies and total numbers of CD3+CD4+IFN-γ+ and CD3+CD8+IFN-γ+ T cells among MNCs were measured by flow cytometry and intracellular staining. To calculate and analyze overall HRV-specific IFN-γ+ T cell responses to vaccination and to simplify the data presentation, the means of IFN-γ+ T cell numbers or frequencies from P[4], P[6], P[8] and AttHRV antigen-stimulated MNCs were subtracted by the numbers or frequencies from mock-stimulated MNCs and assigned as HRV-specific IFN-γ+ T cells from each animal. Mean total numbers and frequencies of CD3+CD4+IFN-γ+ and CD3+CD8+IFN-γ+ T cells among all pigs from the five different treatment groups are presented in Figures 6 and 7.

LS-P2-VP8* vaccines induced higher numbers and frequencies of CD3+CD4+IFNγ+ T cell pre-challenge as compared to control, although they weren't statistically significant (**Figure 6A**). Mean frequencies of CD3+CD8+IFNγ+ pre-challenge were also higher than controls, although again, they were not statistically significant (**Figure 6B**). All groups experienced significant increases in HRV-specific mean frequencies of CD3+CD4+IFN-γ+ T cells after challenge, LS-P2-VP8* (30 μg) primed for significantly higher mean frequencies of HRV-specific CD3+CD4+IFN-γ+ T cells as compared to controls post-challenge (**Figure 7A**). All groups had significant increases in frequencies of CD3+CD8+IFN-γ+ T cells in the blood post-challenge (**Figure 7B**). Higher mean frequencies of this same subset were seen in all vaccinated pigs than in control pigs, although the difference was not statistically significant (**Figure 7B**).

3.6. LS-P2-VP8* Vaccine Primed for Higher Blood and Intestinal ASC Responses and P2-VP8* Vaccine Primed for a Significant Splenic IgG ASC Response to P[8] Post-challenge

MNCs extracted from blood, ileum and spleen were used to evaluate P-type-specific IgG and IgA ASC responses post-challenge by ELISpot assay. All LS-P2-VP8*-vaccinated pigs had higher numbers of P[8]-specific IgG ASCs detected in the blood and ileum post-challenge although not to a significant degree (**Figure 8A**). P2-VP8*-vaccinated pigs had significantly higher numbers of P[8]-specific IgG ASCs in the spleen compared to the controls, indicating the vaccination primed for a systemic memory B cell response upon subsequent virus exposure in the secondary lymphoid tissue (**Figure 8A**). LS-P2-VP8* (30 μg) and protein P2-VP8* vaccination also stimulated production of higher, though not significantly so, numbers of P[8]-specific IgG ASCs in the spleen post-challenge (**Figure 8A**). Similarly, all LS-P2-VP8*-vaccinated pigs were primed for higher numbers of P[8]-specific IgA ASCs in the blood and ileum post-challenge though these numbers were not statistically significant. (**Figure 8B**).

The numbers of P[6]-specific IgG ASC in ileum and spleen were similar across the pig groups post-challenge (**Figure 9A**). But P2-VP8* and LS-P2-VP8* (30 μg) vaccination primed for higher mean numbers of P[6]-specific IgA ASCs in the ileum, though not significantly so due to high varibility (**Figure 9B**). Both LS-P2-VP8* vaccines primed for slightly higher numbers of P[6]- and P[4]-specific IgA ASCs in the blood post-challenge (compare Figures 9A and 10A), while LS-P2-VP8* (30 μg) vaccine only induced higher numbers of P[4]-specific IgG ASCs in the ileum (**Figure 10A**). P2-VP8* and LS-P2-VP8* (30 μg) also induced higher mean numbers of P[4]-specific IgA ASCs in the ileum with high varibility (**Figure 10B**).

3.7 Pigs Vaccinated with both LS-P2-VP8* Candidates were Primed for Significantly Higher P[8]- And P[6]-specific VN Antibody Titers Post-challenge

Serum samples collected prior to each vaccination and pre- and post-challenge were used to evaluate P-type specific neutralizing antibody (nAb) titers in Gn pigs. None of the vaccines induced significant VN titers pre-challenge (**Figure 11A-C**). However, both LS-P2-VP8* doses induced responses against P[4], P[6] and P[8] serotypes on PCD 7. nAb titers were against P[8]- and P[6] viruses were significantly higher in LS-P2-VP8* vaccinated animals- compared to both mock-vaccinated and protein P2-VP8*-vaccinated pigs (**Figure 11A, B**).

4. Discussion

In this study, the immunogenicity and protective efficacy of mRNA vaccines were evaluated using the highly relevant Gn pig challenge model. We demonstrated that LS-P2-VP8* candidates at both doses conferred partial protection against diarrhea upon challenge with the virulent Wa HRV as was evidenced by the significantly reduced mean duration of diarrhea in the LS-P2-VP8* (30 µg) group, as well as the consistently lower mean daily diarrhea scores in both LS-P2-VP8* groups. Along with this, there was one pig each in both LS-P2-VP8* groups that did not experience any diarrhea at any point between PCDs 1-7. We expect with a larger sample size that there would be a greater number of LS-P2-VP8*-vaccinated pigs that experience little to no diarrhea. All mRNA vaccine candidates conferred some degree of protection from virus shedding, as evidenced by the significantly reduced AUC (P2-VP8* and LS-P2-VP8* [12 µg]), duration (P2-VP8* and LS-P2-VP8* [12 µg]), and daily mean titers (both doses of LS-P2-VP8*). LS-P2-VP8* vaccines were also highly immunogenic and induced strong serum IgG antibody responses specific to P[8], P[6],

and P[4] HRVs both pre- and post-challenge, as well as stronger serum IgA to all three P-types post-challenge. Despite the historical use of intestinal IgA as a correlate of protection of live oral attenuated vaccines, it is likely parenteral vaccine protection will be illustrated by other markers, namely HRV-specific serum IgG and IgA (Corthesy, Benureau et al. 2006, Angel, Steele et al. 2014, Baker, Tate et al. 2020, Pollock, Bennett et al. 2022). Evidence supporting this idea includes the fact that hyperimmune serum has been shown to protect nonhuman primates from RV challenge, as well as the long-observed phenomenon of maternal antibody protection from natural HRV infection in the first few months of life, before vaccination can safely occur (Westerman, McClure et al. 2005, Otero, Langel et al. 2020).

Furthermore, LS-P2-VP8* vaccines primed for strong P[8]-specific intestinal IgA and IgG responses post-challenge. This is interesting as inducing mucosal IgA through the use of a parenteral vaccine has proven difficult, and as mentioned above, local mucosal IgA responses are an important correlate of protection in natural HRV infection as well as with the use of live oral attenuated vaccines (Corthesy, Benureau et al. 2006, Angel, Steele et al. 2014, Pollock, Bennett et al. 2022) The immunogenicity seen is consistent with the significant protection from virus shedding seen in these groups. P2-VP8* and 2 (30 µg) vaccines further primed for significantly higher P[8]-specific IgG ASCs in the spleen post-challenge. The spleen is an important secondary lymphoid organ in which B cells rapidly proliferate and transform into ASCs (plasma cells) or memory B cells after various cytokine signals and antigen stimulation (oral homotypic HRV challenge, in this case) (2013). B cells alone have proven to be critical for reduction of HRV-induced diarrhea, which was seen in these groups, but not for complete resolution of disease (Wen, Bui et al. 2016). Along with B cells, CD4+ and CD8+ T cells play a vital, role in the total clearance

of HRV infection and diarrhea, even in the absence of B cells, as evidenced by a study that showed significant increases in both diarrhea and virus shedding severity that persisted past PCD 7 in CD8+ T cell-deficient Gn pigs (Wen, Bui et al. 2016). The current study agrees with this notion, as seen by the significantly reduced severity of virus shedding in our LS-P2-VP8*-vaccinated pigs, in the presence of significantly higher numbers of pre- and post-challenge HRV-specific CD4+IFN-γ+ and CD8+IFN-γ+ T cells in blood. Although we did see significant increases in all groups between pre- and post-challenge timepoints, it is reasonable to assume these groups' post-challenge T cell populations were largely comprised of primary effector T cells stimulated by the virus challenge itself. Elucidating more specific markers for these populations would prove useful in future studies.

Interestingly, the lower dose (12 μg) of LS-P2-VP8* generally performed better than the higher dose (30 μg). One reason why this may be is the lower IFN-α level induced by LS-P2-VP8* (12 μg). Type I IFNs have been shown to have a double-edged effect in terms of mRNA vaccination. A systemic, widespread response is generally not preferable, as it may increase side effects of vaccinations such as headache, fever and fatigue as well as the possibility of stimulating a diffuse antiviral state, preventing antigen uptake and subsequent translation (Fleming 2016). However, a mild, local type I IFN response may be associated with stronger downstream adaptive responses, due to efficient recruitment of APCs to the site (De Beuckelaer, Grooten et al. 2017).

The lack of serum VN antibodies after vaccination and before challenge was unexpected since high titers of P-type specific serum IgG antibodies are detected from PID 14 to PID 35 (PCD 0).

As expected, the protein P2-VP8* vaccine stimulated relatively low IFN-α levels in the pigs. But it did not confer the same protection as reported in a previous study. In previous Gn pig studies using the P2-VP8* vaccine, the protein antigen dose included in the vaccine was 50 µg, and pigs were better protected from Wa HRV-induced diarrhea and virus shedding than they were in the current study, which used only 30 µg dose of each VP8* antigen (Wen, Wen et al. 2014). In regard to the PATH vaccine trial, a recent announcement about the early closure of the ongoing phase III clinical trial due to low protective efficacy was released (Chen, Grow et al. 2022). There is a growing body of evidence that suggests HRV vaccines that express or encode the entire domain of HRV's VP4 may be more immunogenic and result in better protection than those that solely focus on VP8* (Jenni, Li et al. 2022). Combining this novel approach with the mRNA platform is one potential future direction of these technologies. Parenteral vaccines for enteric viruses, especially HRV, remains a subject of further research, and parenteral vaccines continue to show their status as a promising avenue of exploration for improving HRV vaccines.

5. Conclusions

Our study demonstrated the safety, immunogenicity and protective efficacy of three mRNA vaccine candidates against HRV using the neonatal Gn pig model. LS-P2-VP8* vaccines at both doses conferred partial protection from diarrhea and significant protection from virus shedding upon challenge with virulent Wa HRV. Higher numbers of pre-challenge CD4+ IFN-γ+ and CD8+IFN-γ+ T cells in the blood were associated with higher protection post-challenge. LS-P2-VP8* vaccines also induced significant serum IgG to three P-types as early as PID 14, after one vaccination. They also induced significantly higher P-type-specific serum IgA and challenge-strain-specific intestinal IgA post-challenge, which was further associated with protection from diarrhea and virus shedding. Significantly stronger P[8]-specific VN titers were also detected in

these groups. Both humoral and cell-mediated mechanisms appeared to play major roles in the protection seen in LS-P2-VP8*-vaccinated pigs. Ideally, populations of T and B cells would be evaluated in the future to determine how mRNA vaccination modulates the differentiation of these cells and how differing population contribute to protection. Importantly, reliable correlates of protection induced by parenteral HRV vaccines need to be clearly defined. Overall, mRNA vaccination has proven in recent years to be a promising technology for the prevention of several infectious diseases, and further investigation of mRNA-based HRV vaccines is warranted based on these data.

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Conflicts of Interest: S.Ro., B.P., and S.Ra. are employees of CureVac SE, Tübingen, Germany, a publicly listed company developing mRNA-based vaccines and immunotherapeutics and are inventors on several patents on mRNA vaccination and use thereof. S.Ro. and B.P. hold shares in the company. The other authors declare no competing interests. The funder had no role in the design of the study; in the collection, analyses, or interpretation of data, the writing of the manuscript, or in the decision to publish the results.

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Table 1. Allocation of Gn pigs.

Group	n	Vaccine	Challenge
1	8	Irrelevant LNP-formulated mRNA vaccine (**Control**)	Wa HRV (G1P[8])
2	8	Trivalent alum-adjuvanted P2-VP8* protein vaccine (**Protein P2-VP8***)	
3	10	Trivalent LNP-formulated P2-VP8* mRNA vaccine (**mRNA P2-VP8***)	
4	13	Trivalent LNP-formulated LS-P2-VP8* mRNA vaccine (**mRNA LS-P2-VP8* [12 µg]**)	
5	10	Trivalent LNP-formulated LS-P2-VP8* mRNA vaccine (**mRNA LS-P2-VP8* [30 µg]**)	

Table 2. Diarrhea and virus shedding in mRNA vaccinated and control pigs after challenge with virulent Wa HRV (G1P[8])											
		Clinical Signs of Diarrhea[b]					Virus Shedding (CCIF)[c]				
Treatments (vaccine dose µg)[a]	n	Percentage with Diarrhea	Mean Days to Onset	Mean Duration Days	Mean Cumulative Fecal Score	AUC of Diarrhea	Percentage of Shedding Virus	Mean Days to Onset	Mean Duration Days	Mean Peak Titer (FFU/g of feces)[f]	AUC of Virus Shedding
Control	8	8/8 (100%)	2	4.6 (0.53)[A, d, e]	11.5 (0.78)[A]	10.4 (0.65)[A]	8/8 (100%)	1.5	5.4 (0.18)[A]	2.1×10^4 (3645)[A]	4.7×10^4 (6881)[A]
Protein P2-VP8*	8	8/8 (100%)	1.9	3.1 (0.35)[A]	9.9 (0.74)[AB]	8.9 (0.78)[AB]	8/8 (100%)	1.3	4.9 (0.30)[AB]	1.0×10^4 (2182)[ABC]	1.9×10^4 (4030)[AB]
mRNA P2-VP8*	10	10/10 (100%)	2.4	3.1 (0.10)[B]	9.4 (0.48)[AB]	8.2 (0.48)[B]	10/10 (100%)	1.8	4.0 (0.33)[BC]	6.9×10^3 (1849)[CD]	1.6×10^4 (4932)[BC]
mRNA LS-P2-VP8* (12 µg)	13	12/13 (92%)	1.9	3.3 (0.51)[A]	9.1 (0.85)[B]	7.8 (0.72)[B]	13/13 (100%)	1.7	3.6 (0.37)[C]	4.5×10^3 (1182)[D]	8.3×10^3 (2619)[C]
mRNA LS-P2-VP8* (30 µg)	10	9/10 (90%)	2.4	2.5 (0.48)[B]	9.1 (0.91)[B]	7.9 (0.83)[B]	10/10 (100%)	1.7	4.1 (0.43)[BC]	1.2×10^4 (2335)[ABC]	2.1×10^4 (5103)[B]

Note: a. Pigs were immunized three times with an irrelevant mRNA vaccine as control, P2-VP8* protein, or one of two rotavirus mRNA vaccines at 5 (post-inoculation day [PID] 0), 19 days (PID 14) and 33 days (PID 28) of age. On PID 35, all pigs were orally challenged with 1×10^5 FFU of virulent Wa HRV and monitored for diarrhea and virus shedding for 7 days post-challenge. b. Fecal consistency scores were used to assess diarrhea; scores are defined as 0: solid, 1: pasty, 2: semi-liquid, and 3: liquid. Scores of 2 or higher are considered diarrheic. c. Rotavirus shedding titers were determined by rotavirus antigen ELISA (detect viral antigen) and CCIF (determine the number of infectious viral particles). If there is no diarrhea or virus shedding, the mean days to onset were assigned as one day after the pigs were euthanized (PCD 8) for statistical analysis. d. Different letters indicate significant differences between groups (n = 8-13; unadjusted $p < 0.05$), while shared letters or no letters indicate no significant difference. e. Numbers in parentheses represent the standard error of the mean (SEM). f. Mean peak titer and AUC of virus shedding titers were transformed (please indicate which transformation) prior to statistical analysis.

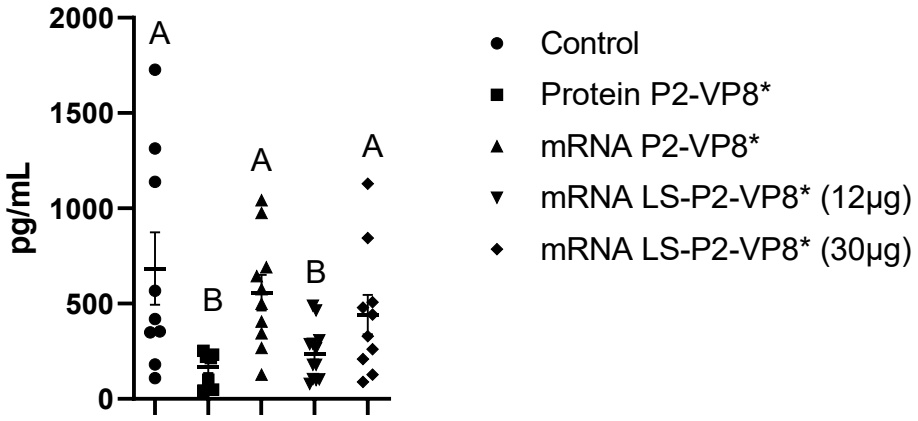


Figure 1. Interferon-α (IFN-α) levels induced by prime vaccination. Serum was taken 14 hours after the first vaccination and evaluated for systemic IFN-α levels using a commercial ELISA kit. Different letters indicate significant differences between groups ($n = 8\text{-}13$; unadjusted $p < 0.05$), while shared letters or no letters indicate no significant difference. Bars indicate means with SEM.

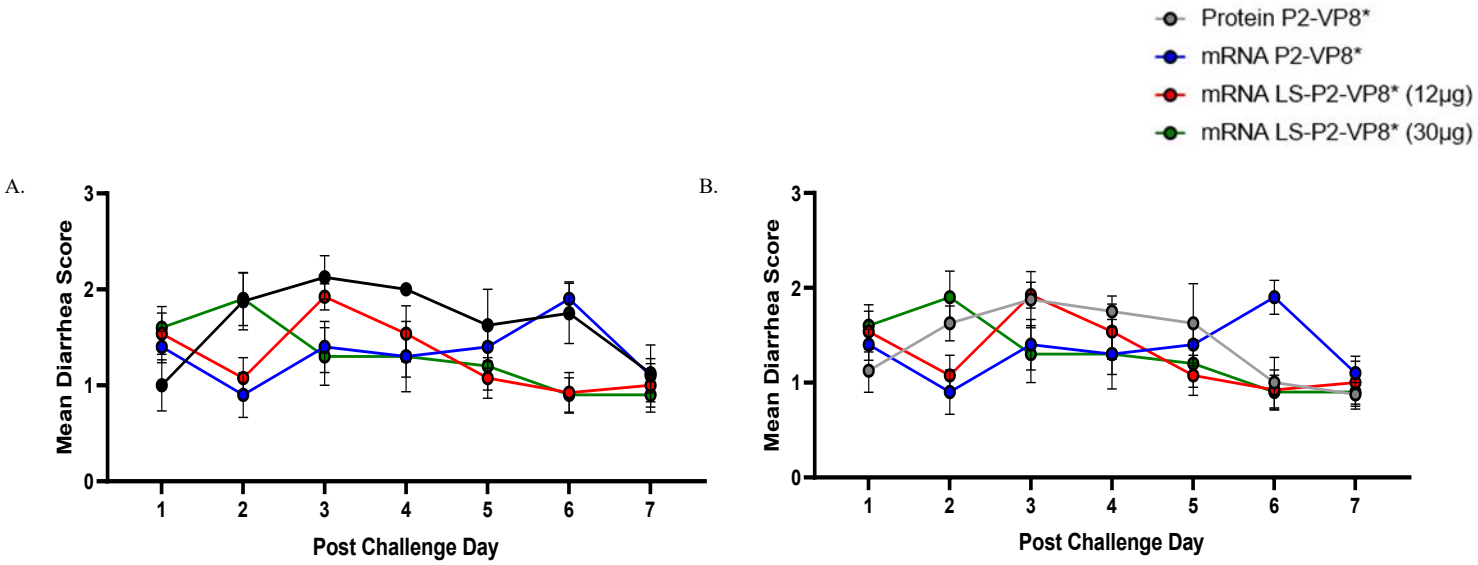


Figure 2. Daily mean diarrhea scores from PCD 0-7 in mRNA P2-VP8*, mRNA LS-P2-VP8* (12µg) and mRNA LS-P2-VP8* (30µg) versus control (A) and protein P2-VP8*-vaccinated pigs (B). Daily rectal swabs were taken for evaluating fecal consistency scores after challenge with Wa HRV. There were no significant differences between groups at any timepoint using two-way ANOVA followed by Holm-Šídák's multiple comparisons test (n=8-13). Circles indicate means with SEM.

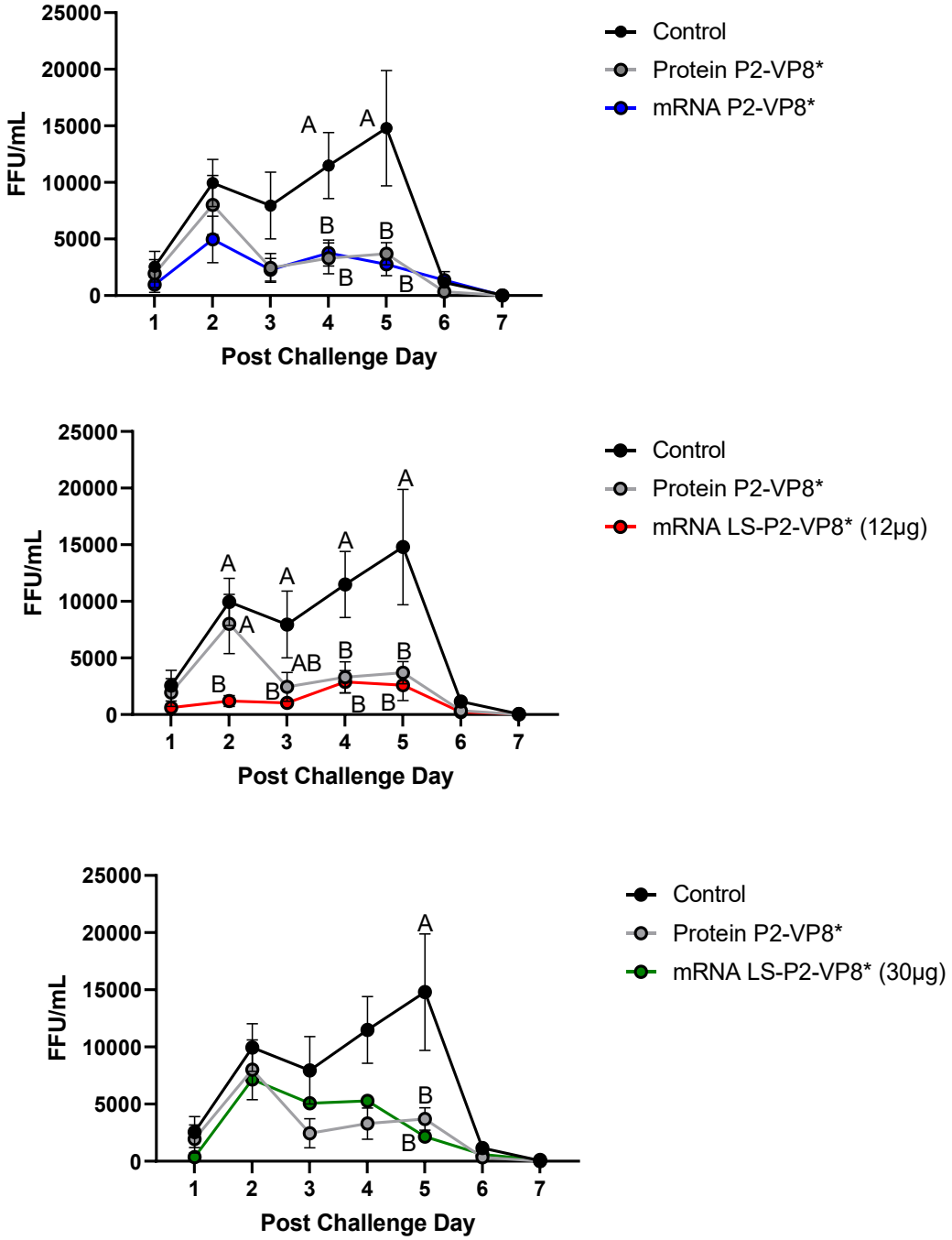


Figure 3. Daily fecal virus shedding as measured by CCIF from PCD 0-7 in mRNA P2-VP8* (A), mRNA LS-P2-VP8* (12µg) (B) and mRNA LS-P2-VP8* (30µg)-vaccinated pigs (C) versus control and protein P2-VP8*-vaccinated pigs. Daily rectal swabs were taken for evaluating fecal virus shedding after challenge with Wa HRV. Fecal infectious virus particles were measured by CCIF and results are expressed as FFU/mL. Fecal samples from mock-infected pigs were used as negative controls. Different capital letters above data points indicate significant differences (adjusted $p < 0.05$) between groups at the same time point, while shared letters or no letters indicate no significant difference, according to two-way ANOVA followed by Tukey's multiple comparisons test. Circles indicate means with SEM. CCIF, cell culture immunofluorescence; FFU, focus forming units.

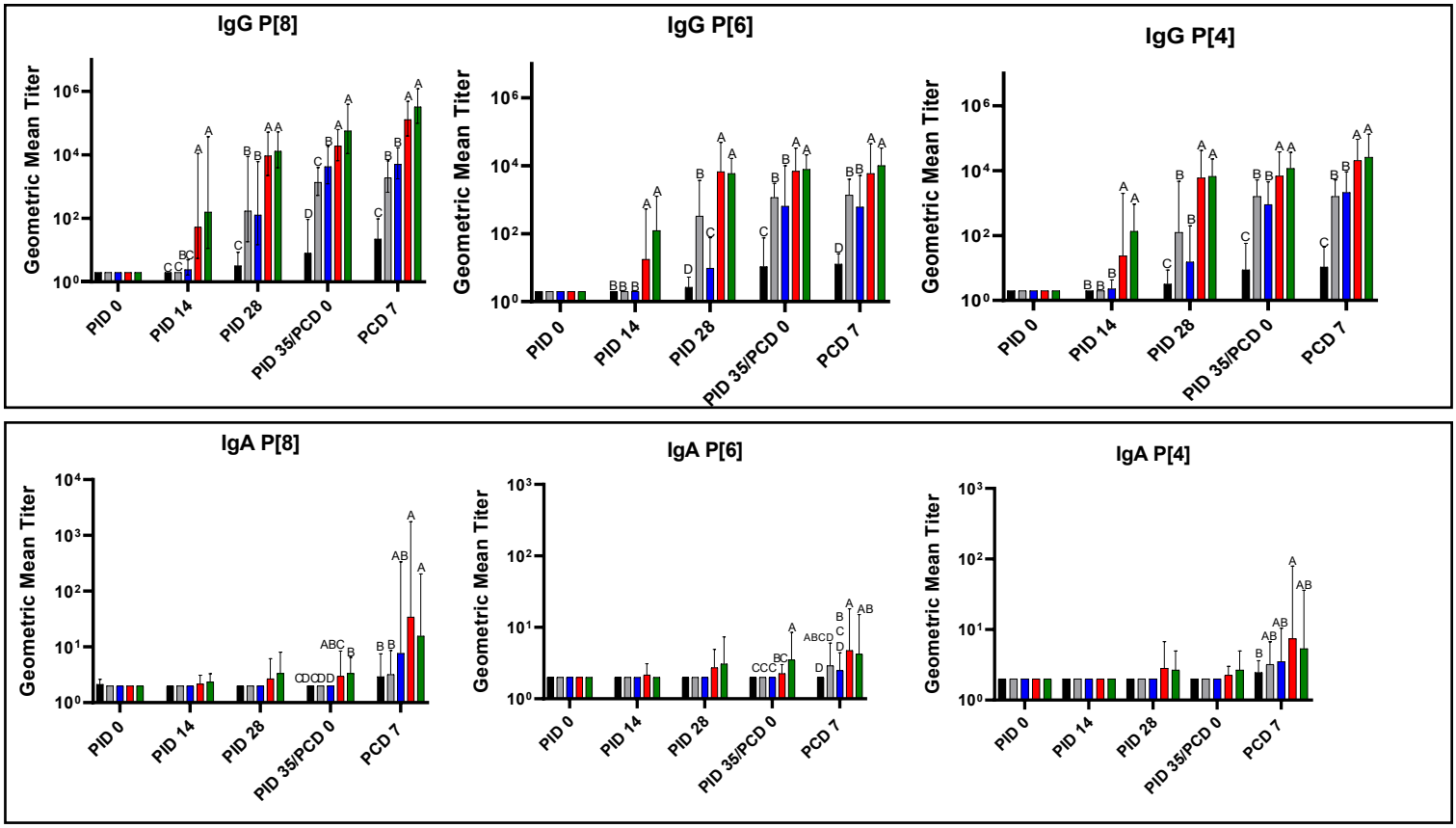


Figure 4. Geometric mean titers of P-type-specific IgG (A) and IgA (B) antibodies in Gn pig serum samples collected at PID 0, PID 14, PID 28, PID 35/PCD 0, and PCD 7. Samples were tested at a series of 4-fold dilutions, beginning at 1:4. All negative samples were given a titer of 2 to allow for data analysis and use in graphical depictions. Different capital letters above bars indicate significant differences (unadjusted $p < 0.05$) between groups, while shared letters or no letters indicate no significant difference. Bars indicate geometric means with geometric SD.

Control
Protein P2-VP8*
mRNA P2-VP8*
mRNA LS-P2-VP8* (12µg)
mRNA LS-P2-VP8* (30µg)

c

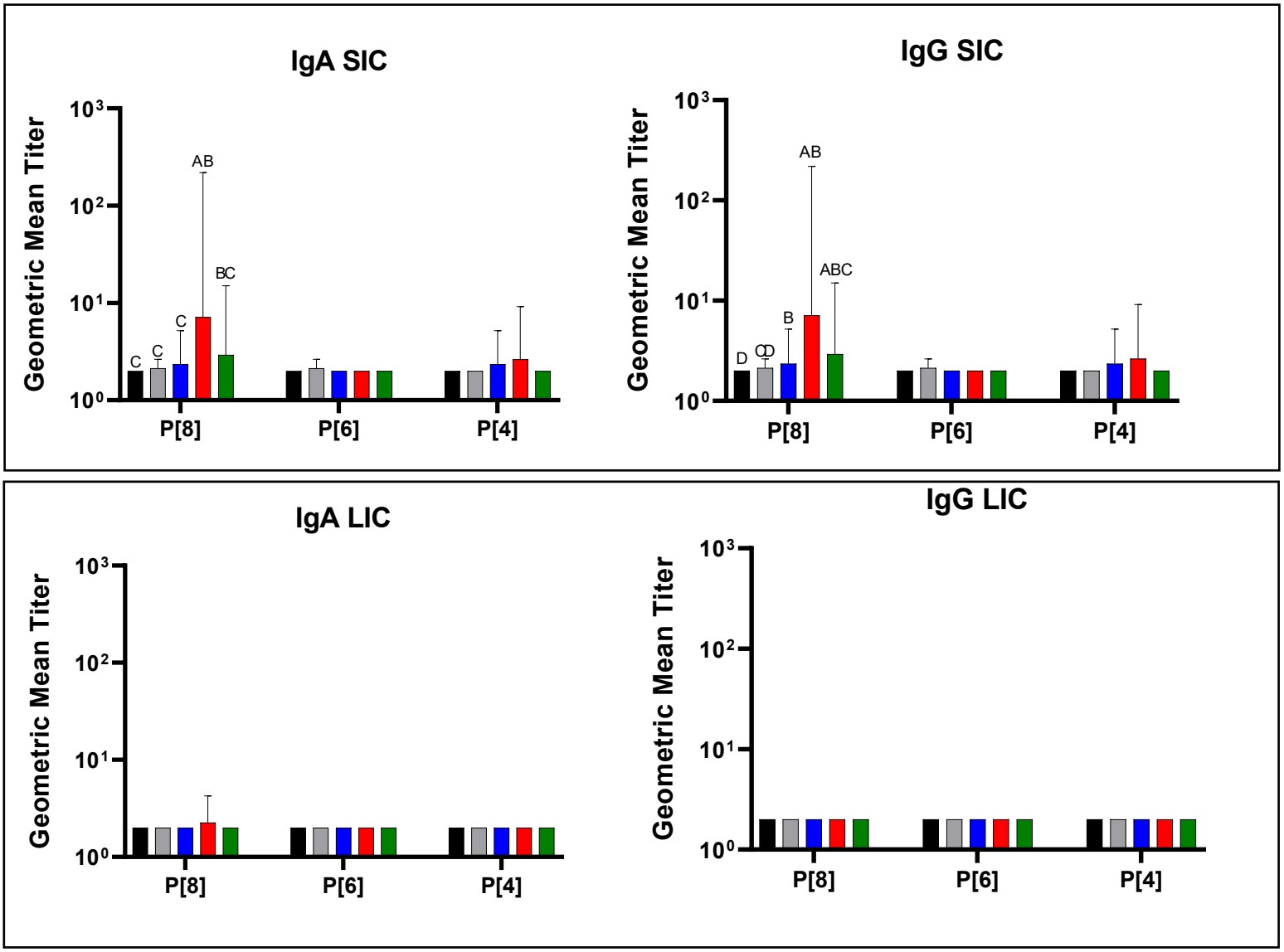


Figure 5. Geometric mean titers of P-type-specific IgG and IgA antibodies in Gn pig intestinal content samples collected at PCD 7. Samples were tested at a series of 4-fold dilutions, beginning at 1:4. All negative samples were given a titer of 2 to allow for data analysis and use in graphical depictions. Different capital letters above bars indicate significant differences (adjusted $p < 0.05$) between groups, while shared letters or no letters indicate no significant difference, according to mixed-effects analysis followed by Tukey's multiple comparisons test. SIC, small intestinal contents; LIC, large intestinal contents. Bars indicate geometric means with geometric SD.

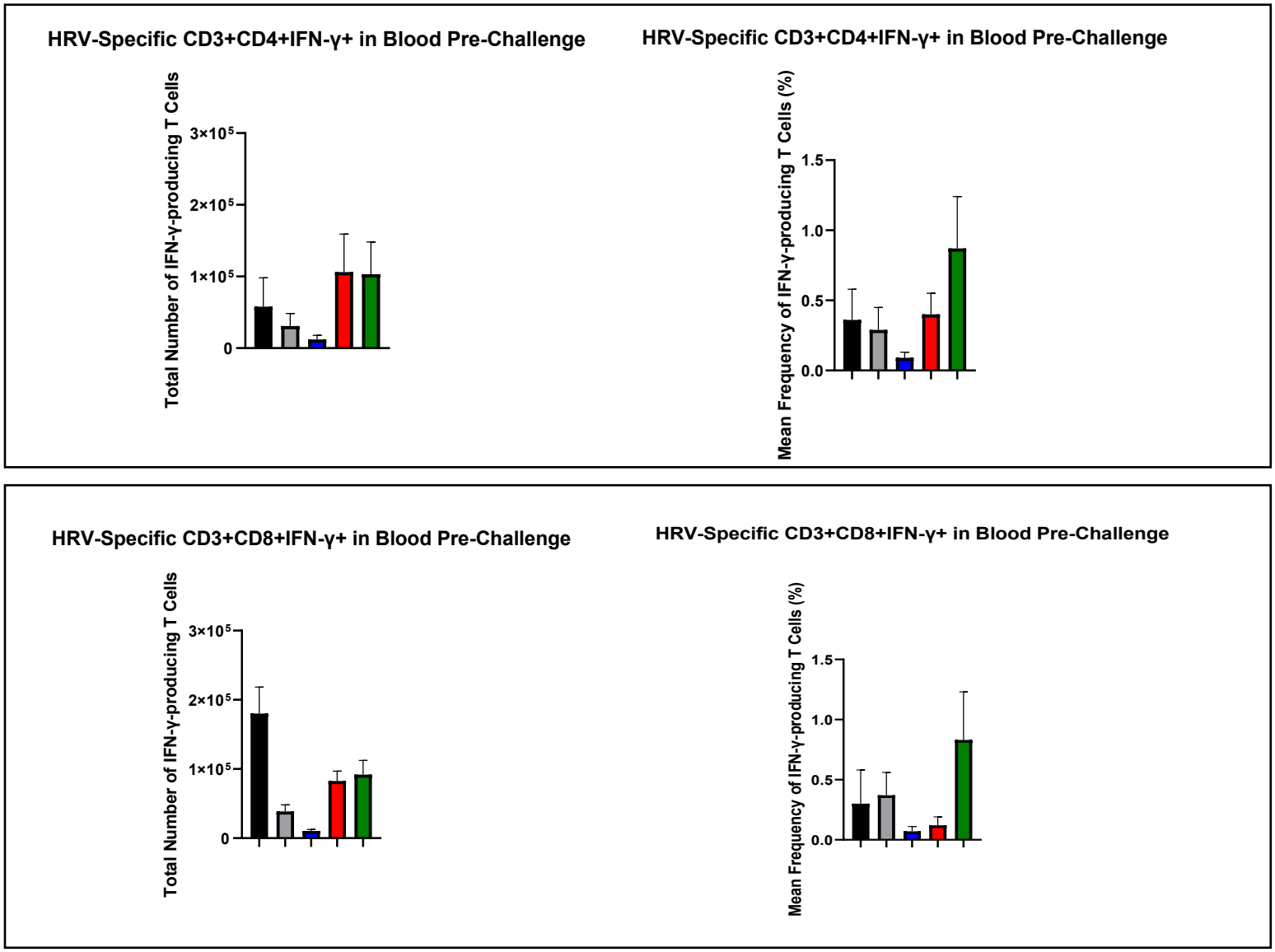
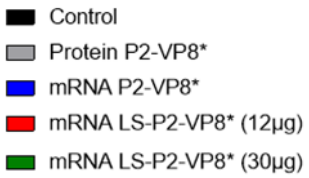
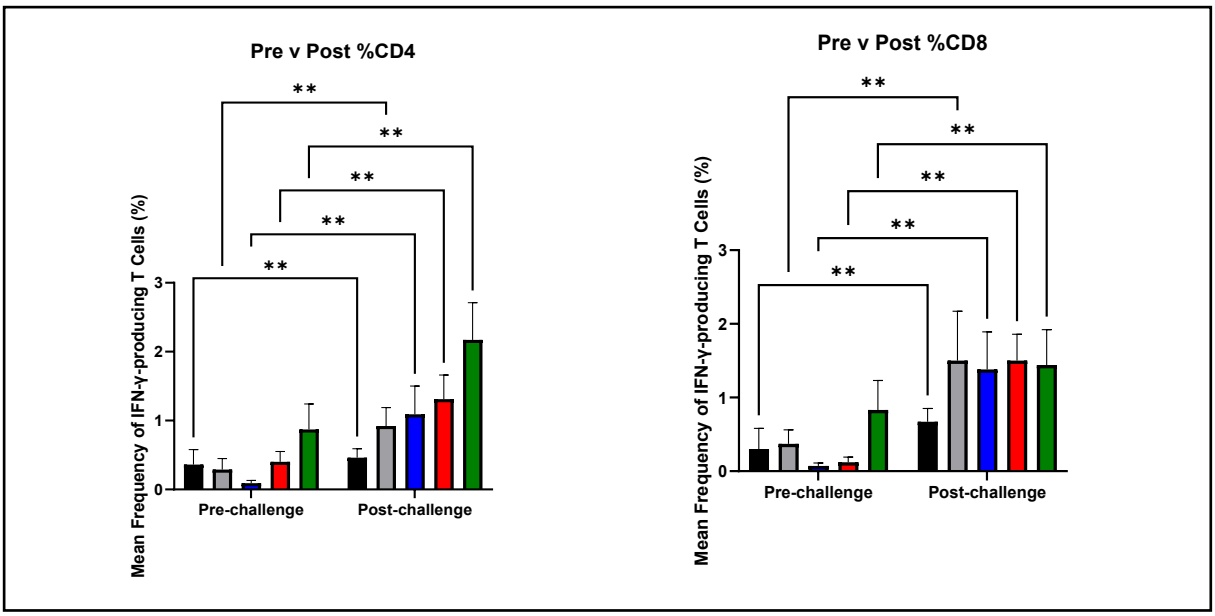


Figure 6. **Total mean numbers and frequencies of CD3+CD4+IFN-γ+ (A) and CD3+CD8+IFN-γ+ (B) in the blood pre-challenge.** There were no significant differences according to ordinary one-way ANOVA followed by Dunnett's multiple comparisons test (n=8-13; * adjusted $p \leq 0.05$). Bars indicate means with SEM.





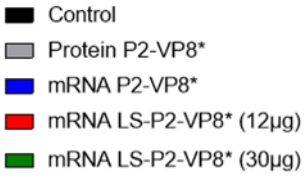


Figure 7. **Mean frequencies (%) of CD3+CD4+IFN-γ+ (A) and CD3+CD8+IFN-γ+ (B) T cells in the blood pre- versus post-challenge**. For comparing between pre- and post-challenge for the same group, two-way ANOVA followed by Tukey's multiple comparisons test was used (n=8-13; * $p \leq 0.05$, ** $p \leq 0.01$). For comparing among groups at the same time point, ordinary one-way ANOVA followed by Dunnett's multiple comparisons test was used (different letters A, B, indicate significant difference; n=8-13; $p \leq 0.05$). Bars indicate means with SEM.

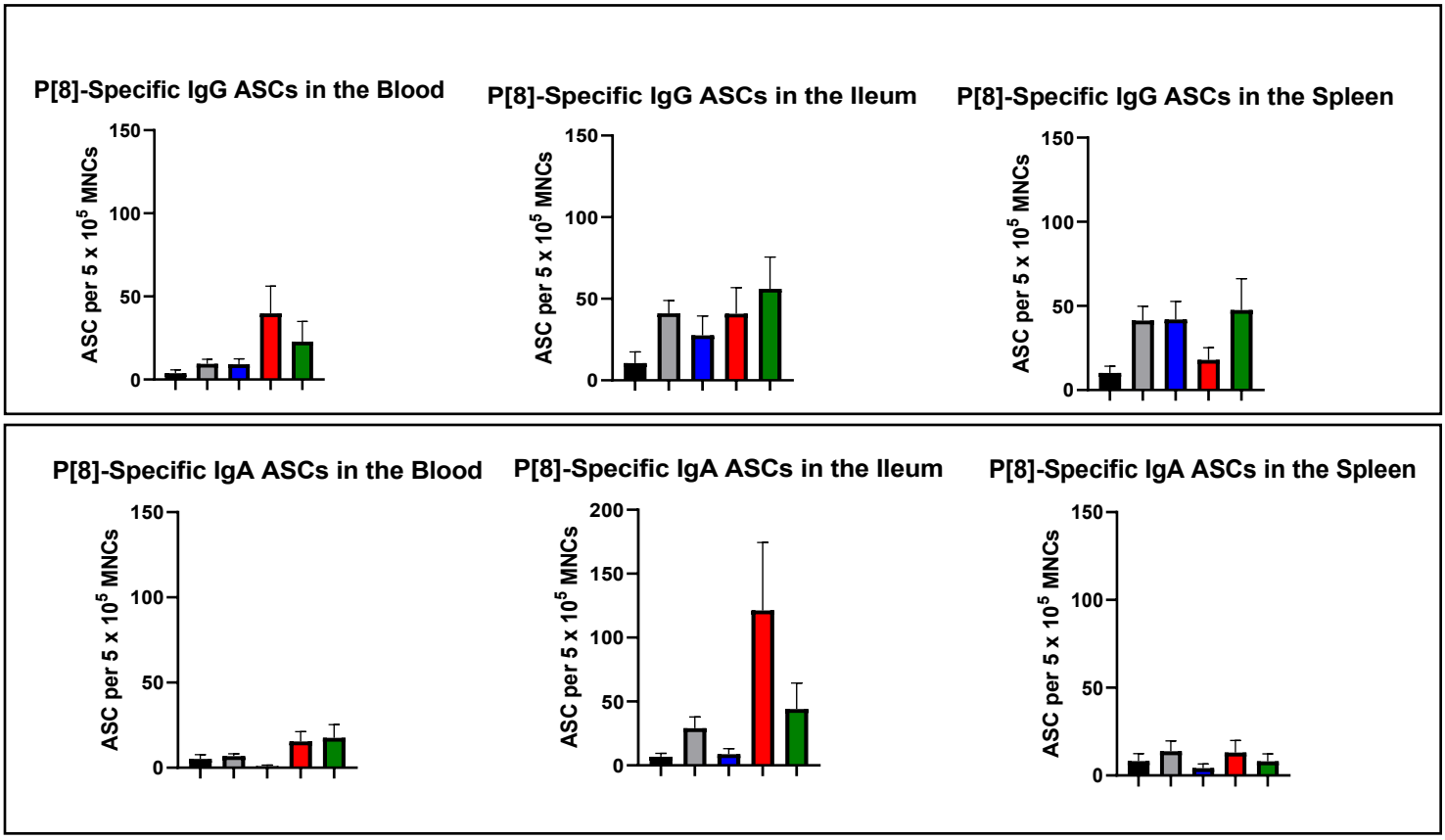


Figure 8. Mean numbers of P[8]-specific ASCs in the tissues of Gn pigs post-challenge. MNCs extracted from post-mortem tissues were evaluated for P-type specific IgG (**A**) or IgA (**B**) ASCs using ELISpot assay. Kruskal-Wallis test followed by Dunn's multiple comparisons test was used for analysis (n=8-13; * $p \leq 0.05$). Bars indicate means with SEM. ELISpot, enzyme-linked immunosorbent spot; ASC, antibody-secreting cells; MNCs, mononuclear cells.

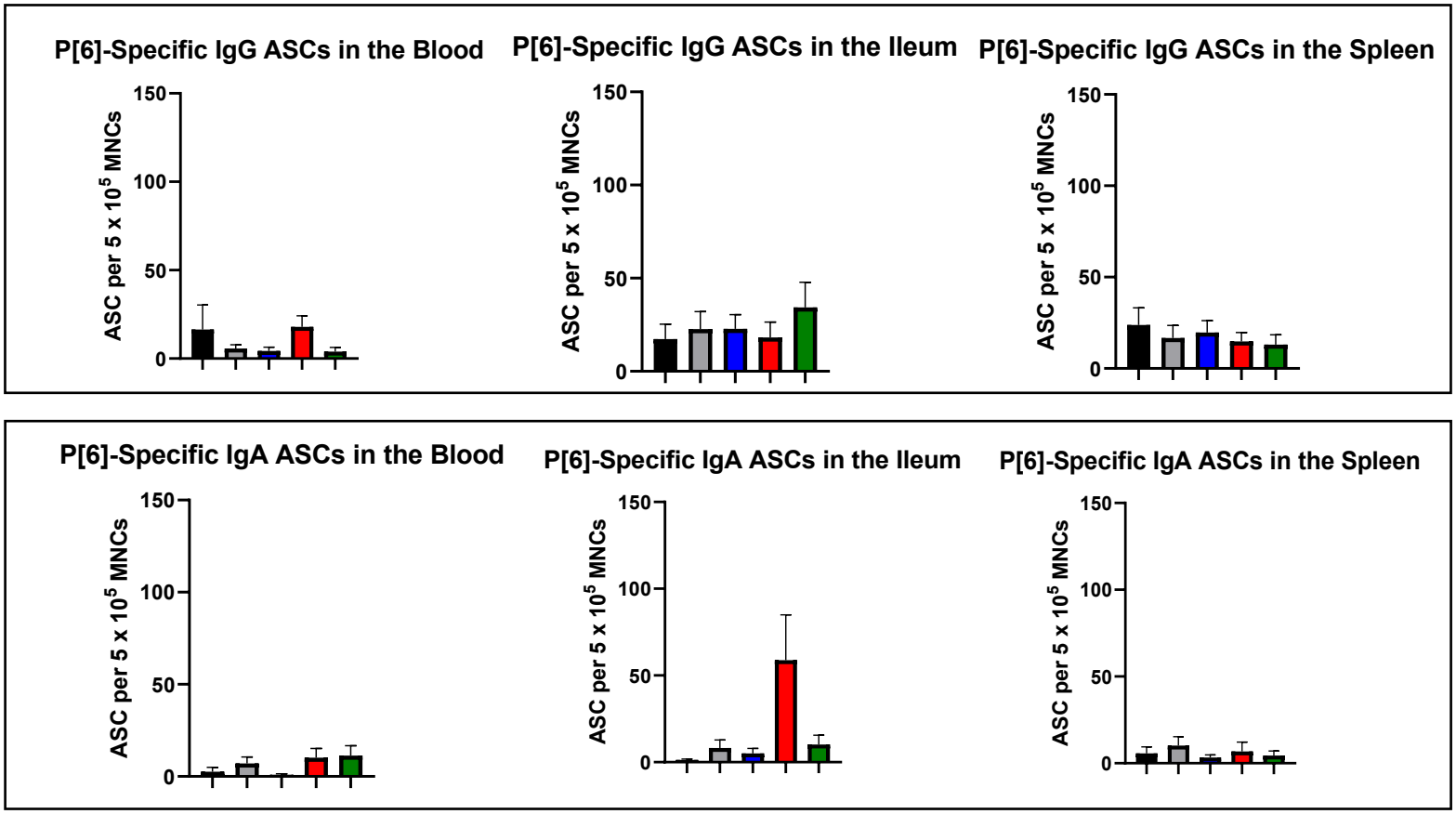


Figure 9. Mean numbers of P[6]-specific ASCs in the tissues of Gn pigs post-challenge. MNCs extracted from post-mortem tissues were evaluated for P-type specific IgG (**A**) or IgA (**B**) ASCs using ELISpot assay. Kruskal-Wallis test followed by Dunn's multiple comparisons test was used for analysis (n=8-13; * $p \leq 0.05$). Bars indicate means with SEM. ELISpot, enzyme-linked immunosorbent spot; ASC, antibody-secreting cells; MNCs, mononuclear cells.

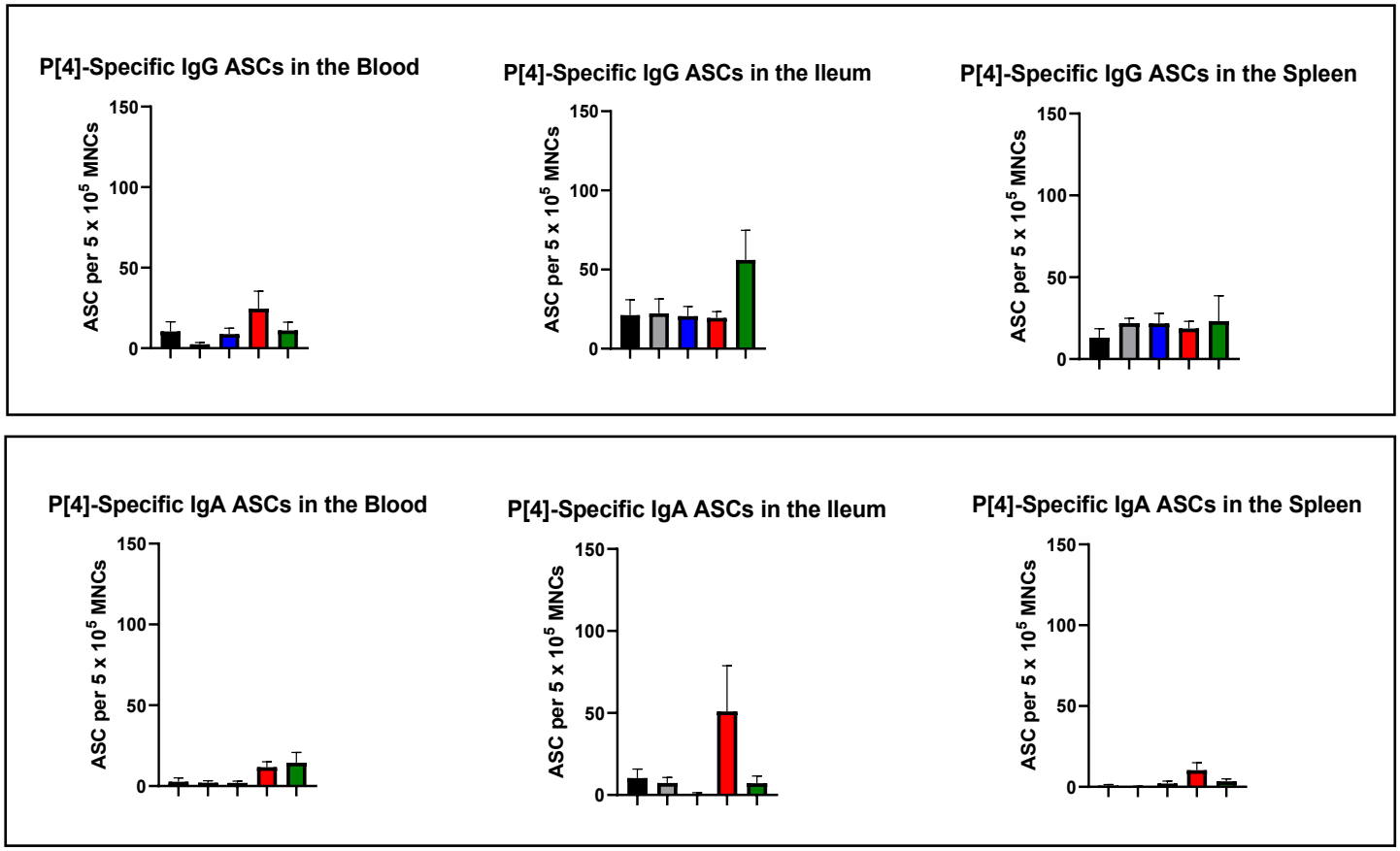


Figure 10. Mean numbers of P[4]-specific ASCs in the tissues of Gn pigs post-challenge. MNCs extracted from post-mortem tissues were evaluated for P-type specific IgG (**A**) or IgA (**B**) ASCs using ELISpot assay. Kruskal-Wallis test followed by Dunn's multiple comparisons test was used for analysis (n=8-13; * $p \leq 0.05$). Bars indicate means with SEM. ELISpot, enzyme-linked immunosorbent spot; ASC, antibody-secreting cells; MNCs, mononuclear cells.

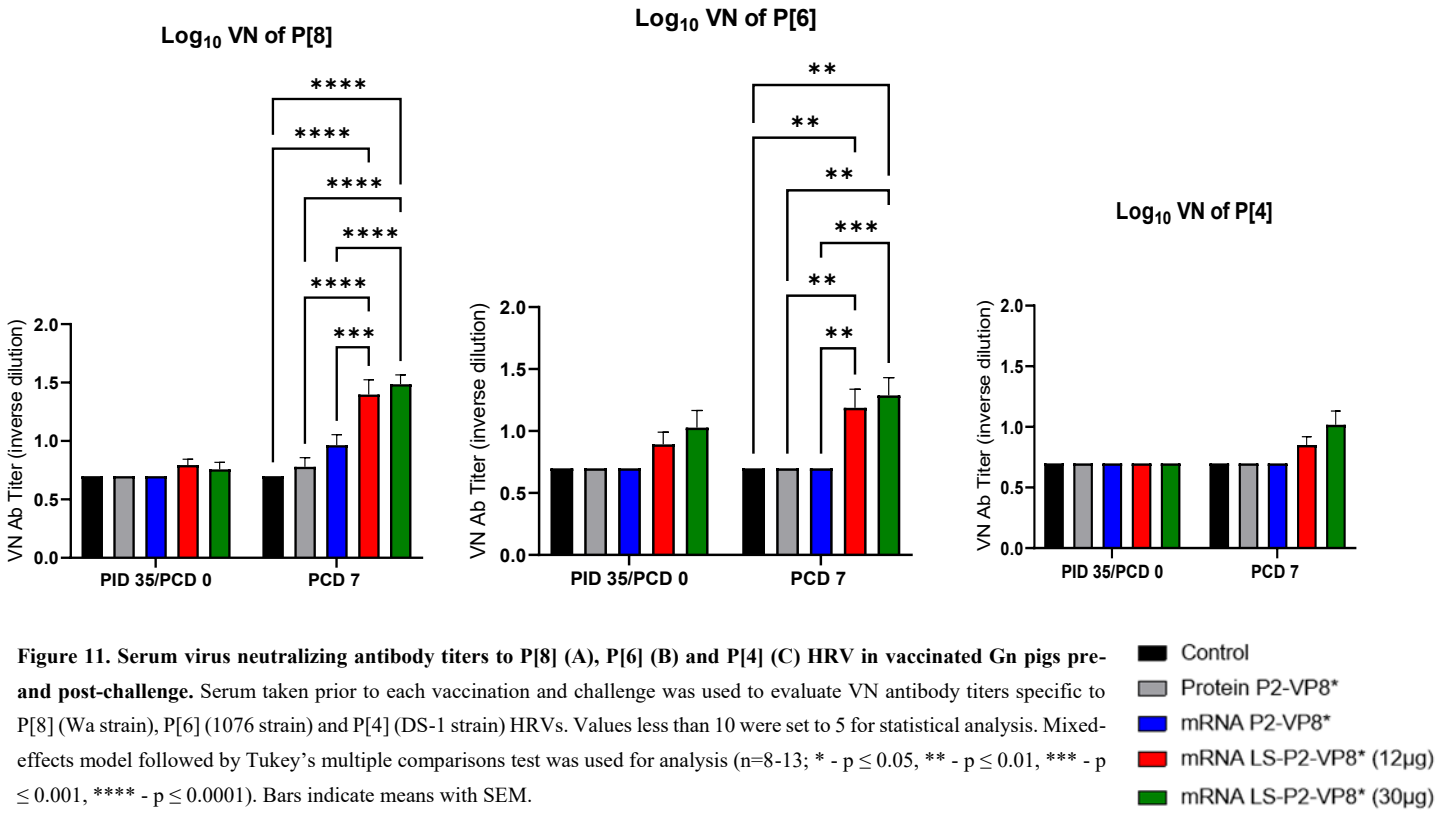


Figure 11. Serum virus neutralizing antibody titers to P[8] (A), P[6] (B) and P[4] (C) HRV in vaccinated Gn pigs pre- and post-challenge. Serum taken prior to each vaccination and challenge was used to evaluate VN antibody titers specific to P[8] (Wa strain), P[6] (1076 strain) and P[4] (DS-1 strain) HRVs. Values less than 10 were set to 5 for statistical analysis. Mixed-effects model followed by Tukey's multiple comparisons test was used for analysis (n=8-13; * - $p \leq 0.05$, ** - $p \leq 0.01$, *** - $p \leq 0.001$, **** - $p \leq 0.0001$). Bars indicate means with SEM.

Chapter 4

Combined live oral priming and intramuscular boosting regimen with Rotarix® and a nanoparticle-based trivalent rotavirus vaccine evaluated in gnotobiotic pig models of G4P[6] and G1P[8] human rotavirus infection

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Abstract

Human rotavirus (HRV) is the causative agent of severe dehydrating diarrhea in children under the age of five, resulting in up to 215,000 deaths each year. With the introduction of live, oral attenuated vaccines nearly two decades ago, these deaths almost exclusively occur in low and middle-income countries where vaccine efficacy is the lowest. Parenteral vaccines for HRV are particularly attractive, as they avoid many of the concerns associated with currently used live oral vaccines (i.e., intussusception). In this study, a two-dose intramuscular (IM) regimen of the trivalent, nanoparticle-based, nonreplicating, HRV vaccine (trivalent S_{60}-VP8*), utilizing the shell (S) domain of the capsid of norovirus (NoV) as an HRV VP8* antigen display platform, was evaluated for safety, immunogenicity and protective efficacy against P[6] and P[8] HRV using Gn pig models. A prime-boost strategy using one dose of the commercial oral Rotarix® vaccine, followed by one dose of the IM trivalent nanoparticle vaccine was also evaluated. Both regimens were highly immunogenic in terms of inducing serum virus neutralizing (VN), IgG, and IgA antibodies. The prime-boost regimen significantly shortened the duration of virus shedding in pigs challenged orally with the virulent Wa (G1P[8]) HRV and significantly shortened the mean duration of virus shedding, mean peak titer, and area under the curve (AUC) of virus shedding after challenge with Arg (G4P[6]) HRV. Prime-boost-vaccinated pigs challenged with P[8] HRV had significantly higher P[8]-specific IgG antibody-secreting cells (ASCs) in the spleen post-challenge. Prime-boost-vaccinated pigs challenged with P[6] HRV had significantly higher numbers of P[6] and P[8]-specific IgG ASCs in the ileum, as well as significantly higher numbers of P[8]-specific IgA ASCs in the spleen post-challenge. These results suggest the promise of and warrant further investigation into the oral priming and parenteral boosting strategy for future HRV vaccines.

Keywords: rotavirus nanoparticle; gnotobiotic pig; priming immunization

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1. Introduction

Human rotavirus (HRV) remains the leading cause of childhood dehydrating diarrhea in low- and middle-income countries (LMICs), despite the use of live attenuated oral HRV vaccines (Carcamo-Calvo, Munoz et al. 2021, Donato and Bines 2021). The two most used, commercially available vaccines are the monovalent Rotarix® (G1P[8]), and pentavalent RotaTeq® (G1-4P[8]). These vaccines, though highly effective in developed countries, do not offer the same protection for children in LMICSs for various reasons, mainly associated with the gastrointestinal (GI) tract (Carcamo-Calvo, Munoz et al. 2021, Donato and Bines 2021). Some of these issues include malnutrition, circulating maternal antibodies, concurrent use of other oral vaccines, and gut dysbiosis. Additionally, P[4] and P[6] HRVs are commonly co-circulating with P[8] strains, highlighting the need for vaccines offering cross-P-type protection, especially as new strains (P[6] in particular) begin to emerge (Vesikari, Matson et al. 2006, Steele, Neuzil et al. 2012, Nyblade, Hensley et al. 2022). Furthermore, these vaccines are associated with a small risk of intussusception (Carcamo-Calvo, Munoz et al. 2021). These reasons, along with the massive yearly economic burden of HRV infection, make parenteral routes of HRV vaccination particularly attractive in LMICs where the HRV burden is the highest (Pitzer, Bennett et al. 2019, Ramesh, Mao et al. 2019). HRV is an enteric pathogen that replicates in the small intestine and the generation of HRV-specific local IgA and virus-specific IFN-γ+ T cells responses are associated with protection from the disease (Yuan, Ward et al. 1996, Yuan, Kang et al. 1998, Yuan and Saif 2002, Yuan, Wen et al. 2008, Yuan 2022). Because of this, live oral attenuated vaccines have been

the focus of vaccine development in the past, due to the vaccines' ability to replicate in the gut and induce similar immune responses to natural HRV infection without inducing severe disease (Yuan and Saif 2002, Yuan 2022). On the contrary, parenteral HRV vaccines have been shown to be highly immunogenic and are now the focus of current efforts to address live oral vaccines' shortcomings (Wen, Wen et al. 2014). Unfortunately, a phase III human trial for a parenteral HRV vaccine was recently terminated due to a lack of improved protective efficacy as compared to currently used live oral attenuated vaccines (Chen, Grow et al. 2022). Despite this, parenteral vaccines still may have an important role in protecting against disease. Priming and boosting regimens for HRV vaccination have been tested in a gnotobiotic (Gn) pig model successfully and have often shown superior results compared to repeated immunization with the same vaccine (Yuan 2022). In a group of studies that investigated various prime-boost strategies in Gn pigs, oral attenuated HRV as a priming immunization (analogous to Rotarix®) and both intranasal (IN) adjuvanted VLPs or intramuscular (IM) DNA plasmid as boosting doses induced higher protection than any other regimen (Yuan, Azevedo et al. 2005, Azevedo, Gonzalez et al. 2010, Yuan 2022).

HRV binding to host cells is mediated by the VP4 protein, which is proteolytically cleaved in the host's gut. This cleavage yields the proximal VP5* and distal VP8*, the latter of which is responsible for facilitating binding and entry, as well as being a major target for host immune responses (Liu, Huang et al. 2012, Knipe and Howley 2013, Tan and Jiang 2014). Because VP8* has been shown to induce virus-neutralizing (VN) antibody responses in several studies, it has remained the target for HRV vaccine development. In this study, a nanoparticle-based nonreplicating HRV vaccine, utilizing the shell (S) domain of the capsid protein (VP1) of norovirus (NoV) as a platform, was developed. The S domain of NoV self-assembles into a 60-valent icosahedral nanoparticle with exposed C-termini, which are amenable to the insertion of

foreign proteins (Xia, Huang et al. 2018, Xia, Hoq et al. 2022). In this vaccine, VP8* from the three most predominant HRV P-types (P[4], P[6], and P[8]) were fused to the exposed C-termini, forming the S_{60}-VP8* trivalent nanoparticle vaccine. This vaccine is easily produced in high quantities using an *E. coli* expression system and has been shown to be highly immunogenic in murine models (Xia, Huang et al. 2019, Xia, Huang et al. 2022).

The goal of this study was to evaluate the safety, immunogenicity, and protective efficacy of the trivalent S_{60}-VP8* vaccine in Gn pigs as both a two-dose IM regimen and as a booster dose preceded by an oral priming dose using the commercially available Rotarix®. Gn pigs have long been used for vaccine efficacy studies, as they develop a similar disease to that of humans after HRV inoculation, including intestinal pathology (Yuan 2022). They are also immunologically similar to humans, and their gnotobiotic status allows for minimal immune-related variables when evaluating vaccine efficacy (Yuan 2022). In this study, we utilized the well-established Gn pig model of P[8] HRV infection and diarrhea as well as the newly characterized P[6] HRV infection and diarrhea model (Nyblade, Hensley et al. 2022). The inclusion of both models allows for the generation of a deeper understanding of potential cross-protection induced by candidate HRV vaccines. This is important as the incidence of P[6] HRVs have been on the rise in recent years and continue to circulate globally, but currently licensed vaccines do not contain P[6] immunogens (Steele, Neuzil et al. 2012).

2. Materials and Methods

2.1 Rotaviruses for Challenge and Immunoassays

The virulent Wa HRV (G1P[8]) and Arg HRV (G4P[6]) inocula consisted of pools composed of small intestinal contents collected from the 29th passage and 6th passages of the viruses in neonatal Gn pigs, respectively. A dose of 10^5 focus-forming units (FFU) of Wa and Arg HRV diluted in

5mL of Diluent #5 (minimal essential media [ThermoFisher Scientific]; 100 IU of penicillin per mL, 0.1 mg of dihydrostreptomycin per ml; and 1% HEPES) was used for the oral challenge of Gn pigs. The median infectious and diarrhea doses of both Wa and Arg HRV were determined previously to be approximately 1 FFU and 10^5 FFU was the optimal challenge dose that will induce diarrhea and virus shedding in 100% of unimmunized Gn pigs (Ward, Rosen et al. 1996, Nyblade, Hensley et al. 2022). The supernatant of attenuated Wa HRV-infected African green monkey kidney MA104 cells (ATCC CRL-2378.1™) was semi-purified by centrifugation and used as a positive control in antigen enzyme-linked immunosorbent assay (ELISA) and cell culture immunofluorescence (CCIF) (Twitchell, Tin et al. 2016).

2.2 Vaccine

Three P types of S_{60}-VP8* nanoparticles (P[8], P[6] and P[4]) were produced and purified as previously described (Xia, Huang et al. 2018, Xia, Huang et al. 2019), mixed as the trivalent vaccine in the liquid formulation in phosphate buffer saline (PBS). The individual nanoparticles and the trivalent vaccine were assessed by 1) SDS-PAGE for protein quality; 2) gel filtration chromatography and EM inspection for nanoparticle formation and structural integrity (Xia, Huang et al. 2018, Xia, Huang et al. 2019). Bacterial endotoxin was removed by using ToxinEraser™ Endotoxin Removal Resin (GenScript, Cat. No L00402. Piscataway, NJ, USA). The vaccines were formulated by mixing each S_{60}-VP8* P[8], P[6] and P[4] or S_{60} alone with 600 µg/dose of aluminum hydroxide adjuvant (Alhydrogel, InvivoGen, San Diego, CA, USA). The vaccine dose was originally designed to contain 200 µg of each P type. However, due to the need for filtration to eliminate contaminating bacteria, the actual amount of antigen in the vaccine was reduced. The actual amount of antigens in the vaccine measured with Nanodrop was 94 µg of P[4],

52 µg of P[6], and 143 µg of P[8]. Sterility of antigens was confirmed using blood agar and thioglycolate broth prior to inoculation of Gn pigs.

2.3 Vaccine Inoculation, Virus Challenge, and Sample Collection of Gn Pigs

Piglets were derived via hysterectomy of near-term sows and maintained in germ-free isolators for the duration of the study (Yuan 2017). Sterility was verified by weekly rectal swabs (RS) on blood agar and in thioglycolate broth. Pigs were fed ultra-high temperature (UHT) treated sterile whole cow's milk (The Hershey Company, Hershey, PA, USA) for the duration of the study. Pigs were inoculated with either the priming dose of trivalent S_{60}-VP8* IM or Rotarix® orally on post-partum day (PPD) 5 (also referred to as post-vaccination day [PVD] 0) and subsequently received the same dose IM boosting of trivalent S60-VP8* at PVD 14 (**Figure 1**). Control pigs were immunized on the same schedule with two IM administrations of the S_{60} nanoparticle without VP8* antigen. Serum was collected before each vaccination at PVD 0, PVD 14 and PVD 28/post-challenge (PCD) 0 and at euthanasia (PCD 7) to evaluate P[8], P[6] and P[4] VP8*-specific IgG and IgA by direct ELISA and virus neutralizing (VN) titers by virus neutralization assay.

A total of 45 pigs were assigned randomly to three groups per challenge strain (**Figure 1**). Pigs were orally challenged on PVD 28/PCD 0 with 1 x 10^5 FFU of either virulent Wa HRV (G1P[8]) or virulent Arg HRV (G4P[6]) and monitored for diarrhea and virus shedding via daily RS from PCD 0-7; RS were processed as described previously (Yang, Twitchell et al. 2015). All pigs were fed 4 mL 200 mM sodium bicarbonate 10 minutes prior to the HRV challenge to neutralize stomach acidity. Pigs were euthanized at PCD 7. At euthanasia, small intestinal contents (SIC) and large intestinal contents (LIC) were collected and processed as previously described, for the detection of intestinal antibody responses and viral antigen presence by direct and sandwich

ELISA, respectively, as well as infectious virus particle counting by CCIF (Parre, Hodgins et al. 1999). Spleen, ileum, and whole blood were collected for extraction of MNCs to be used in the detection of P[8] and P[6] VP8*-specific IgG and IgA ASC responses by enzyme-linked immunosorbent spot (ELISpot) and P[8], P[6] and P[4]VP8*-specific IFN-γ-producing CD4+ and CD8+ T cells by flow cytometry (Yuan, Ward et al. 1996, Yuan, Kang et al. 1998, Yuan, Wen et al. 2008).

2.4 Assessment of Diarrhea and Detection of Fecal Virus Shedding by Antigen ELISA and Virus CCIF

To assess the severity of diarrhea, fecal consistency was recorded, using the RS taken from PCD 0 to 7, as 0: solid, 1: pasty, 2: semi-liquid, and 3: liquid, with any score ≥2 being considered diarrhea (Saif, Ward et al. 1996, Ward, Rosen et al. 1996, Yuan and Saif 2002). Antigen ELISA was used for the detection of HRV VP6 and CCIF for the detection of infectious virus particles, as previously described (Saif, Ward et al. 1996, Saif, Yuan et al. 1997, Yuan and Saif 2002, Vega, Garaicoechea et al. 2021).

2.5 Detection of HRV VP8*-specific Serum and Intestinal IgA and IgG Antibody by ELISA

Anti-HRV VP8* IgA and IgG antibody titers in serum and intestinal contents were measured by using an isotype-specific ELISA as described previously (Xia, Huang et al. 2022), using recombinant GST-VP8* protein of P[8], P[6], or P[4] type as capture antigens that were coated to plates at a concentration of 1.5 µg/mL overnight at 4°C. Both serum and intestinal contents were clarified by centrifugation at 10,000 rpm for 10 min. Coated plates were blocked with 5% non-fat milk in PBS at 37°C, followed by incubation with four-fold serially diluted serum sample (starting from 1:4 to 1:1,048,576) or intestinal content sample (starting from 1:4 to 1:65,536) overnight at

4°C. The bound antibodies were detected using goat anti-pig IgA antibody conjugated with horseradish peroxidase (HRP) (Bethyl Laboratories, Inc. USA) or goat anti-pig IgG heavy/light chain antibody conjugated with HRP (Bethyl Laboratories, Inc.) at 1:8000 diluted in PBST with 1% non-fat milk. Color was developed using TMB Substrate Reagent Set (BD Biosciences) and optical density (OD) was read at 450nm.

2.6 Flow Cytometry for Detection of IFN-γ-producing CD3+CD4+ and CD3+CD8+ T Cells

Mononuclear cells isolated at PCD 7 from peripheral blood (PCD 7), ileum and spleen were diluted to a concentration of 1×10^6 cells/mL and seeded into 12-well plates (Yuan, Wen et al. 2008). Cells were re-stimulated for 17h at 37°C in 5% CO_2 with one of seven antigens: (1) medium (negative control), (2) PHA (10µg/mL; positive control), (3) P[4] protein (12µg/mL), (4) P[6] protein (12µg/mL), and (5) P[8] protein (12µg/mL). Anti-CD49d mAb (0.5µg/mL of clone 9F10, catalog # 555502, BD Biosciences, Franklin Lakes, NJ, US;) was added to all samples before incubation for co-stimulation. After 12h, Brefeldin A (5µg/mL) was added for 5h at 37°C in 5% CO_2. After the total 17h incubation, cells were washed with 2mL of commercial stain buffer and transferred to 5mL Falcon round-bottom polypropylene tubes for 8 min centrifugation at 800xg at 4°C, followed by discarding of the supernatant. Primary antibodies used for staining were mixed in a trivalent that included FITC-conjugated mouse (IgG2b) anti-pig CD4α (clone 74-12-4, catalog #559585, BD Biosciences, Franklin Lakes, NJ, USA), SPRD-conjugated mouse (IgG2a) anti-pig CD8α (clone 76-2-11, catalog #4520-13, Southern Biotech, Birmingham, AL, USA), mouse (IgG1) anti-pig CD3ε (clone PPT3, catalog #4510-01, Southern Biotech, Birmingham AL, USA), and GloCell™ Fixable Viability Dye Violet 450 (catalog #75009, StemCell Technologies, Cambridge, MA, USA) in 100µL of stain buffer per sample. Samples were incubated with the trivalent for 15 mins at 4ºC and subsequently washed with 500µL of wash buffer, followed by 8

min centrifugation at 800xg at 4°C. Then, secondary antibody APC-conjugated rat (IgG1) anti-mouse for CD3ε (clone X56, catalog #550874, BD Biosciences, Franklin Lakes, NJ, USA) diluted in 100µL of stain buffer was added and incubated for another 15 mins at 4ºC. The washing and centrifugation step was repeated, then, 100µL of BD Cytofix/Cytoperm (cat #554714, BD Biosciences, Franklin Lakes, NJ, USA) permeabilizing/fixation solution containing 4.2% formaldehyde was added into each sample and incubated for 30 mins at 4ºC. Washing and centrifugation were then repeated. PE-conjugated mouse (IgG1) anti-pig IFN-γ (clone P2G10, catalog #559812, BD Biosciences, Franklin Lakes, NJ, USA) diluted in 100µL of stain buffer was added and incubated for another 30 mins at 4ºC. A last washing step with 2mL of stain buffer was performed followed by centrifugation. Cells were resuspended in 250µL of stain buffer and stored away from light at 4°C until delivery to Virginia Tech's flow cytometry core for analysis within 24 hours for acquisition on a BD FACSArial™ II flow cytometer.

2.7 Detection of VP8*-specific Antibody-Secreting Cells by ELISpot Assay

Plates (96-well Falcon [Corning], Corning, NY, USA) were coated with 50µL P[8], P[6], or P[4] at the concentration of 5 µg/mL in 50mM carbonate coating buffer (pH 9.6). Plates were incubated at 37ºC for 30 min, then stored at 4ºC overnight. Plates were washed twice with PBST (pH 7.4, with 0.05% Tween 20), blocked with 100 µL/well 4% BSA in PBS (pH 7.4) and incubated at 37ºC for 1 hr. Plates were washed three times with ddH_20. Separately, extracted MNCs were diluted to single-cell suspension at concentrations of 5×10^6 and 5×10^5 cells/mL with E-RPMI and 100µL of each diluted cell suspension was added to duplicate wells. Plates were then centrifuged at 500rpm for 5 min at RT, followed by incubation at 37ºC for 12 hr. Plates were then washed five times with PBST, and 100µL/well biotinylated goat anti-porcine IgA diluted 1:20,000 (Bethyl A100-102B, 1 mg/mL) or IgG (Bethyl A100-104B, 1 mg/mL), diluted 1:20,000 in PBST was

added to each well. Plates were incubated at RT for 2 hr and washed five times with PBST. To ensure cells were completely washed away, the plates were knocked vigorously on paper towels between each wash. Next, 100µL/well HRP-conjugated streptavidin diluted 1:30,000 in PBS was added to the plates followed by incubation at RT for 1 hr. Plates were washed five times with PBST, and 50µL/well KPL TrueBlue substrate (ready-to-use substrate from SeraCare, Milford, MA, USA) was added to each well. Plates were then incubated for 1-2h at RT until spots become dark blue. Spots were counted with ELISpot analyzer S5 (ImmunoSpot, Cleveland, OH, USA) and reported as IgA or IgG ASC per 5 x10^5 MNC.

2.8 Virus Neutralization Assay

HRV neutralization assays were performed using a fluorescence-based plaque reduction assay as described previously (Xia, Huang et al. 2022). Briefly, trypsin-treated HRVs of P[8] (Wa strain, G1P[8]), P[6] (ST-3 strain, G4P[6]), and P[4] (DS-1 strain, G2P[4]) types were incubated with serially diluted serum samples, respectively. The HRVs were then added to MA104 cells on 96-well plates. After further culture for 16 hours, the plated cells were frozen with 80% (v/v) acetone and then blocked with non-fat milk. The HRV-infected cells were stained with a 2KD1 nanobody against VP6 (Garaicoechea, 2008) labeled with AlexaFluor488 at a 1/500 dilution (Garaicoechea, Olichon et al. 2008). The bound antibodies were detected using FITC-labeled goat anti-guinea pig IgG antibody. Fluorescence plaques were photographed using the Cytation 5 imaging reader and then counted. Neutralization titers were defined as the maximum dilutions of the serum samples showing at least 50% reduction in fluorescence-formation plaques.

2.9 Statistical Analysis

Pigs were randomly assigned to treatment groups by animal care staff upon derivation, regardless of sex or body weight. Mean numbers of ASCs in post-mortem tissues in each treatment group were compared using the Kruskal-Wallis non-parametric rank sum test followed by Dunn's multiple comparisons tests. Daily mean diarrhea scores and CCIF titers were evaluated using two-way ANOVA of repeated measures through time, followed by Tukey's multiple comparisons tests. All other diarrhea and virus-shedding parameters were evaluated using one-way ANOVA followed by Tukey's multiple comparisons tests, or Kruskal-Wallis test when homoscedasticity and normality assumptions were not met. Mixed-effects analysis followed by Dunnett's multiple comparisons test was used for comparing serum and intestinal antibody responses, and VN titers were evaluated using mixed-effects analysis with a two-stage linear step-up procedure. Because only two treatment groups were compared, total numbers and mean frequencies of T cell subsets were evaluated using multiple Mann-Whitney tests. All analyses were performed using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA). A p-value lower than 0.05 was accepted as statistically significant.

3. Results

3.1. Prime-Boost Regimen Significantly Reduced Virus Shedding in Both Challenge Groups

Gn pigs were challenged with either virulent Wa or Arg HRV at PVD 28/PCD 0 and clinical signs/virus shedding were monitored via daily RS. These data are summarized in **Tables 1 and 2**, respectively. All pigs in all treatment groups developed diarrhea and virus shedding after challenge with either Wa or Arg HRV. Although no significant differences were detected, trends for both challenge groups showed lower mean duration days, mean cumulative scores, and AUC of diarrhea

for prime-boost vaccinated pigs as compared to controls (**Tables 1, 2**). Significant differences in mean daily diarrhea scores were detected for Wa-HRV challenged prime-boost vaccinated pigs at PCDs 4-6 as compared to controls **(Figure 2A**). Arg HRV-challenged pigs vaccinated with the prime-boost regimen had lower daily mean diarrhea scores from PCDs 1-5, although they were not statistically significant (**Figure 2B**).

Protection against virus shedding was more pronounced. In Wa HRV-challenged pigs, the onset of virus shedding was significantly delayed and the duration of virus shedding was significantly reduced in prime-boost vaccinated pigs as compared to controls (**Figures 3A, B**). In Arg HRV-challenged pigs, the duration of virus shedding, mean peak titers, and AUC of virus shedding were all significantly reduced in prime-boost vaccinated pigs (**Figures 3F-H**). In Wa HRV-challenged pigs, daily mean CCIF titers were significantly lower in prime-boost vaccinated pigs than in other groups on PCD 5 (**Figure 4A**). In Arg HRV-challenged pigs vaccinated with the prime-boost regimen, mean CCIF titers were significantly lower from PCDs 4-6 as compared to controls (**Figure 4B**).

3.2. Both Vaccine Regimens Were Highly Immunogenic and Induced Strong Serum IgG and IgA Responses in Gn Pigs Before and After Challenge with Wa or Arg HRV

Serum samples collected at PVD 0, 14, 28, and PCD 7 were used to evaluate vaccine-induced, P-type specific IgG and IgA antibodies in Gn pigs. In both challenge groups, pigs vaccinated with trivalent nanoparticle 2x had significantly higher titers of P[8], P[6], and P[4]-specific serum IgG as compared to controls after just one dose vaccination (PVD 14), which lasted until PVD 35/PCD 7 (**Figures 5A, 6A**). Gn pigs in both challenge groups who received the prime-boost regimen

developed significantly higher titers for all three P-types as compared to controls at PVD28, which lasted until PVD 35/PCD 7 (**Figures 5A, 6A**).

P[8], P[6], and P[4]-specific serum IgA antibody titers in the Wa HRV challenge group were significantly higher in trivalent nanoparticle 2x-vaccinated pigs at PVD 28/PCD 0 and PVD 35/PCD 7 (**Figure 5B**). P[6]-specific IgA became significantly higher in trivalent nanoparticle 2x-vaccinated Wa-challenged pigs early, after only one dose vaccination (PVD 14), and again lasted until PVD35/PCD 7 (**Figure 5B**). Prime-boost-vaccinated pigs challenged with Wa HRV had significantly higher serum IgA after challenge as compared to controls to all three P-types; these titers were comparable again to trivalent nanoparticle 2x-vaccinated pigs (**Figure 5B**). In the Arg HRV challenge group, P[8]-specific serum IgA titers were significantly higher in both vaccine groups as compared to controls after two vaccinations (**Figure 6B**) However, after challenge, prime-boost pigs had significantly higher P[8]-specific serum IgA titers than controls, with trivalent nanoparticle 2x group had IgA titers only slightly higher than the controls at this timepoint (**Figure 6B**). Both vaccine groups in the Arg HRV challenge group had significantly higher P[6] and P[4]-specific serum IgA titers on PVD 28, which lasted until PVD 35/PCD 7 (**Figure 6B**).

3.3. Prime-boost Vaccinated Gn Pigs Challenged with Arg HRV Had Significantly Higher P[8]-specific IgA in the Small Intestine at Euthanasia

At PVD 35/PCD 7, Arg HRV-challenged pigs who received the prime-boost regimen had significantly higher P[8]-specific IgA antibody titers in SIC as compared to other groups (**Figure 7B**). Wa HRV-challenged pigs had substantially higher titers of P[8]-specific IgA, but they were not statistically significant (**Figure 7A**). Arg HRV-challenged pigs who received the prime-boost

regimen also had higher titers of P[6]-specific IgA in the SIC, but they were not statistically significant either (**Figure 7B**).

3.4. Both Vaccine Regimens Induced Strong P[8] and P[6]-specific Virus Neutralizing Antibody Responses in the Serum of Wa and Arg HRV-challenged Gn Pigs

Both vaccine groups in the Wa HRV-challenge group had significantly higher P[8]-specific VN titers at PVDs 14 and 28 (**Figure 8A**). By PVD 35/PCD 7, there were no significant differences among the three groups, though mean P[8]-specific VN titers were visibly higher in prime-boost vaccinated pigs challenged with Wa HRV. P[6]-specific VN titers were significantly higher in Wa HRV-challenged trivalent nanoparticle 2x-vaccinated pigs after two vaccinations at PID 28, and both vaccine groups had significantly higher mean P[6]-specific VN titers at PID 35 as compared to controls (**Figure 8A**). There were no significant differences in P[4]-specific VN titers among Wa HRV-challenged groups at any time point (**Figure 8A**). P[8]-specific VN titers were higher in both vaccine groups challenged with Arg HRV after two vaccinations and post-challenge, but the differences were not significant (**Figure 8B**). P[6]-specific VN titers were significantly higher in trivalent nanoparticle 2x-vaccinated Gn pigs challgened with Arg HRV at PID 28, but by PID 35/PCD 7, there were no differences between groups (**Figure 8B**). Arg HRV-challenged Gn pigs vaccinated with the prime-boost regimen exhbited higher mean P[4]-specific VN titers at PID 35/PCD 7, but like the Wa HRV-challenged pigs, they were not statistically significant (**Figure 8B**).

3.5. Trivalent Nanoparticle 2x-vaccinated, Wa HRV-challenged Gn Pigs Had Significantly Higher Total Numbers and Frequencies of P[8]-specific CD3+CD4+IFN-γ+ and CD3+CD8+IFN-γ+ T Cells in the Ileum Post-challenge

Lymphocytes extracted from peripheral blood, ileum, and spleen were restimulated *in vitro* with P[8], P[6], or P[4] VP8* proteins and analyzed for their subsequent intracellular production of IFN-γ. Trivalent nanoparticle 2x-vaccinated pigs challenged with Wa HRV had significantly higher total numbers of both CD3+CD4+IFN-γ+ and CD3+CD8+IFN-γ+ T cells, as well as significantly higher frequencies of P[8]-specific CD3+CD4+IFN-γ+ T cells in the ileum compared to the control pigs (**Figures 9A, B**). These T cell responses were also increased in the spleen and blood, but not significantly so (**Figures 9A, B**). Total numbers and mean frequencies of both P[6]-specific CD3+CD4+IFN-γ+ and CD3+CD8+IFN-γ+ T cells were higher in all tissues of Wa HRV-challenged pigs vaccinated with the trivalent nanoparticle 2x regimen than the controls, but not to a significant degree (**Figures 9A, B**). Total numbers and mean frequencies of both P[4]-specific CD3+CD4+IFN-γ+ and CD3+CD8+IFN-γ+ T cells were higher in the ileum of Wa HRV-challenged pigs vaccinated with trivalent nanoparticle 2x, but, again, not significantly so (**Figures 9A, B**). In Arg HRV-challenged pigs, trivalent nanoparticle 2x vaccination also primed for higher total numbers and mean frequencies of P[8]-specific CD3+CD8+IFN-γ+ T cells in all tissues, and CD3+CD4+IFN-γ+ T cells in the ileum and spleen post-challenge, though these values were not statistically significant (**Figures 10A, B**). P[6]-specific total numbers and mean frequencies of both CD3+CD4+IFN-γ+ and CD3+CD8+IFN-γ+ T cells were elevated in all tissues of trivalent nanoparticle 2x-vaccinated pigs challenged with Arg HRV as compared to controls, except for P[6]-specific CD3+CD4+IFN-γ+ mean frequencies in the blood, which were similar to controls

(Figures 10A, B). The T cell responses were unfortunately not determined for the prime-boost vaccinated pigs.

3.6. Both Vaccine Regimens in Both Challenge Groups Induced Significant P-type-specific ASC Responses in the Ileum and Spleen

P[8], P[6], and P[4]-specific IgG and IgA ASC responses in ileum, spleen and blood of all Gn pigs were evaluated at PID 35/PCD 7. Significantly ASC responses in ileum and spleen were primed by both vaccine regimens. In Wa HRV-challenged pigs vaccinated with the trivalent nanoparticle 2x regimen, significantly higher numbers of P[8] and P[4]-specific IgG ASCs were detected in ileum compared to the control pigs (**Figure 11A**). Furthermore, pigs in this challenge group vaccinated with prime-boost regimen, P[8]-specific IgG ASC numbers in the spleen were significantly higher than the controls (**Figure 11A**). In Arg HRV-challenged pigs, P[8]- and P[6]-specific IgG ASC numbers were significantly higher in ileum of prime-boost vaccinated pigs (**Figure 11B**). These pigs also had significantly higher numbers of P[8]-specific IgA ASCs in spleen (**Figure 11B**). Additionally, P[8] and P[6]-specific IgG ASCs of trivalent nanoparticle 2x-vaccinated pigs in the ileum were also higher or significantly higher than the controls (**Figure 11B**). The ASC responses in tissues and vaccine/challenge groups that are not presented here did not differ significantly compared to the control group.

4. Discussion

In this study, we evaluated the immunogenicity and protective efficacy of a trivalent nanoparticle vaccine in two different dosing regimens using Gn pig models of Wa (G1P[8]) and Arg (G4P[6]) HRV infection and diarrhea. The first regimen consisted of two IM administrations and the other an oral priming with a live oral attenuated HRV vaccine followed by one IM boosting with the

trivalent nanoparticle vaccine. We found that neither regimen prevented the incidence of diarrhea upon challenge with 1 x 10^5 FFU of the virulent Wa or Arg HRV. However, there was a significant reduction of virus shedding in both challenge groups vaccinated with the prime-boost regimen, as evidenced by the delayed onset and the significant reduced duration of virus shedding for Wa P[8]-challenged pigs and reduced duration, mean peak titers, and AUC of virus shedding for Arg P[6]-challenged pigs. Furthermore, the trivalent nanoparticle 2x regimen also significantly reduced virus shedding in Arg P[6] HRV-challenged pigs, as evidenced by the significantly reduced mean peak titers and AUC. These results agree with previous studies in mice, where mice vaccinated with S_{60} particles fused with murine rotavirus (EDIM) VP8* had significantly reduced viral antigen shedding after homotypic challenge as compared to controls (Xia, Huang et al. 2019). Our current studies of Gn pigs and studies of mice have also demonstrated similarly the robust immunogenicity of the nanoparticle vaccine platform delivered IM (or subcutaneously) (Xia, Huang et al. 2019, Xia, Huang et al. 2022).

Despite the presence of serum IgA, IgG, and VN antibodies against both P types of the HRV challenge strains at the time of challenge (PVD28/PCD0), trivalent nanoparticle 2x-vaccinated pigs were not protected against diarrhea. A previous study evaluating a similar parenterally administered P24-VP8* vaccine with three IM doses (200 µg/dose) in Gn pigs showed significant protection against diarrhea − shortened the duration from 6.0 to 3.3 days and mean diarrhea scores from 14.3 to 9.1, despite a very similar degree of protection against virus shedding – shortened duration of virus shedding from 5.9 to 2.5 days (Ramesh, Mao et al. 2019). Only one booster and the lower doses in this study may have greatly contributed to the lack of protection against diarrhea by the trivalent nanoparticle 2x regimen. Significantly higher P[8] and P[6]-specific, but not P[4]-specific, serum VN antibody titers were induced by both vaccine regimens. It is plausible that the

P[4]-VP8* component of the trivalent vaccine was less immunogenic due to antigen quality issues (i.e., lower concentration, protein degradation). Further analysis is needed to identify the cause.

In our study, Gn pigs who received an oral priming dose of Rotarix® and an IM boosting dose of S_{60}-VP8* had significantly reduced diarrhea scores from PCD 4-6 after Wa HRV challenge. Intestinal immune responses, namely HRV-specific IgA and IFN-γ producing CD4+ and CD8+ T cells induced by vaccination or natural infection are extremely important in the protection from clinical signs of subsequent HRV infection. Without these immune effectors at the site of viral entry and replication, cells are susceptible to HRV infection, resulting in the various cellular mechanisms that lead to HRV-associated diarrhea. The observed reduction in diarrhea scores is likely due to the priming dose of the replicating vaccine-induced intestinal IgA and effector T cells, which can prevent or reduce infection, in combination with circulating antibody responses, which are responsible for the reduction of viremia to reduce the perpetuation of HRV infection in the intestine (Azevedo, Yuan et al. 2005).

Both challenge groups vaccinated with both regimens showed some cross-P type induction of IgG ASCs in the spleen and ileum. Virus-specific IgG ASCs are not historically correlated with protection against HRV disease, while intestinal IgA ASCs are reported to be a correlate of protection (Yuan, Kang et al. 1998). The lack of intestinal IgA ASCs may also partially explain the lack of protection against diarrhea. HRV-specific IFN-γ+ T cell responses were induced by the trivalent nanoparticle 2x regimen in Wa HRV-challenged pigs, with P[8]-specific responses being significantly higher in the ileum, and substantially higher in all other tissues, including P[6] and P[4]-specific, than the controls. The effector T cell responses should have contributed to the reduction of virus shedding. Previously it was thought that T cell responses, particularly in the mucosa, were difficult to induce through IM vaccination alone; however, this paradigm appears to

be shifting (Clements and Freytag 2016) and is further evidenced by the current study. Furthermore, studies have suggested that the site of antigen entry determines T cell homing capacities (De Calisto, Villablanca et al. 2012). In this case, oral immunization would likely induce T cells' acquisition of homing receptors specific to the gut, whereas parenteral immunization would allow for homing capacities to multiple systemic sites (De Calisto, Villablanca et al. 2012). This would explain the high numbers of HRV-specific T cells in both the ileum, blood, and spleen of the trivalent nanoparticle 2x-vaccinated pigs. We would expect similar if not greater responses to be induced in the prime-boost vaccinated pigs. Further studies are needed to demonstrate the promise of an oral prime/parenteral boost HRV vaccine regimen, as HRV-specific IFN-γ+ T cells in the gut of Gn pigs are strongly associated with protection from challenge (Yuan, Wen et al. 2008). This, along with protection offered by systemic HRV-specific IFN-γ+ T cell responses and antibody/ASC responses, is likely to be highly effective at suppressing primary viremia and intestinal replication of HRV during infection (Ramesh, Mao et al. 2019).

5. Conclusions

The S_{60}-VP8* nanoparticle vaccine candidate is immunogenic and conferred partial protection against virus shedding in Gn pig models of P[6] and P[8] HRV infection both as a two-dose parenteral regimen, as well as in combination with a live oral priming dose of attenuated HRV vaccine. These results are echoed by mouse model studies that have also shown high immunogenicity for all three P-types included in the formulation (Xia, Huang et al. 2022). However, three doses of vaccination are likely needed for the vaccine to realize its potential effectiveness against HRV diarrhea. Our findings support the continued investigation of parenteral nanoparticle-based HRV vaccines as primary and booster vaccines for the improvement of efficacy in areas of the world where HRV is still resulting in significant morbidity and mortality.

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Data Availability Statement: All relevant data are included within the manuscript.

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Conflicts of Interest: Ming Tan has a financial interest in the S particle vaccine platform technology that Cincinnati Children's Hospital Medical Center licenses to Blue Water Vaccines, Inc. The other authors declare no conflict of interest.

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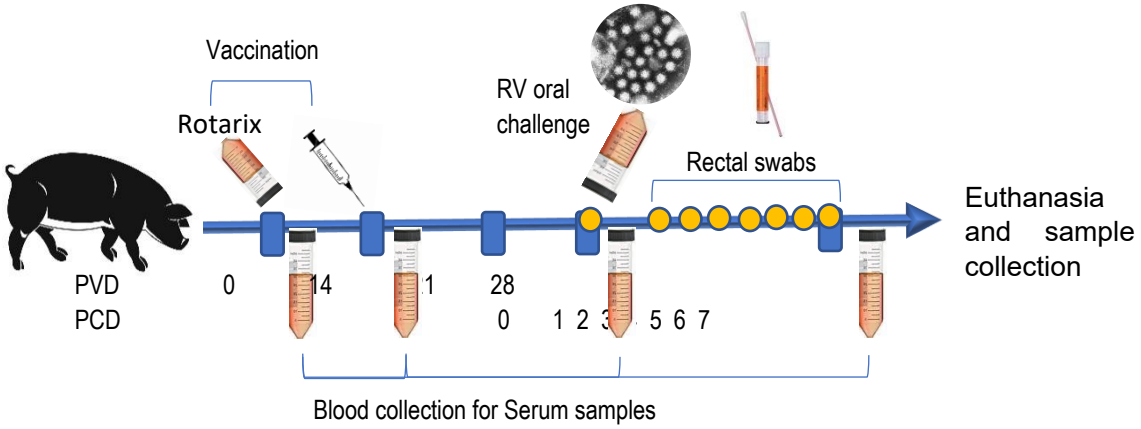
Vaccine Regimen	n	Challenge Strain	n	Challenge Strain
Rotarix® 1x (oral) /Trivalent S_{60}-VP8* 1x (IM; Prime-Boost)	6	Wa HRV (G1P[8])	6	Arg HRV (G4P[6])



Vaccine Regimen	n	Challenge Strain	n	Challenge Strain
Trivalent S_{60}-VP8* 2x (IM; Trivalent Nanoparticle 2x)	12	Wa HRV (G1P[8])	6	Arg HRV (G4P[6])
S_{60}-nanoparticle 2x (IM; Control)	10		5	

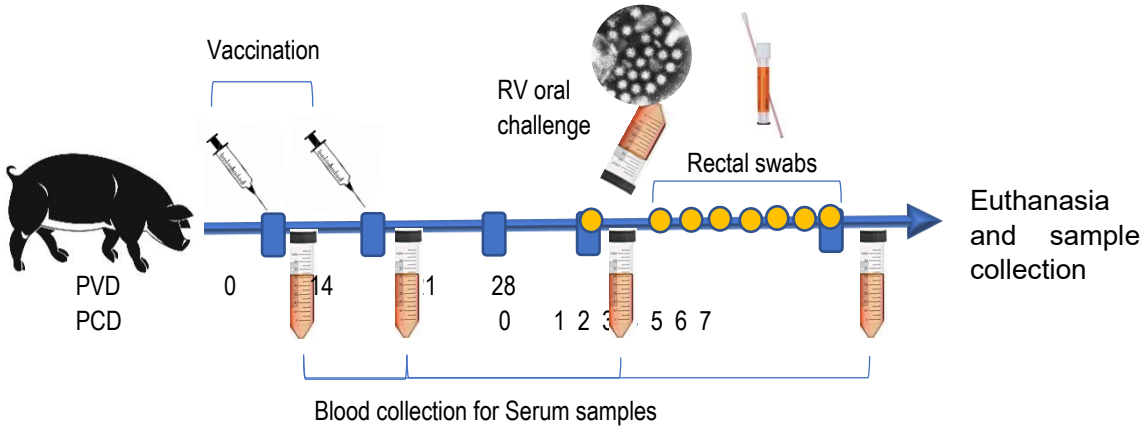


Figure 1. Study design to assess the immune response and protective efficacy of the vaccine regimens in Gn pigs. Pigs were vaccinated with two doses of vaccines on PVD 0 and 14. Blood/serum samples were collected on PVD 0, 14, 28, and PCD 7. The immunized pigs were challenged with virulent Wa HRV or Arg HRV on PCD 0 and rectal swab samples were collected daily post-challenge for seven days for the assessment of diarrhea and virus shedding.

Table 1. Diarrhea and virus shedding in vaccinated and control pigs after challenge with virulent Wa HRV (G1P[8])

		Clinical Signs of Diarrhea [b]					Virus Shedding (CCIF) [b]				
Treatments (vaccine dose µg) [a]	n	Percentage with Diarrhea	Mean Days to Onset	Mean Duration Days	Mean Cumulative Fecal Score	AUC of Diarrhea	Percentage of Shedding Virus	Mean Days to Onset	Mean Duration Days	Mean Peak Titer (FFU/g of feces) [e]	AUC of Virus Shedding [e]
Rotarix 1x/Trivalent Nanoparticle 1x	6	100%	2	3.70	10.3	8.70	100%	2.33 (0.21) [A, c, d]	3.00 (0.4) [B]	18594	51800
Trivalent Nanoparticle 2x	12	100%	2	4.58	12.4	11.1	100%	1.33 (0.14) [B]	5.67 (0.38) [A]	24771	80583
Control	10	100%	2	4.86	13.0	11.4	100%	1.70 (0.33) [AB]	5.00 (0.37) [A]	24552	57440

Note:

a. Pigs were immunized two times with S_{60} nanoparticle control, trivalent nanoparticle vaccine, or once with Rotarix followed by trivalent nanoparticle or control, at 5 (post-vaccination day [PVD] 0), and 19 days (PVD 14) of age. On PVD 28, all pigs were orally challenged with 1×10^5 FFU of virulent Wa HRV and monitored for diarrhea and virus shedding for 7 days post-challenge.

b. Fecal consistency scores were used to assess diarrhea; scores are defined as 0: solid, 1: pasty, 2: semi-liquid, and 3: liquid. Scores of 2 or higher are considered diarrheic. Rotavirus shedding titers were determined by rotavirus antigen ELISA (detect viral antigen) and CCIF (determine the number of infectious viral particles). If there is no diarrhea or virus shedding, the mean days to onset were assigned as one day after the pigs were euthanized (8) for statistical analysis.

c. Numbers in parentheses represent the standard error of the mean (SEM).

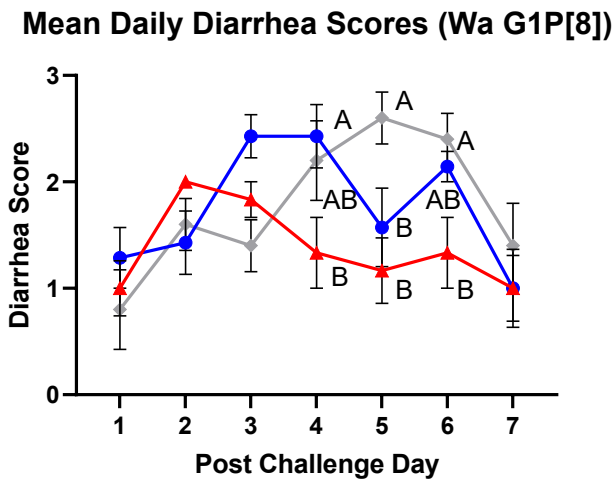
d. Different letters indicate significant differences between groups (n = 6-12; $p \leq 0.05$), while shared letters or no letters indicate no significant difference. e. Mean peak titer and AUC of virus shedding titers were log10 transformed for statistical analysis.

Table 2. Diarrhea and virus shedding in vaccinated and control pigs after challenge with virulent Arg HRV (G4P[6])

		Clinical Signs of Diarrhea [b]					Virus Shedding (CCIF) [b]				
Treatments (vaccine dose µg) [a]	n	Percentage with Diarrhea	Mean Days to Onset	Mean Duration Days	Mean Cumulative Fecal Score	AUC of Diarrhea	Percentage of Shedding Virus	Mean Days to Onset	Mean Duration Days	Mean Peak Titer (FFU/g of feces) [e]	AUC of Virus Shedding [e]
Rotarix 1x/Trivalent Nanoparticle 1x	6	100%	2	4.70	13.0	10.8	100%	1.50	3.33 (0.67) [B, c, d]	15554 (26842) [C]	78200 (41088) [B]
Trivalent Nanoparticle 2x	6	100%	2	5.00	14.2	12.3	100%	1.17	6.00 (0.37) [A]	40778 (15756) [B]	138767 (47474) [B]
Control	5	100%	1	5.20	14.6	12.8	100%	1.00	6.80 (0.20) [A]	107187 (36620) [A]	368600 (93322) [A]

Note: a. Pigs were immunized two times with S_{60} nanoparticle control, trivalent nanoparticle vaccine, or once with Rotarix followed by trivalent nanoparticle or control, at 5 (post-vaccination day [PVD] 0), and 19 days (PVD 14) of age. On PVD 28, all pigs were orally challenged with 1×10^5 FFU of virulent Arg HRV and monitored for diarrhea and virus shedding for 7 days post-challenge. b. Fecal consistency scores were used to assess diarrhea; scores are defined as 0: solid, 1: pasty, 2: semi-liquid, and 3: liquid. Scores of 2 or higher are considered diarrheic. Rotavirus shedding titers were determined by rotavirus antigen ELISA (detect viral antigen) and CCIF (determine the number of infectious viral particles). If there is no diarrhea or virus shedding, the mean days to onset were assigned as one day after the pigs were euthanized (8) for statistical analysis. c. Different letters indicate significant differences between groups (n = 6-10; $p \leq 0.05$), while shared letters or no letters indicate no significant difference. d. Numbers in parentheses represent the standard error of the mean (SEM). e. Mean peak titer and AUC of virus shedding titers were lgo10 transformed for statistical analysis.

A.



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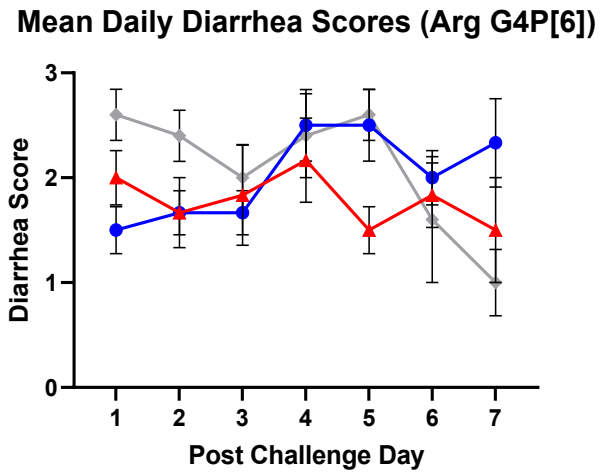
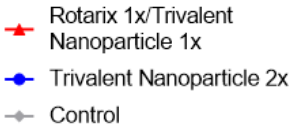
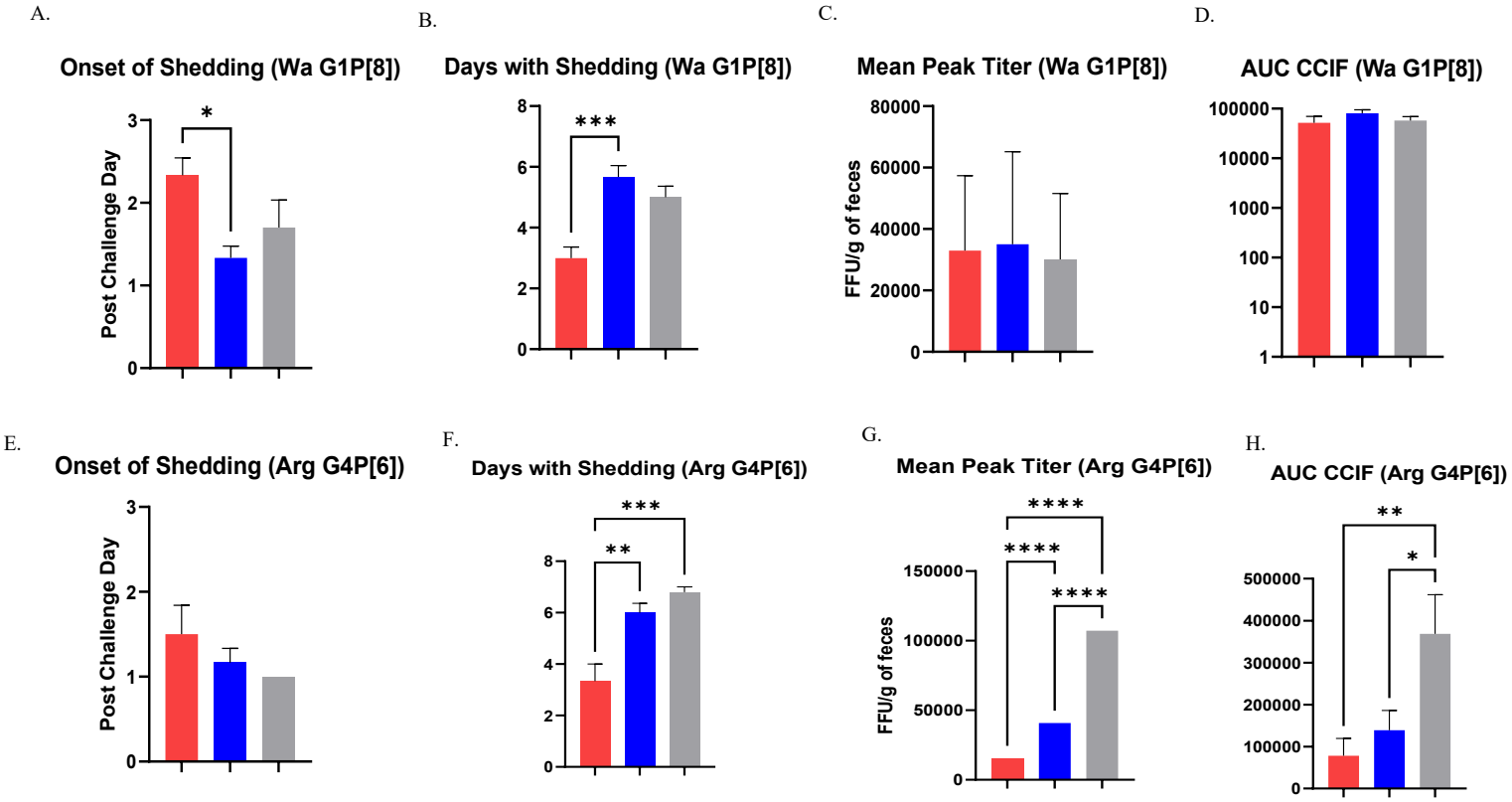


Figure 2. Daily mean diarrhea scores from PCD 0-7 in Wa (A) and Arg HRV-challenged (B) Gn pigs. At PVD 28, Gn pigs were orally challenged with 1 x 10^5 FFU of either Wa or Arg HRV and monitored for diarrhea severity and duration via daily rectal swabs for 7 days. Differing letters indicate significant difference (two-way ANOVA of repeated measures through time, followed by Tukey's multiple comparisons test; $p \leq 0.05$), and error bars indicate SEM.





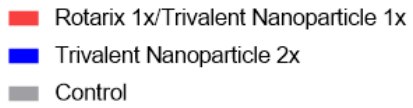


Figure 3. Protection from virus shedding as measured by CCIF in Wa- and Arg HRV-challenged Gn pigs. At PVD 28, pigs were orally challenged with 1 x 10^5 FFU of either Wa or Arg HRV and monitored for virus shedding via daily rectal swabs for 7 days. (**A, E**) Mean onset day of virus shedding in Wa or Arg HRV-challenged pigs; (**B, F**) Mean days with virus shedding in Wa or Arg HRV-challenged pigs; (**C, G**) Mean peak virus shedding titers in Wa or Arg HRV-challenged pigs; (**D, H**) Mean area under the curve of virus shedding in Wa or Arg HRV-challenged pigs. Asterisks indicate degree of statistically significant difference (ordinary one-way ANOVA; * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$). Error bars indicate SEM.

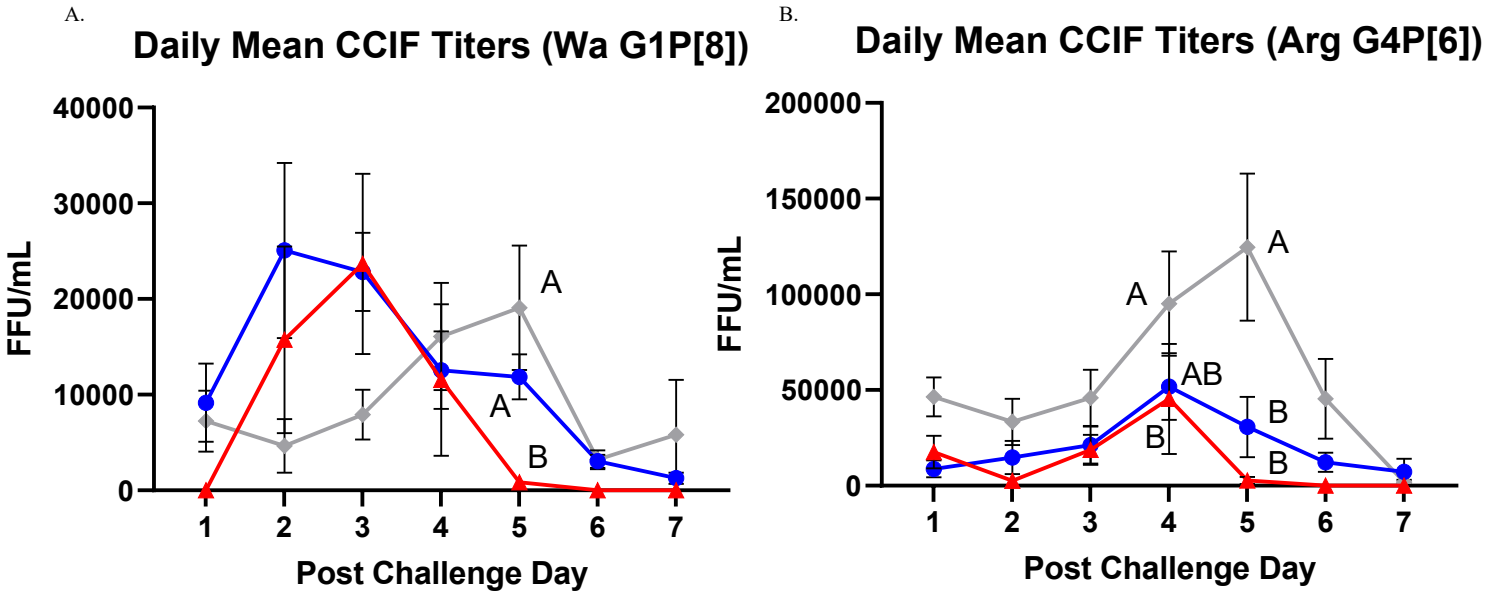


Figure 4. Daily fecal virus shedding as measured by CCIF in prime-boost, trivalent 2x and control-vaccinated pigs from PCDs 0-7. Daily rectal swabs were taken for evaluating fecal virus shedding after challenge with Wa HRV. Infectious virus particles were measured by CCIF and results are expressed as FFU/mL. Fecal samples from mock-infected pigs were used as negative controls. Different capital letters above data points indicate significant differences between groups at the same time point, while shared letters or no letters indicate no significant difference, according to two-way ANOVA of repeated measures through time, followed by Tukey's multiple comparisons test ($p \leq 0.05$) . Bars indicate SEM. CCIF, cell culture immunofluorescence; FFU, focus forming units.

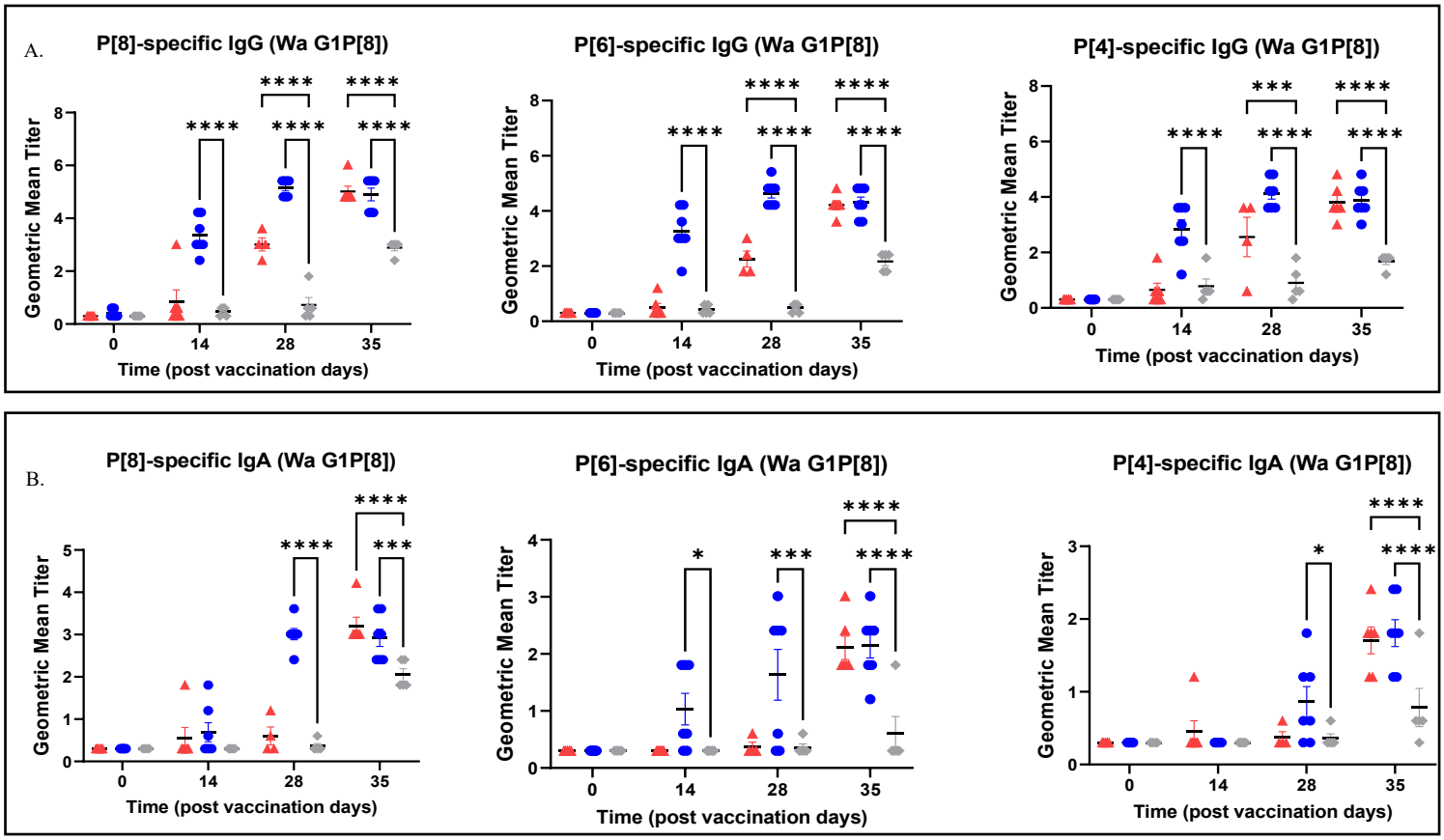


Figure 5. P[8], P[6], and P[4]-specific serum antibody responses in Wa HRV-challenged Gn pigs. Serum was collected at PVD 0, 14, 28 (PCD 0), and 35 (PCD 7) to evaluate systemic P-type-specific IgG and IgA responses using direct ELISA. Asterisks indicate degree of statistically significant difference (mixed-effects analysis with Dunnett's multiple comparisons test; * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$). Lines and error bars indicate mean and SEM, respectively.

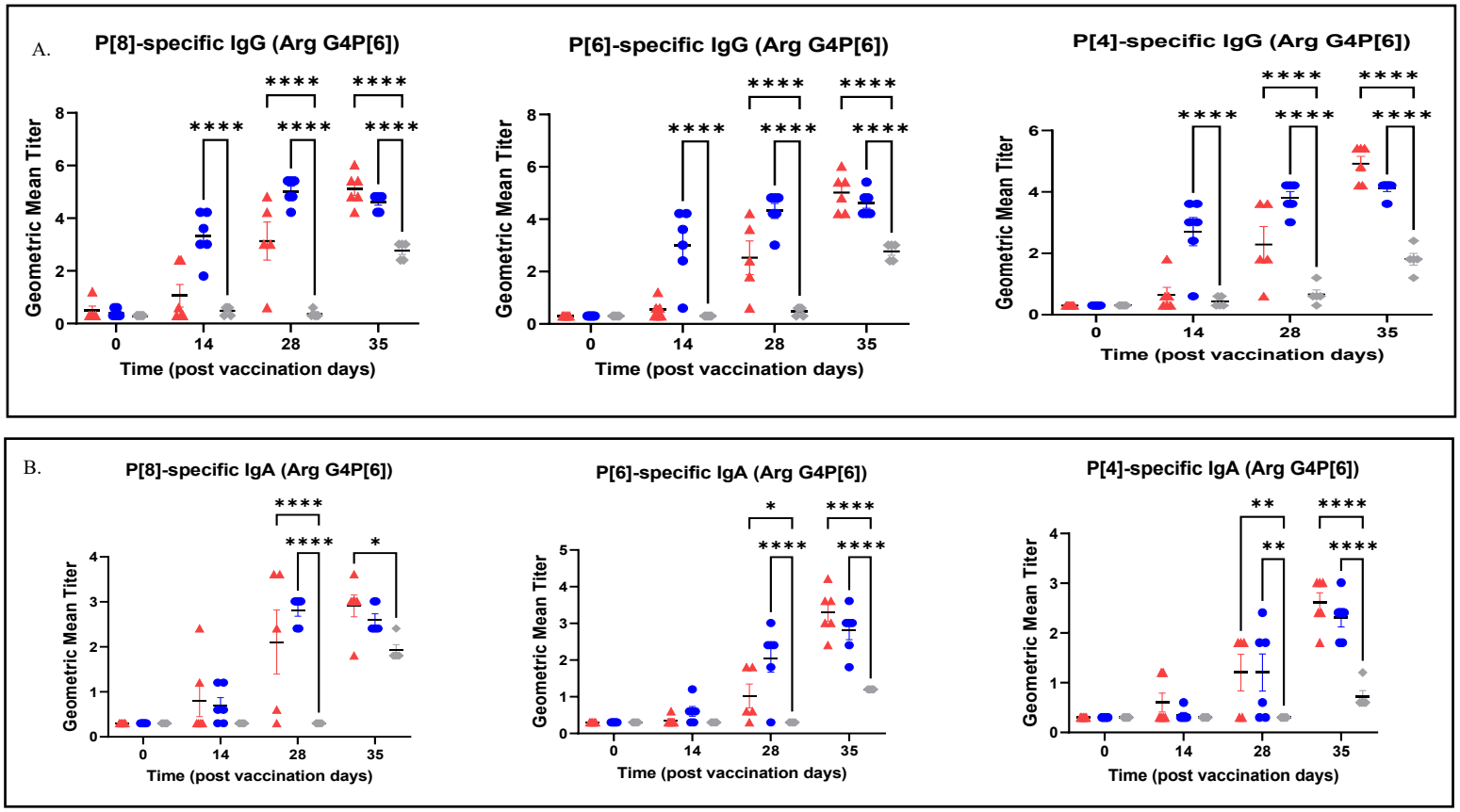


Figure 6. P[8], P[6], and P[4]-specific serum antibody responses in Arg HRV-challenged Gn pigs. Serum was collected at PVD 0, 14, 28 (PCD 0), and 35 (PCD 7) to evaluate systemic P-type-specific IgG and IgA responses using direct ELISA. Asterisks indicate degree of statistically significant difference (mixed-effects analysis with Dunnett's multiple comparisons test; * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$). Lines and error bars indicate mean and SEM, respectively.

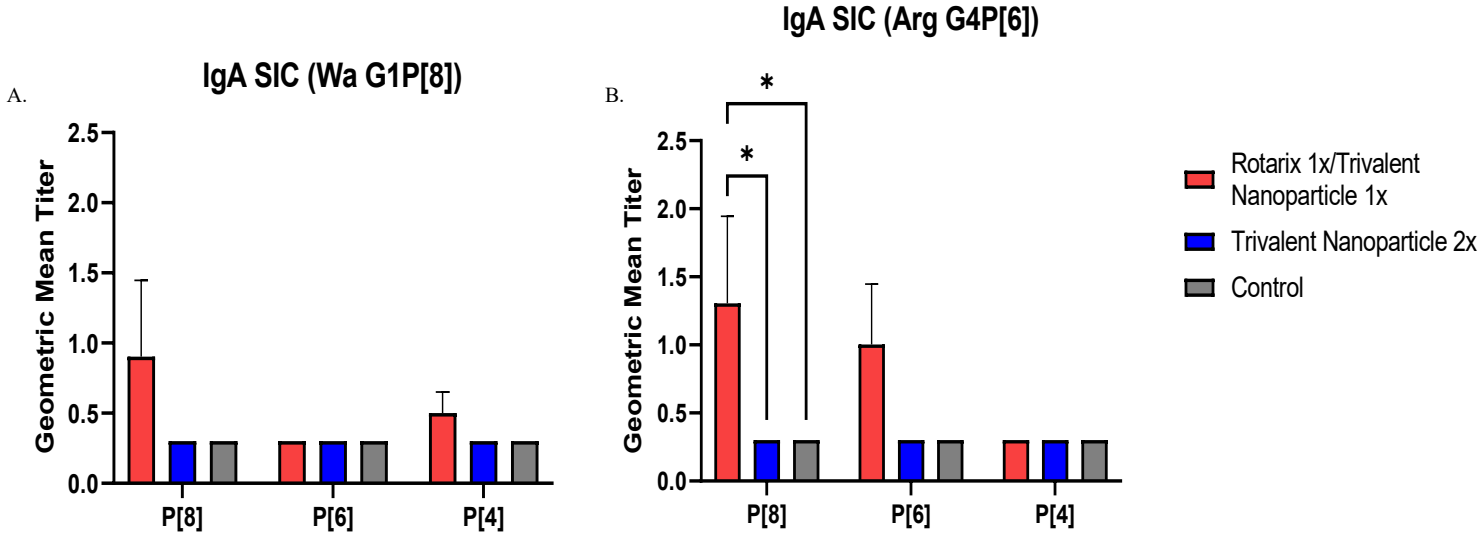
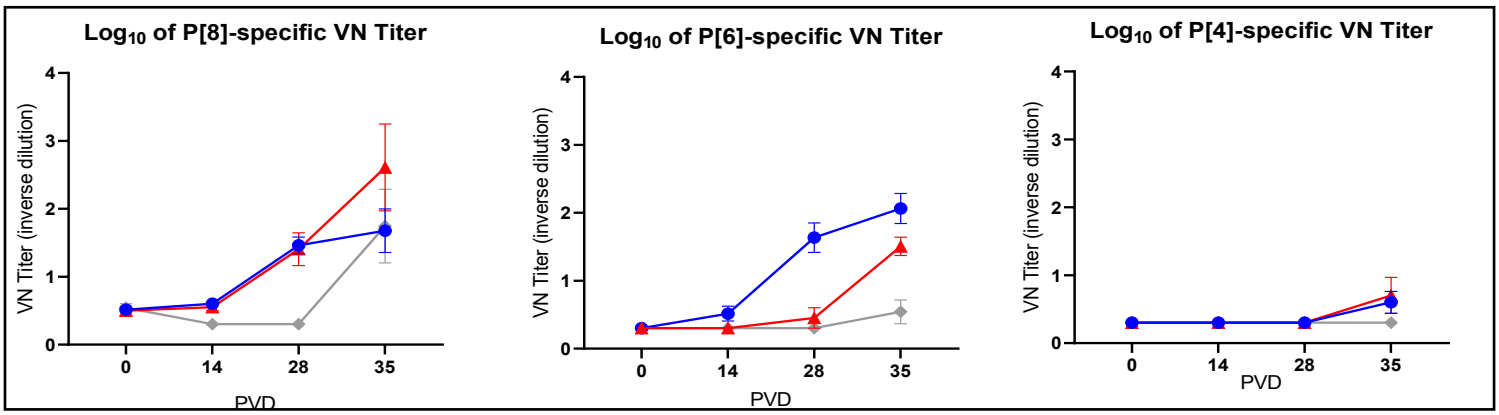


Figure 7. Intestinal IgA antibody titers in vaccinated and control Gn pigs challenged with either Wa (A) or Arg HRV (B). At PVD 35/PCD 7, Gn pigs were euthanized and small intestinal contents were collected and evaluated using direct ELISA to detect P-type specific IgA antibody responses. Asterisks indicate degree of statistically significant difference (mixed-effects analysis with Tukey's multiple comparisons test; * $P \leq 0.05$). Error bars indicate SEM.

A. Wa G1P[8] HRV challenge



B. Arg G4P[6] HRV Challenge

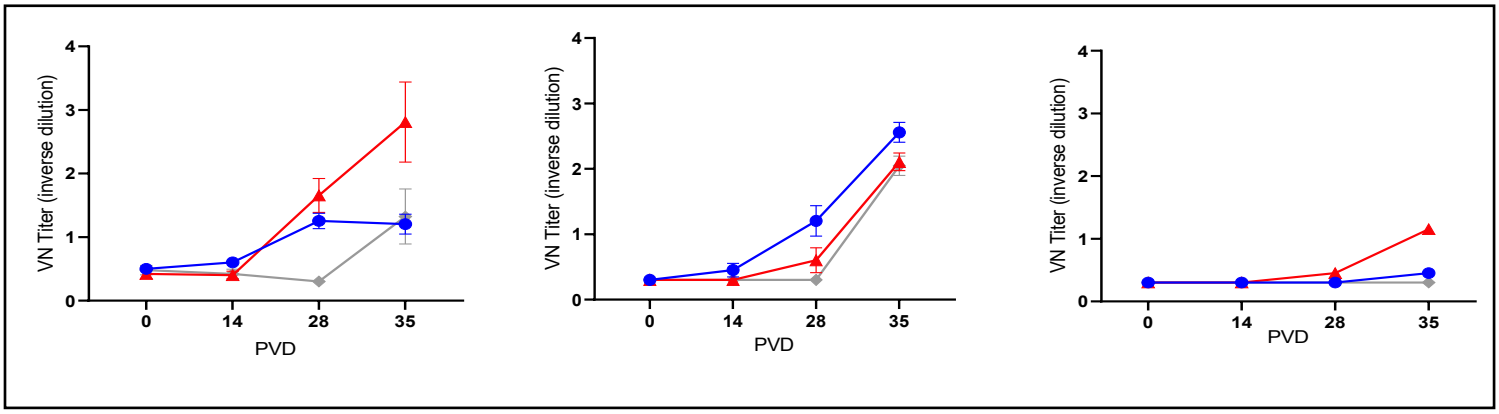
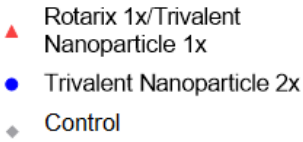


Figure 8. Serum virus neutralization of P[8], P[6], and P[4] HRV in Wa HRV (A) or Arg HRV-challenged Gn pigs (B). Serum was collected at PVD 0, 14, 28 (PCD 0), and 35 (PCD 7) and evaluated for neutralizing antibody activity. Differing letters indicate significant difference (mixed-effects analysis using two-stage linear step-up procedure, $p \leq 0.05$), and error bars indicate SEM.



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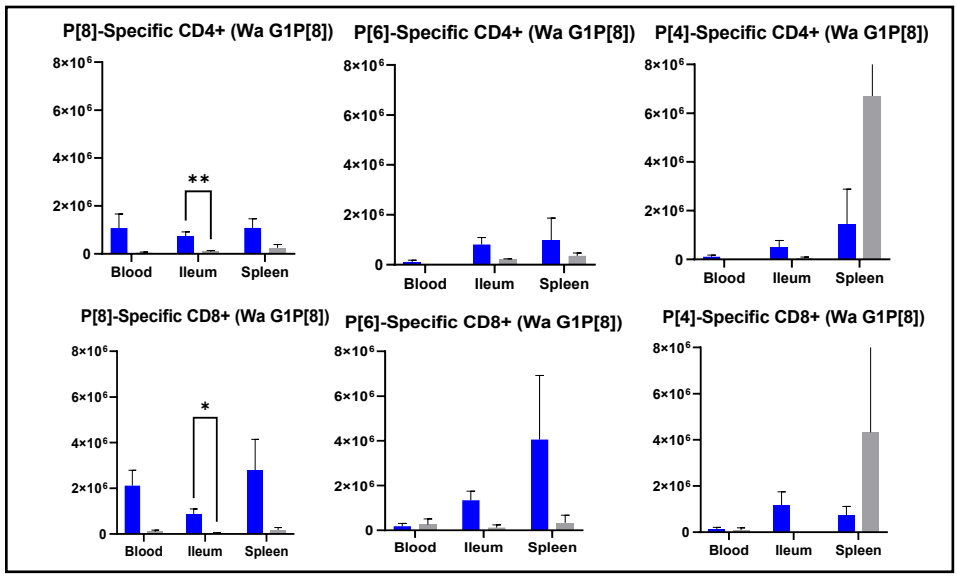

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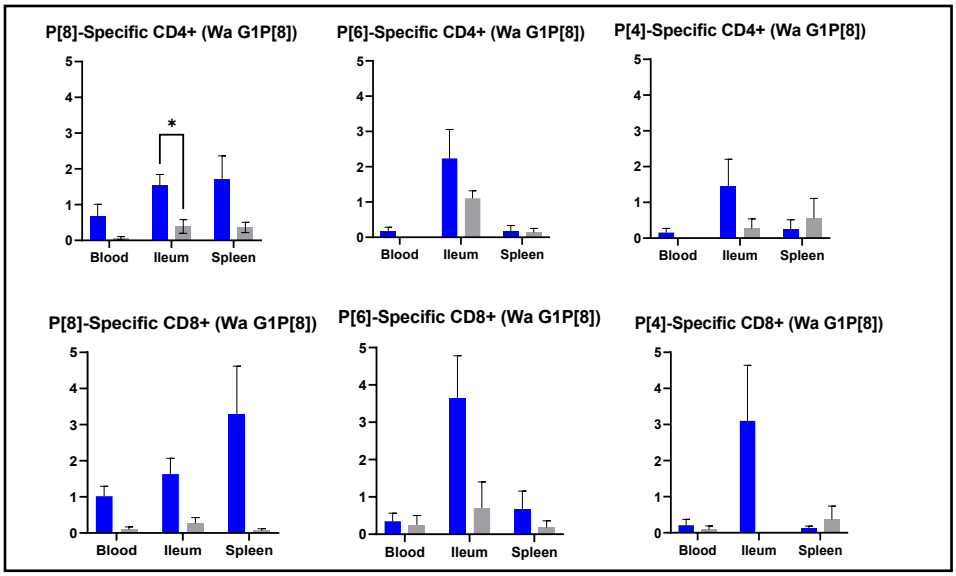
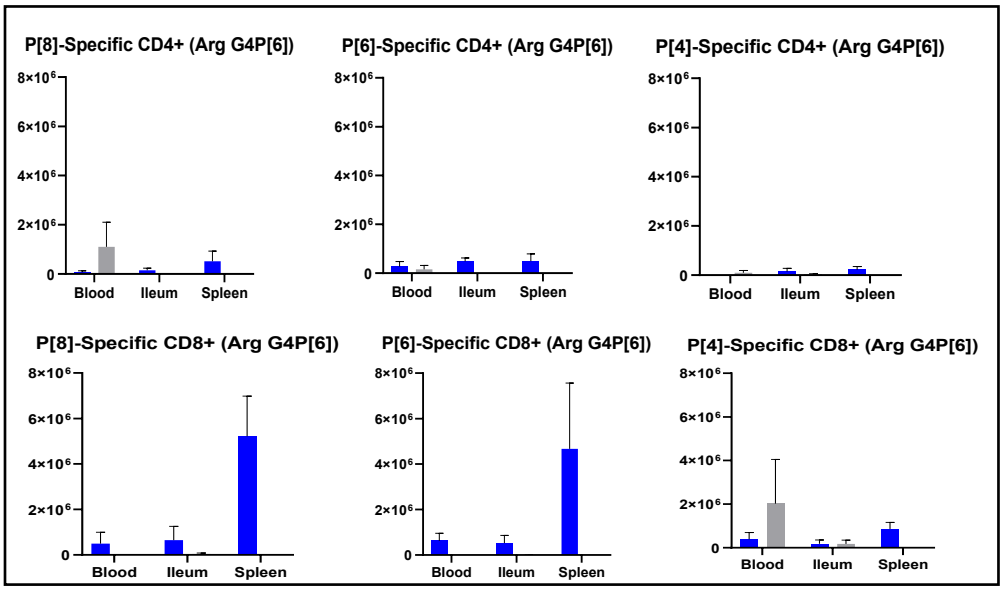


Figure 9. Total numbers (A) and frequencies (B) of P[8], P[6] and P[4]-specific IFN-γ-producing CD3+CD4+ and CD3+CD8+ T cells in blood, ileum and spleen of Wa HRV-challenged Gn pigs at PCD 7 detected by flow cytometry. MNCs were stimulated in vitro for 17h with P[8], P[6] and P[4] S60-VP8* antigen, or mock stimulated. Asterisks indicate degree of statistically significant difference (multiple Mann-Whitney tests; * $P \leq 0.05$, ** $P \leq 0.01$). Error bars indicate SEM.

Trivalent Nanoparticle 2x
Control

A.



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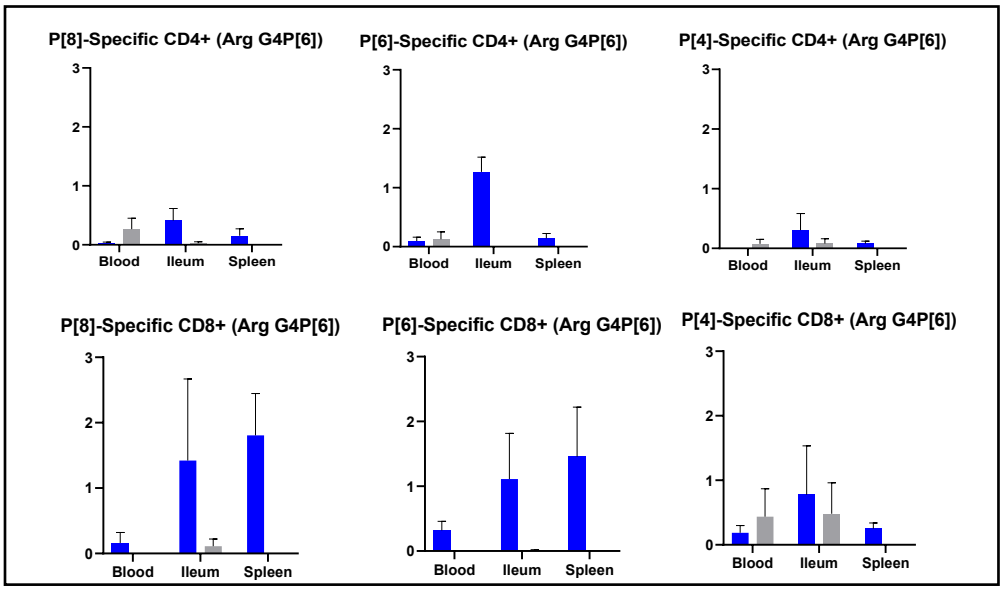

Trivalent Nanoparticle 2x
Control

Figure 10. Total numbers (A) and frequencies (B) of P[8], P[6] and P[4] type-specific IFN-γ-producing CD3+CD4+ and CD3+CD8+ T cells in blood, ileum and spleen of Arg HRV challenged Gn pigs at PCD 7 detected by flow cytometry. MNCs were stimulated in vitro for 17h with P[8], P[6] and P[4] S60-VP8* antigen, or mock stimulated. Asterisks indicate degree of statistically significant difference (multiple Mann-Whitney tests; * $P \leq 0.05$,). Error bars indicate SEM.

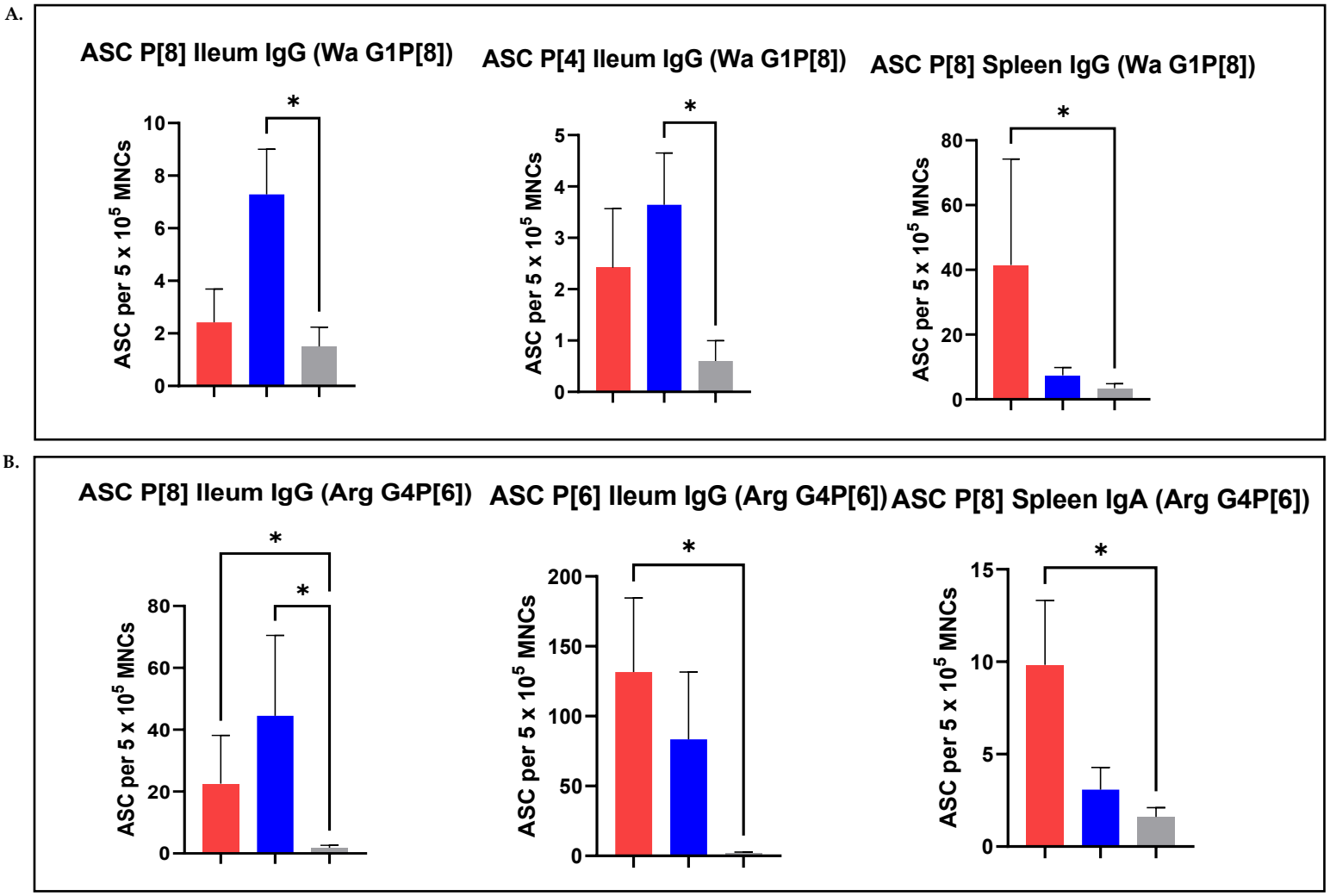

Figure 11. P-type specific antibody-secreting cell (ASC) responses in the ileum and spleen of Wa (A) or Arg (B) HRV-challenged Gn pigs at PCD 7. Asterisks indicate degree of statistically significant difference (Kruskal-Wallis test with Dunn's multiple comparisons test; * $P \leq 0.05$). Error bars indicate SEM. Only ASC responses with significant differences among the groups are presented.

Chapter 5

General Discussion and Future Directions

Casey Hensley

Despite the World Health Organization's recommendation for inclusion of HRV vaccines in national immunization programs, HRV continues to cause high morbidity and mortality in children under the age of five worldwide, mostly in LMICs (Carcamo-Calvo, Munoz et al. 2021). Variable vaccine efficacy, supply chain logistics, and financial constraints in these areas play a large role in the failure to prevent HRV infections in LMICs' vulnerable populations (Carcamo-Calvo, Munoz et al. 2021). Still, vaccination remains the best tool for preventing and/or reducing the severity of HRV disease in children.

This dissertation aimed to address the aforementioned barriers to lessening the burden of HRV in LMICs. First, a thermostable, dissolvable film vaccine was evaluated for its protective efficacy and immunogenicity in the Gn pig model of G1P[8] HRV infection and diarrhea. Currently used live oral vaccines require reliable and consistent cold-chain storage. Without continuous low temperature storage, these vaccines may lose potency over time, resulting in decreased replication in the gut of recipients, and ultimately less robust immune responses. The need for consistent low temperature is a major financial concern in regard to distribution of vaccines in LMICs, even in those funded through GAVI (Pecenka, Debellut et al. 2018). Not only is maintaining cold-chain expensive, but its failure can result in massive wasting of vaccine products. Our vaccine was developed by collaborators at Universal Stabilization Technologies, using their state-of-the-art preservation-by-vaporization technology. This allowed the conversion of a cost-effective HRV vaccine strain from a liquid formulation into a dissolvable oral film that can be administered easily on the buccal mucosa and is proven stable in temperatures of up to 37°C for 3 months (Hensley, Zhou et al. 2021). Two doses of this vaccine in the Gn pig model significantly reduced diarrhea and fecal virus shedding in film-vaccinated pigs as compared to pigs vaccinated with the liquid formulation (Hensley, Zhou et al. 2021). The vaccine also

induced strong IgG, IgA, and VN titers in the serum, and perhaps most importantly, intestinal IgA (Hensley, Zhou et al. 2021). The thermostable film potentially solves a number of issues associated with the decreased efficacy. Along with eliminating the enormous cost of cold-chain transport and storage needed for the current vaccines, this vaccine is comprised of vaccine strains derived from human-rhesus reassortants that have been proven cost-effective and safe, including from risk of intussusception, a potentially fatal bowel pathology that has been previously associated with live oral HRV vaccines (Bines 2005, Armah, Kapikian et al. 2013). In the future, improving the plasticity of the film to ease administration is the first step to advancing this vaccine candidate.

The second aim of this dissertation was to address the need for next-generation HRV vaccines, particularly for use in LMICs. To do so, the immunogenicity and protective efficacy of two parenteral vaccines, one mRNA-based and one nanoparticle-based, were evaluated using the Gn pig model of G1P[8] and G1P[8] in parallel with the G4P[6] HRV infection and diarrhea model, respectively. Currently used live oral vaccines are highly effective (>90%) at preventing moderate to severe disease in high-income countries; however, efficacy can be as low as less than 40% in LMICs (Carcamo-Calvo, Munoz et al. 2021). The reason for this is likely multifactorial, and includes chronic malnutrition, gut dysbiosis, circulating maternal antibodies, concomitant enteric viral infection, and simultaneous use of other live oral vaccines (Carcamo-Calvo, Munoz et al. 2021). Because these issues are mainly confined to the gastrointestinal (GI) tract, usage of live oral vaccines for HRV, which work mainly through the mounting of protective immune responses in the GI tract, has several drawbacks. This circumstance makes parenteral routes of administration appealing for these populations. Research focusing on mRNA therapeutics is not new; however, its potential as a tool against infectious diseases has been

highlighted significantly since the first Food and Drug Administration licensure of two mRNA vaccines for COVID-19 in the last three years (Schlake, Thess et al. 2012). Our first parenteral vaccine study evaluated three mRNA-based vaccine candidates generated by German biotech company, CureVac. These vaccines encode for a universal CD4+ T cell epitope derived from tetanus toxin, fused with three different VP8* proteins (from P[4], P[6] and P[8] HRV) with the additional inclusion of LS fusion in two of the three candidates (Roier et al., unpublished data). Roier et al. demonstrated the high immunogenicity of these vaccines in mice and guinea pigs, and these data were mirrored by our study in Gn pig model of G1P[8] HRV infection and diarrhea (Roier et al., unpublished data). These responses were also associated with significant protection from virus shedding, but only slight protection from diarrhea. The mRNA vaccines also failed to induce neutralizing antibodies. With the exception of the inclusion of the LS fusion in two of the three candidates, all candidates used in this study were equivalent to the P2-VP8* subunit vaccine developed by NIH/PATH (Roier et al., unpublished data). The P2-VP8* subunit vaccine has been the most advanced parenteral HRV vaccine, proceeding to phase III trials in humans (Groome, Fairlie et al. 2020). It was shown to be highly immunogenic in rodent and Gn pig models, conferring significant protection from disease in the latter challenge model (Kovacs-Nolan and Mine 2006, Wen, Wen et al. 2014, Wen, Cao et al. 2015, Lakatos, McAdams et al. 2020). Unfortunately, human clinical trials did not show protection to be superior to currently licensed live oral vaccines, effectively terminating the trial (Chen, Grow et al. 2022). This, coupled with the lack of protection from clinical signs in Gn pigs by the mRNA vaccines, suggests that more work needs to be done in developing subunit and mRNA-based vaccines for HRV, especially in terms of induction of mucosal responses in the intestine. CureVac has recently announced their change of focus from unmodified nucleotides in their mRNA products

to the use of modified nucleotides. Reformulating this vaccine with their updated technology is one potential next step in improving the protection conferred by the mRNA HRV vaccine candidates.

The second parenteral vaccine evaluated in this dissertation was a nanoparticle-based vaccine developed by collaborators at the Cincinnati Children's Hospital Medical Center. This vaccine utilizes the domain of human norovirus (NoV) capsid protein (VP1) as an antigen display platform. The S domain is easily and cost-effectively expressed in *Escherichia coli*. It self-assembles into a 60-valent nanoparticle with exposed C-termini, which permit insertion of foreign proteins (Xia, Huang et al. 2018). In this case, VP8* from P[4], P[6] and P[8] HRV were fused to the nanoparticle and mixed to create a cocktail vaccine. We used this vaccine as both a two-dose IM administration regimen or as an IM booster preceded by an oral priming dose of the commercially available monovalent Rotarix®. Both regimens were highly immunogenic in the Gn pig models of both G1P[8] and G4P[6] HRV infection and diarrhea, inducing cross-protective antibody responses in both the serum and intestine, with the prime-boost regimen outperforming the IM-only regimen in terms of protection from disease. These data confirm the promise of prime-boost regimens for HRV vaccines, which have performed better than parenteral-only regimens in past studies in Gn pigs (Gonzalez, Nguyen et al. 2004, Yuan, Azevedo et al. 2005, Azevedo, Gonzalez et al. 2010). The lack of protection from diarrhea in this study indicated immune responses were not adequate. One future direction to be explored is the inclusion of the entire VP4 protein, instead of just VP8*. Though VP8* has been historically hailed as the major target for neutralizing antibody responses, VP5*'s role in the molecular mechanisms required for virus entry into the host cell are well documented, and likely induce more protective immune responses (Zarate, Espinosa et al. 2000, Zarate, Cuadras et al. 2003).

In summary, the knowledge generated from the studies within this work demonstrate the endless possibilities and the difficulties for developing next-generation HRV vaccines. With new technologies being validated routinely, and more specialized animal models such as the Gn pig becoming more refined through tools like genetic manipulation, the search for a reliably efficacious HRV vaccine is quite attainable. From a public health perspective, it is absolutely essential to simultaneously address the social determinants of health that impact HRV vaccine efficacy as well, as this will likely have a major impact on the usefulness of current and future HRV vaccines.

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