Study of enteric virus infection and parenteral vaccines in the gnotobiotic pig model

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Academic Abstract

Human rotavirus (HRV) and human norovirus (HuNoV) are the most common causative agents of acute gastroenteritis- (AGE) related morbidity and mortality around the world. Gnotobiotic (Gn) pigs are the ideal large-animal model that allows for accurate, and precise, preclinical evaluation of vaccine efficacy. Similarities in gastrointestinal anatomy, physiology, and immune system allows for direct translation of results from Gn pigs to humans. Commercially available HRV vaccines perform significantly poorer in low- and middle- income countries as compared with developed countries. Non-replicating rotavirus vaccines (NRRVs) have been proposed as a viable solution to the problems facing currently available live-, attenuated oral vaccines and evaluation of a NRRV was the first research project in this dissertation. Three doses of a novel parenterally administered nanoparticle-based RV vaccine, P24-VP8*, adjuvanted with $Al(OH)_3$ adjuvant, was able to prime VP8*-specific mucosal and systemic T cell responses (IFN-γ producing CD4+ and CD8+ T cells), and to induce strong systemic B cell responses (IgA, IgG and serum neutralizing antibodies). A significant reduction in the mean diarrhea duration, fecal virus shedding titers, and significantly lower fecal cumulative consistency scores was observed among vaccinated pigs demonstrating the efficacy of the vaccine against RV infection and diarrhea.

Next, we determined the median infectious dose (ID_{50}) and median diarrhea dose (DD_{50}) of the GII.4/2003 Cin-1 variant of HuNoV in Gn pigs to better standardize the pig model for HuNoV vaccine evaluation. Gn pigs were inoculated with 7 different doses of Cin-1 at 33-34 days of age. Pigs were monitored daily from post-inoculation day (PID) 1 to 7, for fecal virus shedding and fecal consistency to evaluate the virus infectiousness and associated diarrhea. The Log_{10} ID_{50} and DD_{50} were determined based on various mathematical models to be between 3.11 to 3.76, and 3.37 to 4.87 RNA copies, respectively. The Beta-Poisson was identified to be the best-fitting statistical model for estimating both the ID_{50} and DD_{50} of Cin-1. Determining the ID_{50} of the challenge virus strain is crucial for identifying the true infectiousness of HuNoVs and for accurate evaluation of protective efficacies in pre-clinical studies of therapeutics, vaccines and other prophylactics using this reliable animal model.

The lack of an easily reproducible cell culture model for HuNoV has significantly delayed the development of effective vaccines. There is still no HuNoV vaccine available. Currently, the vaccine development efforts are mostly based on genetically engineered virus-like particles (VLPs) comprised of the major HuNoV capsid protein VP1. We tested the immunogenicity of a novel tetravalent VLP vaccine containing 4 major HuNoV genotypes (GI.1, GII.3, GII.4 and GII.17) using Gn pigs and evaluated its protective efficacy when challenged with GII.4 Cin-1 HuNoV. Three doses of the VLP vaccine with $Al(OH)_3$ adjuvant administered to Gn pigs intramuscularly (IM), induced high levels of VLP-specific serum IgA and IgG antibody and hemagglutination inhibition antibody responses in the vaccinated pigs. VLP-specific IFN-γ producing CD4+ and CD8+ T cells were also elevated among vaccinated pigs at post-challenge day (PCD) 7 in the spleen and blood, but not in the ileum. However, the vaccinated pigs were not protected from infection and diarrhea when challenged with any one of the three different doses

(2 x 10^5, 8 x 10^4, and 2 x 10^4 genome RNA copies) of Cin-1 HuNoV. These results indicated that the IM tetravalent VLP vaccine was highly immunogenic, but the presence of high levels of immune effectors induced by the vaccine were not sufficient for protecting the Gn pigs from Cin-1 challenge. Amino acid (aa) sequence analysis showed that the GII.4 Sydney 2012 strain which was included in the VLP vaccine, had 23 aa substitutions in the major receptor binding domain (P2) compared to the Cin-1, a GII.4 Farmington Hills 2002 strain. Our findings, for the first time, provided *in vivo* experimental evidence for the total lack of cross-genogroup, cross-genotype and cross-variant protection among HuNoV. This finding has importance implications for HuNoV vaccine development. HuNoV vaccines have to include multiple variants and have to be routinely updated in order to ensure sustained protection among the population.

Together these three studies in this dissertation demonstrate the versatility of Gn pigs as a reliable large animal model for studying the pathogenesis and immunity of enteric viruses and the evaluation of immunogenicity and protective efficacy of novel enteric viral vaccines.

Study of enteric virus infection and parenteral vaccines in the gnotobiotic pig model

Ashwin Kumar Ramesh

General Audience Abstract

People of all age groups are susceptible to acute gastroenteritis (AGE), a condition characterized by sudden onset of diarrhea, nausea and abdominal cramps. The two most important viral pathogens responsible for causing AGE are rotavirus (RV) and norovirus (NoV). Gnotobiotic (Gn) pigs have been valuable in helping us understand the mechanism of infection, pathogenesis, immunity and have played a key role in the expediting development of novel vaccines and therapeutics against both of these viruses. Live oral RV vaccines are available but they are not very effective in low income countries where the vaccines are needed the most. Next generation parenteral vaccines are proposed to improve the RV vaccine efficacy. Our first study showed that a nanoparticle-based intramuscular (IM) RV vaccine effectively reduced the duration and severity of human RV infection and diarrhea in Gn pigs. Secondly, we examined in detail the infectivity of HuNoV and identified accurately using different mathematical models on how much virus would be required to infect and cause diarrhea in naïve Gn pigs. This knowledge would greatly help in the accurate assessment of the efficacy of NoV vaccines. Third, we evaluated the immunogenicity and protective efficacy of a tetravalent IM NoV vaccine in Gn pigs. Although the vaccine was highly immunogenic, it did not confer any protection against infection and diarrhea upon challenge with the NoV at different doses. NoVs are so diverse that one year we might be infected with one strain and a few years later, we might be infected again with another strain, even though they

belong to the same genotype, and experience the same symptoms. This is because, changes brought about due to mutation in the virus capsid protein allow the viruses to hide from neutralizing antibodies induced by previous infection or vaccination as we have revealed in this study. NoV diversity and lack of cross protection need to be taken into consideration during vaccine development. This thesis shows how Gn pigs can be used to study these components in order to further maximize our ability to understand and combat enteric viral diseases.

Dedicated to my parents

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List of Abbreviations

Abbreviations	Definitions
3PLL	three-parameter log logistic
AGE	Acute gastroenteritis
AIC	Akiake Inference Criterion
$Al(OH)_3$	Aluminum hydroxide
ANOVA	analysis of variance
ASC	antibody secreting cells
AttHRV	attenuated HRV
AUC	area under curve
CCIF	cell culture immunofluorescence
Cin-1	HuNoV GII.4/2003 Cin-1 virus
DAPI	4,6-diamidino-2-phenylindole
DB	Dragstedt-Behrens
DD50	median diarrhea dose
dsRNA	double-stranded RNA
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
FFU	fluorescent focus-forming unit
GE	Gastroenteritis
GLM	general linear model
Gn	gnotobiotic
HAI	Hemagglutination inhibition
HBGA	histo-blood group antigen
HIE	human intestinal enteroid
HuNoV	human rotavirus
IACUC	Institutional Animal Care and Use Committee
ID50	median infectious dose
IFN-γ	Interferon gamma
Ig	immunoglobulin
IM	intramuscular
LIC	large intestinal contents
LIPS	Luciferase Immunoprecipitation Systems
LMIC	low- and middle-income countries
MEM	minimum essential media
MNCs	mononuclear cells

MNV	murine norovirus
MPL	monoposphoryl lipoprotein
mRNA	messenger RNA
NoV	norovirus
NRRV	nonreplicating rotavirus vaccine
NSP	non-structural protein
ORF	open reading frame
PCD	post-challenge day
PCV	Porcine circovirus
PD	proportionate distance
PID	post-inoculation day
PPD	post-partum day
PTA	phosphotungstate
RdRp	RNA dependent RNA polymerase
RM	Reed-Muench
RT-qPCR	Quantitative reverse transcription PCR
RV	rotavirus
SCID	severe combined immunodeficiency
sf-EMEM	serum free EMEM
SIC	small intestinal contents
SK	Spearman-Karber
VirHRV	virulent HRV
VLP	virus like particles
VN	virus-neutralizing
VP	viral protein

Chapter 1

Literature Review

Current state of vaccine development for rotavirus and norovirus infection and disease

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i. Chapter 11. Rotavirus. In *Handbook of Foodborne Diseases*, Food Microbiology Series, Edited by Donyou Liu. CRC Press/Taylor & Francis Group. p121-136. (2018).

ii. Chapter 22. Molecular Mechanisms of Host Immune Responses to Rotavirus. In *Molecular Food Microbiology*, Edited by Donyou Liu. CRC Press/Taylor & Francis Group (in press).

1. Acute Gastroenteritis

Acute gastroenteritis (AGE) is a condition that is often characterized by a sudden onset of diarrhea, nausea and abdominal cramps, that affects people of all age groups. Diarrhea is the second leading cause of death among children under 5 years of age, with an estimated annual global mortality rate of 526,000 in 2015 (Keusch, Walker *et al*. 2016). Limited access to safe and clean drinking water, basic sanitation, poor hygiene practices, and lack of medical care are the main contributing factors to AGE in low-income countries (GBD 2016). The most common etiological agents responsible for AGE-related morbidity and mortality around the world are norovirus (NoV), rotavirus (RV) and enteropathogenic *Escherichia coli* (Lanata, Fischer-Walker *et al*. 2013). Diarrheal disease caused by rotavirus primarily affects children <5 years old, but NoV AGE affects people of all ages (Monroe 2011). Together both these viruses demand a unified interest among researchers in the field to understand the pathogenesis and to develop effective prophylactic and therapeutic approaches against these preventable diseases. This review will focus on the key aspects of RV and NoV infection in humans, briefly outline current research efforts focused on understanding the pathogenesis and immunity, and the efforts in vaccine development to reduce the disease burdens in the world. This work also introduces the gnotobiotic (Gn) pig model system and highlights their value to enteric viral research.

2. Rotavirus

Rotaviruses belong to the genus *Rotavirus* within the family *Reoviridae*, which are characterized by a segmented, double-stranded RNA (dsRNA) genome encapsulated within a non-enveloped, triple-layered icosahedral capsid. RV is the leading cause of severe, dehydrating diarrhea among children under 5 years of age globally (Tate, Burton *et al*. 2016). The RV genome consists of 11 segments that encode for six viral structural proteins (VP1 to VP4, VP6 and VP7) and six nonstructural proteins (NSP1 to NSP6). Each genome segment encodes a single protein (monocistronic), except for segment 11 which encodes two proteins (NSP5 and NSP6) (Mattion, Mitchell *et al*. 1991).

2.1 Virus Structure

Three concentric layers of protein, an inner core layer, a middle layer, and an outer layer form the icosahedral capsid of the viral particle (McClain, Settembre *et al*. 2010, Green 2013). The core of the virion is made up of 120 molecules of VP2 arranged as 60 dimers that enclose the dsRNA genome together with the viral RNA dependent RNA polymerase (RdRp) (VP1) (Valenzuela, Pizarro *et al*. 1991, Zeng, Wentz *et al*. 1996) and the mRNA capping enzyme (VP3) (Chen, Luongo *et al*. 1999). The middle layer is formed by 260 trimers of VP6 protein. The inner core and the middle layer form double-layered particles (DLP). The outer surface of the RV virion is composed of 260 trimers of VP7 glycoprotein and 60 VP4 spike proteins also presented as trimers. The spike proteins are divided into four regions (head, body, stalk and foot) which are formed by two VP4 trypsin cleavage fragments VP5* and VP8*.

2.2 RV-host cell binding and entry

The main capsid proteins of rotavirus are VP4 and VP7, with VP7 comprising the outer shell of the capsid and VP4 forming the spike protein (Greenberg and Estes 2009). As such, VP4 is primarily responsible for rotavirus attaching to enterocytes. It is enzymatically cleaved by trypsin, found in the host gastrointestinal tract, into VP5* and VP8*, with VP5* forming the main stem and VP8* forming the head of the spike that directly encounters receptors on small intestinal cells (Liu, Huang *et al*. 2012, Desselberger 2014).

Initially, sialic acid moieties were thought to be involved in rotavirus binding (Ciarlet, Ludert *et al*. 2002). Recent multidisciplinary studies involving structural biology and glycobiology, have revealed that rotavirus utilizes histo-blood group antigens (HBGAs) as cellular receptors (Hu, Crawford *et al*. 2012, Huang, Xia *et al*. 2012, Liu, Huang *et al*. 2012, Böhm, Fleming *et al*. 2015, Ma, Li *et al*. 2015, Ramani, Hu *et al*. 2016). These glycan ligands have previously been determined to be responsible for the cellular binding of another enteric virus, norovirus (Marionneau, Ruvoën *et al*. 2002, Huang, Farkas *et al*. 2003). HBGAs are carbohydrates found on RBCs and epithelial surfaces throughout the body. In certain individuals, known as secretors, they are also found in biologic fluids including blood, milk, saliva and intestinal contents

(Huang, Xia *et al*. 2012, Ma, Li *et al*. 2015). *In vitro* assays using human saliva, milk and synthetic oligosaccharides have thus demonstrated that VP8* binds to HBGAs (Huang, Xia *et al*. 2012). Several HBGA phenotypes exist based on varied expression of ABO, Lewis and secretor genes; current knowledge indicates rotavirus strain-specific binding to A, H type 1 and/or Lewis^{b} antigens (Hu, Crawford *et al*. 2012, Huang, Xia *et al*. 2012). Most studies have also determined increased susceptibility to symptomatic infection in secretor-positive individuals (Imbert-Marcille, Barbé *et al*. 2014, Nordgren, Sharma *et al*. 2014, Van Trang, Vu *et al*. 2014, Zhang, Long *et al*. 2016), though conflicting evidence of infection in non-secretors does exist (Ayouni, Sdiri-Loulizi *et al*. 2015). Since the expression of HBGA is determined genetically and regulated developmentally, variations in glycan binding between different RV strains have significant impacts on multiple aspects of RV pathogenesis, including interspecies transmission, host range restriction, and tissue tropism, as well as on the susceptibility of the host to different RV strains and vaccine viruses (Ramani, Hu *et al*. 2016).

HBGAs are not the only glycans that have been demonstrated to interact with rotavirus. VP8* also interacts with gangliosides in a strain-dependent manner (Coulson 2015). As stated previously, some animal strains do bind sialic acid as confirmed by their sensitivity to sialidases. These strains are able to bind the sialic acid found as a terminal moiety on the ganglioside GM3 (Haselhorst, Fiebig *et al*. 2011, Yu, Coulson *et al*. 2011) and as terminal and internal moieties on ganglioside GD1a (Haselhorst, Fleming *et al*. 2009). In contrast, sialidase-insensitive rotaviruses, which include most human strains, appear to interact with the ganglioside GM1a that lacks a terminal sialic acid (Haselhorst, Fleming *et al*. 2009). Of note, GM1a contains an internal sialic acid moiety that can interact with VP8* and it is not affected by sialidases.

In addition to VP8*, VP5* and VP7 have also been implicated for their role in cellular binding. VP5* has been demonstrated to bind integrins $\alpha 2\beta 1$, $\alpha 4\beta 1$ and $\alpha 4\beta 7$ (Coulson, Londrigan *et al*. 1997, Graham, Halasz *et al*. 2003, Graham, Fleming *et al*. 2005), while VP7 binds integrins $\alpha x\beta 2$, $\alpha v\beta 3$ and $\alpha 4\beta 1$ found on surface of epithelial cells (Coulson, Londrigan *et al*. 1997, Graham, Fleming *et al*. 2005). Heat shock

cognate protein hsc70 (Guerrero, Bouyssounade *et al*. 2002) and tight junction protein JAM-A (Torres-Flores, Silva-Ayala *et al*. 2015) are also involved in RV-host cell interaction.

Once bound, rotavirus has been shown to enter enterocytes via endocytosis, though the exact mechanism appears to be strain dependent (Gutiérrez, Isa *et al*. 2010, Díaz-Salinas, Romero *et al*. 2013, Arias, Silva-Ayala *et al*. 2015). Several RV strains have been known to spread to other sites extraintestinally, in humans as well as animal models, suggesting a wider receptor as well as host tissue range than previously hypothesized (Kraft 1958, Uhnoo, Riepenhoff-Talty *et al*. 1990, Mossel and Ramig 2003, Azevedo, Yuan *et al*. 2005, Jaimes, Feng *et al*. 2005, Zhao, Xia *et al*. 2005, Blutt, Fenaux *et al*. 2006, Crawford, Patel *et al*. 2006, Fenaux, Cuadras *et al*. 2006, Blutt, Matson *et al*. 2007, Graham, O'Donnell *et al*. 2007, Minami, Tamura *et al*. 2007, Green 2013).

2.3 RV Classification

The *Rotavirus* genus currently is comprised of ten different species (groups) designated from A to J (Matthijnssens, Otto *et al*. 2012, Mihalov-Kovacs, Gellert *et al*. 2015, Banyai, Kemenesi *et al*. 2017, Crawford, Ramani *et al*. 2017). Strains belonging to RVA, RVB and RVC groups are known to infect both humans and animals, whereas strains within RVD-RVJ groups have only been known to infect animals. Within groups, RV are further classified into different serotypes or genotypes. Serotypes are defined by reactivity of virus in plaque reduction (or fluorescent foci reduction) neutralization assays using polyclonal or monoclonal antibodies (Estes and Cohen 1989). Genotypes are defined by comparative sequence analysis (and/or nucleic acid hybridization in the old days). The two outer capsid proteins VP7 and VP4 carry the serotype neutralizing epitopes that are encoded by genomic segment 7 (VP7 glycoprotein and thus G serotype) and segment 4 (VP4 protease sensitive protein and thus P serotype). Since these two proteins are encoded by separate gene segments, RVs within the same genogroup can generate new G-P serotype antigen combinations through reassortment after dual infections of single cells (Gentsch, Laird *et al*. 2005).

The RVA group contains at least 36 G genotypes, and 51 P genotypes (RCWG 2019), with increasing

numbers of variants discovered each year. A standardized nucleotide (nt) sequence-based characterization scheme for RVA genotypes has been developed by the Rotavirus Classification Working Group (RCWG) to maintain, evaluate and develop the RV genotype classification system, in particular to aid in the designation of new genotypes (RCWG 2015). The genotypes based on complete genome sequence of VP7–VP4–VP6–VP1–VP2–VP3–NSP1–NSP2–NSP3–NSP4–NSP5/6 of circulating RV strains are being continuously identified and differentiated according to particular cut-off points of nt sequence identities, allowing for accurate identification (Matthijnssens, Ciarlet *et al*. 2008, Matthijnssens, Ciarlet *et al*. 2008, Matthijnssens, Ciarlet *et al*. 2011).

2.4 Pathogenesis of RV infection

A low dose of 100-1000 infectious particles is sufficient to cause the clinical manifestation of disease presented as AGE (Ward, Bernstein *et al*. 1986). RV utilizes integrins, sialic acids, and HBGAs to trigger cellular entry of the viral particle (Lopez and Arias 2006, Coulson 2015). The virus invades the mature enterocytes lining the middle and apical portions of the villi, by direct membrane penetration or through endocytosis. RV NSP4s are able to increase intracellular Ca^{2+} levels, which degrades tight junctions allowing paracellular water transportation. This can trigger the enteric nervous system's release of additional Ca^{2+} as well as Cl^-, contributing to the RV secretary diarrhea mechanism (Ball, Tian *et al*. 1996, Greenberg and Estes 2009). The classic histopathological markers of RV infections include villus blunting, crypt hyperplasia, and increased numbers of intraepithelial lymphocytes, but no significant inflammation (Ramig 2004). Infectious virions are released from non-polarized cells (e.g. MA104) by lysis (McNulty, Curran *et al*. 1976), but from epithelial cells (e.g. Caco-2) by a budding process that does not immediately kill the cell (Gardet, Breton *et al*. 2006). During RV-associated diarrhea, stools of infected individuals are known to contain large amounts of infectious virus particles (Crawford, Ramani *et al*. 2017).

2.5 Epidemiology of RV infection

RV is the most common cause of severe, dehydrating diarrhea among children under 5 years of age globally. Annual mortality rates due to RV infections have declined from 528,000 (range, 465,000–591,000) in 2000 to 215,000 (range, 197,000–233,000) in 2013 (Tate, Burton *et al*. 2016) owing to the implementation of RV vaccines in the national immunization program in many countries. RV is highly contagious and transmitted through a fecal-oral route. It is commonly spread by contaminated food, water, hands, and fomites. A respiratory mode of transmission has also been proposed (Prince, Astry *et al*. 1986). It has been found to cause disease in the young of many different animal species including, but not limited to: humans, cats, dog, cattle, pigs, horses, rats, birds, and various exotic animal species such as giant pandas, raccoon dogs, emus, sugar gliders, salmon and sea lions to name a few (Ghosh and Kobayashi 2014). In humans, the median age when the first RV episode may occur ranges from 6 to 9 months old with 80% of infections occurring among infants less than 1-year-old, whereas the median age in high income countries is 2-5 years (WHO 2013). Mortality rates due to RV infections in high income countries are rare due to the availability of prompt rehydration measures. RV disease typically manifests with nausea, malaise, headache, abdominal cramping, diarrhea and fever (Anderson and Weber 2004). In conditions where access to healthcare is limited, excessive diarrhea due to RV-infections cause an electrolyte imbalance that ultimately leads to death (Desselberger 2014).

RV infection accounts for 38.2% of all the diarrhea cases that require hospitalization each year worldwide (Lamberti, Ashraf *et al*. 2016). Prior to the implementation of RV vaccination, RV disease in the United State accounted for 3 million episodes per year, requiring 500,000 outpatient visits, and 60,000 hospitalizations, leading to 20-40 deaths (Fischer, Viboud *et al*. 2007, Esposito, Holman *et al*. 2011). Similar numbers were reported in European studies apart from a higher mortality rate of 200 deaths per year (Soriano-Gabarro, Mrukowicz *et al*. 2006, Van Damme, Giaquinto *et al*. 2007). RV disease in sub-Saharan African and some south Asian countries has been associated with significantly high disease episodes usually accompanied with death at high numbers (Parashar, Burton *et al*. 2009, Tate, Burton *et al*.

2012). Patterns of year-round disease were more evident in low- and low-middle income countries compared with upper-middle and high-income countries where disease was more likely to be seasonal (Patel, Pitzer *et al*. 2013). High mortality rates in developing countries in sub-Saharan Africa and Asia are predominantly due to lack of access to adequate health care and oral or intravenous rehydration therapies (Tate, Burton *et al*. 2012).

Multiple occurrences of RVs happen throughout an individual's lifetime, leading to the development of life-long immunity over time (severe disease does not occur in individuals who have had more than 2 RV infections), hence lowering the risk of serious outbreaks among the healthy (Burke, Tate *et al*. 2018). After the initial sets of RV infection and once sufficient memory immunity has been generated, RV-memory is lifelong (Velazquez, Matson *et al*. 1996).

2.6 Vaccines against RV: Rotarix and RotaTeq

RotaTeq® (Merck), a pentavalent human-bovine reassortant oral vaccine, and Rotarix® (GlaxoSmithKline), a monovalent attenuated human RV (HRV) oral vaccine, are two currently licensed, commercially available, RV vaccines worldwide. In 2009, the World Health Organization (WHO) recommended that all countries, in particular those with high prevalence of diarrhea associated deaths in children, introduce RV vaccines into their national immunization programs (WHO 2009). RV vaccination programs have now been implemented in over 100 countries (WHO 2018). Both these vaccines are highly efficacious (80-90%) in high income countries against severe RV AGE, but display only a moderate degree of protection (40-60%) in low income countries (Ruiz-Palacios, Perez-Schael *et al*. 2006, Vesikari, Matson *et al*. 2006, Armah, Sow *et al*. 2010, Zaman, Dang *et al*. 2010, Vesikari 2012, De Oliveira, Giglio *et al*. 2013, Pendleton, Galic *et al*. 2013, Glass, Parashar *et al*. 2014, Gilmartin and Petri 2015, Lamberti, Ashraf *et al*. 2016). Multiple factors have been associated with this discrepancy between vaccine efficacies in the two-income resource settings (Clarke and Desselberger 2015). The composition and diversity of intestinal microbiota, especially bacteria, have recently been shown to play a role on the immunogenicity of the oral RV and other vaccines. Particularly, members of gut bacteria belonging to *Actinobacteria* such as *Bifidobacterium longum*

subspecies *infantis*, were present in higher abundance and positively correlated with T cell responses to oral polio (OP), Bacillus Calmette-Guérin (BCG) and tetanus toxoid (TT) vaccines. On the other hand, the presence of *Clostridiales, Enterobacteriaceae,* and *Pseudomonadales*, were associated with poor vaccine responses (Huda, Lewis *et al*. 2014).

The majority of countries currently implementing RV vaccines as part of their national immunization programs are countries with low RV-related mortality, so the real impact of the vaccines on global estimates of RV mortality has been limited (Tate, Burton *et al*. 2016). HRV disease is most prevalent among the African, southern- and southeastern- Asian countries. A case study in 2013 analyzed the etiological causes of moderate to severe diarrhea in seven developing countries in sub-Saharan Africa and Southern Asia, and found that the vast majority of the diarrhea cases within the first two years of life were caused by HRV (Kotloff, Nataro *et al*. 2013). While WHO advises that all countries should implement RV vaccines and over 100 countries have licensed RotaTeq and/or Rotarix (WHO 2018), the countries with the highest rates of diarrheal deaths have seen the lowest rate of RV vaccine seroconversions, averaging 43% (Patel, Glass *et al*. 2013, WHO 2013, Kompithra, Paul *et al*. 2014). The reasons for lower vaccine efficacy in low income settings are not fully determined. A combination of factors has been proposed to be responsible for the differences in vaccine efficacy in high vs low income countries (Zaman, Dang *et al*. 2010, Valdez, Brown *et al*. 2014, Valdez, Brown *et al*. 2014, Becker-Dreps, Vilchez *et al*. 2015, Gilmartin and Petri 2015, Twitchell, Tin *et al*. 2016).

2.7 Risks associated with live RV vaccines: intussusception linked to RV vaccines and virus contaminations

Intussusception is a condition where the intestine telescopes within itself leading to intestinal obstruction. If left untreated, it could lead to internal hernias and even sepsis and can be fatal (Jain and Haydel 2019). There are no definitive underlying causes of intussusception although an increase in gut motility during viral infections have been positively associated (Koch, Harder *et al*. 2017).

The very first vaccine that was manufactured and licensed in the United States, RotaShield® (Wyeth), only circulated in the market between September 1998 – July 1999 before its withdrawal due to its association with intussusception (CDC 1999). Due to this potential adverse effect, both RotaTeq and Rotarix underwent rigorous prelicensure clinical trials involving larger cohorts of 60,000-70,000 infants each, designed specifically to evaluate the safety of these vaccines. There was no difference in the risk of intussusception between vaccine, and placebo, recipients subsequently allowing for the vaccines to be licensed.

Post-licensure surveillance studies have revealed that children vaccinated with RotaTeq and Rotarix are prone to an increased risk of intussusception, potentially due to the high titers of replicating attenuated RV in the intestines (Parashar, Cortese *et al*. 2015). Rotarix vaccine stocks were identified to be contaminated with low levels of live porcine circovirus type 1 (PCV) viral particles, as well as viral DNA causing a safety concern in some countries (Victoria, Wang *et al*. 2010). This led to the temporarily withdrawal of Rotarix from circulation until it was confirmed that there was no immunological or clinical evidence of PCV1 infection among vaccine recipients pre- and post-licensure (Dubin, Toussaint *et al*. 2013). Benefit-risk studies have been carried out throughout the world in order to assess vaccine safety and efficacy (Clark, Jit *et al*. 2014, Ledent, Lieftucht *et al*. 2016, Lamrani, Tubert-Bitter *et al*. 2017, Ledent, Arlegui *et al*. 2018). These studies have shown that the benefits of currently licensed RV vaccines far outweigh the risks associated with their use.

2.8 Other live, attenuated oral RV vaccines

With varying degrees of vaccine efficacies among different populations worldwide, local organizations funded by their governments have developed vaccines targeting rotavirus strains endemic among their populations.

Recently, two more live oral vaccines Rotasiil® (pentavalent bovine-human reassortant) and Rotavac® (monovalent G9P[11]), have been added to the WHO pre-qualification list for distribution (Pecenka, Debellut *et al*. 2018). Clinical trials carried out in India on Rotavac demonstrated an efficacy of 56% against

severe RV gastroenteritis (GE) requiring hospitalization in the first year of life and 49% in the second year of life (Bhandari, Rongsen-Chandola *et al*. 2014) while the efficacy of Rotasiil was observed to be 33% during the first year of life in India (Kulkarni, Desai *et al*. 2017) and 67% in Niger (Coldiron, Guindo *et al*. 2018). The complete post-market safety and reactogenicity of the recently introduced Rotasiil and Rotavac are still under evaluation, with current understanding is that there is no difference in rates of intussusception between infants receiving the vaccines and placebo (Burke, Tate *et al*. 2019).

Rotavin-M1 vaccine (Polyvac), is a live, attenuated oral vaccine, based on G1P[8] strain isolated from a child hospitalized for diarrhea in Nha Trang, Vietnam. This vaccine showed 73% seroconversion in an efficacy trial carried out using 160 infants. Based on these studies, Rotavin-M1 has been licensed in Vietnam and is available in the private market. Phase III immunogenicity trials are currently underway to further evaluate the liquid formulation of the vaccine.

Another live, attenuated oral vaccine, Lanzhou lamb rotavirus vaccine (Lanzhou Institute of Biological Products), has been licensed and available exclusively in China since 2000. One post-marketing study found that a single dose of the LLR vaccine confers ~50% protection rate when given to children between 9 and 35 months old (Fu, He *et al*. 2012).

RV3-BB (PT BioFarma, Bandung, Indonesia) is a naturally attenuated oral vaccine developed by the Murdoch Children's Research Institute based on G3P[6] RV strain which was isolated from the stool of asymptomatic infants. The RV3 strain is naturally adapted to the newborn gut allowing its replication despite the presence of maternal antibodies from breastfeeding. A study conducted in Indonesia administered 3 doses of this vaccine in two ways: (i) neonatal schedule when the vaccine was administered at 0-5 days, 8 and 14 weeks; or (ii) infant schedule when the vaccine was administered on 8, 14 and 18 weeks. Among the cohort that received the first dose of the vaccine immediately after birth (neonatal schedule), 75% of the infants were protected from severe RV infection while 51% the infants that were administered the vaccine based on the infant schedule were protected from severe RVGE. The vaccine was well tolerated among both vaccine groups and showed similar incidence of adverse events to the placebo

recipients (Bines, At Thobari *et al*. 2018). Further studies evaluating the safety and immunogenicity of this vaccine are being carried out in African neonates and infants (Burke, Tate *et al*. 2019).

2.9 Nonreplicating rotavirus vaccines

Underperformance of oral RV vaccines in low income countries has been hypothesized to be caused by multiple factors, mostly centered around the inability of vaccine strains to reach their target cells in the intestine. Passively transferred anti-RV antibodies, in particular, IgA via breast milk, and transplacentally acquired IgG, have been associated with negative impacts on the immunogenicity of oral RV vaccines (Velasquez, Parashar *et al*. 2018). Co-administration of polio vaccine together with oral RV vaccine have shown to decrease RV-specific IgA seroconversion rates (Emperador, Velasquez *et al*. 2016, Ramani, Mamani *et al*. 2016). High stomach acidity has also been linked with virus degradation prior to accessing the intestines leading to a decreased immunogenicity of vaccines (Patel, Shane *et al*. 2009, Glass, Jiang *et al*. 2018). Nonreplicating rotavirus vaccines (NRRV) have been considered as suitable alternatives to mucosal-administered RV vaccines. In addition to being unaffected by a lower vaccine immunogenicity due to reasons hypothesized, NRRVs also completely eliminate the risk of intussusception making them safer than the currently available vaccines. Several new NRRV candidates are currently under various stages of development (Glass, Jiang *et al*. 2018).

2.10 Development of a VP8*-based vaccine

The binding of RV VP8* proteins to host cell surface glycans initiates the process of virus entry into host cell leading to a successful infection. VP8* proteins have been identified as important target epitopes for virus neutralization. Recombinant VP8* proteins, produced using an *E. coli* expression system, were initially reported to elicit the production of high levels of VP8*-specific IgG and/or neutralizing antibodies in intramuscularly vaccinated guinea pigs (Wen, Cao *et al*. 2012). The inclusion of a tetanus toxoid T cell epitope P2 sequence, and administering it together with an alum-based adjuvant further boosted the vaccine potency and enhanced the immunogenicity of VP8* proteins originated from P[8] and P[6] strains, when

evaluated using guinea pig and Gn pig models (Wen, Wen *et al*. 2014). With ongoing Phase III human clinical trials, the P2-VP8* vaccine has showed significant promise and is furthest along towards licensure. Phase I and Phase II clinical trials carried out to evaluate the effects of P2-VP8* vaccine demonstrated that participants who received the full course (three doses) of vaccination, shed fewer attenuated rotavirus in feces as compared to trial participants who received either the placebo or two doses of P2-VP8* after Rotarix oral inoculation (Fix, Harro *et al*. 2015, Groome, Koen *et al*. 2017).

2.11 Correlates of protection against RV

The mechanisms responsible for generating protective immunity to rotavirus infections and illness following vaccination or natural infection are not completely understood, especially in humans where detailed examination of the acquired cellular immune response (B cells, T cells) in young children has been limited because of the difficulty in obtaining timely and sufficient specimens. RV infections elicit both nonspecific (innate) (Gonzalez, Azevedo *et al*. 2010, Holloway and Coulson 2013) and acquired virus-specific humoral and cellular immune responses (Angel, Franco *et al*. 2012). However, there has been no established correlate of vaccine-induced protection. The presence of certain levels (>20U/ml or 3-fold increase) of humoral RV-specific antibodies such as the presence of IgA in fecal contents, as well as the serum IgA and IgG have been accepted as correlates of protection (Angel, Franco *et al*. 2012, Patel, Glass *et al*. 2013). This general consensus is due to RV-specific serum IgA antibody levels generally following the same trend as, other, less-easily measured immune effector functions such as mucosal antibody titers or levels of virus-specific memory B cells in the gut associated lymphoid tissues. Fecal IgA titers of >1:80, serum IgA titers of >1:200, and serum IgG titers of >1:800 are correlated with protection in high income countries (Desselberger 2014). Interestingly, it has been demonstrated that high serum IgA titers do not confer the same degree of protection in low income countries as compared to initial studies carried out in high income countries (Lee, Carmolli *et al*. 2018).

2.12 Gn pig models and their utility in RV research

Animal models are very useful for the preclinical evaluations of vaccine candidates. The neonatal Gn pig is a valuable model for its contribution to the understanding of the underlying mechanisms of RV infection (Azevedo, Yuan *et al*. 2005), pathogenesis (Ward, Rosen *et al*. 1996, Azevedo, Yuan *et al*. 2006), and defining the correlates of protection (Saif, Ward *et al*. 1996, Yuan, Ward *et al*. 1996) against RV disease. Gn pigs have also been used for evaluating the protective efficacy of novel vaccines against RV infection (Yuan, Wen *et al*. 2008, Wen, Wen *et al*. 2014). RV infections in pigs manifest within 12-18 hours after infection with virus shedding lasting up to 4-7 days.

Signs of infection and intestinal pathology in Gn pigs due to RV infections are very similar to that in humans, making them a suitable model to study the course of RV disease (Ward, Rosen *et al*. 1996). Gn pigs that were fully protected from RV disease when challenged with virulent HRV had significantly different RV-specific intestinal IgA ASC numbers and intestinal IFN-γ frequencies during the time of challenge as compared to unprotected pigs. Using Gn pig models, RV-specific intestinal IgA antibody secreting cells (ASC) and IFN-γ producing T cells have been identified as important determinants of protective immunity (Yuan, Ward *et al*. 1996, Yuan, Wen *et al*. 2008). High frequencies of regulatory T-cells (CD4+CD25- Treg cells in particular) have been correlated with poor protective immunity against RV infections in Gn pig models (Wen, Li *et al*. 2012). On the other hand, frequencies of intestinal IFN-γ producing CD4+ and CD8+ T cells induced by HRV infection or live attenuated HRV vaccination were significantly correlated with a significant protection rate against HRV diarrhea (Yuan *et al*., 2008). Gn pigs have been instrumental for the validation of RV vaccines prior to their clinical trials.

Gnotobiotic (Gn) piglet models transplanted with unhealthy human gut microbiome to study rotavirus vaccine efficacy have demonstrated low effector T cell response (interferon γ producing T cells) to the oral RV vaccine (Twitchell, Tin *et al*. 2016). Conversely, Gn pigs colonized with healthy gut microbiome obtained from children with low gut enteropathy scores had a robust immune response to RV vaccine. Recently, humanized pig models have been developed to study the impacts of nutrition status (nutrition /

malnutrition) on the severity of RV infection (Kumar, Vlasova *et al*. 2018). The benefit of having a large animal model that closely mimics the infection and disease observed in humans provides the ability to modulate different aspects of the model, allowing for a better understanding of the disease in a way that cannot be carried out in humans. Continued research using this animal model will help with the understanding of many unanswered questions.

3. Norovirus

With more RV vaccines being implemented as part of national immunization programs around the world, human noroviruses (HuNoV) are now the leading causative agents of AGE among all age groups (Hall, Glass *et al*. 2016, Mattison, Cardemil *et al*. 2018). HuNoV causes over 20% of all AGE cases annually (Ahmed, Hall *et al*. 2014). Among susceptible individuals, very low inoculum doses are sufficient to generate a full course of infection with high titers of virus shed in feces (Teunis, Moe *et al*. 2008, Atmar, Opekun *et al*. 2014, Messner, Berger *et al*. 2014, Schmidt 2015). HuNoVs are transmitted through a fecal-oral route and epidemics primarily occur most in healthcare facilities, restaurants, and schools among other settings. HuNoV outbreaks primarily occur due to individuals coming in contact with contaminated food and water (CDC 2011, Brennan, Cavallo *et al*. 2018, Free, Buss *et al*. 2019).

3.1 Classification of NoV

NoVs are single-stranded positive sense RNA viruses that belong to the genus *Norovirus* within the family *Caliciviridae*. NoV genome is composed of 3 open reading frames (ORFs). NoV classification is based on the genetic sequence of the entire virus genome or, the sequence of an individual virus gene (Kroneman, Vega *et al*. 2013). Each genogroup is further sub-classified into genotypes based on phylogenetic analysis of the capsid (VP1) and the RNA dependent RNA polymerase (RdRp) protein sequences (De Graaf *et al*., 2016). NoV is currently categorized into 10 genogroups (G1-GX) which are further classified into 49 genotypes based on VP1 gene (Chhabra, de Graaf *et al*. 2019). There are 29 different virus strains spread

over 3 different genogroups (genogroup I, II, and IV), that are responsible for AGE in humans (Kroneman, Vega *et al*. 2013, Vinje 2015, Cortes-Penfield, Ramani *et al*. 2017).

3.2 Structure of NoV

The NoV capsid is made up of two structural proteins, VP1 (major) and VP2 (minor), coded by ORF 2 and 3 respectively. VP1 is divided into a shell (S) domain and the protruding (P) domain that provide form and stability to the virus (Tan and Jiang 2005). The VP1 has been extensively studied for its antigenic properties and ability to be easily produced using different *in vitro* expression systems. The VP1 has been observed to self-assemble leading to the formation of virus-like particles (VLPs) that have been the basis of NoV vaccines (Tan and Jiang 2019). Recently, using a feline calicivirus model, the VP2 has been discovered to function as trans-capsid channel that is responsible for the delivery of viral genome upon host cell binding (Conley, McElwee *et al*. 2019).

3.3 Pathogenesis of NoV

Over 80% of all HuNoV-associated GE around the world is caused by GII.4 NoVs (Riddle and Walker 2016). The absence of a cell culture system has delayed the progress made on the understanding of NoV pathogenesis and immunity and the development of effective vaccines or therapeutics against NoV disease in humans (Duizer, Schwab *et al*. 2004). The site of replication for NoVs has not been well established, but it is assumed that they replicate in the upper intestinal tract. Biopsies collected from the jejunum of volunteers who develop gastrointestinal illness following an oral administration of the Norwalk (GI.1) or Hawaii virus (GII.1) have exhibited histopathologic lesions (Karandikar, Crawford *et al*. 2016). Interestingly, broadening and blunting of the villi of the proximal small intestine has been observed, although the mucosa itself remains histologically intact (Glass, Parashar *et al*. 2009).

Jones *et al*, reported that a human derived continuous B-cell line, BJAB, is capable of supporting HuNoV replication (Jones, Watanabe *et al*. 2014). Infectivity of a GII.4 HuNoV in the cell culture system was stimulated by the presence of enteric bacteria or HBGAs, which were proposed to facilitate attachment to

the B cells. The same group also showed that murine norovirus (MNV) were capable of replicating in continuous B-cell lines (M12 and WEHI-231). The poor repeatability and low viral titers yielded from using the BJAB cell line gave rise to new challenges that need to be overcome in order to establish a successful cell culture model. This theory of B-cells being permissive for HuNoV infections was tested in human patients with severe combined immunodeficiency (SCID). Two groups, those with intact (but sometimes dysfunctional) B cells (n = 10) and those without (n = 8), and all were tested for the presence of NoV RNA in stool. The viral RNA titers were similar between the two groups, leading to the conclusion that NoV infections do occur in the absence of B-cells (Brown, Gilmour *et al*. 2016). In a study involving severe combined immunodeficiency (SCID) Gn pigs that were deficient in B cells (and in T cells and NK cells by *RAG2*/*IL2RG* gene knockout), after an oral inoculation with HuNoV, fecal virus shedding was observed for a longer duration and the virus-tissue distribution was wider compared to the wildtype Gn pigs (Lei, *et al* 2016). This could either mean that B-cells are not the primary targets of HuNoV infection or that HuNoVs are capable of infecting a broader range of cell-types similar to the findings in RVs. The results of this study were further supported by data recently published by another group that showed no difference between rates and severity of NoV infection among SCID and non-SCID Gn pigs indicating that the innate immune mechanism might be crucial for NoV clearance (Annamalai, Lu *et al*. 2019).

Successful NoV cell-culture has been achieved using a stem-cell derived human enteroid system. Bile acid and the presence of HBGA receptors on the cell surfaces were identified to be key determinants of virus entry and propagation (Ettayebi, Crawford *et al*. 2016). Enteroids derived from all three segments of the small intestine (duodenum, jejunum, and ileum) were permissive for NoV replication up to 96 hours post inoculation. Immunocytochemistry analysis on intestinal biopsies collected from a transplant patient who was suffering from severe GII.4 GE revealed that epithelial cells are the primary site of replication for NoV (Green, Kaufman *et al*. 2019). In another study, duodenal biopsies taken from an individual suffering from common variable immune deficiency who was chronically infected with HuNoV also showed viral antigens (capsid proteins and RdRp) within enterocytes (Estes, Ettayebi *et al*. 2019). The conclusion that epithelial

cells is the predominate target cell type for HuNoV in these studies of humans is consistent with the observation in Gn pigs infected with HuNoV (Bui, Kocher *et al*. 2013, Lei, Ramesh *et al*. 2016, Lei, Samuel *et al*. 2016).

3.4 Correlates of protection against NoV infections

Current knowledge on immune responses to HuNoV comes predominantly from experimental human infection models and clinical studies with VLP vaccines. Similar to some RV species as mentioned earlier, HBGAs are host susceptibility determinants for NoV infections. HBGA found on the surface of intestinal epithelial cells of humans with the secretor phenotype (Marionneau, Ruvoen *et al*. 2002) have been determined as key regulators of susceptibility to HuNoV binding and entry in humans (Lindesmith, Moe *et al*. 2003, Hutson, Airaud *et al*. 2005). Furthermore, the ABO blood group antigens have also been linked to susceptibility to Norwalk (HuNoV GI.1 prototype) infection, with AB, and B blood group antigen individuals showing a lower incidence of infection and illness with HuNoV (Lindesmith, Moe *et al*. 2003). Understanding the binding capacity of HuNoV VLPs to blood group antigens allowed for the development of Hemagglutination Inhibition Assay (Czakó, Atmar *et al*. 2012). Hemagglutination inhibition (HAI) titers have since been used as a correlate of protection by determining the antibody titers that can effectively block the binding of blood group antigens with HuNoV (Ramani, Estes *et al*. 2016).

Antibodies found in serum that block NoV-HBGA binding (HBGA blocking antibodies) have been suggested as a direct correlate of protection from clinical illness (vomiting and diarrhea) following GII.4 HuNoV vaccination or challenge. In a HuNoV human volunteer challenge study, pre-challenge serum titer levels (>1:500) of functional antibodies that block binding of HuNoV VLPs to HBGAs correlated with lower risk of illness (Reeck, Kavanagh *et al*. 2010). High serum IgA but not serum IgG titers have also been linked with protection from HuNoV-associated illness (Atmar, Bernstein *et al*. 2015).

NoV-specific salivary IgA and NoV-specific memory IgG cells have also been identified as additional correlates of protection against NoV GE (Ramani, Neill *et al*. 2015). Pre-challenge, NoV-specific salivary

IgA levels correlated with reduced severity of GE. Rapid salivary IgA response following NoV challenge was previously demonstrated to be protective against infection. Pre-challenge, NoV-specific fecal IgA levels are associated with a lower peak viral load, while levels on day seven post-infection correlate with a shorter duration of virus shedding (Ramani, Neill *et al*. 2015).

A Luciferase Immunoprecipitation Systems (LIPS) assay, recently developed by Tin *et al*, has made it possible to screen NoV genotype-specific IgG antibodies in the serum against the VP1 capsid protein (Tin, Yuan *et al*. 2017). The benefit of having an assay that is capable of identifying genotype specific serum or fecal antibody response would enhance the accuracy in determining the immunogenicity of vaccine candidates.

Interferon-γ (IFN-γ) secreted by CD4+ and CD8+ T cells in response to NoV infections have been correlated with protection in humans (Lindesmith, Moe *et al*. 2005), mice (Zhu, Regev *et al*. 2013, Rodriguez, Monte *et al*. 2014), and Gn pig models (Kocher, Bui *et al*. 2014, Lei, Ramesh *et al*. 2016).

The host factors associated with HuNoV infection are poorly understood due to their host-specificity and the limited access to laboratory models to study its pathogenesis. Incremental advancements have been made by studying different aspects of NoV infection using different animal models and collating the results together to form a better understanding of the NoV infection. The importance of non-human models for the study of NoV pathogenesis has been reviewed in detail elsewhere (Wobus, Cunha *et al*. 2016). Cell-culture research using MNV, a surrogate virus model, has been ongoing and key highlights include the discovery of the MNV that grows in dendritic cells and murine macrophage-like cell lines (Wobus, Karst *et al*. 2004); the demonstration that transfection of a full-length cDNA clone of the NV genome (under control of the T7 promoter) into modified vaccinia Ankara (MVA)-T7 infected cells allowing the expression of viral proteins and subsequent NV RNA replication (Asanaka, Atmar *et al*. 2005); the expression of self-replicating NoV RNAs following the transfection of NoV RNA in a hepatoma cell line (Chang, Sosnovtsev *et al*. 2006); and most recently, cells expressing CD300lf, namely tuft cells, in the presence of secondary bile acids, have been identified as the target cells capable of facilitating MNV infection and replication (Wilen, Lee *et al*.

2018). A more recent study concluded that CD300lf is the primary physiologic receptor for MNV, but not for HuNoV (Graziano, Walker *et al*. 2019).

3.5 Animal models for NoV research

Currently, there is no animal model that can directly recapitulate the full range of NoV disease symptoms that are observed in humans, but animals in which evidence for infection has been reported following challenge with HuNoV include Gn piglets and calves (Cheetham, Souza *et al*. 2006, Souza, Azevedo *et al*. 2008, Jung, Wang *et al*. 2012, Bui, Kocher *et al*. 2013, Lei, Samuel *et al*. 2016), monkeys and chimpanzees (Bok, Parra *et al*. 2011), and immunodeficient mice (Taube, Kolawole *et al*. 2013). MNV has been used as a surrogate to study the pathogenesis, immunology, and replication of HuNoV infections, and a large amount of data has been generated (Wobus, Karst *et al*. 2004). However, the limitations of this model are obvious due to the difference between the two viruses in clinical manifestations (without diarrhea/vomiting), host receptors (sialic acid versus HBGAs), infected cell types (dendritic/macrophages versus digestive epithelial cells), and pathogenesis (Tan and Jiang 2010).

Pigs share several characteristics with humans in their gastrointestinal anatomy, physiology, immune system, the presence of FUT2 gene (pigs are all secretors) and the presence of HBGAs, such as the A and H antigens on mucosal surfaces making them susceptible to HuNoV infections. In the neonatal Gn pig model, several different NoVs belonging to GII.4 and GII.12, including GII.4 HS66/2001 (Souza, Cheetham *et al*. 2007), GII.4 KU131206 (Park, Jung *et al*. 2018), GII.4 strain 766 (Craig, Dai *et al*. 2019), GII.4 2006b (Bui, Kocher *et al*. 2013, Kocher, Bui *et al*. 2014, Lei, Ramesh *et al*. 2016, Lei, Ryu *et al*. 2016, Lei, Samuel *et al*. 2016, Lei, Twitchell *et al*. 2019), GII.4 2003 (see chapters 3 and 4 of this dissertation), GII.4 HS194 (Jung, Wang *et al*. 2012), and GII.12 HS206 (Takanashi, Wang *et al*. 2011) have all resulted in diarrhea, virus shedding, seroconversion, immuno-cytopathic change in the intestinal sections, transient viremia and/or activation of innate and adaptive immune responses (Cheetham, Souza *et al*. 2006, Cheetham, Souza *et al*. 2007, Souza, Cheetham *et al*. 2007, Bui, Kocher *et al*. 2013, Kocher, Bui *et al*. 2014, Lei, Ramesh *et al*. 2016, Lei, Ryu *et al*. 2016, Lei, Samuel *et al*. 2016, Lei, Twitchell *et al*.

2019). Similar results have also been observed in Gn calves (Souza, Azevedo *et al*. 2008), making these animal models useful for studying immunology and pathogenesis, and the assessment of vaccines and antivirals against HuNoV infections prior to being tested on humans.

It is important to identify the most appropriate animal model for biomedical research. They have to be carefully determined based on their ability to recapitulate disease pathogenesis. An ideal animal model needs to reliably mimic the normal anatomy and physiology of human organs and tissues of interest, as well as accurately reflect the morphological and biochemical aspects of disease pathogenesis. Meticulous planning and organization are required for the establishment of successful Gn pig facilities (Yuan, Jobst *et al*. 2017). They are usually associated with high maintenance costs, specialized equipment and training, but prove to be a reliable tool for answering many of the questions regarding HuNoV infection and disease without using a human model (Wobus, Cunha *et al*. 2016).

3.6 Current HuNoV vaccine pipeline

No commercial NoV vaccines or therapeutics are currently available, although several vaccine candidates are in various stages of human clinical trials (Lucero, Vidal *et al*. 2018). Treatment for NoV infections is centered over palliative care. This is particularly difficult in developing countries where access to healthcare is limited, and in some locations, clean water is scarce. Due to these reasons, NoV vaccines are urgently needed and they need to be safe, affordable, and efficacious, with a reasonable shelf-life.

Vaccines developed to confer protection against NoVs need to account for the genetically and antigenically diverse nature of NoVs (Bernstein, Atmar *et al*. 2015). Because of the lack of any efficient cell culture system for NoV, live attenuated NoV vaccine is out of reach at this time. VLPs have been most widely explored as a candidate for NoV vaccines. They are readily produced using various expression systems (Jiang, Wang *et al*. 1992, Tamminen, Huhti *et al*. 2012, Tan and Jiang 2012) and self-assemble to a confirmation that is structurally and antigenically similar to an infectious NoV particle.

VLPs based on GI.1 NoVs were initially developed as a monovalent vaccine (LigoCyte Pharmaceuticals, now Takeda Vaccines) candidate and evaluated for its safety, immunogenicity and efficacy in a proof-of-principle study (Atmar, Bernstein *et al*. 2011). They were administered intranasally together with monophosphoryl lipoprotein (MPL) and chitosan adjuvants. Study participants who received the vaccine had a reduced incidence rate (37%) of GI.1-associated GE compared to participants who received the placebo vaccine (69%). Infection characterized by the presence of viral RNA detected by RT-PCR was observed among 61% of vaccine recipients compared to 82% who received the placebo (Atmar, Bernstein *et al*. 2011). Protection conferred by the vaccine was synonymous with the presence of Norwalk virus-specific serum IgA and IgG levels, along with the induction of IgA ASCs and memory B cells (El-Kamary, Pasetti *et al*. 2010, Ramirez, Wahid *et al*. 2012). It was soon identified that a bivalent intramuscular (IM) vaccine (GI.1 and GII.4) adjuvanted with aluminum hydroxide elicited a stronger immune response compared to the intranasal administration (El-Kamary, Pasetti *et al*. 2010, Treanor, Atmar *et al*. 2014, Bernstein, Atmar *et al*. 2015, Sundararajan, Sangster *et al*. 2015). This bivalent IM VLP vaccine is currently under Phase III clinical evaluations and is leading the race for licensure for a NoV vaccine.

Another vaccine that is under clinical evaluations is the adenovirus-vectored G1.1 VP1 vaccine which recently concluded its Phase I clinical trials. This vaccine is orally administered in the form of a tablet and is comprised of a non-replicating adenovirus vector expressing the NoV GI.1 VP1 gene adjuvanted with a double-stranded RNA. The vaccine generated a 2-fold increase in blocking titers among 78% of the vaccinated individuals and generated strong systemic and mucosal IgA and IgG responses (Kim, Liebowitz *et al*. 2018).

P particles (Kocher *et al*., 2014), VLPs (Cheetham, Souza *et al*. 2006) and lactic acid bacteria-based vaccine candidates (Craig, Dai *et al*. 2019) have been evaluated for their immunogenicity and protective efficacy in Gn pig model of HuNoV infection and diarrhea. Several other candidate vaccines that are still under development include: a RV-VP6 intermediate capsid expressing the NoV GI.3 and GII.4 VLPs as a trivalent vaccine (Malm, Tamminen *et al*. 2016); a bivalent GII.4 NoV and enterovirus 71 VLP-based vaccine

(Wang, Ku *et al*. 2015); and a Newcastle disease virus vector expressing a GII.4 VP1 gene (Kim, Chen *et al*. 2014).

4. Concluding remarks

Animal models have been extensively used, and have been very useful in bridging the knowledge gaps to facilitate the understanding of the viral pathogenesis and immune correlates of protective immunity against enteric viral infections (Saif, Ward *et al*. 1996). AGE caused by HRV and HuNoV is an important vaccine-preventable issue that causes severe morbidity and mortality especially among poverty-stricken countries (Mattison, Cardemil *et al*. 2018, WHO 2018). Successful vaccination campaigns that targets children at the earliest possible time of intervention will greatly benefit these communities (Bines, At Thobari *et al*. 2018, Burke, Tate *et al*. 2019). Vaccines are the most effective way of reducing morbidity and mortality and have proven in many occasions to save lives (Rappuoli, Pizza *et al*. 2014). Research focused on the development of more protective and affordable NRRVs are required in order to provide a wider coverage for RV disease while this needs to be kept in mind while developing novel HuNoV vaccines.

It remains to be seen, the success of the HuNoV candidate vaccines as they enter the human clinical trials. The success of these vaccines will help us understand NoV immunity and bring us closer to developing an effective method of defeating the burden of NoV infections worldwide.

References

Ahmed, S. M., A. J. Hall, A. E. Robinson, L. Verhoef, P. Premkumar, U. D. Parashar, M. Koopmans and B. A. Lopman (2014). "Global prevalence of norovirus in cases of gastroenteritis: a systematic review and meta-analysis." Lancet Infect Dis **14**(8): 725-730.

Anderson, E. J. and S. G. Weber (2004). "Rotavirus infection in adults." Lancet Infect Dis **4**(2): 91-99.

Angel, J., M. A. Franco and H. B. Greenberg (2012). "Rotavirus immune responses and correlates of protection." Curr Opin Virol **2**(4): 419-425.

Annamalai, T., Z. Lu, K. Jung, S. N. Langel, C. K. Tuggle, J. C. M. Dekkers, E. H. Waide, S. Kandasamy and L. J. Saif (2019). "Infectivity of GII.4 human norovirus does not differ between T-B-NK(+) severe combined immunodeficiency (SCID) and non-SCID gnotobiotic pigs, implicating the role of NK cells in mediation of human norovirus infection." Virus Res **267**: 21-25.

Arias, C. F., D. Silva-Ayala and S. López (2015). "Rotavirus Entry: a Deep Journey into the Cell with Several Exits." Journal of Virology **89**(2): 14.

Armah, G. E., S. O. Sow, R. F. Breiman, M. J. Dallas, M. D. Tapia, D. R. Feikin, F. N. Binka, A. D. Steele, K. F. Laserson, N. A. Ansah, M. M. Levine, K. Lewis, M. L. Coia, M. Attah-Poku, J. Ojwando, S. B. Rivers, J. C. Victor, G. Nyambane, A. Hodgson, F. Schodel, M. Ciarlet and K. M. Neuzil (2010). "Efficacy of pentavalent rotavirus vaccine against severe rotavirus gastroenteritis in infants in developing countries in sub-Saharan Africa: a randomised, double-blind, placebo-controlled trial." Lancet **376**(9741): 606-614.

Asanaka, M., R. L. Atmar, V. Ruvolo, S. E. Crawford, F. H. Neill and M. K. Estes (2005). "Replication and packaging of Norwalk virus RNA in cultured mammalian cells." Proc Natl Acad Sci U S A **102**(29): 10327-10332.

Atmar, R. L., D. I. Bernstein, C. D. Harro, M. S. Al-Ibrahim, W. H. Chen, J. Ferreira, M. K. Estes, D. Y. Graham, A. R. Opekun, C. Richardson and P. M. Mendelman (2011). "Norovirus Vaccine against Experimental Human Norwalk Virus Illness." New England Journal of Medicine **365**(23): 2178-2187.

Atmar, R. L., D. I. Bernstein, G. M. Lyon, J. J. Treanor, M. S. Al-Ibrahim, D. Y. Graham, J. Vinjé, X. Jiang, N. Gregoricus, R. W. Frenck, C. L. Moe, W. H. Chen, J. Ferreira, J. Barrett, A. R. Opekun, M. K. Estes, A. Borkowski, F. Baehner, R. Goodwin, A. Edmonds and P. M. Mendelman (2015). "Serological Correlates of Protection against a GII.4 Norovirus." Clinical and Vaccine Immunology **22**(8): 923.

Atmar, R. L., A. R. Opekun, M. A. Gilger, M. K. Estes, S. E. Crawford, F. H. Neill, S. Ramani, H. Hill, J. Ferreira and D. Y. Graham (2014). "Determination of the 50% human infectious dose for Norwalk virus." J Infect Dis **209**(7): 1016-1022.

Ayouni, S., K. Sdiri-Loulizi, A. de Rougemont, M. Estienney, K. Ambert-Balay, S. Aho, S. Hamami, M. Aouni, M. Neji-Guediche, P. Pothier and G. Belliot (2015). "Rotavirus P[8] Infections in Persons with Secretor and Nonsecretor Phenotypes, Tunisia." Emerging infectious diseases **21**(11): 2055-2058.

Azevedo, M. S., L. Yuan, K. I. Jeong, A. Gonzalez, T. V. Nguyen, S. Pouly, M. Gochnauer, W. Zhang, A. Azevedo and L. J. Saif (2005). "Viremia and Nasal and Rectal Shedding of Rotavirus in Gnotobiotic Pigs Inoculated with Wa Human Rotavirus." Journal of Virology **79**(9): 5428.

Azevedo, M. S., L. Yuan, K. I. Jeong, A. Gonzalez, T. V. Nguyen, S. Pouly, M. Gochnauer, W. Zhang, A. Azevedo and L. J. Saif (2005). "Viremia and Nasal and Rectal Shedding of Rotavirus in Gnotobiotic Pigs Inoculated with Wa Human Rotavirus." Journal of Virology **79**(9): 5428-5436.

Azevedo, M. S. P., L. Yuan, S. Pouly, A. M. Gonzales, K. I. Jeong, T. V. Nguyen and L. J. Saif (2006). "Cytokine Responses in Gnotobiotic Pigs after Infection with Virulent or Attenuated Human Rotavirus." Journal of Virology **80**(1): 372.

Ball, J. M., P. Tian, C. Q. Zeng, A. P. Morris and M. K. Estes (1996). "Age-dependent diarrhea induced by a rotaviral nonstructural glycoprotein." Science **272**(5258): 101-104.

Banyai, K., G. Kemenesi, I. Budinski, F. Foldes, B. Zana, S. Marton, R. Varga-Kugler, M. Oldal, K. Kurucz and F. Jakab (2017). "Candidate new rotavirus species in Schreiber's bats, Serbia." Infect Genet Evol **48**: 19-26.

Becker-Dreps, S., S. Vilchez, D. Velasquez, S. S. Moon, M. G. Hudgens, L. E. Zambrana and B. Jiang (2015). "Rotavirus-specific IgG antibodies from mothers' serum may inhibit infant immune responses to the pentavalent rotavirus vaccine." Pediatr Infect Dis J **34**(1): 115-116.

Bernstein, D. I., R. L. Atmar, G. M. Lyon, J. J. Treanor, W. H. Chen, X. Jiang, J. Vinje, N. Gregoricus, R. W. Frenck, Jr., C. L. Moe, M. S. Al-Ibrahim, J. Barrett, J. Ferreira, M. K. Estes, D. Y. Graham, R. Goodwin, A. Borkowski, R. Clemens and P. M. Mendelman (2015). "Norovirus vaccine against experimental human GII.4 virus illness: a challenge study in healthy adults." J Infect Dis **211**(6): 870-878.

Bernstein, D. I., R. L. Atmar, G. M. Lyon, J. J. Treanor, W. H. Chen, X. Jiang, J. Vinjé, N. Gregoricus, R. W. Frenck, Jr., C. L. Moe, M. S. Al-Ibrahim, J. Barrett, J. Ferreira, M. K. Estes, D. Y. Graham, R. Goodwin, A. Borkowski, R. Clemens and P. M. Mendelman (2015). "Norovirus vaccine against experimental human GII.4 virus illness: a challenge study in healthy adults." The Journal of infectious diseases **211**(6): 870-878.

Bhandari, N., T. Rongsen-Chandola, A. Bavdekar, J. John, K. Antony, S. Taneja, N. Goyal, A. Kawade, G. Kang, S. S. Rathore, S. Juvekar, J. Muliyil, A. Arya, H. Shaikh, V. Abraham, S. Vrati, M. Proschan, R. Kohberger, G. Thiry, R. Glass, H. B. Greenberg, G. Curlin, K. Mohan, G. V. Harshavardhan, S. Prasad, T. S. Rao, J. Boslego and M. K. Bhan (2014). "Efficacy of a monovalent human-bovine (116E) rotavirus vaccine in Indian children in the second year of life." Vaccine **32 Suppl 1**: A110-116.

Bines, J. E., J. At Thobari, C. D. Satria, A. Handley, E. Watts, D. Cowley, H. Nirwati, J. Ackland, J. Standish, F. Justice, G. Byars, K. J. Lee, G. L. Barnes, N. S. Bachtiar, A. Viska Icanervilia, K. Boniface, N. Bogdanovic-Sakran, D. Pavlic, R. F. Bishop, C. D. Kirkwood, J. P. Buttery and Y. Soenarto (2018). "Human Neonatal Rotavirus Vaccine (RV3-BB) to Target Rotavirus from Birth." The New England journal of medicine **378**(8): 719-730.

Blutt, S. E., M. Fenaux, K. L. Warfield, H. B. Greenberg and M. E. Conner (2006). "Active viremia in rotavirus-infected mice." J Virol **80**(13): 6702-6705.

Blutt, S. E., D. O. Matson, S. E. Crawford, M. A. Staat, P. Azimi, B. L. Bennett, P. A. Piedra and M. E. Conner (2007). "Rotavirus antigenemia in children is associated with viremia." PLoS Med **4**(4): e121.

Böhm, R., F. E. Fleming, A. Maggioni, V. T. Dang, G. Holloway, B. S. Coulson, M. von Itzstein and T. Haselhorst (2015). "Revisiting the role of histo-blood group antigens in rotavirus host-cell invasion." Nature communications **6**: 5907.

Bok, K., G. I. Parra, T. Mitra, E. Abente, C. K. Shaver, D. Boon, R. Engle, C. Yu, A. Z. Kapikian, S. V. Sosnovtsev, R. H. Purcell and K. Y. Green (2011). "Chimpanzees as an animal model for human norovirus infection and vaccine development." Proc Natl Acad Sci U S A **108**(1): 325-330.

Brennan, J., S. J. Cavallo, K. Garman, K. Lewis, D. J. Irving, C. Moore, L. Thomas, J. Hill, R. Villegas, J. F. Norman, J. R. Dunn, W. Schaffner and T. F. Jones (2018). "Notes from the Field: Multiple Modes of Transmission During a Thanksgiving Day Norovirus Outbreak - Tennessee, 2017." MMWR Morb Mortal Wkly Rep **67**(46): 1300-1301.

Brown, J. R., K. Gilmour and J. Breuer (2016). "Norovirus Infections Occur in B-Cell-Deficient Patients." Clin Infect Dis **62**(9): 1136-1138.

Bui, T., J. Kocher, Y. Li, K. Wen, G. Li, F. Liu, X. Yang, T. LeRoith, M. Tan, M. Xia, W. Zhong, X. Jiang and L. Yuan (2013). "Median infectious dose of human norovirus GII.4 in gnotobiotic pigs is decreased by simvastatin treatment and increased by age." J Gen Virol **94**(Pt 9): 2005-2016.

Burke, R. M., J. E. Tate, N. Barin, C. Bock, M. D. Bowen, D. Chang, R. Gautam, G. Han, J. Holguin, T. Huynh, C. Y. Pan, R. Quenelle, C. Sallenave, C. Torres, D. Wadford and U. Parashar (2018). "Three Rotavirus Outbreaks in the Postvaccine Era - California, 2017." MMWR Morb Mortal Wkly Rep **67**(16): 470-472.

Burke, R. M., J. E. Tate, C. D. Kirkwood, A. D. Steele and U. D. Parashar (2019). "Current and new rotavirus vaccines." Current Opinion in Infectious Diseases **32**(5): 435-444.

CDC (1999). "Intussusception among recipients of rotavirus vaccine--United States, 1998-1999." MMWR Morb Mortal Wkly Rep **48**(27): 577-581.

CDC (2011). "Updated norovirus outbreak management and disease prevention guidelines." MMWR Recomm Rep **60**(Rr-3): 1-18.

Chang, K.-O., S. V. Sosnovtsev, G. Belliot, A. D. King and K. Y. Green (2006). "Stable expression of a Norwalk virus RNA replicon in a human hepatoma cell line." Virology **353**(2): 463-473.

Cheetham, S., M. Souza, R. McGregor, T. Meulia, Q. Wang and L. J. Saif (2007). "Binding Patterns of Human Norovirus-Like Particles to Buccal and Intestinal Tissues of Gnotobiotic Pigs in Relation to A/H Histo-Blood Group Antigen Expression." Journal of Virology **81**(7): 3535-3544.

Cheetham, S., M. Souza, T. Meulia, S. Grimes, M. G. Han and L. J. Saif (2006). "Pathogenesis of a genogroup II human norovirus in gnotobiotic pigs." J Virol **80**(21): 10372-10381.

Chen, D., C. L. Luongo, M. L. Nibert and J. T. Patton (1999). "Rotavirus open cores catalyze 5'-capping and methylation of exogenous RNA: evidence that VP3 is a methyltransferase." Virology **265**(1): 120-130.

Chhabra, P., M. de Graaf, G. I. Parra, M. C.-W. Chan, K. Green, V. Martella, Q. Wang, P. A. White, K. Katayama, H. Vennema, M. P. G. Koopmans and J. Vinjé (2019). "Updated classification of norovirus genogroups and genotypes." Journal of General Virology **100**(10): 1393-1406.

Ciarlet, M., J. E. Ludert, M. Iturriza-Gomara, F. Liprandi, J. J. Gray, U. Desselberger and M. K. Estes (2002). "Initial interaction of rotavirus strains with N-acetylneuraminic (sialic) acid residues on the cell surface correlates with VP4 genotype, not species of origin." J Virol **76**(8): 4087-4095.

Clark, A., M. Jit, N. Andrews, C. Atchison, W. J. Edmunds and C. Sanderson (2014). "Evaluating the potential risks and benefits of infant rotavirus vaccination in England." Vaccine **32**(29): 3604-3610.

Clarke, E. and U. Desselberger (2015). "Correlates of protection against human rotavirus disease and the factors influencing protection in low-income settings." Mucosal Immunol **8**(1): 1-17.

Coldiron, M. E., O. Guindo, R. Makarimi, I. Soumana, A. Matar Seck, S. Garba, E. Macher, S. Isanaka and R. F. Grais (2018). "Safety of a heat-stable rotavirus vaccine among children in Niger: Data from a phase 3, randomized, double-blind, placebo-controlled trial." Vaccine **36**(25): 3674-3680.

Conley, M. J., M. McElwee, L. Azmi, M. Gabrielsen, O. Byron, I. G. Goodfellow and D. Bhella (2019). "Calicivirus VP2 forms a portal-like assembly following receptor engagement." Nature **565**(7739): 377-381.

Cortes-Penfield, N. W., S. Ramani, M. K. Estes and R. L. Atmar (2017). "Prospects and Challenges in the Development of a Norovirus Vaccine." Clin Ther **39**(8): 1537-1549.

Coulson, B. S. (2015). "Expanding diversity of glycan receptor usage by rotaviruses." Current opinion in virology **15**: 90-96.

Coulson, B. S., S. L. Londrigan and D. J. Lee (1997). "Rotavirus contains integrin ligand sequences and a disintegrin-like domain that are implicated in virus entry into cells." Proceedings of the National Academy of Sciences of the United States of America **94**(10): 5389-5394.

Craig, K., X. Dai, A. Li, M. Lu, M. Xue, L. Rosas, T. Z. Gao, A. Niehaus, R. Jennings and J. Li (2019). "A Lactic Acid Bacteria (LAB)-Based Vaccine Candidate for Human Norovirus." Viruses **11**(3): 213.

Crawford, S. E., D. G. Patel, E. Cheng, Z. Berkova, J. M. Hyser, M. Ciarlet, M. J. Finegold, M. E. Conner and M. K. Estes (2006). "Rotavirus viremia and extraintestinal viral infection in the neonatal rat model." J Virol **80**(10): 4820-4832.

Crawford, S. E., S. Ramani, J. E. Tate, U. D. Parashar, L. Svensson, M. Hagbom, M. A. Franco, H. B. Greenberg, M. O'Ryan, G. Kang, U. Desselberger and M. K. Estes (2017). "Rotavirus infection." Nature reviews. Disease primers **3**: 17083-17083.

Czakó, R., R. L. Atmar, A. R. Opekun, M. A. Gilger, D. Y. Graham and M. K. Estes (2012). "Serum hemagglutination inhibition activity correlates with protection from gastroenteritis in persons infected with Norwalk virus." Clinical and vaccine immunology : CVI **19**(2): 284-287.

De Oliveira, L. H., N. Giglio, A. Ciapponi, S. Garcia Marti, M. Kuperman, N. J. Sanwogou, C. Ruiz-Matus and M. F. Marinho de Sousa (2013). "Temporal trends in diarrhea-related hospitalizations and deaths in children under age 5 before and after the introduction of the rotavirus vaccine in four Latin American countries." Vaccine **31 Suppl 3**: C99-108.

Desselberger, U. (2014). "Rotaviruses." Virus Res **190**: 75-96.

Díaz-Salinas, M. A., P. Romero, R. Espinosa, Y. Hoshino, S. López and C. F. Arias (2013). "The spike protein VP4 defines the endocytic pathway used by rotavirus to enter MA104 cells." Journal of virology **87**(3): 1658-1663.

Dubin, G., J.-F. Toussaint, J.-P. Cassart, B. Howe, D. Boyce, L. Friedland, R. Abu-Elyazeed, S. Poncelet, H. H. Han and S. Debrus (2013). "Investigation of a regulatory agency enquiry into potential porcine circovirus type 1 contamination of the human rotavirus vaccine, Rotarix: approach and outcome." Human vaccines & immunotherapeutics **9**(11): 2398-2408.

Duizer, E., K. J. Schwab, F. H. Neill, R. L. Atmar, M. P. Koopmans and M. K. Estes (2004). "Laboratory efforts to cultivate noroviruses." J Gen Virol **85**(Pt 1): 79-87.

El-Kamary, S. S., M. F. Pasetti, P. M. Mendelman, S. E. Frey, D. I. Bernstein, J. J. Treanor, J. Ferreira, W. H. Chen, R. Sublett, C. Richardson, R. F. Bargatze, M. B. Sztein and C. O. Tacket (2010). "Adjuvanted Intranasal Norwalk Virus-Like Particle Vaccine Elicits Antibodies and Antibody-Secreting Cells That Express Homing Receptors for Mucosal and Peripheral Lymphoid Tissues." The Journal of Infectious Diseases **202**(11): 1649-1658.

Emperador, D. M., D. E. Velasquez, C. F. Estivariz, B. Lopman, B. Jiang, U. Parashar, A. Anand and K. Zaman (2016). "Interference of Monovalent, Bivalent, and Trivalent Oral Poliovirus Vaccines on Monovalent Rotavirus Vaccine Immunogenicity in Rural Bangladesh." Clin Infect Dis **62**(2): 150-156.

Esposito, D. H., R. C. Holman, D. L. Haberling, J. E. Tate, L. J. Podewils, R. I. Glass and U. Parashar (2011). "Baseline estimates of diarrhea-associated mortality among United States children before rotavirus vaccine introduction." Pediatr Infect Dis J **30**(11): 942-947.

Estes, M. K. and J. Cohen (1989). "Rotavirus gene structure and function." Microbiol Rev **53**(4): 410-449.

Estes, M. K., K. Ettayebi, V. R. Tenge, K. Murakami, U. Karandikar, S.-C. Lin, B. V. Ayyar, N. W. Cortes-Penfield, K. Haga, F. H. Neill, A. R. Opekun, J. R. Broughman, X.-L. Zeng, S. E. Blutt, S. E. Crawford, S. Ramani, D. Y. Graham and R. L. Atmar (2019). "Human Norovirus Cultivation in Nontransformed Stem Cell-Derived Human Intestinal Enteroid Cultures: Success and Challenges." Viruses **11**(7): 638.

Ettayebi, K., S. E. Crawford, K. Murakami, J. R. Broughman, U. Karandikar, V. R. Tenge, F. H. Neill, S. E. Blutt, X.-L. Zeng, L. Qu, B. Kou, A. R. Opekun, D. Burrin, D. Y. Graham, S. Ramani, R. L. Atmar and M. K. Estes (2016). "Replication of human noroviruses in stem cell-derived human enteroids." Science (New York, N.Y.) **353**(6306): 1387-1393.

Fenaux, M., M. A. Cuadras, N. Feng, M. Jaimes and H. B. Greenberg (2006). "Extraintestinal spread and replication of a homologous EC rotavirus strain and a heterologous rhesus rotavirus in BALB/c mice." J Virol **80**(11): 5219-5232.

Fischer, T. K., C. Viboud, U. Parashar, M. Malek, C. Steiner, R. Glass and L. Simonsen (2007). "Hospitalizations and Deaths from Diarrhea and Rotavirus among Children <5 Years of Age in the United States, 1993–2003." The Journal of Infectious Diseases **195**(8): 1117-1125.

Fix, A. D., C. Harro, M. McNeal, L. Dally, J. Flores, G. Robertson, J. W. Boslego and S. Cryz (2015). "Safety and immunogenicity of a parenterally administered rotavirus VP8 subunit vaccine in healthy adults." Vaccine **33**(31): 3766-3772.

Free, R. J., B. F. Buss, S. Koirala, M. Ulses, A. Carlson, B. Loeck and T. Safranek (2019). "Successive Norovirus Outbreaks at an Event Center - Nebraska, October-November, 2017." MMWR Morb Mortal Wkly Rep **68**(28): 627-630.

Fu, C., Q. He, J. Xu, H. Xie, P. Ding, W. Hu, Z. Dong, X. Liu and M. Wang (2012). "Effectiveness of the Lanzhou lamb rotavirus vaccine against gastroenteritis among children." Vaccine **31**(1): 154-158.

Gardet, A., M. Breton, P. Fontanges, G. Trugnan and S. Chwetzoff (2006). "Rotavirus Spike Protein VP4 Binds to and Remodels Actin Bundles of the Epithelial Brush Border into Actin Bodies." J. Virol. **80**(8): 3947-3956.

GBD (2016). "Global, regional, and national life expectancy, all-cause mortality, and cause-specific mortality for 249 causes of death, 1980-2015: a systematic analysis for the Global Burden of Disease Study 2015." Lancet **388**(10053): 1459-1544.

Gentsch, J. R., A. R. Laird, B. Bielfelt, D. D. Griffin, K. Banyai, M. Ramachandran, V. Jain, N. A. Cunliffe, O. Nakagomi, C. D. Kirkwood, T. K. Fischer, U. D. Parashar, J. S. Bresee, B. Jiang and R. I. Glass (2005). "Serotype diversity and reassortment between human and animal rotavirus strains: implications for rotavirus vaccine programs." J Infect Dis **192 Suppl 1**: S146-159.

Ghosh, S. and N. Kobayashi (2014). "Exotic rotaviruses in animals and rotaviruses in exotic animals." Virusdisease **25**(2): 158-172.

Gilmartin, A. A. and W. A. Petri, Jr. (2015). "Exploring the role of environmental enteropathy in malnutrition, infant development and oral vaccine response." Philos Trans R Soc Lond B Biol Sci **370**(1671).

Glass, R. I., B. Jiang and U. Parashar (2018). "The future control of rotavirus disease: Can live oral vaccines alone solve the rotavirus problem?" Vaccine **36**(17): 2233-2236.

Glass, R. I., U. Parashar, M. Patel, J. Gentsch and B. Jiang (2014). "Rotavirus vaccines: successes and challenges." J Infect **68 Suppl 1**: S9-18.

Glass, R. I., U. D. Parashar and M. K. Estes (2009). "Norovirus gastroenteritis." The New England journal of medicine **361**(18): 1776-1785.

Gonzalez, A. M., M. S. Azevedo, K. Jung, A. Vlasova, W. Zhang and L. J. Saif (2010). "Innate immune responses to human rotavirus in the neonatal gnotobiotic piglet disease model." Immunology **131**(2): 242-256.

Graham, K. L., F. E. Fleming, P. Halasz, M. J. Hewish, H. S. Nagesha, I. H. Holmes, Y. Takada and B. S. Coulson (2005). "Rotaviruses interact with alpha4beta7 and alpha4beta1 integrins by binding the same integrin domains as natural ligands." J Gen Virol **86**(Pt 12): 3397-3408.

Graham, K. L., P. Halasz, Y. Tan, M. J. Hewish, Y. Takada, E. R. Mackow, M. K. Robinson and B. S. Coulson (2003). "Integrin-using rotaviruses bind alpha2beta1 integrin alpha2 I domain via VP4 DGE sequence and recognize alphaXbeta2 and alphaVbeta3 by using VP7 during cell entry." Journal of virology **77**(18): 9969-9978.

Graham, K. L., J. A. O'Donnell, Y. Tan, N. Sanders, E. M. Carrington, J. Allison and B. S. Coulson (2007). "Rotavirus infection of infant and young adult nonobese diabetic mice involves extraintestinal spread and delays diabetes onset." J Virol **81**(12): 6446-6458.

Graziano, V. R., F. C. Walker, E. A. Kennedy, J. Wei, K. Ettayebi, M. S. Simões, R. B. Filler, E. Hassan, L. L. Hsieh, A. O. Kolawole, C. E. Wobus, L. C. Lindesmith, R. S. Baric, M. K. Estes, R. C. Orchard, M. T. Baldridge and C. B. Wilen (2019). "CD300lf is the primary physiologic receptor of murine norovirus but not human norovirus. bioRxiv. doi: https://doi.org/10.1101/859025."

Green, K. (2013). Caliciviridae: The Noroviruses. Fields Virology. D. M. Knipe and P. M. Howley. Philadelphia, PA, Wolters Kluwer Health/Lippincott Williams & Wilkins. **1:** 582-608.

Green, K., S. Kaufman, B. Nagata, N. Chaimongkol, D. Kim, E. Levenson, C. Aburto, A. Berhele-Yardley, J. Johnson, A. Ferreira, I. Moore and S. Sosnovtsev (2019). Human norovirus targets epithelial cells in the small intestine. The 7th International Calicivirus Conference.

Greenberg, H. B. and M. K. Estes (2009). "Rotaviruses: from pathogenesis to vaccination." Gastroenterology **136**(6): 1939-1951.

Groome, M. J., A. Koen, A. Fix, N. Page, L. Jose, S. A. Madhi, M. McNeal, L. Dally, I. Cho, M. Power, J. Flores and S. Cryz (2017). "Safety and immunogenicity of a parenteral P2-VP8-P[8] subunit rotavirus vaccine in toddlers and infants in South Africa: a randomised, double-blind, placebo-controlled trial." The Lancet Infectious Diseases **17**(8): 843-853.

Guerrero, C. A., D. Bouyssounade, S. Zárate, P. Iša, T. López, R. Espinosa, P. Romero, E. Méndez, S. López and C. F. Arias (2002). "Heat Shock Cognate Protein 70 Is Involved in Rotavirus Cell Entry." Journal of Virology **76**(8): 4096-4102.

Gutiérrez, M., P. Isa, C. Sánchez-San Martin, J. Pérez-Vargas, R. Espinosa, C. F. Arias and S. López (2010). "Different rotavirus strains enter MA104 cells through different endocytic pathways: the role of clathrin-mediated endocytosis." Journal of virology **84**(18): 9161-9169.

Hall, A. J., R. I. Glass and U. D. Parashar (2016). "New insights into the global burden of noroviruses and opportunities for prevention." Expert Rev Vaccines: 1-3.

Haselhorst, T., T. Fiebig, J. C. Dyason, F. E. Fleming, H. Blanchard, B. S. Coulson and M. von Itzstein (2011). "Recognition of the GM3 ganglioside glycan by Rhesus rotavirus particles." Angewandte Chemie (International ed. in English) **50**(5): 1055-1058.

Haselhorst, T., F. E. Fleming, J. C. Dyason, R. D. Hartnell, X. Yu, G. Holloway, K. Santegoets, M. J. Kiefel, H. Blanchard, B. S. Coulson and M. von Itzstein (2009). "Sialic acid dependence in rotavirus host cell invasion." Nature chemical biology **5**(2): 91-93.

Holloway, G. and B. S. Coulson (2013). "Innate cellular responses to rotavirus infection." J Gen Virol **94**(Pt 6): 1151-1160.

Hu, L., S. E. Crawford, R. Czako, N. W. Cortes-Penfield, D. F. Smith, J. Le Pendu, M. K. Estes and B. V. V. Prasad (2012). "Cell attachment protein VP8* of a human rotavirus specifically interacts with A-type histo-blood group antigen." Nature **485**(7397): 256-259.

Huang, P., T. Farkas, S. Marionneau, W. Zhong, N. Ruvoën-Clouet, A. L. Morrow, M. Altaye, L. K. Pickering, D. S. Newburg, J. LePendu and X. Jiang (2003). "Noroviruses bind to human ABO, Lewis, and secretor histo-blood group antigens: identification of 4 distinct strain-specific patterns." The Journal of infectious diseases **188**(1): 19-31.

Huang, P., M. Xia, M. Tan, W. Zhong, C. Wei, L. Wang, A. Morrow and X. Jiang (2012). "Spike protein VP8* of human rotavirus recognizes histo-blood group antigens in a type-specific manner." Journal of virology **86**(9): 4833-4843.

Huda, M. N., Z. Lewis, K. M. Kalanetra, M. Rashid, S. M. Ahmad, R. Raqib, F. Qadri, M. A. Underwood, D. A. Mills and C. B. Stephensen (2014). "Stool Microbiota and Vaccine Responses of Infants." Pediatrics.

Hutson, A. M., F. Airaud, J. LePendu, M. K. Estes and R. L. Atmar (2005). "Norwalk virus infection associates with secretor status genotyped from sera." J Med Virol **77**(1): 116-120.

Imbert-Marcille, B.-M. M., L. Barbé, M. Dupé, B. Le Moullac-Vaidye, B. Besse, C. Peltier, N. Ruvoën-Clouet and J. Le Pendu (2014). "A FUT2 gene common polymorphism determines resistance to rotavirus A of the P[8] genotype." The Journal of infectious diseases **209**(8): 1227-1230.

Jaimes, M. C., N. Feng and H. B. Greenberg (2005). "Characterization of Homologous and Heterologous Rotavirus-Specific T-Cell Responses in Infant and Adult Mice." Journal of Virology **79**(8): 4568-4579.

Jain, S. and M. J. Haydel (2019). Child Intussusception. StatPearls [Internet], Treasure Island (FL): StatPearls Publishing.

Jiang, X., M. Wang, D. Y. Graham and M. K. Estes (1992). "Expression, self-assembly, and antigenicity of the Norwalk virus capsid protein." Journal of virology **66**(11): 6527-6532.

Jones, M. K., M. Watanabe, S. Zhu, C. L. Graves, L. R. Keyes, K. R. Grau, M. B. Gonzalez-Hernandez, N. M. Iovine, C. E. Wobus, J. Vinjé, S. A. Tibbetts, S. M. Wallet and S. M. Karst (2014). "Enteric bacteria promote human and mouse norovirus infection of B cells." Science **346**(6210): 755-759.

Jung, K., Q. Wang, Y. Kim, K. Scheuer, Z. Zhang, Q. Shen, K.-O. Chang and L. J. Saif (2012). "The Effects of Simvastatin or Interferon-α on Infectivity of Human Norovirus Using a Gnotobiotic Pig Model for the Study of Antivirals." PLOS ONE **7**(7): e41619.

Jung, K., Q. Wang, Y. Kim, K. Scheuer, Z. Zhang, Q. Shen, K. O. Chang and L. J. Saif (2012). "The effects of simvastatin or interferon-alpha on infectivity of human norovirus using a gnotobiotic pig model for the study of antivirals." PLoS One **7**(7): e41619.

Karandikar, U. C., S. E. Crawford, N. J. Ajami, K. Murakami, B. Kou, K. Ettayebi, G. A. Papanicolaou, U. Jongwutiwes, M.-A. Perales, J. Shia, D. Mercer, M. J. Finegold, J. Vinjé, R. L. Atmar and M. K. Estes (2016). "Detection of human norovirus in intestinal biopsies from immunocompromised transplant patients." The Journal of general virology **97**(9): 2291-2300.

Keusch, G. T., C. F. Walker, J. K. Das, S. Horton and D. Habte (2016). Diarrheal Diseases. Reproductive, Maternal, Newborn, and Child Health: Disease Control Priorities, Third Edition (Volume 2). R. E. Black, R. Laxminarayan, M. Temmerman and N. Walker. Washington (DC).

Kim, L., D. Liebowitz, K. Lin, K. Kasparek, M. F. Pasetti, S. J. Garg, K. Gottlieb, G. Trager and S. N. Tucker (2018). "Safety and immunogenicity of an oral tablet norovirus vaccine, a phase I randomized, placebo-controlled trial." JCI Insight **3**(13).

Kim, S.-H., S. Chen, X. Jiang, K. Y. Green and S. K. Samal (2014). "Newcastle disease virus vector producing human norovirus-like particles induces serum, cellular, and mucosal immune responses in mice." Journal of virology **88**(17): 9718-9727.

Koch, J., T. Harder, R. von Kries and O. Wichmann (2017). "Risk of Intussusception After Rotavirus Vaccination." Deutsches Arzteblatt international **114**(15): 255-262.

Kocher, J., T. Bui, E. Giri-Rachman, K. Wen, G. Li, X. Yang, F. Liu, M. Tan, M. Xia, W. Zhong, X. Jiang and L. Yuan (2014). "Intranasal P particle vaccine provided partial cross-variant protection against human GII.4 norovirus diarrhea in gnotobiotic pigs." J Virol **88**(17): 9728-9743.

Kompithra, R. Z., A. Paul, D. Manoharan, S. Babji, R. Sarkar, L. G. Mathew and G. Kang (2014). "Immunogenicity of a three dose and five dose oral human rotavirus vaccine (RIX4414) schedule in south Indian infants." Vaccine **32**: A129-A133.

Kotloff, K. L., J. P. Nataro, W. C. Blackwelder, D. Nasrin, T. H. Farag, S. Panchalingam, Y. Wu, S. O. Sow, D. Sur and R. F. Breiman (2013). "Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study." Lancet **382**(9888): 209-222.

Kraft, L. M. (1958). "Observations on the control and natural history of epidemic diarrhea of infant mice (EDIM)." Yale J Biol Med **31**(3): 121-137.

Kroneman, A., E. Vega, H. Vennema, J. Vinje, P. A. White, G. Hansman, K. Green, V. Martella, K. Katayama and M. Koopmans (2013). "Proposal for a unified norovirus nomenclature and genotyping." Arch Virol **158**(10): 2059-2068.

Kroneman, A., E. Vega, H. Vennema, J. Vinjé, P. A. White, G. Hansman, K. Green, V. Martella, K. Katayama and M. Koopmans (2013). "Proposal for a unified norovirus nomenclature and genotyping." Archives of virology **158**(10): 2059-2068.

Kulkarni, P. S., S. Desai, T. Tewari, A. Kawade, N. Goyal, B. S. Garg, D. Kumar, S. Kanungo, V. Kamat, G. Kang, A. Bavdekar, S. Babji, S. Juvekar, B. Manna, S. Dutta, R. Angurana, D. Dewan, A. Dharmadhikari, J. K. Zade, R. M. Dhere, A. Fix, M. Power, V. Uprety, V. Parulekar, I. Cho, T. R. Chandola, V. K. Kedia, A. Raut and J. Flores (2017). "A randomized Phase III clinical trial to assess the efficacy of a bovine-human reassortant pentavalent rotavirus vaccine in Indian infants." Vaccine **35**(45): 6228-6237.

Kumar, A., A. N. Vlasova, L. Deblais, H.-C. Huang, A. Wijeratne, S. Kandasamy, D. D. Fischer, S. N. Langel, F. C. Paim, M. A. Alhamo, L. Shao, L. J. Saif and G. Rajashekara (2018). "Impact of nutrition and

rotavirus infection on the infant gut microbiota in a humanized pig model." BMC gastroenterology **18**(1): 93-93.

Lamberti, L. M., S. Ashraf, C. L. Walker and R. E. Black (2016). "A Systematic Review of the Effect of Rotavirus Vaccination on Diarrhea Outcomes Among Children Younger Than 5 Years." Pediatr Infect Dis J **35**(9): 992-998.

Lamrani, A., P. Tubert-Bitter, C. Hill and S. Escolano (2017). "A benefit-risk analysis of rotavirus vaccination, France, 2015." Euro surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin **22**(50): 17-00041.

Lanata, C. F., C. L. Fischer-Walker, A. C. Olascoaga, C. X. Torres, M. J. Aryee and R. E. Black (2013). "Global causes of diarrheal disease mortality in children <5 years of age: a systematic review." PLoS One **8**(9): e72788.

Ledent, E., H. Arlegui, H. Buyse, P. Basile, N. Karkada, N. Praet and G. Nachbaur (2018). "Benefit Versus Risk Assessment of Rotavirus Vaccination in France: A Simulation and Modeling Analysis." BioDrugs **32**(2): 139-152.

Ledent, E., A. Lieftucht, H. Buyse, K. Sugiyama, M. McKenna and K. Holl (2016). "Post-Marketing Benefit-Risk Assessment of Rotavirus Vaccination in Japan: A Simulation and Modelling Analysis." Drug Saf **39**(3): 219-230.

Lee, B., M. Carmolli, D. M. Dickson, E. R. Colgate, S. A. Diehl, M. I. Uddin, S. Islam, M. Hossain, T. A. Rafique, T. R. Bhuiyan, M. Alam, U. Nayak, J. C. Mychaleckyj, M. M. McNeal, W. A. Petri, F. Qadri, R. Haque and B. D. Kirkpatrick (2018). "Rotavirus-Specific Immunoglobulin A Responses Are Impaired and Serve as a Suboptimal Correlate of Protection Among Infants in Bangladesh." Clin Infect Dis **67**(2): 186-192.

Lei, S., A. Ramesh, E. Twitchell, K. Wen, T. Bui, M. Weiss, X. Yang, J. Kocher, G. Li, E. Giri-Rachman, N. V. Trang, X. Jiang, E. P. Ryan and L. Yuan (2016). "High Protective Efficacy of Probiotics and Rice Bran against Human Norovirus Infection and Diarrhea in Gnotobiotic Pigs." Frontiers in microbiology **7**: 1699-1699.

Lei, S., J. Ryu, K. Wen, E. Twitchell, T. Bui, A. Ramesh, M. Weiss, G. Li, H. Samuel, S. Clark-Deener, X. Jiang, K. Lee and L. Yuan (2016). "Increased and prolonged human norovirus infection in RAG2/IL2RG deficient gnotobiotic pigs with severe combined immunodeficiency." Scientific Reports **6**(1): 25222.

Lei, S., H. Samuel, E. Twitchell, T. Bui, A. Ramesh, K. Wen, M. Weiss, G. Li, X. Yang, X. Jiang and L. Yuan (2016). "Enterobacter cloacae inhibits human norovirus infectivity in gnotobiotic pigs." Sci Rep **6**: 25017.

Lei, S., E. L. Twitchell, A. K. Ramesh, T. Bui, E. Majette, C. M. Tin, R. Avery, G. Arango-Argoty, L. Zhang, S. Becker-Dreps, M. A. Azcarate-Peril, X. Jiang and L. Yuan (2019). "Enhanced GII.4 human norovirus infection in gnotobiotic pigs transplanted with a human gut microbiota." Journal of General Virology **100**(11): 1530-1540.

Lindesmith, L., C. Moe, J. LePendu, J. A. Frelinger, J. Treanor and R. S. Baric (2005). "Cellular and Humoral Immunity following Snow Mountain Virus Challenge." Journal of Virology **79**(5): 2900.

Lindesmith, L., C. Moe, S. Marionneau, N. Ruvoen, X. Jiang, L. Lindblad, P. Stewart, J. LePendu and R. Baric (2003). "Human susceptibility and resistance to Norwalk virus infection." Nat Med **9**(5): 548-553.

Liu, Y., P. Huang, M. Tan, Y. Liu, J. Biesiada, J. Meller, A. A. Castello, B. Jiang and X. Jiang (2012). "Rotavirus VP8*: phylogeny, host range, and interaction with histo-blood group antigens." Journal of virology **86**(18): 9899-9910.

Lopez, S. and C. F. Arias (2006). "Early steps in rotavirus cell entry." Curr Top Microbiol Immunol **309**: 39-66.

Lucero, Y., R. Vidal and M. O'Ryan G (2018). "Norovirus vaccines under development." Vaccine **36**(36): 5435-5441.

Ma, X., D.-d. D. Li, X.-M. M. Sun, Y.-Q. Q. Guo, J.-Y. Y. Xiang, W.-H. H. Wang, L.-X. X. Zhang, Q.-J. J. Gu and Z.-J. J. Duan (2015). "Binding Patterns of Rotavirus Genotypes P[4], P[6], and P[8] in China with Histo-Blood Group Antigens." PloS one **10**(8): e0134584.

Malm, M., K. Tamminen, S. Lappalainen, T. Vesikari and V. Blazevic (2016). "Rotavirus Recombinant VP6 Nanotubes Act as an Immunomodulator and Delivery Vehicle for Norovirus Virus-Like Particles." Journal of Immunology Research **2016**: 13.

Marionneau, S., N. Ruvoen, B. Le Moullac-Vaidye, M. Clement, A. Cailleau-Thomas, G. Ruiz-Palacois, P. Huang, X. Jiang and J. Le Pendu (2002). "Norwalk virus binds to histo-blood group antigens present on gastroduodenal epithelial cells of secretor individuals." Gastroenterology **122**(7): 1967-1977.

Marionneau, S., N. Ruvoën, B. Moullac–Vaidye, M. Clement, A. Cailleau–Thomas, G. Ruiz–Palacois, P. Huang, X. Jiang and J. Pendu (2002). "Norwalk virus binds to histo-blood group antigens present on gastroduodenal epithelial cells of secretor individuals." Gastroenterology **122**(7): 1967-1977.

Matthijnssens, J., M. Ciarlet, E. Heiman, I. Arijs, T. Delbeke, S. M. McDonald, E. A. Palombo, M. Iturriza-Gomara, P. Maes, J. T. Patton, M. Rahman and M. Van Ranst (2008). "Full genome-based classification of rotaviruses reveals a common origin between human Wa-Like and porcine rotavirus strains and human DS-1-like and bovine rotavirus strains." J Virol **82**(7): 3204-3219.

Matthijnssens, J., M. Ciarlet, S. M. McDonald, H. Attoui, K. Banyai, J. R. Brister, J. Buesa, M. D. Esona, M. K. Estes, J. R. Gentsch, M. Iturriza-Gomara, R. Johne, C. D. Kirkwood, V. Martella, P. P. Mertens, O. Nakagomi, V. Parreno, M. Rahman, F. M. Ruggeri, L. J. Saif, N. Santos, A. Steyer, K. Taniguchi, J. T. Patton, U. Desselberger and M. Van Ranst (2011). "Uniformity of rotavirus strain nomenclature proposed by the Rotavirus Classification Working Group (RCWG)." Arch Virol **156**(8): 1397-1413.

Matthijnssens, J., M. Ciarlet, M. Rahman, H. Attoui, K. Banyai, M. K. Estes, J. R. Gentsch, M. Iturriza-Gomara, C. D. Kirkwood, V. Martella, P. P. Mertens, O. Nakagomi, J. T. Patton, F. M. Ruggeri, L. J. Saif, N. Santos, A. Steyer, K. Taniguchi, U. Desselberger and M. Van Ranst (2008). "Recommendations for the classification of group A rotaviruses using all 11 genomic RNA segments." Arch Virol **153**(8): 1621-1629.

Matthijnssens, J., P. H. Otto, M. Ciarlet, U. Desselberger, M. Van Ranst and R. Johne (2012). "VP6-sequence-based cutoff values as a criterion for rotavirus species demarcation." Arch Virol **157**(6): 1177-1182.

Mattion, N. M., D. B. Mitchell, G. W. Both and M. K. Estes (1991). "Expression of rotavirus proteins encoded by alternative open reading frames of genome segment 11." Virology **181**(1): 295-304.

Mattison, C. P., C. V. Cardemil and A. J. Hall (2018). "Progress on norovirus vaccine research: public health considerations and future directions." Expert review of vaccines **17**(9): 773-784.

McClain, B., E. Settembre, B. R. Temple, A. R. Bellamy and S. C. Harrison (2010). "X-ray crystal structure of the rotavirus inner capsid particle at 3.8 A resolution." J Mol Biol **397**(2): 587-599.

McNulty, M. S., W. L. Curran and J. B. McFerran (1976). "The morphogenesis of a cytopathic bovine rotavirus in Madin-Darby bovine kidney cells." J Gen Virol **33**(3): 503-508.

Messner, M. J., P. Berger and S. P. Nappier (2014). "Fractional poisson--a simple dose-response model for human norovirus." Risk Anal **34**(10): 1820-1829.

Mihalov-Kovacs, E., A. Gellert, S. Marton, S. L. Farkas, E. Feher, M. Oldal, F. Jakab, V. Martella and K. Banyai (2015). "Candidate new rotavirus species in sheltered dogs, Hungary." Emerg Infect Dis **21**(4): 660-663.

Minami, K., A. Tamura, Y. Komori, T. Yanagawa, G. Izumi, N. Kioka and N. Yoshikawa (2007). "Acute encephalopathy and rhabdomyolysis following rotavirus gastroenteritis." J Paediatr Child Health **43**(1-2): 90-91.

Monroe, S. S. (2011). "Control and prevention of viral gastroenteritis." Emerging infectious diseases **17**(8): 1347-1348.

Mossel, E. C. and R. F. Ramig (2003). "A lymphatic mechanism of rotavirus extraintestinal spread in the neonatal mouse." J Virol **77**(22): 12352-12356.

Nordgren, J., S. Sharma, F. Bucardo, W. Nasir, G. Günaydın, D. Ouermi, L. W. Nitiema, S. Becker-Dreps, J. Simpore, L. Hammarström, G. Larson and L. Svensson (2014). "Both Lewis and secretor status mediate susceptibility to rotavirus infections in a rotavirus genotype-dependent manner." Clinical infectious diseases : an official publication of the Infectious Diseases Society of America **59**(11): 1567-1573.

Parashar, U. D., A. Burton, C. Lanata, C. Boschi-Pinto, K. Shibuya, D. Steele, M. Birmingham and R. I. Glass (2009). "Global Mortality Associated with Rotavirus Disease among Children in 2004." The Journal of Infectious Diseases **200**(Supplement_1): S9-S15.

Parashar, U. D., M. M. Cortese, D. C. Payne, B. Lopman, C. Yen and J. E. Tate (2015). "Value of post-licensure data on benefits and risks of vaccination to inform vaccine policy: The example of rotavirus vaccines." Vaccine **33**: D55-D59.

Park, B. J., S. T. Jung, C. S. Choi, J. Myoung, H. S. Ahn, S. H. Han, Y. H. Kim, H. J. Go, J. B. Lee, S. Y. Park, C. S. Song, S. W. Lee and I. S. Choi (2018). "Pathogenesis of Human Norovirus Genogroup II Genotype 4 in Post-Weaning Gnotobiotic Pigs." J Microbiol Biotechnol **28**(12): 2133-2140.

Patel, M., R. I. Glass, B. Jiang, M. Santosham, B. Lopman and U. Parashar (2013). "A systematic review of anti-rotavirus serum IgA antibody titer as a potential correlate of rotavirus vaccine efficacy." J Infect Dis **208**(2): 284-294.

Patel, M., R. I. Glass, B. Jiang, M. Santosham, B. Lopman and U. Parashar (2013). "A systematic review of anti-rotavirus serum IgA antibody titer as a potential correlate of rotavirus vaccine efficacy." J Infect Dis **208**(2): 284-294.

Patel, M., A. L. Shane, U. D. Parashar, B. Jiang, J. R. Gentsch and R. I. Glass (2009). "Oral rotavirus vaccines: how well will they work where they are needed most?" The Journal of infectious diseases **200 Suppl 1**(0 1): S39-S48.

Patel, M. M., V. Pitzer, W. J. Alonso, D. Vera, B. Lopman, J. Tate, C. Viboud and U. D. Parashar (2013). "Global Seasonality of Rotavirus Disease." The Pediatric infectious disease journal **32**(4): e134-e147.

Pecenka, C., F. Debellut, N. Bar-Zeev, P. Anwari, J. Nonvignon, M. Shamsuzzaman and A. Clark (2018). "Re-evaluating the cost and cost-effectiveness of rotavirus vaccination in Bangladesh, Ghana, and Malawi: A comparison of three rotavirus vaccines." Vaccine **36**(49): 7472-7478.

Pendleton, A., M. Galic, C. Clarke, S. P. Ng, E. Ledesma, G. Ramakrishnan and Y. Liu (2013). "Impact of rotavirus vaccination in Australian children below 5 years of age: a database study." Hum Vaccin Immunother **9**(8): 1617-1625.

Prince, D. S., C. Astry, S. Vonderfecht, G. Jakab, F. M. Shen and R. H. Yolken (1986). "Aerosol transmission of experimental rotavirus infection." Pediatr Infect Dis **5**(2): 218-222.

Ramani, S., M. K. Estes and R. L. Atmar (2016). "Correlates of Protection against Norovirus Infection and Disease-Where Are We Now, Where Do We Go?" PLoS pathogens **12**(4): e1005334-e1005334.

Ramani, S., L. Hu, B. V. Venkataram Prasad and M. K. Estes (2016). "Diversity in Rotavirus-Host Glycan Interactions: A "Sweet" Spectrum." Cell Mol Gastroenterol Hepatol **2**(3): 263-273.

Ramani, S., N. Mamani, R. Villena, A. S. Bandyopadhyay, C. Gast, A. Sato, D. Laucirica, R. Clemens, M. K. Estes and M. L. O'Ryan (2016). "Rotavirus Serum IgA Immune Response in Children Receiving Rotarix Coadministered With bOPV or IPV." Pediatr Infect Dis J **35**(10): 1137-1139.

Ramani, S., F. H. Neill, A. R. Opekun, M. A. Gilger, D. Y. Graham, M. K. Estes and R. L. Atmar (2015). "Mucosal and Cellular Immune Responses to Norwalk Virus." The Journal of Infectious Diseases **212**(3): 397-405.

Ramig, R. F. (2004). "Pathogenesis of intestinal and systemic rotavirus infection." J Virol **78**(19): 10213-10220.

Ramirez, K., R. Wahid, C. Richardson, R. F. Bargatze, S. S. El-Kamary, M. B. Sztein and M. F. Pasetti (2012). "Intranasal vaccination with an adjuvanted Norwalk virus-like particle vaccine elicits antigen-specific B memory responses in human adult volunteers." Clin Immunol **144**(2): 98-108.

Rappuoli, R., M. Pizza, G. Del Giudice and E. De Gregorio (2014). "Vaccines, new opportunities for a new society." Proceedings of the National Academy of Sciences of the United States of America **111**(34): 12288-12293.

RCWG (2015). Minutes of the 7th Rotavirus Classification Working Group (RCWG) Meeting. International Double Stranded RNA Virus Symposium, Goa, India.

RCWG. (2019). "Rotavirus Classification Working Group: RCWG." Retrieved 09/15/2019, 2019, from https://rega.kuleuven.be/cev/viralmetagenomics/virus-classification/rcwg.

Reeck, A., O. Kavanagh, M. K. Estes, A. R. Opekun, M. A. Gilger, D. Y. Graham and R. L. Atmar (2010). "Serologic Correlate of Protection against Norovirus-Induced Gastroenteritis." The Journal of infectious diseases **202**(8): 1212-1218.

Riddle, M. S. and R. I. Walker (2016). "Status of vaccine research and development for norovirus." Vaccine **34**(26): 2895-2899.

Rodriguez, M. R., K. Monte, L. B. Thackray and D. J. Lenschow (2014). "ISG15 Functions as an Interferon-Mediated Antiviral Effector Early in the Murine Norovirus Life Cycle." Journal of Virology **88**(16): 9277.

Ruiz-Palacios, G. M., I. Perez-Schael, F. R. Velazquez, H. Abate, T. Breuer, S. C. Clemens, B. Cheuvart, F. Espinoza, P. Gillard, B. L. Innis, Y. Cervantes, A. C. Linhares, P. Lopez, M. Macias-Parra, E. Ortega-Barria, V. Richardson, D. M. Rivera-Medina, L. Rivera, B. Salinas, N. Pavia-Ruz, J. Salmeron, R. Ruttimann, J. C. Tinoco, P. Rubio, E. Nunez, M. L. Guerrero, J. P. Yarzabal, S. Damaso, N. Tornieporth, X. Saez-Llorens, R. F. Vergara, T. Vesikari, A. Bouckenooghe, R. Clemens, B. De Vos and M. O'Ryan (2006). "Safety and efficacy of an attenuated vaccine against severe rotavirus gastroenteritis." N Engl J Med **354**(1): 11-22.

Saif, L., L. Ward, L. Yuan, B. Rosen and T. To (1996). "The gnotobiotic pig as a model for studies of disease pathogenesis and immunity to rotavirus." Archives of virology. Supplementum **12**: 153-161.

Saif, L. J., L. A. Ward, L. Yuan, B. I. Rosen and T. L. To (1996). The gnotobiotic piglet as a model for studies of disease pathogenesis and immunity to human rotaviruses. Viral Gastroenteritis, Vienna, Springer Vienna.

Schmidt, P. J. (2015). "Norovirus Dose-Response: Are Currently Available Data Informative Enough to Determine How Susceptible Humans Are to Infection from a Single Virus?" Risk Anal **35**(7): 1364-1383.

Soriano-Gabarro, M., J. Mrukowicz, T. Vesikari and T. Verstraeten (2006). "Burden of rotavirus disease in European Union countries." Pediatr Infect Dis J **25**(1 Suppl): S7-S11.

Souza, M., M. S. Azevedo, K. Jung, S. Cheetham and L. J. Saif (2008). "Pathogenesis and immune responses in gnotobiotic calves after infection with the genogroup II.4-HS66 strain of human norovirus." J Virol **82**(4): 1777-1786.

Souza, M., S. M. Cheetham, M. S. P. Azevedo, V. Costantini and L. J. Saif (2007). "Cytokine and Antibody Responses in Gnotobiotic Pigs after Infection with Human Norovirus Genogroup II.4 (HS66 Strain)." Journal of Virology **81**(17): 9183-9192.

Sundararajan, A., M. Y. Sangster, S. Frey, R. L. Atmar, W. H. Chen, J. Ferreira, R. Bargatze, P. M. Mendelman, J. J. Treanor and D. J. Topham (2015). "Robust mucosal-homing antibody-secreting B cell responses induced by intramuscular administration of adjuvanted bivalent human norovirus-like particle vaccine." Vaccine **33**(4): 568-576.

Takanashi, S., Q. Wang, N. Chen, Q. Shen, K. Jung, Z. Zhang, M. Yokoyama, L. C. Lindesmith, R. S. Baric and L. J. Saif (2011). "Characterization of emerging GII.g/GII.12 noroviruses from a gastroenteritis outbreak in the United States in 2010." J Clin Microbiol **49**(9): 3234-3244.

Tamminen, K., L. Huhti, T. Koho, S. Lappalainen, V. P. Hytönen, T. Vesikari and V. Blazevic (2012). "A comparison of immunogenicity of norovirus GII-4 virus-like particles and P-particles." Immunology **135**(1): 89-99.

Tan, M. and X. Jiang (2005). "The P Domain of Norovirus Capsid Protein Forms a Subviral Particle That Binds to Histo-Blood Group Antigen Receptors." Journal of Virology **79**(22): 14017.

Tan, M. and X. Jiang (2010). "Norovirus Gastroenteritis, Carbohydrate Receptors, and Animal Models." PLOS Pathogens **6**(8): e1000983.

Tan, M. and X. Jiang (2012). "Norovirus P particle: a subviral nanoparticle for vaccine development against norovirus, rotavirus and influenza virus." Nanomedicine (London, England) **7**(6): 889-897.

Tan, M. and X. Jiang (2019). "Norovirus Capsid Protein-Derived Nanoparticles and Polymers as Versatile Platforms for Antigen Presentation and Vaccine Development." Pharmaceutics **11**(9): 472.

Tate, J. E., A. H. Burton, C. Boschi-Pinto and U. D. Parashar (2016). "Global, Regional, and National Estimates of Rotavirus Mortality in Children <5 Years of Age, 2000-2013." Clin Infect Dis **62 Suppl 2**: S96-s105.

Tate, J. E., A. H. Burton, C. Boschi-Pinto, A. D. Steele, J. Duque and U. D. Parashar (2012). "2008 estimate of worldwide rotavirus-associated mortality in children younger than 5 years before the introduction of

universal rotavirus vaccination programmes: a systematic review and meta-analysis." The Lancet Infectious Diseases **12**(2): 136-141.

Taube, S., A. O. Kolawole, M. Hohne, J. E. Wilkinson, S. A. Handley, J. W. Perry, L. B. Thackray, R. Akkina and C. E. Wobus (2013). "A mouse model for human norovirus." MBio **4**(4).

Teunis, P. F., C. L. Moe, P. Liu, S. E. Miller, L. Lindesmith, R. S. Baric, J. Le Pendu and R. L. Calderon (2008). "Norwalk virus: how infectious is it?" J Med Virol **80**(8): 1468-1476.

Tin, C. M., L. Yuan, R. J. Dexter, G. I. Parra, T. Bui, K. Y. Green and S. V. Sosnovtsev (2017). "A Luciferase Immunoprecipitation System (LIPS) assay for profiling human norovirus antibodies." Journal of Virological Methods **248**: 116-129.

Torres-Flores, J. M., D. Silva-Ayala, M. A. Espinoza, S. Lopez and C. F. Arias (2015). "The tight junction protein JAM-A functions as coreceptor for rotavirus entry into MA104 cells." Virology **475**: 172-178.

Treanor, J. J., R. L. Atmar, S. E. Frey, R. Gormley, W. H. Chen, J. Ferreira, R. Goodwin, A. Borkowski, R. Clemens and P. M. Mendelman (2014). "A Novel Intramuscular Bivalent Norovirus Virus-Like Particle Vaccine Candidate—Reactogenicity, Safety, and Immunogenicity in a Phase 1 Trial in Healthy Adults." The Journal of Infectious Diseases **210**(11): 1763-1771.

Twitchell, E. L., C. Tin, K. Wen, H. Zhang, S. Becker-Dreps, M. A. Azcarate-Peril, S. Vilchez, G. Li, A. Ramesh, M. Weiss, S. Lei, T. Bui, X. Yang, S. Schultz-Cherry and L. Yuan (2016). "Modeling human enteric dysbiosis and rotavirus immunity in gnotobiotic pigs." Gut Pathogens **8**: 51.

Twitchell, E. L., C. Tin, K. Wen, H. Zhang, S. Becker-Dreps, M. A. Azcarate-Peril, S. Vilchez, G. Li, A. Ramesh, M. Weiss, S. Lei, T. Bui, X. Yang, S. Schultz-Cherry and L. Yuan (2016). "Modeling human enteric dysbiosis and rotavirus immunity in gnotobiotic pigs." Gut Pathog **8**: 51.

Uhnoo, I., M. Riepenhoff-Talty, T. Dharakul, P. Chegas, J. E. Fisher, H. B. Greenberg and P. L. Ogra (1990). "Extramucosal spread and development of hepatitis in immunodeficient and normal mice infected with rhesus rotavirus." J Virol **64**(1): 361-368.

Valdez, Y., E. M. Brown and B. B. Finlay (2014). "Influence of the microbiota on vaccine effectiveness." Trends Immunol **35**(11): 526-537.

Valdez, Y., E. M. Brown and B. B. Finlay (2014). "Influence of the microbiota on vaccine effectiveness." Trends in Immunology **35**(11): 526-537.

Valenzuela, S., J. Pizarro, A. M. Sandino, M. Vasquez, J. Fernandez, O. Hernandez, J. Patton and E. Spencer (1991). "Photoaffinity labeling of rotavirus VP1 with 8-azido-ATP: identification of the viral RNA polymerase." J Virol **65**(7): 3964-3967.

Van Damme, P., C. Giaquinto, F. Huet, L. Gothefors, M. Maxwell and M. Van der Wielen (2007). "Multicenter prospective study of the burden of rotavirus acute gastroenteritis in Europe, 2004-2005: the REVEAL study." J Infect Dis **195 Suppl 1**: S4-s16.

Van Trang, N., H. T. Vu, N. T. Le, P. Huang, X. Jiang and D. D. Anh (2014). "Association between norovirus and rotavirus infection and histo-blood group antigen types in Vietnamese children." Journal of clinical microbiology **52**(5): 1366-1374.

Velasquez, D. E., U. Parashar and B. Jiang (2018). "Decreased performance of live attenuated, oral rotavirus vaccines in low-income settings: causes and contributing factors." Expert Rev Vaccines **17**(2): 145-161.

Velazquez, F. R., D. O. Matson, J. J. Calva, L. Guerrero, A. L. Morrow, S. Carter-Campbell, R. I. Glass, M. K. Estes, L. K. Pickering and G. M. Ruiz-Palacios (1996). "Rotavirus infection in infants as protection against subsequent infections." N Engl J Med **335**(14): 1022-1028.

Vesikari, T. (2012). "Rotavirus vaccination: a concise review." Clin Microbiol Infect **18 Suppl 5**: 57-63.

Vesikari, T., D. O. Matson, P. Dennehy, P. Van Damme, M. Santosham, Z. Rodriguez, M. J. Dallas, J. F. Heyse, M. G. Goveia, S. B. Black, H. R. Shinefield, C. D. C. Christie, S. Ylitalo, R. F. Itzler, M. L. Coia, M. T. Onorato, B. A. Adeyi, G. S. Marshall, L. Gothefors, D. Campens, A. Karvonen, J. P. Watt, K. L. O'Brien, M. J. DiNubile, H. F. Clark, J. W. Boslego, P. A. Offit and P. M. Heaton (2006). "Safety and Efficacy of a Pentavalent Human–Bovine (WC3) Reassortant Rotavirus Vaccine." New England Journal of Medicine **354**(1): 23-33.

Victoria, J. G., C. Wang, M. S. Jones, C. Jaing, K. McLoughlin, S. Gardner and E. L. Delwart (2010). "Viral nucleic acids in live-attenuated vaccines: detection of minority variants and an adventitious virus." Journal of virology **84**(12): 6033-6040.

Vinje, J. (2015). "Advances in laboratory methods for detection and typing of norovirus." J Clin Microbiol **53**(2): 373-381.

Wang, X., Z. Ku, W. Dai, T. Chen, X. Ye, C. Zhang, Y. Zhang, Q. Liu, X. Jin and Z. Huang (2015). "A bivalent virus-like particle based vaccine induces a balanced antibody response against both enterovirus 71 and norovirus in mice." Vaccine **33**(43): 5779-5785.

Ward, L. A., B. I. Rosen, L. Yuan and L. J. Saif (1996). "Pathogenesis of an attenuated and a virulent strain of group A human rotavirus in neonatal gnotobiotic pigs." Journal of General Virology **77**(7): 1431-1441.

Ward, R. L., D. I. Bernstein, E. C. Young, J. R. Sherwood, D. R. Knowlton and G. M. Schiff (1986). "Human rotavirus studies in volunteers: determination of infectious dose and serological response to infection." J Infect Dis **154**(5): 871-880.

Wen, K., G. Li, X. Yang, T. Bui, M. Bai, F. Liu, J. Kocher and L. Yuan (2012). "CD4+ CD25- FoxP3+ regulatory cells are the predominant responding regulatory T cells after human rotavirus infection or vaccination in gnotobiotic pigs." Immunology **137**(2): 160-171.

Wen, X., D. Cao, R. W. Jones, J. Li, S. Szu and Y. Hoshino (2012). "Construction and characterization of human rotavirus recombinant VP8* subunit parenteral vaccine candidates." Vaccine **30**(43): 6121-6126.

Wen, X., K. Wen, D. Cao, G. Li, R. W. Jones, J. Li, S. Szu, Y. Hoshino and L. Yuan (2014). "Inclusion of a universal tetanus toxoid CD4(+) T cell epitope P2 significantly enhanced the immunogenicity of recombinant rotavirus ΔVP8* subunit parenteral vaccines." Vaccine **32**(35): 4420-4427.

WHO (2009). "Rotavirus vaccines:an update." Wkly Epidemiol Rec **84**(50): 533-540.

WHO (2013) "Weekly Epidemiological Record;2013 IIS 4640-P3;ISSN 0049-8114."

WHO (2013). "World Health Organization. Rotavirus vaccines: WHO position paper." Wkly Epidemiol Rec **88**(05): 49-64.

WHO. (2018, 3/8/2018). "Data, statistics and graphics." Immunization, Vaccines and Biologicals Retrieved 4/15/2018, 2018, from http://www.who.int/immunization/monitoring_surveillance/data/en/.

Wilen, C. B., S. Lee, L. L. Hsieh, R. C. Orchard, C. Desai, B. L. Hykes, Jr., M. R. McAllaster, D. R. Balce, T. Feehley, J. R. Brestoff, C. A. Hickey, C. C. Yokoyama, Y. T. Wang, D. A. MacDuff, D. Kreamalmayer,

M. R. Howitt, J. A. Neil, K. Cadwell, P. M. Allen, S. A. Handley, M. van Lookeren Campagne, M. T. Baldridge and H. W. Virgin (2018). "Tropism for tuft cells determines immune promotion of norovirus pathogenesis." Science **360**(6385): 204-208.

Wobus, C. E., J. B. Cunha, M. D. Elftman and A. O. Kolawole (2016). Chapter 3.4 - Animal Models of Norovirus Infection A2 - Svensson, Lennart. Viral Gastroenteritis. U. Desselberger, H. B. Greenberg and M. K. Estes. Boston, Academic Press**:** 397-422.

Wobus, C. E., S. M. Karst, L. B. Thackray, K.-O. Chang, S. V. Sosnovtsev, G. Belliot, A. Krug, J. M. Mackenzie, K. Y. Green and H. W. Virgin (2004). "Replication of Norovirus in Cell Culture Reveals a Tropism for Dendritic Cells and Macrophages." PLoS Biology **2**(12): e432.

Yu, X., B. S. Coulson, F. E. Fleming, J. C. Dyason, M. von Itzstein and H. Blanchard (2011). "Novel structural insights into rotavirus recognition of ganglioside glycan receptors." Journal of molecular biology **413**(5): 929-939.

Yuan, L., P. M. Jobst and M. Weiss (2017). Gnotobiotic Pigs. Gnotobiotics. T. R. Schoeb and K. A. Eaton, Academic Press**:** 349-368.

Yuan, L., L. A. Ward, B. I. Rosen, T. L. To and L. J. Saif (1996). "Systematic and intestinal antibody-secreting cell responses and correlates of protective immunity to human rotavirus in a gnotobiotic pig model of disease." Journal of Virology **70**(5): 3075.

Yuan, L., L. A. Ward, B. I. Rosen, T. L. To and L. J. Saif (1996). "Systematic and intestinal antibody-secreting cell responses and correlates of protective immunity to human rotavirus in a gnotobiotic pig model of disease." J Virol **70**(5): 3075-3083.

Yuan, L., K. Wen, M. S. Azevedo, A. M. Gonzalez, W. Zhang and L. J. Saif (2008). "Virus-specific intestinal IFN-gamma producing T cell responses induced by human rotavirus infection and vaccines are correlated with protection against rotavirus diarrhea in gnotobiotic pigs." Vaccine **26**(26): 3322-3331.

Zaman, K., D. A. Dang, J. C. Victor, S. Shin, M. Yunus, M. J. Dallas, G. Podder, D. T. Vu, T. P. Le, S. P. Luby, H. T. Le, M. L. Coia, K. Lewis, S. B. Rivers, D. A. Sack, F. Schodel, A. D. Steele, K. M. Neuzil and M. Ciarlet (2010). "Efficacy of pentavalent rotavirus vaccine against severe rotavirus gastroenteritis in

infants in developing countries in Asia: a randomised, double-blind, placebo-controlled trial." Lancet **376**(9741): 615-623.

Zeng, C. Q., M. J. Wentz, J. Cohen, M. K. Estes and R. F. Ramig (1996). "Characterization and replicase activity of double-layered and single-layered rotavirus-like particles expressed from baculovirus recombinants." Journal of Virology **70**(5): 2736-2742.

Zhang, X.-F. F., Y. Long, M. Tan, T. Zhang, Q. Huang, X. Jiang, W.-F. F. Tan, J.-D. D. Li, G.-F. F. Hu, S. Tang and Y.-C. C. Dai (2016). "P[8] and P[4] Rotavirus Infection Associated with Secretor Phenotypes Among Children in South China." Scientific reports **6**: 34591.

Zhao, W., M. Xia, T. Bridges-Malveo, M. Cantu, M. M. McNeal, A. H. Choi, R. L. Ward and K. Sestak (2005). "Evaluation of rotavirus dsRNA load in specimens and body fluids from experimentally infected juvenile macaques by real-time PCR." Virology **341**(2): 248-256.

Zhu, S., D. Regev, M. Watanabe, D. Hickman, N. Moussatche, D. M. Jesus, S. M. Kahan, S. Napthine, I. Brierley, R. N. Hunter, D. Devabhaktuni, M. K. Jones and S. M. Karst (2013). "Identification of Immune and Viral Correlates of Norovirus Protective Immunity through Comparative Study of Intra-Cluster Norovirus Strains." PLoS Pathogens **9**(9): e1003592.

Chapter 2

Parenterally Administered P24-VP8* Nanoparticle Vaccine Conferred Strong Protection against Rotavirus Diarrhea and Virus Shedding in Gnotobiotic Pigs

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Abstract

Current live rotavirus vaccines are costly with increased risk of intussusception due to vaccine replication in the gut of vaccinated children. New vaccines with improved safety and cost-effectiveness are needed. In this study, we assessed the immunogenicity and protective efficacy of a novel P24-VP8* nanoparticle vaccine using the gnotobiotic (Gn) pig model of human rotavirus infection and disease. Three doses of P24-VP8* (200 μg/dose) intramuscular vaccine with $Al(OH)_3$ adjuvant (600 μg) conferred significant protection against infection and diarrhea after challenge

with virulent Wa strain rotavirus. This was indicated by the significant reduction in the mean duration of diarrhea, virus shedding in feces, and significantly lower fecal cumulative consistency scores in post-challenge day (PCD) 1–7 among vaccinated pigs compared to the mock immunized controls. The P24-VP8* vaccine was highly immunogenic in Gn pigs. It induced strong VP8*-specific serum IgG and Wa-specific virus-neutralizing antibody responses from post-inoculation day 21 to PCD 7, but did not induce serum or intestinal IgA antibody responses or a strong effector T cell response, which are consistent with the immunization route, the adjuvant used, and the nature of the non-replicating vaccine. The findings are highly translatable and thus will facilitate clinical trials of the P24-VP8* nanoparticle vaccine.

Keywords: rotavirus nanoparticle vaccine; gnotobiotic pigs

1. Introduction

Human rotavirus (HRV) is a leading cause of severe, dehydrating gastroenteritis in children under five years of age. Although two live-attenuated oral vaccines, RotaTeq® and Rotarix®, have been implemented as part of national vaccination programs in over 98 different countries [1], their vaccine efficacy was reported to be lower in low- and middle- income countries (LMICs [39%–70%]) compared to that in high-income countries (80%–90%) [2–7]. A combination of factors have been suggested to be responsible for the lower efficacy, which have been reviewed and discussed in detail by Arnold [8] and Desselberger [9]. Both these live vaccines have also been linked to a low risk of intussusception (1–7 cases per 100,000 vaccinated infants) as a result of the vaccine rotavirus (RV) strains replicating in the intestines [10,11], and have remained expensive even after Gavi, the Vaccine Alliance, subsidization [12], particularly in resource-deprived countries. These scenarios have increased the demand for a safer, more cost-effective, and more efficacious vaccine, especially in LMICs that can be easily administered.

Parenteral intramuscular (IM) vaccines have been preferred to oral vaccines due to their increased immunogenicity. They are also not directly affected by microbiome composition or gut enteropathy, both of which have been known to affect the efficacy of oral vaccines in LMICs [13]. Non-replicating rotavirus vaccines (NRRV) have been proposed as safer alternatives to live-attenuated vaccines as they do not lead to intussusception due to their parenteral immunization route [14].

The VP4 region of RV constitutes surface spike proteins that are cleaved by host intestinal proteases into two fragments, VP5* and VP8*. VP8* forms the distal portion of the VP4 spikes, interacting with glycan receptors to facilitate viral attachment and entry [15,16]. VP8* expressed in various culture systems has been explored as an immunogen in rotavirus vaccine development [17–

22]. VP8*-containing vaccine candidates have been shown to induce rotavirus-specific neutralizing antibodies and/or protection in mouse, guinea pigs, and gnotobiotic (Gn) pig models [23–28]. Among those, the leading candidate is the P2-VP8 (VP8* fused to a universal tetanus toxin CD4+ T cell epitope P2) vaccine, adsorbed with aluminum hydroxide for IM administration, which has progressed to phase 1/2 human clinical trials [19,29].

The norovirus (NoV) P particle, referred to as the P24 particle, is an octahedral nanoparticle (≈840 kDa) composed of 24 copies of the protrusion (P) domain of the NoV capsid protein. It can be easily produced in large quantities using an *E. coli* expression system. The distal surface of each P domain, corresponding to the outermost surface of the P particle, contains three surface loops, which can tolerate large sequence insertions. Based on this concept, a nanoparticle vaccine was developed by inserting the HRV VP8* antigen into the loop sections of the P domains. The P24-VP8* nanoparticle consists of a 24-valent core of NoV P particle and 24 surface-displayed HRV VP8*s. The P24-VP8* nanoparticle shares the features of the P24 particle in self-formation, easy production, and high stability over a wide range of temperatures [30]. Efficacy studies in mice revealed that the P24-VP8* nanoparticle vaccine is highly immunogenic and capable of inducing a significantly higher VP8* specific antibody response as compared with free VP8* particles even without adjuvant [30].

The main objectives of this study were to assess the immunogenicity and protective efficacy of a novel P24-VP8* nanoparticle vaccine using the gnotobiotic (Gn) pig model of human rotavirus infection and disease. The Gn pig model of HRV (Wa, G1P [8]) infection and diarrhea has been well established and used in the pre-clinical evaluation of HRV vaccine efficacies [31]. No other conventional lab animals develop diarrhea after HRV inoculation [32]. Pigs are genetically, physiologically, anatomically, and immunologically similar to humans [33–35],

allowing data from Gn pigs to be translated to humans. The immunogenicity and protective efficacy of the P24-VP8* nanoparticle vaccine were determined using the Gn pig model of HRV infection and disease. High serum IgA, IgG, and virus-neutralizing (VN) antibody titers, as well as HRV-specific IFN-γ producing T cells, have been correlated with protection from HRV infection and disease, and data has been demonstrated to be comparable in Gn pigs and human studies [24,36,37].

2. Materials and Methods

2.1. Human Rotavirus

The virulent HRV (VirHRV) inoculum consisted of a pool composed of intestinal contents collected from the 27th passage in Gn pigs of the Wa strain HRV based on successive passages carried out in Gn pigs. A total of 1×10^5 fluorescent focus-forming units (FFUs) of VirHRV were diluted in 5 mL of Diluent #5 [minimal essential media (MEM, ThermoFisher Scientific); 100 IU of penicillin per mL, 0.1 mg of dihydrostreptomycin per ml; and 1% HEPES] for the inoculation of each Gn pig. The median infectious dose (ID_{50}) and median diarrhea dose (DD_{50}) of the VirHRV in Gn pigs were determined as approximately 1 FFU [38].

The cell culture-adapted HRV Wa strain (AttHRV), derived from the 35th passage in African green monkey kidney cells (MA104, ATCC# CRL-2378.1) [38,39], were used as the positive control for the assessment of RV antigens in feces using enzyme-linked immunosorbent assay (ELISA). The origination and passage history of the VirHRV and AttHRV have been explained by Wentzel *et al*. [40].

2.2. Vaccine

The P24-VP8* vaccine was comprised of 200 μg of P24-VP8* proteins and 600 μg Aluminum hydrogel [$Al(OH)_3$] adjuvant. The vaccine was stored at 4 °C (up to 8 months) until administered to Gn pigs (Supplementary Figure S1). The dosage of the P24-VP8* vaccine was selected based on a similar VP8* molar amount of the P2-VP8 subunit vaccine used in the clinical trial [19,27,29]. The VP8* region used in this vaccine was designed based on the sequence of Wa HRV. As the negative control, the $Al(OH)_3$ adjuvant (G-Biosciences, St. Louis, MO, USA) was diluted in sterile PBS to form a final concentration of 600 μg/mL and stored at room temperature, as per manufacturer instructions, until administered.

The purified P24-VP8* proteins were used as the detector antigen in ELISA for the detection of serum IgA and IgG antibody responses [41] and as stimulating antigen in the intracellular IFN-γ staining assay [39,42].

2.3. Gn Pigs and Treatments

Pigs (Large white cross breed) used in this study were surgically derived by hysterectomy and maintained in sterile isolators, as described previously [43]. The sterility status of the pigs housed in the gnotobiotic isolators was confirmed by culturing isolator swabs and pig rectal swabs on blood agar plates and in thioglycolate broth first at 3 days after derivation and then repeated once a week until the end of the study. Pigs were fed on a diet that solely consisted of commercial UHT sterile whole cow's milk (The Hershey Company, Hershey, PA, USA) until post-inoculation day (PID) 21, and were switched over to Similac® baby formula (Abbott Laboratories, Chicago, IL, USA) until the end of the study.

A total of 25 pigs were assigned to two groups, and a subset of pigs in each group were euthanized either pre-challenge (PID 28) or at post-challenge day (PCD) 7 (Table 1).

Pigs were administered IM with an equal volume (1 mL) of either P24-VP8* vaccine formulated with adjuvant or adjuvant alone at 5 days of age (PID 0), followed by two booster doses at PID10 and PID21. The Phase I and Phase II clinical trials carried out to evaluate the effects of P2-VP8* vaccine demonstrated that participants who received a 3-dose vaccination regime shed fewer attenuated rotavirus in feces as compared to trial participants who received two doses [19,29]. Based on this rationale, we opted to use the 3-dose regimen in this current study. The timing of 3 injections in Gn pigs are established in previous studies [27,37] based on the time needed to prime and boost immune responses in Gn pigs. Serum was collected at PID 0, PID 10, PID 21, PID 28, and PCD 7 for the detection of VP8*-specific IgA, IgG, and Wa HRV-specific neutralizing antibody responses.

One subset of pigs (n = 3–7) from each group was euthanized before the challenge at PID 28. Another subset of pigs (n = 7–8) was orally challenged with 1×10^5 FFU of VirHRV Wa strain and monitored from PCD 0 to PCD 7 to assess the protection against virus shedding and diarrhea conferred by the vaccine before euthanasia on PCD 7. The pathogenesis of the Wa VirHRV infection has been studied in detail in Gn pigs; diarrhea and virus shedding persisted between 4 to 7 days post infection [33,38,44,45]. Based on these observations, we limited the study duration to 7 days post-challenge in order to assess the immediate protection conferred by the vaccine against VirWa challenge. Four milliliters of 200 mM $NaHCO_3$ were given orally 15–20 min before the VirHRV challenge to reduce stomach acidity to allow for rotavirus inoculum to pass through the stomach without being degraded due to low pH in the stomach.

At euthanasia, small and large intestinal contents (SIC and LIC) were collected from all pigs and processed, as described [46], for the detection of intestinal antibody responses by ELISA.

Ileum, blood, and spleen were collected, and mononuclear cells (MNCs) were isolated from them for the detection of effector T cell responses by flow cytometry as described [42].

2.4. Assessment of Diarrhea and Detection of RV Shedding in Feces by Rotavirus Antigen ELISA and CCIF

The pigs were on a milk-based diet throughout the duration of the study, making their fecal consistency resemble that of a newborn infant. For the assessment of diarrhea, fecal consistency was recorded daily from PCD 0–7 and categorized as follows: 0: normal; 1: pasty; 2: semi-liquid; 3: liquid. The fecal scoring system used here has been well established and used for multiple Gn pigs studies [32,33,38,44,45,47,48]. Pigs were considered to be having diarrhea when their daily fecal consistency scores were recorded to be 2 or greater (≥2).

Rectal swabs were collected daily to monitor virus shedding by ELISA (for the detection of RV antigens) and cell culture immunofluorescence (CCIF; for the detection of infectious virions) from PCD 0–7. Rectal swabs were processed, as reported previously [49]. ELISA and CCIF assays for the detection and titration of VirHRV antigen in rectal swabs were carried out as previously described [33,38,44,45,47,50–52]. CCIF titers [fluorescent focus units (FFU)/mL)] were determined by Equation (1):

$$\frac{ffu}{mL} = \frac{\#\ Plaques\ counted}{d\ x\ V} \qquad (1)$$

where d = dilution factor, and V = volume of virus added.

2.5. RV-Specific Serum VN, and VP8*-Specific Serum and Intestinal IgA and IgG Antibody Titration

VN antibody titers in serum samples were determined based on methods described previously [47]. MA104 cells were cultured in 96-well plates until an even monolayer was formed (≈3–4 days).

Cells were washed once with sf-EMEM, and enriched with 100 µL of sf-EMEM and incubated at 37 °C for 2 h. The media was then discarded, and the cells were inoculated with trypsin-activated AttHRV (4×10^3 FFU in 10 µg/mL trypsin) in the absence or presence of 4-fold decreasing concentrations of Gn pig serum samples. The inoculum was discarded, and the plates were incubated at 37 °C for 18 h in 5% CO_2 containing fresh sf-EMEM. The remainder of the steps has been described in detail in a previous publication [47]. The VN antibody titer was expressed as the reciprocal of the serum dilution, which reduced the number of fluorescent cell-forming units by >80% compared to the negative control serum. VP8*-specific IgA and IgG antibody titers in serum and intestinal contents were measured by using isotype-specific antibody ELISA with purified P24-VP8* as detector antigen at the plate coating concentration of 6.63 µg/mL, following methods described elsewhere [46,47,53]. When loading the testing samples on ELISA plates, four-fold serial dilutions of each sample started from 1:4 to 1:16384 for IgA, SIC, and LIC and 1:256 to 1:1048576 for IgG.

2.6. Flow Cytometry

Mononuclear cells (MNCs) collected from the ileum, blood, and spleen were diluted to a concentration of 2×10^6 cells/mL and were seeded into 12-well plates and stimulated with 12 µg/mL of P24-VP8* antigen for 17 h at 37 °C in 5% CO_2 as determined previously [42]. CD3+CD4+ and CD3+CD8+ cell surface marker staining and IFN-γ intracellular staining have been described in previous publications [42,47,54,55]. All samples were stored in 0.05 mL of stain buffer and were maintained at 4 °C. A minimum of 100,000 events were acquired using a FACSAria flow cytometer (BD Biosciences, San Jose, CA, USA). Flow cytometry data were analyzed using FlowJo X software (Tree Star, Ashland, OR, USA).

2.7. Statistical Analysis

Gn pigs were randomly assigned into treatment groups upon derivation regardless of gender and body weight by animal care technicians. Student's *t*-test was used for comparisons of virus shedding and diarrhea data between the treatment groups. One-way analysis of variance (ANOVA) (General linear model) was used to compare rotavirus-specific IgA, IgG, virus-neutralizing (VN) antibody titers between the treatment groups. Tukey-Kramer HSD was used for the comparison of different time points within the same treatment group. Two-way ANOVA, followed by a Multiple *t*-test, was used for comparisons of frequencies of IFN-γ producing T cells between treatment groups. ANOVA analyses were carried out using JMP 14.0 (SAS Institute, Kerry, NC, USA), and all other statistical analyses were performed using GraphPad Prism 7.0 (GraphPad Software, San Diego, CA, USA). A *p* value lower than 0.05 was accepted to be statistically significant.

Results

3.1. 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 (diarrhea) and virus shedding from PCD 1 to PCD 7. Gn pigs that were administered with P24-VP8* vaccine had a significantly delayed onset of diarrhea (from 1.6 to 4.4 days), a significantly reduced duration of diarrhea (from 6.0 to 3.3 days), significantly lower mean diarrhea scores on PID 1 and 2, and a significantly lower cumulative fecal consistency score (from 14.3 to 9.1) as compared to the mock-vaccinated control group (Table 2 and Figure 1A). A delayed onset of virus shedding, a reduced peak titer, a reduced cumulative virus titer (presented as the area under the curve, AUC), and a significantly reduced duration (from 5.9 to 2.5 days) of virus shedding were observed in P24-VP8* vaccinated pigs when compared to the control group (Table

2). In addition, the mean daily virus shedding titer in the vaccinated pigs was significantly reduced at PCD 2 (Figure 1B), and the reduction of total virus shed (AUC) was 2.27-fold compared to the control pigs (Table 2). However, the vaccine did not significantly reduce the incidence (%) of diarrhea and virus shedding (Table 2).

3.2. Strong VP8*-Specific IgG and Virus Neutralizing, but Lack of IgA, Antibody Responses in Serum

In order to monitor the development of VP8* specific humoral immunity, serum samples were collected during the time of vaccine administration (PID 0, PID 10, and PID 21) at the VirHRV challenge (PID 28) and upon euthanasia (PCD 7). Serum IgG and IgA antibody responses were quantified using ELISA and depicted in Figures 2A and 2B, respectively. P24-VP8*-specific IgG antibody titers in serum were significantly higher ($p < 0.001$) in vaccinated pigs at PID 10, PID 21, PID 28, and PCD 7 when compared to pigs in the control group (Figure 2A). However, serum IgA titers were only detectable after challenge (PCD 7) with VirHRV (Figure 2B).

HRV neutralizing antibodies were detected in the serum of P24-VP8* vaccinated pigs starting from PID 21 and were observed to increase similarly with VP8*-specific IgG titers until euthanasia at PCD 7. In control pigs, VN antibodies were only detectable after challenge with VirHRV and were at significantly lower levels compared to the vaccinated pigs (Figure 2C).

3.3. Lack of P24-VP8* Specific Antibody Responses in the Intestines

P24-VP8*-specific IgA and IgG antibody titers in SIC and LIC, collected at the time of euthanasia (PID 28 and PCD 7), were measured by ELISA. The P24-VP8* vaccine did not induce any detectable intestinal IgA or IgG antibody responses before the challenge at PID 28. After the challenge, among the eight vaccinated and challenged pigs, only VP8*-specific IgG antibodies

were detected (ELISA titers ranging from 256 to 1024) in the SIC of three pigs at PCD 7 (Supplementary Figure S2). However, the SIC IgG titers were not associated with the severity of diarrhea or the amount of virus shed in the three pigs throughout the challenge period.

3.4. P24-VP8* Vaccine did not Induce Strong VP8*-Specific Effector T Cell Responses in Intestinal and Systemic Lymphoid Tissues

Frequencies of IFN-γ+CD8+ and IFN-γ+CD4+ T cells in ileum, peripheral blood (PBL), and spleen at PID 28, and PCD 7 are summarized in Figure 3. At PID 28, slightly higher (not statistically significant) IFN-γ producing CD4+ and CD8+ T cell responses to the P24-VP8* antigen was detected in vaccinated pigs as compared to control pigs (Figure 3A). P24-VP8* vaccinated pigs had higher frequencies of IFN-γ+CD4+ T cells in ileum and blood and higher IFN-γ+CD8+ T cells in ileum, blood, and spleen compared to the mock-vaccinated control pigs. Upon the VirHRV challenge, there was still no significant difference in the frequencies of IFN-γ secreting CD4+ and CD8+ T cells between the two groups in the intestinal (ileum) or the systemic tissues (PBL and spleen) (Figure 3B).

Discussion

In this study, the immunogenicity (antibody and T cell responses) and protective efficacy of the P24-VP8* nanoparticle vaccine were evaluated in Gn pigs. We first demonstrated that the IM P24-VP8* vaccine conferred significant protection against infection and diarrhea when challenged with the homotypic virulent strain Wa of HRV. This was indicated by the significant reduction in the mean duration of diarrhea, virus shedding in feces, and significantly lower fecal cumulative consistency scores recorded from PCD 1–7 in vaccinated pigs compared to the controls. However, the vaccine did not significantly reduce the incidence (%) of diarrhea and virus shedding,

indicating that there was a lack of protective immune effectors at the site of infection (small intestine) at the time of challenge, which is consistent with the observed intestinal immune responses. The IM P24-VP8* vaccine with $Al(OH)_3$ adjuvant was highly immunogenic in Gn pigs. It induced strong VP8*-specific serum IgG and virus-neutralizing antibody responses from PID 21 to PCD 7 but did not induce serum or intestinal IgA antibody responses or a strong effector T cell response. These results are consistent with the IM immunization route, the $Al(OH)_3$ adjuvant, and the nature of the non-replicating vaccine. Non-replicating vaccines are typically ineffective in inducing effector T cell responses. The $Al(OH)_3$ adjuvant is characteristic for its ability to enhance a Th2 type immune response, promoting strong humoral responses and suppressing effector T cell responses [56].

The observed protection and immune responses data together suggest that the protection conferred by the P24-VP8* vaccine against diarrhea and virus shedding upon challenge with the virulent Wa HRV was mediated by the vaccine-induced antibodies in the serum. Although there were no antibodies present at the lumen of the small intestine, the site of HRV infection, at the time of challenge to totally prevent the initiation of RV infection, the viruses disseminated into blood from the infected small intestinal epithelial cells could have been neutralized by the high titers of VP8*-specific IgG and virus-neutralizing antibodies during the phase of viremia. Such mechanisms can reduce the chance of infection of more epithelial cells by the virus from the basolateral side [45]. Studies showing that passively transferred serum antibodies can suppress or delay viral infection in RV-challenged pigtailed macaques [57], and an inactivated IM HRV vaccine (CDC-9) reduced virus shedding in Gn pigs upon challenge with Wa VirHRV [48] likely share the same protection mechanism with the P24-VP8* vaccine. The serum IgG and virus-neutralizing antibody responses induced by the P24-VP8* IM nanoparticle vaccine had similar

dynamics and magnitude as the aluminum phosphate adjuvanted inactivated CDC-9 and P2-VP8* IM vaccines in Gn pigs [27,48]. The P24-VP8* vaccine demonstrated a similar degree of protection against diarrhea but a stronger protection against virus shedding in Gn pigs as compared to the P2-VP8* vaccine [27].

There was a trend of inverse correlation between serum VP8*-specific IgG titers at PID 28 and cumulative diarrhea scores post-challenge in the vaccinated pigs (Pearson's rank correlation, $r = -0.6699$ and $p = 0.0691$), suggesting that vaccinated pigs with higher serum VP8*-specific IgG responses are more likely to be protected against severe diarrhea, which is in agreement with the study of serum IgG antibody in human adults showing that VP4-specific IgG titer was correlated with resistance to HRV infection [58]. The presence of high preexisting IgG titers was also correlated with less severe or shorter duration of diarrhea among children under three years of age [59]. As reviewed by Jiang *et al*., serum antibodies, if present at critical levels, are either protective themselves or are an important and powerful correlate of protection against rotavirus disease [60].

Additional investigations are required to explore the full potential of P24-VP8* vaccine efficacy. First, P24-VP8* is a candidate dual-vaccine against both NoV and RV, but we only examined the immune responses and protection against HRV, not human norovirus (HuNoV). Further studies in the Gn pig model of HuNoV infection are needed to evaluate its efficacy against NoV. Second, we only examined the protection against challenge with a homotypic HRV, and it remains to be determined whether the P24-VP8* vaccine would be effective in protecting against heterotypic HRV, as the monovalent P[8] HRV vaccine Rotarix showed significant efficacy against P[4] (70.9%) and P[6] (55.2%) HRV associated gastroenteritis in African infants [61]. One of the important potential advantages of the novel P24-VP8* nanoparticle dual vaccine is that the vaccine can be formulated as a cocktail vaccine to cover multiple types of RVs and NoVs for broad

protection. Thus far, the Gn pig model of HRV infection and diarrhea has only been evaluated using the P [8] Wa HRV, requiring the need to test the effectiveness of Gn pigs as a suitable model for additional HRV P types to evaluate the broadness of protection of the novel P24-VP8* nanoparticle vaccine.

Conclusion

The P24-VP8* vaccine candidate is a typical nanoparticle vaccine with 24 copies of the major RV surface neutralizing antigen VP8* displayed on the self-assembled norovirus P24 particles. The P24-VP8* nanoparticles are easily produced in *E. coli* with a high yield and simple purification procedures at a low cost. Significant enhancement of the immunogenicity of both VP8* and P domain backbone have been demonstrated in mouse immunization studies. In this current study, the usefulness of the P24-VP8* vaccine was assessed in a Gn pig model, followed by the challenge of HRV. Three doses of IM immunization of Gn pigs demonstrated the nanoparticle vaccine's effectiveness to significantly shorten the duration of HRV diarrhea and virus shedding, reduce the severity of diarrhea, and lower the amount of virus shed when challenged. Immune responses associated with protection include high titers of VP8*-specific serum IgG antibodies and virus-neutralizing antibodies induced by the vaccine after the second and third booster doses. These findings will facilitate clinical trials of this vaccine candidate into a useful, safe, non-replicating, parental vaccine against RVs.

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Ethical Approval: All animal experiments were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee at Virginia Tech (IACUC protocol: 16-214-CVM).

Reference

1. IVAC. Current Vaccine Intro Status. Available online: www.view-hub.org (accessed on 11 June 2019).
2. Gilmartin, A.A.; Petri, W.A. Exploring the role of environmental enteropathy in malnutrition, infant development and oral vaccine response. *Philos. Trans. R. Soc. B Biol. Sci.* **2015**, *370*, 20140143.
3. Boom, J.A.; Tate, J.E.; Sahni, L.C.; Rench, M.A.; Hull, J.J.; Gentsch, J.R.; Patel, M.M.; Baker, C.J.; Parashar, U.D. Effectiveness of Pentavalent Rotavirus Vaccine in a Large Urban Population in the United States. *Pediatrics* **2010**, *125*, 199–207.
4. Vesikari, T.; Karvonen, A.; Prymula, R.; Schuster, V.; Tejedor, J.C.; Cohen, R.; Meurice, F.; Han, H.; Damaso, S.; Bouckenooghe, A. Efficacy of human rotavirus vaccine against rotavirus gastroenteritis during the first 2 years of life in European infants: Randomised, double-blind controlled study. *Lancet* **2007**, *370*, 1757–1763.
5. Armah, G.E.; O Sow, S.; Breiman, R.F.; Dallas, M.J.; Tapia, M.D.; Feikin, D.R.; Binka, F.N.; Steele, A.D.; Laserson, K.F.; Ansah, N.A.; *et al*. Efficacy of pentavalent rotavirus vaccine against severe rotavirus gastroenteritis in infants in developing countries in sub-Saharan Africa: A randomised, double-blind, placebo-controlled trial. *Lancet* **2010**, *376*, 606–614.
6. Patel, M.M.; Glass, R.; Desai, R.; E Tate, J.; Parashar, U.D. Fulfilling the promise of rotavirus vaccines: How far have we come since licensure? *Lancet Infect. Dis.* **2012**, *12*, 561–570.
7. Lamberti, L.M.; Ashraf, S.; Walker, C.L.F.; Black, R.E. A Systematic Review of the Effect of Rotavirus Vaccination on Diarrhea Outcomes among Children Younger Than 5 Years. *Pediatr. Infect. Dis. J.* **2016**, *35*, 992–998.

8. Arnold, M.M. Rotavirus Vaccines: Why Continued Investment in Research Is Necessary. *Curr. Clin. Microbiol. Rep.* **2018**, *5*, 73–81.
9. Desselberger, U. Differences of Rotavirus Vaccine Effectiveness by Country: Likely Causes and Contributing Factors. *Pathogens* **2017**, *6*, 65.
10. Aliabadi, N.; Tate, J.; Parashar, U. Potential safety issues and other factors that may affect the introduction and uptake of rotavirus vaccines. *Clin. Microbiol. Infect.* **2016**, *22*, S128–S135.
11. Safety of rotavirus vaccines: Postmarketing surveillance in the WHO Region of the Americas. *Relev. Epidemiol. Hebd.* **2011**, *86*, 66–72.
12. Pecenka, C.; Debellut, F.; Bar-Zeev, N.; Anwari, P.; Nonvignon, J.; Shamsuzzaman, M.; Clark, A. Re-evaluating the cost and cost-effectiveness of rotavirus vaccination in Bangladesh, Ghana, and Malawi: A comparison of three rotavirus vaccines. *Vaccine* **2018**, *36*, 7472–7478.
13. Velasquez, D.E.; Parashar, U.; Jiang, B. Decreased performance of live attenuated, oral rotavirus vaccines in low-income settings: Causes and contributing factors. *Expert Rev. Vaccines* **2018**, *17*, 145–161, doi:10.1080/14760584.2018.1418665.
14. O'Ryan, M.; A Lopman, B. Parenteral protein-based rotavirus vaccine. *Lancet Infect. Dis.* **2017**, *17*, 786–787.
15. Liu, Y.; Huang, P.; Tan, M.; Liu, Y.; Biesiada, J.; Meller, J.; Castello, A.A.; Jiang, B.; Jiang, X. Rotavirus VP8: Phylogeny, Host Range, and Interaction with Histo-Blood Group Antigens. *J. Virol.* **2012**, *86*, 9899–9910.
16. Tan, M.; Jiang, X. Histo-blood group antigens: A common niche for norovirus and rotavirus. *Expert Rev. Mol. Med.* **2014**, *16*, e5.

17. Andres, I.; Rodriguez-Diaz, J.; Buesa, J.; Zueco, J. Yeast expression of the VP8* fragment of the rotavirus spike protein and its use as immunogen in mice. *Biotechnol. Bioeng.* **2006**, *93*, 89–98.

18. Dunn, S.J.; Fiore, L.; Werner, R.L.; Cross, T.L.; Broome, R.L.; Ruggeri, F.M.; Greenberg, H.B. Immunogenicity, antigenicity, and protection efficacy of baculovirus expressed VP4 trypsin cleavage products, VP5(1) and VP8 from rhesus rotavirus. *Arch. Virol.* **1995**, *140*, 1969–1978.

19. Fix, A.D.; Harro, C.; McNeal, M.; Dally, L.; Flores, J.; Robertson, G.; Boslego, J.W.; Cryz, S. Safety and immunogenicity of a parenterally administered rotavirus VP8 subunit vaccine in healthy adults. *Vaccine* **2015**, *33*, 3766–3772.

20. Gil, M.T.; De Souza, C.O.; Asensi, M.; Buesa, J. Homotypic Protection Against Rotavirus-Induced Diarrhea in Infant Mice Breast-Fed by Dams Immunized with the Recombinant VP8 Subunit of the VP4 Capsid Protein. *Viral Immunol.* **2000**, *13*, 187–200.

21. Marelli, B.; Pérez, A.R.; Banchio, C.; De Mendoza, D.; Magni, C. Oral immunization with live Lactococcus lactis expressing rotavirus VP8* subunit induces specific immune response in mice. *J. Virol. Methods* **2011**, *175*, 28–37.

22. Rodríguez-Díaz, J.; Montava, R.; Viana, R.; Buesa, J.; Pérez-Martínez, G.; Monedero, V. Oral immunization of mice with Lactococcus lactis expressing the rotavirus VP8* protein. *Biotechnol. Lett.* **2011**, *33*, 1169–1175.

23. Wen, X.; Cao, D.; Jones, R.W.; Li, J.; Szu, S.; Hoshino, Y. Construction and characterization of human rotavirus recombinant VP8* subunit parenteral vaccine candidates. *Vaccine* **2012**, *30*, 6121–6126.

24. Wen, X.; Cao, D.; Jones, R.W.; Hoshino, Y.; Yuan, L. Tandem truncated rotavirus VP8* subunit protein with T cell epitope as non-replicating parenteral vaccine is highly immunogenic. *Hum. Vaccines Immunother.* **2015**, *11*, 2483–2489.
25. Kang, D.K.; Kim, P.H.; Ko, E.J.; Seo, J.Y.; Seong, S.Y.; Kim, Y.H.; Kwon, I.C.; Jeong, S.Y.; Yang, J.M. Peroral immunization of microencapsulated human VP8 in combination with cholera toxin induces intestinal antibody responses. *Mol. Cells* **1999**, *9*, 609–616.
26. Lentz, E.; Mozgovoj, M.; Bellido, D.; Santos, M.D.; Wigdorovitz, A.; Bravo-Almonacid, F. VP8* antigen produced in tobacco transplastomic plants confers protection against bovine rotavirus infection in a suckling mouse model. *J. Biotechnol.* **2011**, *156*, 100–107.
27. Wen, X.; Wen, K.; Cao, D.; Li, G.; Jones, R.W.; Li, J.; Szu, S.; Hoshino, Y.; Yuan, L. Inclusion of a universal tetanus toxoid CD4(+) T cell epitope P2 significantly enhanced the immunogenicity of recombinant rotavirus DeltaVP8* subunit parenteral vaccines. *Vaccine* **2014**, *32*, 4420–4427.
28. Xue, M.; Yu, L.; Che, Y.; Lin, H.; Zeng, Y.; Fang, M.; Li, T.; Ge, S.; Xia, N. Characterization and protective efficacy in an animal model of a novel truncated rotavirus VP8 subunit parenteral vaccine candidate. *Vaccine* **2015**, *33*, 2606–2613.
29. Groome, M.J.; Koen, A.; Fix, A.; Page, N.; Jose, L.; Madhi, S.A.; McNeal, M.; Dally, L.; Cho, I.; Power, M.; *et al*. Safety and immunogenicity of a parenteral P2-VP8-P[8] subunit rotavirus vaccine in toddlers and infants in South Africa: A randomised, double-blind, placebo-controlled trial. *Lancet Infect. Dis.* **2017**, *17*, 843–853.
30. Tan, M.; Huang, P.; Xia, M.; Fang, P.A.; Zhong, W.; McNeal, M.; Wei, C.; Jiang, W.; Jiang, X. Norovirus P Particle, a Novel Platform for Vaccine Development and Antibody Production. *J. Virol.* **2011**, *85*, 753–764.

31. Yuan, L.; Wen, K. Rotavirus. In *Laboratory Models for Foodborne Infections*; Liu, D., Ed.; CRC Press, Taylor & Francis: Boca Raton, FL, USA, 2017; pp. 95–107.
32. Yuan, L.; Saif, L.J. Induction of mucosal immune responses and protection against enteric viruses: Rotavirus infection of gnotobiotic pigs as a model. *Veter. Immunol. Immunopathol.* **2002**, *87*, 147–160.
33. Saif, L.J.; Ward, L.A.; Yuan, L.; Rosen, B.I.; To, T.L. The gnotobiotic piglet as a model for studies of disease pathogenesis and immunity to human rotaviruses. *Viral Gastroenteritis* **1996**, *12*, 153–161.
34. Birchall, M.A.; Bailey, M.; Barker, E.V.; Rothkötter, H.-J.; Otto, K.; Macchiarini, P. Model for experimental revascularized laryngeal allotransplantation. *BJS* **2002**, *89*, 1470–1475.
35. Rothkötter, H.J.; Sowa, E.; Pabst, R. The pig as a model of developmental immunology. *Hum. Exp. Toxicol.* **2002**, *21*, 533–536.
36. Desselberger, U.; Huppertz, H.I. Immune responses to rotavirus infection and vaccination and associated correlates of protection. *J. Infect. Dis.* **2011**, *203*, 188–195.
37. Yuan, L.; Kang, S.Y.; Ward, L.A.; To, T.L.; Saif, L.J. Antibody-Secreting Cell Responses and Protective Immunity Assessed in Gnotobiotic Pigs Inoculated Orally or Intramuscularly with Inactivated Human Rotavirus. *J. Virol.* **1998**, *72*, 330–338.
38. Ward, L.A.; Rosen, B.I.; Yuan, L.; Saif, L.J. Pathogenesis of an attenuated and a virulent strain of group A human rotavirus in neonatal gnotobiotic pigs. *J. Gen. Virol.* **1996**, *77*, 1431–1441.
39. Wen, K.; Tin, C.; Wang, H.; Yang, X.; Li, G.; Giri-Rachman, E.; Kocher, J.; Bui, T.; Clark-Deener, S.; Yuan, L. Probiotic Lactobacillus rhamnosus GG Enhanced Th1 Cellular Immunity

but Did Not Affect Antibody Responses in a Human Gut Microbiota Transplanted Neonatal Gnotobiotic Pig Model. *PLoS ONE* **2014**, *9*, e94504.

40. Wentzel, J.F.; Yuan, L.; Rao, S.; Van Dijk, A.A.; O'Neill, H.G. Consensus sequence determination and elucidation of the evolutionary history of a rotavirus Wa variant reveal a close relationship to various Wa variants derived from the original Wa strain. *Infect. Genet. Evol.* **2013**, *20*, 276–283.
41. Liu, F.; Wen, K.; Li, G.; Yang, X.; Kocher, J.; Bui, T.; Jones, D.; Pelzer, K.; Clark-Deener, S.; Yuan, L. Dual Functions of Lactobacillus acidophilus NCFM at The Intermediate Dose in Protection Against Rotavirus Diarrhea in Gnotobiotic Pigs Vaccinated with a Human Rotavirus Vaccine. *J. Pediatric Gastroenterol. Nutr.* **2014**, *58*, 171.
42. Yuan, L.; Wen, K.; Azevedo, M.S.; Gonzalez, A.M.; Zhang, W.; Saif, L.J. Virus-specific intestinal IFN-gamma producing T cell responses induced by human rotavirus infection and vaccines are correlated with protection against rotavirus diarrhea in gnotobiotic pigs. *Vaccine* **2008**, *26*, 3322–3331.
43. Yuan, L.J.; Jobst, P.M.; Weiss, M. Gnotobiotic Pigs: From Establishing Facility to Modeling Human Infectious Diseases. In *Gnotobiotics*; Schoeb, T.R., Eaton, K.A., Eds.; Acamedic Press: Cambridge, MA, USA, 2017; pp. 349–368.
44. Saif, L.; Yuan, L.; Ward, L.; To, T. Comparative Studies of the Pathogenesis, Antibody Immune Responses, and Homologous Protection to Porcine and Human Rotaviruses in Gnotobiotic Piglets. *Adv. Exp. Med. Biol.* **1997**, *412*, 397–403.
45. Azevedo, M.S.; Yuan, L.; Jeong, K.I.; Gonzalez, A.; Nguyen, T.V.; Pouly, S.; Gochnauer, M.; Zhang, W.; Azevedo, A.; Saif, L.J. Viremia and Nasal and Rectal Shedding of Rotavirus in Gnotobiotic Pigs Inoculated with Wa Human Rotavirus. *J. Virol.* **2005**, *79*, 5428–5436.

46. Parre, V.; Hodgins, D.C.; Saif, L.J.; Yuan, L.; Kang, S.Y.; Yuan, L.; Ward, L.A.; De Arriba, L. Serum and intestinal isotype antibody responses to Wa human rotavirus in gnotobiotic pigs are modulated by maternal antibodies. *J. Gen. Virol.* **1999**, *80*, 1417–1428.
47. Twitchell, E.L.; Tin, C.; Wen, K.; Zhang, H.; Becker-Dreps, S.; Azcarate-Peril, M.A.; Vilchez, S.; Li, G.; Ramesh, A.; Weiss, M.; *et al*. Modeling human enteric dysbiosis and rotavirus immunity in gnotobiotic pigs. *Gut Pathog.* **2016**, *8*, 51.
48. Wang, Y.; Azevedo, M.; Saif, L.J.; Gentsch, J.R.; Glass, R.I.; Jiang, B. Inactivated rotavirus vaccine induces protective immunity in gnotobiotic piglets. *Vaccine* **2010**, *28*, 5432–5436.
49. Yang, X.; Twitchell, E.; Li, G.; Wen, K.; Weiss, M.; Kocher, J.; Lei, S.; Ramesh, A.; Ryan, E.P.; Yuan, L. High protective efficacy of rice bran against human rotavirus diarrhea via enhancing probiotic growth, gut barrier function, and innate immunity. *Sci. Rep.* **2015**, *5*, 15004.
50. Liu, F.; Li, G.; Wen, K.; Bui, T.; Cao, D.; Zhang, Y.; Yuan, L. Porcine Small Intestinal Epithelial Cell Line (IPEC-J2) of Rotavirus Infection as a New Model for the Study of Innate Immune Responses to Rotaviruses and Probiotics. *Viral Immunol.* **2010**, *23*, 135–149.
51. Arnold, M.; Patton, J.T.; McDonald, S.M. Culturing, storage, and quantification of rotaviruses. *Curr. Protoc. Microbiol.* **2009**, Chapter 15, Unit 15C.13
52. Shao, L.; Fischer, D.D.; Kandasamy, S.; Rauf, A.; Langel, S.N.; Wentworth, D.E.; Stucker, K.M.; Halpin, R.A.; Lam, H.C.; Marthaler, D.; *et al*. Comparative In Vitro and In Vivo Studies of Porcine Rotavirus G9P[13] and Human Rotavirus Wa G1P. *J. Virol.* **2015**, *90*, 142–151.
53. Saif, L.J.; Yuan, L.; Ward, L.A. Serum and intestinal isotype antibody responses and correlates of protective immunity to human rotavirus in a gnotobiotic pig model of disease. *J. Gen. Virol.* **1998**, *79*, 2661–2672.

54. Yuan, L.; Ward, L.A.; Rosen, B.I.; To, T.L.; Saif, L.J. Systematic and intestinal antibody-secreting cell responses and correlates of protective immunity to human rotavirus in a gnotobiotic pig model of disease. *J. Virol.* **1996**, *70*, 3075–3083.

55. Lei, S.; Ryu, J.; Wen, K.; Twitchell, E.; Bui, T.; Ramesh, A.; Weiss, M.; Li, G.; Samuel, H.; Clark-Deener, S.; *et al*. Increased and prolonged human norovirus infection in RAG2/IL2RG deficient gnotobiotic pigs with severe combined immunodeficiency. *Sci. Rep.* **2016**, *6*, 25222.

56. HogenEsch, H. Mechanism of Immunopotentiation and Safety of Aluminum Adjuvants. *Front. Immunol.* **2013**, *3*, 406.

57. Westerman, L.E.; McClure, H.M.; Jiang, B.; Almond, J.W.; Glass, R.I. Serum IgG mediates mucosal immunity against rotavirus infection. *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 7268–7273.

58. Yuan, L.; Honma, S.; Kim, I.; Kapikian, A.Z.; Hoshino, Y. Resistance to rotavirus infection in adult volunteers challenged with a virulent G1P1A[8] virus correlated with serum immunoglobulin G antibodies to homotypic viral proteins 7 and 4. *J. Infect. Dis.* **2009**, *200*, 1443–1451.

59. Xu, J.; Dennehy, P.; Keyserling, H.; Westerman, L.E.; Wang, Y.; Holman, R.C.; Gentsch, J.R.; Glass, R.I.; Jiang, B. Serum Antibody Responses in Children with Rotavirus Diarrhea Can Serve as Proxy for Protection. *Clin. Diagn. Lab. Immunol.* **2005**, *12*, 273–279.

60. Jiang, B.; Gentsch, J.R.; Glass, R.I. The Role of Serum Antibodies in the Protection against Rotavirus Disease: An Overview. *Clin. Infect. Dis.* **2002**, *34*, 1351–1361.

61. Steele, A.D.; Neuzil, K.M.; Cunliffe, N.A.; Madhi, S.A.; Bos, P.; Ngwira, B.; Witte, D.; Todd, S.; Louw, C.; Kirsten, M.; *et al*. Human rotavirus vaccine Rotarix™ provides protection

against diverse circulating rotavirus strains in African infants: A randomized controlled trial. *BMC Infect. Dis.* **2012**, *12*, 213.

Table 1. Assignment of treatment groups for gnotobiotic (Gn) pigs.

Group	Number of Pigs	Challenge	Time of Euthanasia
Control	3	No	PID 28
Control	7	Yes	PCD 7
P24-VP8* Vaccine	7	No	PID 28
P24-VP8* Vaccine	8	Yes	PCD 7

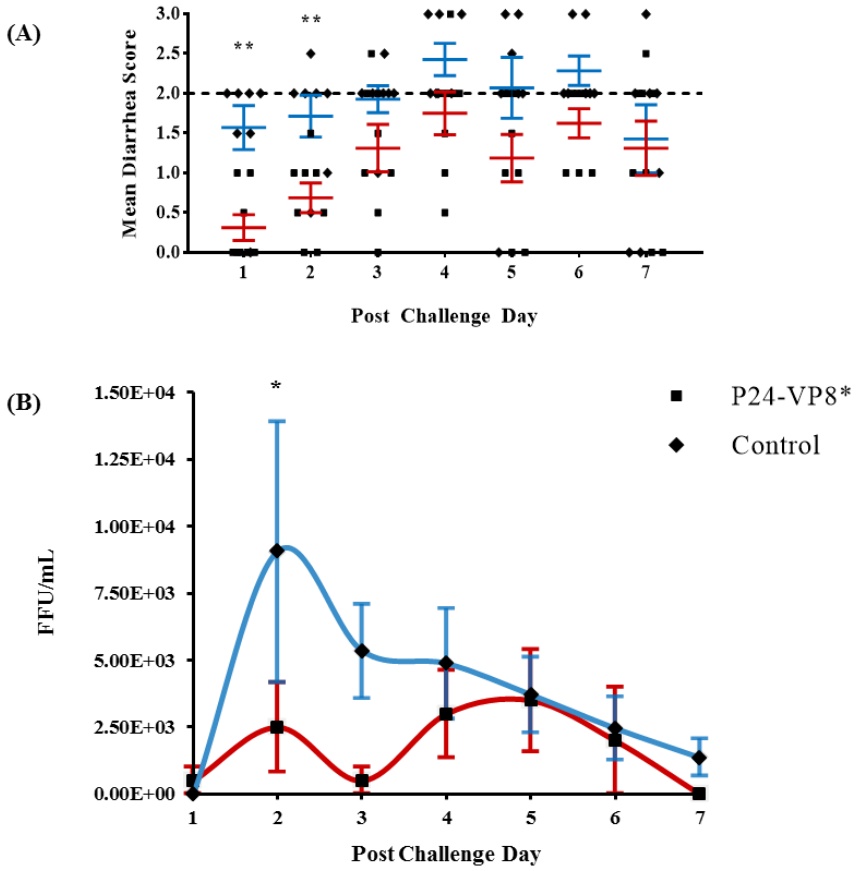

Figure 1. P24-VP8* vaccine protected against VirHRV diarrhea and reduced overall virus shed among vaccinated pigs. Fecal consistency (A) and virus shedding (B) were monitored daily from post challenge day (PCD) 1 to PCD 7 after the challenge with VirHRV. Fecal consistency scores ≥2 were considered to be diarrheic (dashed line indicates the threshold of diarrhea). Statistical significance between vaccinated and control groups, determined by multiple t tests, are indicated by asterisks (*, $p < 0.05$; **, $p < 0.01$).

Table 2. Diarrhea and rotavirus fecal virus shedding in Gn pigs after the VirHRV challenge.

		Diarrhea				Fecal Virus Shedding				
Treatment	***n***	**% with Diarrhea [a]**	**Mean Days to Onset [b]**	**Mean Duration Days [c,§]**	**Mean Cumulative Fecal Score [c]**	**% Shedding Virus [a]**	**Mean Days to Onset [b]**	**Mean Duration Days[c]**	**Mean Peak Titer (FFU/mL)**	**AUC**
P24-VP8*	8	87.5	4.4 (0.5) [d,]*	3.3 (0.75) *	9.1 (1.23) *	75	4.8 (1.0)	2.5 (0.89) *	8500 (2196) *	11,750 (3172)
Control	7	100	1.6 (0.3)	6.0 (0)	14.3 (0.44)	85.7	1.9 (0.14)	5.9 (0.14)	11,492 (4300)	26,664 (10,489)

[a] Gn pigs were orally inoculated with 1×10^5 FFU/mL of VirHRV at post-innoculation day (PID) 28. Rectal swabs were collected daily after the challenge from PCD 1 to PCD 7 to monitor for clinical signs and virus shedding. Pigs with daily fecal scores of ≥2 were considered diarrheic. Fecal consistency was scored as follows: 0, normal; 1, pasty; 2, semi-liquid; and 3, liquid. Fecal virus shedding data was determined by ELISA and/or CCIF. [b] An arbitrary designation of Day 8 was assigned to pigs that did not develop diarrhea or shed virus in feces for calculating the mean days to onset. [c] For the purposes of calculating diarrhea and virus shedding duration, if no diarrhea or virus shedding was observed in pigs until euthanasia day (PCD 7), the duration days were recorded as 0. [d] Standard error of the mean. [§] Student's *t*-test was used for comparison between vaccine and control groups. Asterisk indicates statistical significance between the groups (n = 7–8; *, $p < 0.05$).

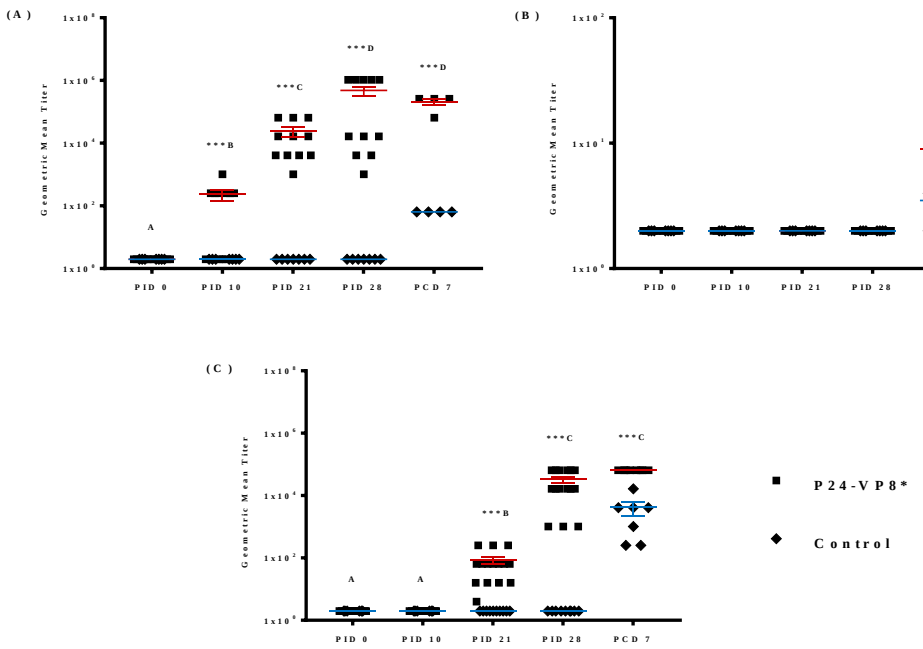

Figure 2. Geometric mean VP8*-specific IgG (**A**) and IgA (**B**) and Wa-HRV neutralizing (**C**) antibody titers in serum collected from Gn pigs at PID 0, 10, 21, 28, and PCD 7. Pigs were vaccinated with P24-VP8* vaccine or $Al(OH)_3$ adjuvant only. Each serum specimen was tested at an initial dilution of 1:4. Negative samples were assigned an arbitrary value of 2 for calculation and graphical illustration purposes. Comparisons between groups at the same time points were carried out using Student's *t*-test and significant differences are identified by *** (n = 10–15; $p < 0.001$). Tukey-Kramer HSD was used for the comparison of different time points within the same group, where different capital letters (A, B, C, D) indicate a significant difference, $p < 0.01$, and shared letters indicate no significant difference.

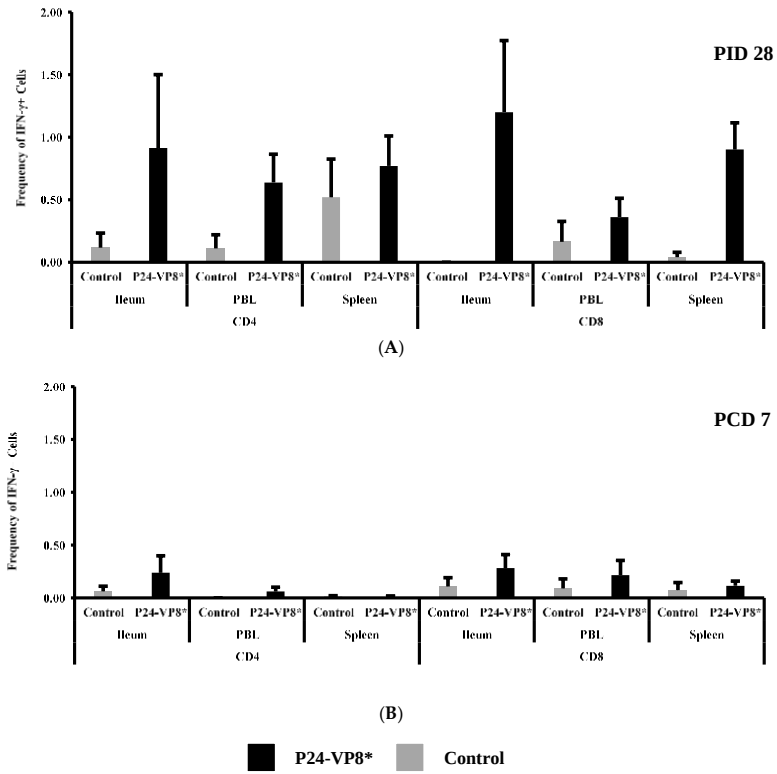

Figure 3. Frequencies of IFN-γ+CD8+ and IFN-γ+CD4+ T cells in ileum, peripheral blood (PBL), and spleen at PID 28 (A) and PCD 7 (B). Two-way ANOVA followed by Multiple t-tests were carried out for comparisons. ($n = 3–8$; $p < 0.05$). There were no significant differences.

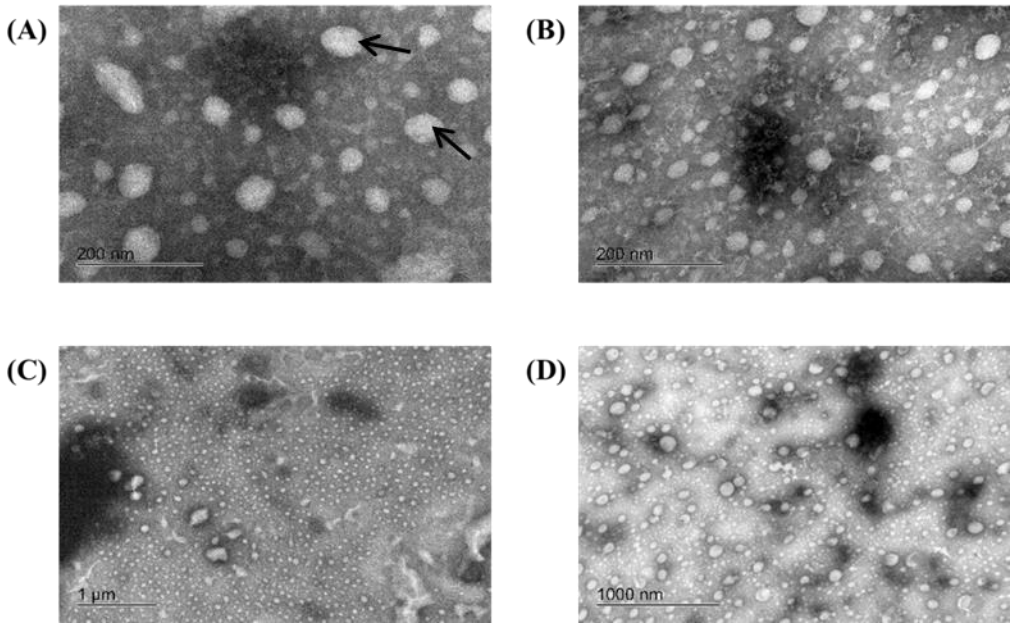

Figure S1. Negative stain TEM images of P24-VP8* particles (indicated by black arrow) taken over two different time points. Panels (**A**) and (**C**) were taken 3 months after vaccine preparation and panels (**B**) and (**D**) were imaged 8 months after vaccine preparation.

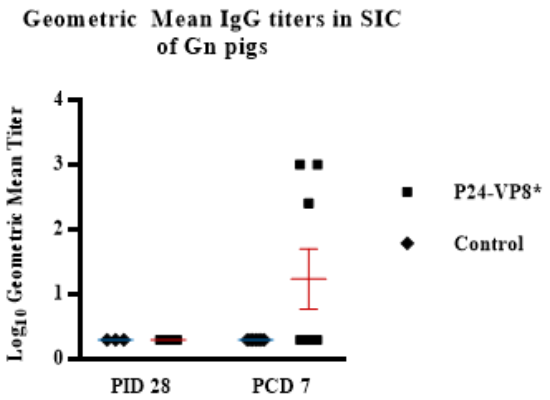

Figure S2. Geometric mean VP8*-specific IgG titers in SIC samples collected upon euthanasia at PID 28 and PCD 7. Negative samples were assigned an arbitrary value of 2 for statistical calculations and graphical illustration. Three out of 8 pigs were observed to be positive for VP8*-specific IgG antibodies. There were no significant differences.

Chapter 3

Evaluation of different dose-response models for the calculation of 50% infectious dose of human norovirus Cin-1 in gnotobiotic pigs

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Key words: median infection dose, norovirus, gnotobiotic pigs, dose-response models

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Abstract

Human noroviruses (HuNoVs) are the leading causative agents of epidemic and sporadic acute gastroenteritis that affect people of all ages worldwide. However, very few dose-response studies have been carried out to determine the median infectious dose of HuNoVs. In this study, we determined the median infectious (ID_{50}) and diarrhea (DD_{50}) dose of GII.4/2003 variant of HuNoV (Cin-1) in the gnotobiotic (Gn) pig model of HuNoV infection and disease. Gn pigs were inoculated with 7 different doses (ranging from 8×10^2 to 2×10^6 genomic RNA copies) of Cin-1 at 33-34 days of age. Pigs were monitored daily from post-inoculation day (PID) 1 to 7, for fecal virus shedding and fecal consistency to evaluate the virus infectiousness and associated diarrhea. Using various mathematical models, we estimated the Log_{10} ID_{50} and DD_{50} to be between 3.11 to 3.76, and 3.37 to 4.87 RNA copies, respectively. Of the different models used in this study, the exponential and Beta-Poisson model were most extensively used by other dose-response studies carried out for enteric pathogens such as rotavirus and *E. coli*. Comparison of Akaike information criterion values showed that Beta-Poisson is the best-fitting mathematical model to estimate both the ID_{50} and DD_{50} of Cin-1 when using the rate of animals with virus shedding and diarrhea in each dose group. Adjusting a 3-parameter log logistic regression model to area under the curve values of virus shedding and fecal consistency scores resulted in the Log_{10} of ID_{50} to be 3.89 and the Log_{10} DD_{50} to be 5.03 RNA copies, within the range obtained from the counting data. Determining the ID_{50} of the challenge virus strain is crucial for identifying the true infectiousness of HuNoVs and for accurate evaluation of protective efficacies in pre-clinical studies of therapeutics, vaccines and other prophylactics using this reliable animal model.

1. Introduction

Human noroviruses (HuNoVs) are non-enveloped RNA viruses with a positive-sense single-stranded genome in the *Caliciviridae* family. They are the most common etiological agents of epidemic and sporadic acute gastroenteritis in people of all age groups (Payne, Vinjé *et al*. 2013, Pringle, Lopman *et al*. 2015, Rouhani, Peñataro Yori *et al*. 2016) and are responsible for around 125 million cases of foodborne illnesses worldwide each year (Kirk, Pires *et al*. 2015). There are currently no licensed vaccines available for HuNoVs, but several virus-like particle (VLP) vaccine candidates are currently undergoing various stages of clinical trials and have thus far shown some promise in both immunogenicity and protective efficacy studies, although the correlates of protection to norovirus infections are yet to be fully determined (Treanor, Atmar *et al*. 2014, Bernstein, Atmar *et al*. 2015, Sundararajan, Sangster *et al*. 2015, Atmar, Baehner *et al*. 2019). The lack of a widely available animal model has made it difficult for the study of viral pathogenesis, as well as the underlying immune mechanisms of protection against HuNoV infections (Ernst 2016, Wobus, Cunha *et al*. 2016). Although the stem cell-derived, human intestinal enteroid (HIE) monolayer culture system has been successful in cultivating several HuNoV strains (Ettayebi, Crawford *et al*. 2016, Ramani, Crawford *et al*. 2018), the magnitude of virus amplification is low and large quantities of infectious viral particles still can only be acquired from stool samples of infected human patients.

The ideal animal model to study HuNoV infection and disease would be one that is capable of replicating the biological and clinical features associated with human disease. Among the different models used in HuNoV research (Cheetham, Souza *et al*. 2007, Souza, Azevedo *et al*. 2008, Bok, Parra *et al*. 2011, Taube, Kolawole *et al*. 2013, Todd and Tripp 2019), gnotobiotic (Gn) pig models provide a suitable solution for studying HuNoV infection and disease due to the close similarities

in physiology, immune development, virus binding patterns, histo-blood group antigens (HBGA) phenotypes between pigs and humans (Cheetham, Souza *et al*. 2007). Since their introduction into research in the early 1960s, Gn pigs have been utilized extensively for the study of enteric virus and bacteria (Yuan, Jobst *et al*. 2017, Lei, Twitchell *et al*. 2018). Gn pig infection model for two different HuNoV GII.4 variants (GII.4/HS66 and GII.4/2006b) have been established for evaluating HuNoV vaccines (Cheetham, Souza *et al*. 2006, Souza, Costantini *et al*. 2007, Bui, Kocher *et al*. 2013, Kocher, Bui *et al*. 2014), displaying similar levels of infectivity compared to HuNoV GI.1/Norwalk and GII.4/2003 Cin-1 in human challenge studies and vaccine trials (Atmar, Bernstein *et al*. 2011, Treanor, Atmar *et al*. 2014, Bernstein, Atmar *et al*. 2015).

HuNoVs are believed to be highly infectious. A few studies have explored the infection risk (Le Guyader, Neill *et al*. 2003, Le Guyader, Krol *et al*. 2010, Lowther, Gustar *et al*. 2012) and calculated the median infectious dose (ID_{50}) (Teunis, Moe *et al*. 2008, Atmar, Opekun *et al*. 2014) for Norwalk virus in humans. It was identified that around 2800 RNA copy numbers were sufficient to successfully infect an adult human volunteer (Treanor, Atmar *et al*. 2014). A previous study of the ID_{50} of a GII.4/2006b variant in newborn (4-5 days of age) and older (33-34 days of age) Gn pigs was the only ID_{50} study of HuNoV in Gn pigs (Bui, Kocher *et al*. 2013). Dose-response data provide valuable information that are used to make the most informed risk assessments for viral infections in humans and is critically important for the standardization of the animal challenge model used in pre-clinical efficacy evaluation of vaccines and anti-viral agents.

The current study uses a different HuNoV, a GII.4/2003 variant Cin-1. The Cin-1 inoculum has been well characterized and used in human volunteer challenge studies with 5×10^4 RT-PCR units (viral RNA copies) per dose (Frenck, Bernstein *et al*. 2012, Bernstein, Atmar *et al*. 2015). The objectives of this study are to determine the median infectious and diarrhea doses, that is, the

dilution of virus inoculum at which 50% of the Gn pigs are infected (i.e., the ID_{50}) or developed diarrhea (i.e., DD_{50}) for the GII.4/2003 variant. The significance of this study includes: i). Identifying the most appropriate Cin-1 challenge dose in 33-34 day old Gn pigs to standardize the model for HuNoV vaccine evaluation; ii) Comparing different dose-response models used for estimating the ID_{50} and DD_{50} of Cin-1 in Gn pigs to determine the best fit model to the outcome variables expressed as a counting variable (using the rate or frequencies of animals affected) or as a continuous variable (the area under the curve of virus shed/diarrhea score) and; iii) To compare the obtained value with the dose used in human volunteers. These statistical analyses will maximize the internal validity and reproducibility of this animal model of HuNoV infection and disease. Comparing the infectiousness of the same GII.4 variant challenge pool in Gn pigs and humans (objective iii) will lead to a better understanding of the zoonotic potential of NoVs between different species and further validate the Gn pig model of HuNoV infection as a proper predictive tool of the future efficacy of vaccines and other antiviral strategies to control NoV diarrhea in humans.

2. Materials and Methods

2.1 Virus inoculum

A pool of human stool specimens containing GII.4 strain, Cin-1 (Hu/GII.4/Cin-1/2003/US), a 2002 Farmington Hills-like variant, was isolated by Xi Jiang's laboratory at Cincinnati Children's Hospital Medical Center collected from a man who developed a 3-day illness characterized by diarrhea, vomiting, nausea, abdominal cramps, and fever in 2003. This sample underwent extensive quality control prior to being used as a challenge pool in various human studies (Frenck, Bernstein *et al*. 2012, Bernstein, Atmar *et al*. 2015). The HuNoV concentration of the challenge

pool was determined by quantitative real-time RT-PCR (RT-qPCR) and as approximately 2 x 10^6 viral genomic RNA copies/mL of stool. The challenge pool was stored at -80°C in individual 1 mL aliquots until the day of Gn pig inoculation.

2.2 Gnotobiotic pigs and treatments

Near-term pigs (Large white cross breed) were derived by aseptic hysterectomy and maintained in sterile isolator units as described previously (Yuan, Jobst *et al*. 2017). To determine the sterility status of the isolators that the pigs were housed in, rectal swabs collected from pigs were cultured on blood agar plates (Hardy Diagnostics) and in Thioglycollate media (Hardy Diagnostics) 3 days after derivation and repeated once a week until the end of the study. Pigs were fed commercial ultra-high temperature-treated sterile cow milk throughout the study. A total of 28 pigs were assigned to seven groups. Pigs were randomly divided into groups upon derivation regardless of gender and body weight. Each group of pigs received a different dose of Cin-1 inoculum at postpartum day (PPD) 33 or 34. Prior to inoculation with HuNoV, 4 ml of 200 mM sodium bicarbonate was given orally 15 min to neutralize stomach acids. The Cin-1 inoculum was mixed with 5 ml of Diluent #5 (minimal essential medium with 1% penicillin-streptomycin and 1% HEPES) on the day of inoculation. All pigs received oral doses of Cin-1 ranging from 8 x 10^2 to 2 × 10^6 genomic RNA copies at PID (post-inoculation day) 0. Clinical signs and fecal virus shedding were monitored daily until euthanasia. All pigs were euthanized on PID 7. All experiments involving the use of Gn pigs were approved by the Institutional Animal Care and Use Committee at Virginia Tech (IACUC protocol: 17-110-CVM). All experimental procedures were carried out in compliance with federal and university regulations.

2.3 HBGA-typing of Gn pigs by immunofluorescence assay

Prior to inoculation, all Gn pigs were screened for their HBGA type by immunofluorescence assay. Epithelial cells collected from the cheeks were prepared as described previously (Bui, Kocher *et al*. 2013). HBGA phenotypes were detected from these cells using fluorescent labeled antibodies described elsewhere (Lei, Ryu *et al*. 2016). All slides were prepared with VECTASHIELD® Antifade Mounting Medium with 4,6-diamidino-2-phenylindole (DAPI) which was used as a nuclear counterstain (Vector Laboratories) for fluorescent microscopy. Based on this screening technique, HBGA type A^- and H^- pigs were excluded from this study because A^- or H^- pigs are less susceptible to NoV infection than A^+ or H^+ pigs (Cheetham, Souza *et al*. 2007, Bui, Kocher *et al*. 2013).

2.4 Detection of fecal consistency and HuNoV shedding by RT-qPCR

Fecal consistency and virus shedding were monitored at 24-hour intervals at the same time daily, after HuNoV inoculation at PID 0, by rectal swab sampling. The following scoring system was used to assess fecal consistency: 0, solid; 1, pasty; 2, semiliquid; 3, liquid. Pigs with fecal consistency scores of 2 or greater were considered to be diarrheic. Presence of HuNoV genomes in rectal swab samples collected after inoculation with different doses of Cin-1 were evaluated using RT-qPCR protocol as described previously (Lei, Ryu *et al*. 2016). Briefly, once collected, swabs were swirled in 1 ml PBS to release the feces and centrifuged at 10000 g for 5 min. 250 µl of the supernatant collected was mixed with 750 µl TRIzol (Invitrogen) following manufacturer's instructions for viral RNA extraction. The extracted RNA was then dissolved in 40 µl autoclaved dd-H_2O. Five microliters of RNA were used in 20 µl RT-qPCR reaction with a SensiFAST Probe No-ROX One-Step Kit (Bioline) to detect HuNoV genomes. Primers COG2F, COG2R, and probe

RING2 were used with cycling conditions as described in a previous study (Kageyama, Kojima *et al*. 2003). A standard curve was generated using COG2 amplicon-containing cDNA expression plasmids in serially diluted tenfold from 2 x 10^6 to 2 genomic RNA copies. Amplification was performed on CFX96 Real-Time System (Bio-Rad), and data were collected and analyzed with Bio-Rad CFX Manager 2.0.

2.5 Electron Microscopy

Negative-stain electron microscopy (EM) was carried out to determine the aggregation status of Cin-1 inoculum. Briefly, TEM grids were treated with 1% aqueous Alcian blue (Sigma-Aldrich) solution for 5 min and washed with a few drops of distilled water until the dye is removed. Samples were diluted as mentioned in Section 2.2 to ensure that the visualized particles correspond to the dilution administered to Gn pigs. A droplet of sample was added to each grid and allowed to sit for 1 minute to ensure adsorption onto the grid. 3% Sodium (K) phosphotungstate (PTA; Sigma-Aldrich) were then added and allowed to stain for 1 min. Upon removing the treatment solution using filter paper, the grids were allowed to air dry. Zeiss 10A transmission electron microscope was then used to visualize and image the stained Cin-1 NoV particles at 200 k to 250 k magnitudes.

2.6 Statistical analysis

2.6.1 Analysis of variance among challenge doses

The analyses of variances were carried out using Infostat® connected to R (https://www.infostat.com.ar/index.php?mod=page&id=46) and further confirmed using scripts from the R software (R Core Team) in R Studio. The variables related to virus shedding were analyzed using a general linear model (GLM), considering the treatment (pigs receiving different doses of HuNoV) as a fixed factor and the Gn pigs within the groups as a random factor. The

variance was modelated using a varIdent structure for the variance-covariance matrix. The variables associated to diarrhea were analyzed by one-way ANOVA prior to testing the normality and homoscedasticity assumptions. In all cases, multiple post ANOVA comparisons were carried out using the Tukey method for comparisons between the 7 different doses. Statistical significance was considered at $p < 0.05$ for all comparisons.

2.6.2 ID_{50} and DD_{50} calculations using different dose-response models

For a specific pathogen, in a specific host, using a specific exposure route for a particular endpoint (response or outcome: in this case virus shedding and diarrhea), there is a unique dose response relationship that can be described using different mathematical functions that allows for selecting the model with the best fit.

i. Reed-Muench, Dragstedt-Behrens and Spearman-Karber method

The Reed-Muench (RM) method, first described in 1938 (Reed and Muench 1938), is an old fashion method that has been widely used for the calculation of 50% end point in experimental biology. The RM method employs a two-step equation that involves the calculation of the proportionate distance (PD) between dilutions above and below the 50% end point as calculated using Equation (1).

$$Proportionate\ distance\ (PD) = \frac{Percentage\ infected\ at\ dilution\ next\ above\ 50\% - 50\%}{(Percentage\ infected\ at\ dilution\ next\ above\ 50\% - Percentage\ infected\ at\ dilution\ next\ below\ 50\%} \quad (1)$$

$$log 50\ \%\ end\ point = (\log dilution\ above\ 50\%) - (PD * \log dilution\ factor) \quad (2)$$

The dilution factor used in this equation describes the fold difference between the two inoculum titers above and below 50% response rate. The median dose was then calculated based on Equation

(2). The RM method has been used previously for the determination of the ID_{50} of a different HuNoV variant (GII.4/2006b) in Gn pigs (Bui, Kocher *et al*. 2013).

The Dragstedt-Behrens (DB) method (Dragstedt and Lang 1928, James 1959) is very similar to the RM method but instead of working with the cumulative sums directly, it uses the fraction of the cumulative sums that are positive at each dose. The ID_{50} is estimated by interpolating the line that connects the hypothetical fractions of the bracketing doses (Siev 2018).

Both methods rely on data to be equally-spaced, with each group containing equal "n" numbers, and the overall data to be symmetric in nature for an accurate estimate of the dose-response. Although primarily used for their historical relevance, the RM and DB methods are still used extensively in modern scientific research as the primary model for calculating the end-point dilution dose (Bui, Kocher *et al*. 2013, Ramakrishnan 2016, Elikaei, Hosseini *et al*. 2017, Zhao, Xie *et al*. 2017, Han, Chen *et al*. 2018, Spackman, Malladi *et al*. 2019, Sudeep, Vyas *et al*. 2019).

Spearman-Karber is another method that is widely used and specially suggested by the regulatory agencies for the calculation of virus titers and mean infectious doses. These non-parametric methods produce accurate estimates of the mean but not the median. An important criteria for being able to use this method would be the presence of dose response data with 0% and 100% response in order to generate a symmetrical distribution of data with well-defined upper and lower plateaus (Siev 2018).

ii. Exponential dose-response model and Beta-Poisson generalized linear model (GLM)

Both Exponential and Beta-Poisson models have been identified to be the best-fit models for the calculation of ID_{50} of various viral infections in humans and other animal models.

The exponential dose response model is derived by assuming that the parameter **r** representing the survival of the pathogen in the host is a constant parameter. The mechanistic microbial dose response model is the exponential equation (3) from which there is a binomial likelihood of **r** pathogens that survive to initiate an infection. This means that either the pathogen survives or does not to initiate an infection.

$$P(r) = 1 - exp(-r \cdot dose) \qquad (3)$$

The Beta Poisson dose response model is an expansion from the exponential model. In this case, it is assumed that the survival of the pathogen in the host is a random variable with a Beta Poisson distribution for the average of the bolus dose. This allows for an increased level of realism in modeling the dose response relationship in the host.

$$P(r) = 1 - \left[1 + \frac{dose \cdot 2^{1/\alpha} - 1}{N_{50}}\right]^{-\alpha} \qquad (4)$$

In equation (4), N_{50} is an estimate of the median infectious/diarrhea dose with units of counts (i.e. genome copy numbers) and **α** is a unitless model parameter. Equation (4) represents an approximation of the exact form of the Beta Poisson model (Weir, Mitchell *et al*. 2017). This approximated form retains the linearity at low doses, a fundamental characteristic of microbial dose response models (Haas, Rose *et al*. 2014, Weir, Mitchell *et al*. 2017).

Assumptions of the model:

a. *The number of aggregates in the virus suspension are random Poisson-distributed.*

This assumes that a Poisson distribution of NoV aggregated among Gn pigs (replicates) receiving the same challenge doses. This assumption helps to explain that some pigs are highly infected and others do not in the same dose group due to this random chance to receive one or several aggregates.

b. *Aggregates size vary according to a log-series distribution but it is not a problem*

c. *The dilution procedure do not modify the aggregation condition of the sample*

d. *One organism is capable of producing an infection if it arrives at an appropriate site*.

Once the viruses enter the host, the viruses become disaggregated and act (infect) independently. Organisms have independent and identical probability *P(r)* of surviving to reach and infect at an appropriate site.

Survival probabilities *P(r)* are given by the beta distribution.

Online tools are available for both models and can be found here:

1. Exponential Model: AATBioquest® - https://www.aatbio.com/tools/ed50-calculator
2. Beta-Poisson Model: NCBI Statistical Computational Biology Infectious Dose or Dilution (ID_{50}) Server - https://www.ncbi.nlm.nih.gov/CBBresearch/Spouge/html_ncbi/html/id50/id50.cgi (Spouge 2015). This tool was based on VACMAN, a computational program that calculates statistics for *in vitro* and *in vivo* infectivity data (Spouge 1992).

Calculations of ID_{50} and DD_{50} were also carried out using the R scripts published by Weir 2017 (Weir, Mitchell *et al*. 2017) for Exponential and Beta Poisson (R Core Team). Briefly, the script uses the maximum likelihood estimation (MLE) to fit the counting data (rate of animals with virus

shedding/diarrhea) to both theoretical distributions, either Exponential or Beta-Poisson due to their biologic plausibility. The process calculates the probability of obtaining the observed data given a theoretical distribution by minimizing the deviance (Y) and the Akaike information criterion (AIC) of each of these fitted models are defined by equations (5) and (6) respectively.

$$\text{Deviance} = Y = -2\,[\ln M2 - \ln M1] \qquad (5)$$

Where lnM1, lnM2 are the MLE for the full (M2) and restricted (M1) models. Optimized deviance follows a Chi-squared distribution with k – p degrees of freedom, where k is the number of doses and p is the number of dose-response parameters of a given model. The model is rejected if $Y > X^2(k\text{-}p,\alpha)$.

$$AIC = 2p + Y \qquad (6)$$

Where p is the value of the parameter of the model and Y is the deviance. The AIC adds a penalty for adding parameters to the model to avoid model overfitting (increasing the number of parameters in the model almost always improves the goodness of the fit). If two or more models are significant, the model with the lowest deviance (and AIC) is chosen. Bootstrapping is performed to characterize the uncertainty of parameter estimates of the distribution, most commonly by generating confidence intervals. The estimates from this approach approximate the uncertainty associated with the "true" distribution by repeatedly sampling the data and re-computing a statistic (Weir, Mitchell *et al*. 2017).

iii. ID_{50} and DD_{50} estimated by the adjustment of a three-parameter log logistic (3PLL) dose-response model on the shedding and diarrhea AUC values

Area under the curves (AUC) of virus shedding (Log10 genomic copies) and diarrhea severity (fecal score) for each individual pig were calculated. These two continuous variables were based

on a 3PLL model and the ID_{50} and DD_{50} estimated using the "dr package" in R described previously (Ritz, Baty *et al*. 2016).

3. Results

3.1 Assessment of infection status in Gn pigs

Infection caused by HuNoV Cin-1 was defined by the presence of viral RNA quantified using RT-qPCR carried out on samples isolated from rectal swabs on any day from PID 1 to 7. The percentage of affected animals, mean days to onset, duration, peak titer and AUC of fecal virus shedding, in pigs in each of 7 dose groups are summarized in Table 1. An increase in inoculation dose was positively associated with a shorter incubation period, which coincided with the observed increased duration (Figure 1A), as well as an increased overall virus shed in feces measured by AUC (Figure 1B). All pigs belonging to dose groups 1 to 4 shed detectable titers of virus in their feces, while 67% of pigs shed virus when inoculated at doses between 3.2×10^3 to 2×10^4 RNA copies. Only 25% of the pigs that were inoculated with 8×10^2 RNA shed virus. Important to note, pigs that were administered a dose of 2×10^5 RNA copies shed significantly higher amount of virus in their feces ($p<0.0001$) and shed virus for a significantly longer duration than pigs in the other groups ($p<0.0001$) (Figure 1A and 1B).

3.2 Assessment of diarrhea status in Gn pigs

After inoculation, pigs were monitored daily for diarrhea status. Fecal consistency scores were determined based on previous studies carried out by our group (Lei, Ramesh *et al*. 2016, Lei, Ryu *et al*. 2016, Lei, Samuel *et al*. 2016): 0 solid; 1 pasty; 2 semi-liquid; or 3 liquid. A score of 2 or greater (≥ 2) was considered to be diarrheic. The percentage of pigs with diarrhea, the mean duration, the AUC and mean onset day of diarrhea from Cin-1 inoculated pigs are summarized in

Table 1. A longer duration of diarrhea (Figure 1C) and a higher cumulative diarrhea score (Figure 1D) were observed in Gn pigs that were inoculated with higher doses of Cin-1. Pigs that were inoculated with 2 x 10^5 RNA copies of Cin-1 that had the highest virus titers shed in their feces also experienced diarrhea for a longer duration of time than the pigs inoculated with the other doses. Pigs belonging to dose group 1 experienced diarrhea for the longest duration of time (5 out of 7 days) and had the quickest onset (within 1.5 days after inoculation), indicating severe clinical onset mimicking that in humans (Cheetham, Souza *et al*. 2006, Cheetham, Souza *et al*. 2007), while HuNoV-associated gastroenteritis typically lasts for 3-5 days after infection among susceptible populations (Green 2013). Pigs in dose groups 5 and 6 had a delayed onset to diarrhea together with lower cumulative fecal consistency scores, demonstrating a milder diarrhea burden. No diarrhea was observed among pigs in dose group 7. In summary, the third dose with 2 x 10^5 RNA copies of Cin-1 was also the optimal dose in terms of severity and mean duration of diarrhea.

3.3 Determination of ID_{50} and DD_{50} using various dose-response models

The parameters used for conventional RM method is displayed in supplementary material Supplementary Table S1. The cumulative frequencies (%) of pigs shedding virus and experiencing diarrhea were used to calculate the ID_{50} and DD_{50} doses in conventional RM method. The ID_{50} and DD_{50} of Cin-1 were calculated to be log dose 3.11 and 4.86, respectively. The ID_{50} and DD_{50} calculated using the exponential and the beta-Poisson dose-response models are presented in Table 2. AUC-based estimates of ID_{50} and DD_{50} using the 3PLL model were log 3.89 and 5.02 respectively (Table 2 and Figure 3). From the estimates calculated using the Spearman-Karber (SK) method, only the DD_{50} can be considered reliable, while the ID_{50} even within the range of the estimates obtained by the other method is not reliable due to the lack of sufficient dose-response data in the lower limit.

3.4 Comparison of infectiousness of HuNoV Cin-1 in humans and Gn pigs.

We compared the percentage, mean duration and peak day of virus shedding between humans and Gn pigs after inoculation with the same HuNoV Cin-1 inoculum (Table 3). In the human adult challenge study (Frenck, Bernstein *et al*. 2012), after oral inoculation with 5 x 10^4 RNA copies of HuNoV Cin-1, 16 out of 23 secretors (70%) were infected, which was defined by the detection of HuNoV in stool samples by RT-qPCR same as in the Gn pig study. In Gn pigs, an inoculation dose of 2 x 10^4 infected 67% of pigs, whereas 8 x 10^4 and 2 x 10^5 RNA copies infected 100% pigs (Table 1). Among pigs inoculated with 2 x 10^5 RNA copies, the duration and range of days of virus shedding are comparable to those of humans and the peak day of virus shedding is almost identical (PID 3). These data indicate that the infectiousness of HuNoV Cin-1 in Gn pigs and humans are very similar and the Gn pig model when inoculated with the optimal virus dose (2 x 10^5 RNA copies) accurately replicates the course of HuNoV infection in humans.

3.5 Identification of best-fitting method for dose-response analysis of Cin-1

Both Exponential and Beta-Poisson are routinely used as interchangeable logit models for the determination of median infectious- and median diarrhea- doses for different pathogens due to their biological plausibility. Comparisons of goodness of fit data demonstrated that both models are equally effective for the determination of ID_{50} (Figure 2A and 2B; and Table 4a) but the Beta-Poisson method, based on a lower deviance and AIC values, was determined to be the best-fitting model for determining the DD_{50} of Cin-1 (Figure 2C and 2D; and Table 4b). The ID_{50} and DD_{50} values determined by beta-Poisson model was 2.57 x 10^3 RNA copies and 2.09 x 10^4 RNA copies respectively.

3.6 Determination of an optimal challenge dose

Based on the virus shedding and diarrhea status of infected pigs, we determined the optimal challenge dose of Cin-1 to be 2 x 10^5 (Table 5). Our data shows that pigs infected at this dose had a mean onset day of 1.3, with virus shed in feces in large quantities (AUC) for almost the whole duration of the infection period (6.3 days out of 7; Table 1). Moreover, pigs in this dose group also experienced the highest diarrhea burden among all dose groups, with diarrhea starting at 2.8 days after inoculation and occurring for a duration of 4 days. Pigs in this group also had the highest mean cumulative diarrhea score of 9.31 (Table 2).

3.7 Aggregation of Cin-1

The Cin-1 virus pool has been previously demonstrated to be aggregated (Messner, Berger *et al*. 2014), but no evidence was provided with regards to the size of each aggregate. In an attempt to ascertain the amount of virus particles within each aggregate, TEM micrographs were taken of Cin-1 inoculum at 8 x 10^4 and at 2 x 10^5 RNA copies. The average number of virus particles within each observed aggregate was determined to be 175 and 390 respectively. Figure 4 shows representative images of Cin-1 norovirus inoculum pool at 8 x 10^4 and 2 x 10^5 RNA copies.

4. Discussion

In this study, we determined the ID_{50} and DD_{50} of HuNoV GII.4/2003 Cin-1 variant in Gn pigs, a large animal model that has proven to be capable of replicating HuNoV GII.4-associated disease in humans (Cheetham, Souza *et al*. 2006, Bui, Kocher *et al*. 2013). The ID_{50} and DD_{50} calculated with the best fitting, beta-Poisson model, are 2.57 x 10^3 RNA copies and 2.09 x 10^4 RNA copies, respectively. Our data was analyzed with different dose response models, and all the ID_{50} estimates were within 1 log of each of the methods, confirming the accuracy of the experiments.

The GII.4/2006b variant of HuNoV was primarily observed to cause illness among the pediatric population in day cares and hospitals (Tu, Bull *et al*. 2008, Chen, Feng *et al*. 2015, Kumazaki and Usuku 2015). Bui *et al* determined the ID_{50} of GII.4/2006b variant in neonatal (4-5 days of age) and older (33-34 day old) Gn pigs using RM calculation method. An ID_{50} of 6.43 x 10^4 RNA copies was identified based on the infection status of older pigs (Bui, Kocher *et al*. 2013). The Farmington Hills virus was responsible for around 64% of cruise ship outbreaks and 45% of the land-based outbreaks, most of which occurred in long-term care facilities, schools, and restaurants, in the US in 2002 (Isakbaeva, Widdowson *et al*. 2005, Bull, Tu *et al*. 2006, Johnston, Qiu *et al*. 2007). In the current study, using the RM method, we determined the ID_{50} of Cin-1, a variant of the Farmington Hills virus, to be 1.84 x 10^3 RNA copies in similar aged pigs (Table 2). A 35-fold higher virus titer is required to establish infection among 50% of the pigs inoculated with the GII.4/2006b virus as compared to the Cin-1 (Table 5), indicating that Cin-1 is a more infectious strain in Gn pigs.

In a previous human clinical study, administering a dose of 5 x 10^4 RNA copies of Cin-1 from the same Cin-1 challenge pool caused infection among 70% of secretor positive individuals (Frenck, Bernstein *et al*. 2012). A phase I vaccine study in human volunteers carried out by Bernstein and colleagues used a challenge dose of 4.4 x 10^3 RNA copies (Bernstein, Atmar *et al*. 2015) based on a pilot study showing similar rates of infection (26/34 [76.5%]) and illness (19/34 [55.9%]) compared to the 5 x 10^4 RNA copies used by Frenck (Frenck, Bernstein *et al*. 2012, Bernstein, Atmar *et al*. 2015). However, this challenge dose (4.4 x 10^3 RNA copies) was too low and caused infection only in 62.5% and disease (vomiting or diarrhea of mild or greater severity) in 37.5% of the volunteers among the placebo control group. This data compared to the slightly reduced incidence of infection (54%) and disease (20%) among the vaccine recipients, leading to the

inconclusive protective effect of the vaccine due to the lack of statistical significance (Bernstein, Atmar *et al*. 2015). Similarly, in the present study, in dose group 5 (2 x 10^4 RNA copies), only 67% of the pigs shed virus and 33% had diarrhea, whereas 100% of the pigs in dose groups 1-4 shed virus and 75% to 100% had diarrhea. This data shows an observable trend that corroborates the data from Frenck and Bernstein (Frenck, Bernstein *et al*. 2012, Bernstein, Atmar *et al*. 2015). Collectively, these data highlight the importance of identifying the optimal virus challenge dose for the evaluation of protective efficacy of vaccines in animal models and in humans.

Diarrhea status in this study was determined based on the fecal scores that are assigned by the researchers conducting the study working in blind regarding the dose administered to each group. The conditions established within the gnotobiotic lab setting are well controlled (Yuan, Jobst *et al*. 2017), preventing the interference of extraneous pathogens or dietary changes that can cause diarrhea or influence the state of infection. The presence of other pathogens/microorganisms in the environment can lead to discrepancies regarding the true cause of diarrhea (Lei, Twitchell *et al*. 2019).

Regardless of their universal usage, the RM and DB methods are heavily reliant on a sequential progression of responses, since they use only information from two points around the potential titer completely overlooking any random distribution of data (Siev 2018). Nonetheless, there has been continued debate, among the research community, on the reliability of RM and DB and their use (particularly due to the ease of methodology) for equally spaced dose levels, making them valuable tools for a rapid and/or rough calculation of ID_{50} and DD_{50} for non-statisticians. When handling sequentially spaced dose levels such as the ones in this study, RM and DB have been described to be highly reliable and have been known to provide a high coverage rate (> 95% CI), but on the other hand when data is more randomly distributed, carrying out RM and DB method

could complicate the inference due to inaccuracies, questioning the internal validity of the data and results.

The Spearman-Karber (SK) method is another non-parametric method that can be easily calculated by hand with more accurate estimates of the mean dose (Miller 1973, Miller and Ulrich 2001). It relies on the symmetric nature of dose response data to calculate the median dose, meaning that it requires a dose with 0% and 100% responses to calculate the median dose. Some dose-response studies are not capable of generating data fulfilling the criterion. In this study, this is observable with the ID_{50} data where all the dose groups had animals that shed virus. On the other hand, the SK method would be applicable for calculating the DD_{50}, since dose groups contained all (100%) or none (0%) of the pigs infected.

With the increasing availability of powerful statistical tools, logistic regression-based models have been more heavily relied on for dose-response calculations for their accuracy and robustness compared to non-parametric methods (Spackman, Malladi *et al*. 2019). Both the exponential and Beta-Poisson are routinely used for the quantitative determination of dose-response relationships (Haas 2002). The exponential equation takes the symmetric nature of the curve around the inflection point into account when calculating the median dose, while reducing the effects of variability, due to large differences between the doses used. Using a logistic regression model (exponential model), the ID_{50} of the original Norwalk virus (GI.1, 8fIIa isolate) challenge pool was predicted to be around 2.8×10^3 RNA copies in all secretor positive individuals (Atmar, Opekun *et al*. 2014). Similarly, in the current study, we determined the ID_{50} of Cin-1 to be around 2.57×10^3 RNA copies. These data show a similar infection potential between the prototypic GI.1 Norwalk virus and the Cin-1. With the availability of more dose-response studies and the relatability between human studies and Gn pig studies, it could eventually be possible to compare

the pathogenesis of different HuNoV genogroups to help further identify similarities and/or differences between different HuNoVs.

More than generating an ID_{50}, Beta-Poisson dose-response models, can describe the full range of probability of response (ID_{20} or ID_{80}), and it is accurate at low doses as well (Roederer 2015). It allows for greater flexibility and a wider range of understanding in the estimated probability of infection. AIC is an estimate of the expected relative distance between the fitted model and the unknown true mechanism that actually generated the observed data (Korner-Nievergelt, Roth *et al*. 2015). Comparing both these models using AIC showed the closeness of the two models for calculating the ID_{50} while the beta-Poisson model was the ideal model for DD_{50} estimation.

Using the 3PLL model, it allows for the estimation of the ID_{50} and DD_{50} based on cumulative data (AUC), a continuous variable. The closeness of these ID_{50} and DD_{50} estimates demonstrates the reliability of the model for calculating accurate dose response. Overall, both approaches prove their value to dose-response studies and can be used separately to simply determine the median infectious doses, or together for a more concrete understanding of the infectious potential of the pathogen. AIC was used to identify the most reliable model that can be used for calculating the ID_{50} and DD_{50} based on the data collected. We showed that Beta-Poisson model is the one for the counting variable while 3PLL is suitable for the AUC continuous variable, reinforcing the validity of the estimated ID_{50} and DD_{50} in Gn pigs.

Interestingly, there were three values of AUC of virus shedding in the two lower doses that are very high, this result could be due to aggregation effect that was confirmed by EM. Since the very first visualization of HuNoV, samples isolated from stool filtrates without extensive processing have always been identified as aggregates (Kapikian, Wyatt *et al*. 1972, Teunis, Moe *et al*. 2008, Otto, Clarke *et al*. 2011). A recent study showed that a large fraction of norovirus shed into stool

reside inside membrane-bound vesicles and such vesicles increase virus stability within the gastrointestinal tract and enhance the virus infectivity (Santiana, Ghosh *et al*. 2018). Although EM is not routinely used for laboratory diagnosis of HuNoV infections, they provide a good understanding whether the virus retains its natural qualities as an aggregate which defines its natural state of existence. As demonstrated here (Figure 4), determining the aggregation status of HuNoV at different inoculum doses allow for the understanding of HuNoV infectivity as a single virion compared to an aggregate. As shown by Teunis, *et.al*, the difference in the infectivity of 8ffa and 8ffb strains of HuNoV is hypothesized due to the aggregation status of the viruses (Teunis, Moe *et al*. 2008). Based on this calculation method, we estimated here that, a dose of 2 x 10^5 RNA copies contained around 512.8 infectious virus particles in 100 µl of Cin-1 inoculum, translating to 5.128 x 10^3 infectious particles / mL.

Our studies have demonstrated that Gn pigs are a well-suited model for studying the infectivity of GII.4 HuNoV strains. We have showed its closeness to humans regarding their susceptibility to HuNoV infection. Gn pigs are capable of exhibiting the full course of HuNoV infection and develop disease very similar to the one presented in humans. In conclusion, we have established a reliable model that can be used to test candidate prophylactics and therapeutics prior to clinical trials in humans, and the model is ready to be used for the evaluation of immunogenicity and protective efficacy of candidate HuNoV vaccines.

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References

Atmar, R. L., F. Baehner, J. P. Cramer, E. Lloyd, J. Sherwood, A. Borkowski, P. M. Mendelman and N. O. R. S. Group (2019). "Antibody persistence to two Virus-Like Particle norovirus vaccine candidate formulations in healthy adults: one-year follow-up with memory probe vaccination." J Infect Dis.

Atmar, R. L., D. I. Bernstein, C. D. Harro, M. S. Al-Ibrahim, W. H. Chen, J. Ferreira, M. K. Estes, D. Y. Graham, A. R. Opekun, C. Richardson and P. M. Mendelman (2011). "Norovirus Vaccine against Experimental Human Norwalk Virus Illness." New England Journal of Medicine **365**(23): 2178-2187.

Atmar, R. L., A. R. Opekun, M. A. Gilger, M. K. Estes, S. E. Crawford, F. H. Neill, S. Ramani, H. Hill, J. Ferreira and D. Y. Graham (2014). "Determination of the 50% human infectious dose for Norwalk virus." J Infect Dis **209**(7): 1016-1022.

Bernstein, D. I., R. L. Atmar, G. M. Lyon, J. J. Treanor, W. H. Chen, X. Jiang, J. Vinje, N. Gregoricus, R. W. Frenck, Jr., C. L. Moe, M. S. Al-Ibrahim, J. Barrett, J. Ferreira, M. K. Estes, D. Y. Graham, R. Goodwin, A. Borkowski, R. Clemens and P. M. Mendelman (2015). "Norovirus vaccine against experimental human GII.4 virus illness: a challenge study in healthy adults." J Infect Dis **211**(6): 870-878.

Bernstein, D. I., R. L. Atmar, G. M. Lyon, J. J. Treanor, W. H. Chen, X. Jiang, J. Vinjé, N. Gregoricus, R. W. Frenck, Jr., C. L. Moe, M. S. Al-Ibrahim, J. Barrett, J. Ferreira, M. K. Estes, D. Y. Graham, R. Goodwin, A. Borkowski, R. Clemens and P. M. Mendelman (2015). "Norovirus vaccine against experimental human GII.4 virus illness: a challenge study in healthy adults." The Journal of infectious diseases **211**(6): 870-878.

Bok, K., G. I. Parra, T. Mitra, E. Abente, C. K. Shaver, D. Boon, R. Engle, C. Yu, A. Z. Kapikian, S. V. Sosnovtsev, R. H. Purcell and K. Y. Green (2011). "Chimpanzees as an animal model for human norovirus infection and vaccine development." Proceedings of the National Academy of Sciences of the United States of America **108**(1): 325-330.

Bui, T., J. Kocher, Y. Li, K. Wen, G. Li, F. Liu, X. Yang, T. LeRoith, M. Tan, M. Xia, W. Zhong, X. Jiang and L. Yuan (2013). "Median infectious dose of human norovirus GII.4 in gnotobiotic pigs is decreased by simvastatin treatment and increased by age." J Gen Virol **94**(Pt 9): 2005-2016.

Bull, R. A., E. T. V. Tu, C. J. McIver, W. D. Rawlinson and P. A. White (2006). "Emergence of a New Norovirus Genotype II.4 Variant Associated with Global Outbreaks of Gastroenteritis." Journal of Clinical Microbiology **44**(2): 327-333.

Cheetham, S., M. Souza, R. McGregor, T. Meulia, Q. Wang and L. J. Saif (2007). "Binding Patterns of Human Norovirus-Like Particles to Buccal and Intestinal Tissues of Gnotobiotic Pigs in Relation to A/H Histo-Blood Group Antigen Expression." Journal of Virology **81**(7): 3535-3544.

Cheetham, S., M. Souza, T. Meulia, S. Grimes, M. G. Han and L. J. Saif (2006). "Pathogenesis of a genogroup II human norovirus in gnotobiotic pigs." J Virol **80**(21): 10372-10381.

Chen, S. Y., Y. Feng, H. C. Chao, M. W. Lai, W. L. Huang, C. Y. Lin, C. N. Tsai, C. L. Chen and C. H. Chiu (2015). "Emergence in Taiwan of novel norovirus GII.4 variants causing acute gastroenteritis and intestinal haemorrhage in children." J Med Microbiol **64**(Pt 5): 544-550.

Dragstedt, C. A. and V. F. Lang (1928). "RESPIRATORY STIMULANTS IN ACUTE COCAINE POISONING IN RABBITS." Journal of Pharmacology and Experimental Therapeutics **32**(3): 215.

Elikaei, A., S. M. Hosseini and Z. Sharifi (2017). "Inactivation of model viruses and bacteria in human fresh frozen plasma using riboflavin and long wave ultraviolet rays." Iranian journal of microbiology **9**(1): 50-54.

Ernst, W. (2016). "Humanized mice in infectious diseases." Comparative Immunology, Microbiology and Infectious Diseases **49**: 29-38.

Ettayebi, K., S. E. Crawford, K. Murakami, J. R. Broughman, U. Karandikar, V. R. Tenge, F. H. Neill, S. E. Blutt, X. L. Zeng, L. Qu, B. Kou, A. R. Opekun, D. Burrin, D. Y. Graham, S. Ramani, R. L. Atmar and M. K. Estes (2016). "Replication of human noroviruses in stem cell-derived human enteroids." Science **353**(6306): 1387-1393.

Frenck, R., D. I. Bernstein, M. Xia, P. Huang, W. Zhong, S. Parker, M. Dickey, M. McNeal and X. Jiang (2012). "Predicting susceptibility to norovirus GII.4 by use of a challenge model involving humans." J Infect Dis **206**(9): 1386-1393.

Green, K. (2013). Caliciviridae: The Noroviruses. Fields Virology. D. M. Knipe and P. M. Howley. Philadelphia, PA, Wolters Kluwer Health/Lippincott Williams & Wilkins. **1:** 582-608.

Haas, C. N. (2002). "Conditional dose-response relationships for microorganisms: development and application." Risk Anal **22**(3): 455-463.

Haas, C. N., J. B. Rose and C. P. Gerba (2014). "Quantitative microbial risk assessment."

Han, N., Z. Chen, H. Wan, G. Huang, J. Li and B. R. Jin (2018). "A rapid method for titration of ascovirus infectivity." Journal of Virological Methods **255**: 101-106.

Isakbaeva, E. T., M.-A. Widdowson, R. S. Beard, S. N. Bulens, J. Mullins, S. S. Monroe, J. Bresee, P. Sassano, E. H. Cramer and R. I. Glass (2005). "Norovirus transmission on cruise ship." Emerging infectious diseases **11**(1): 154-158.

James, G. S. (1959). "The Behrens-Fisher Distribution and Weighted Means." Journal of the Royal Statistical Society. Series B (Methodological) **21**(1): 73-90.

Johnston, C. P., H. Qiu, J. R. Ticehurst, C. Dickson, P. Rosenbaum, P. Lawson, A. B. Stokes, C. J. Lowenstein, M. Kaminsky, S. E. Cosgrove, K. Y. Green and T. M. Perl (2007). "Outbreak Management and Implications of a Nosocomial Norovirus Outbreak." Clinical Infectious Diseases **45**(5): 534-540.

Kageyama, T., S. Kojima, M. Shinohara, K. Uchida, S. Fukushi, F. B. Hoshino, N. Takeda and K. Katayama (2003). "Broadly reactive and highly sensitive assay for Norwalk-like viruses based on real-time quantitative reverse transcription-PCR." J Clin Microbiol **41**(4): 1548-1557.

Kapikian, A. Z., R. G. Wyatt, R. Dolin, T. S. Thornhill, A. R. Kalica and R. M. Chanock (1972). "Visualization by immune electron microscopy of a 27-nm particle associated with acute infectious nonbacterial gastroenteritis." J Virol **10**(5): 1075-1081.

Kirk, M. D., S. M. Pires, R. E. Black, M. Caipo, J. A. Crump, B. Devleesschauwer, D. Döpfer, A. Fazil, C. L. Fischer-Walker, T. Hald, A. J. Hall, K. H. Keddy, R. J. Lake, C. F. Lanata, P. R. Torgerson, A. H. Havelaar and F. J. Angulo (2015). "World Health Organization Estimates of the Global and Regional Disease Burden of 22 Foodborne Bacterial, Protozoal, and Viral Diseases, 2010: A Data Synthesis." PLOS Medicine **12**(12): e1001921.

Kocher, J., T. Bui, E. Giri-Rachman, K. Wen, G. Li, X. Yang, F. Liu, M. Tan, M. Xia, W. Zhong, X. Jiang and L. Yuan (2014). "Intranasal P particle vaccine provided partial cross-variant protection against human GII.4 norovirus diarrhea in gnotobiotic pigs." Journal of virology **88**(17): 9728-9743.

Korner-Nievergelt, F., T. Roth, S. von Felten, J. Guélat, B. Almasi and P. Korner-Nievergelt (2015). Chapter 11 - Model Selection and Multimodel Inference. Bayesian Data Analysis in

Ecology Using Linear Models with R, BUGS, and STAN. F. Korner-Nievergelt, T. Roth, S. von Felten *et al*. Boston, Academic Press**:** 175-196.

Kumazaki, M. and S. Usuku (2015). "Genetic Analysis of Norovirus GII.4 Variant Strains Detected in Outbreaks of Gastroenteritis in Yokohama, Japan, from the 2006-2007 to the 2013-2014 Seasons." PLOS ONE **10**(11): e0142568.

Le Guyader, F. S., J. Krol, K. Ambert-Balay, N. Ruvoen-Clouet, B. Desaubliaux, S. Parnaudeau, J.-C. Le Saux, A. Ponge, P. Pothier, R. L. Atmar and J. Le Pendu (2010). "Comprehensive Analysis of a Norovirus-Associated Gastroenteritis Outbreak, from the Environment to the Consumer." Journal of Clinical Microbiology **48**(3): 915-920.

Le Guyader, F. S., F. H. Neill, E. Dubois, F. Bon, F. Loisy, E. Kohli, M. Pommepuy and R. L. Atmar (2003). "A semiquantitative approach to estimate Norwalk-like virus contamination of oysters implicated in an outbreak." Int J Food Microbiol **87**(1-2): 107-112.

Lei, S., A. Ramesh, E. Twitchell, K. Wen, T. Bui, M. Weiss, X. Yang, J. Kocher, G. Li, E. Giri-Rachman, N. V. Trang, X. Jiang, E. P. Ryan and L. Yuan (2016). "High Protective Efficacy of Probiotics and Rice Bran against Human Norovirus Infection and Diarrhea in Gnotobiotic Pigs." Front Microbiol **7**: 1699.

Lei, S., J. Ryu, K. Wen, E. Twitchell, T. Bui, A. Ramesh, M. Weiss, G. Li, H. Samuel, S. Clark-Deener, X. Jiang, K. Lee and L. Yuan (2016). "Increased and prolonged human norovirus infection in RAG2/IL2RG deficient gnotobiotic pigs with severe combined immunodeficiency." Sci Rep **6**: 25222.

Lei, S., H. Samuel, E. Twitchell, T. Bui, A. Ramesh, K. Wen, M. Weiss, G. Li, X. Yang, X. Jiang and L. Yuan (2016). "Enterobacter cloacae inhibits human norovirus infectivity in gnotobiotic pigs." Sci Rep **6**: 25017.

Lei, S., E. Twitchell and L. Yuan (2018). Pathogenesis, Immunity and the Role of Microbiome/Probiotics in Enteric Virus Infections in Humans and Animal Models. Mechanisms Underlying Host-Microbiome Interactions in Pathophysiology of Human Diseases. J. Sun and P. K. Dudeja. Boston, MA, Springer US**:** 55-78.

Lei, S., E. L. Twitchell, A. K. Ramesh, T. Bui, E. Majette, C. M. Tin, R. Avery, G. Arango-Argoty, L. Zhang, S. Becker-Dreps, M. A. Azcarate-Peril, X. Jiang and L. Yuan (2019). "Enhanced GII.4 human norovirus infection in gnotobiotic pigs transplanted with a human gut microbiota." J Gen Virol.

Lowther, J. A., N. E. Gustar, R. E. Hartnell and D. N. Lees (2012). "Comparison of norovirus RNA levels in outbreak-related oysters with background environmental levels." J Food Prot **75**(2): 389-393.

Messner, M. J., P. Berger and S. P. Nappier (2014). "Fractional poisson--a simple dose-response model for human norovirus." Risk Anal **34**(10): 1820-1829.

Miller, J. and R. Ulrich (2001). "On the analysis of psychometric functions: The Spearman-Kärber method." Perception & Psychophysics **63**(8): 1399-1420.

Miller, R. G. (1973). "Nonparametric Estimators of the Mean Tolerance in Bioassay." Biometrika **60**(3): 535-542.

Otto, P. H., I. N. Clarke, P. R. Lambden, O. Salim, J. Reetz and E. M. Liebler-Tenorio (2011). "Infection of Calves with Bovine Norovirus GIII.1 Strain Jena Virus: an Experimental Model To Study the Pathogenesis of Norovirus Infection." Journal of Virology **85**(22): 12013.

Payne, D. C., J. Vinjé, P. G. Szilagyi, K. M. Edwards, M. A. Staat, G. A. Weinberg, C. B. Hall, J. Chappell, D. I. Bernstein, A. T. Curns, M. Wikswo, S. H. Shirley, A. J. Hall, B. Lopman and U.

D. Parashar (2013). "Norovirus and Medically Attended Gastroenteritis in U.S. Children." New England Journal of Medicine **368**(12): 1121-1130.

Pringle, K., B. Lopman, E. Vega, J. Vinje, U. D. Parashar and A. J. Hall (2015). "Noroviruses: epidemiology, immunity and prospects for prevention." Future Microbiol **10**(1): 53-67.

Ramakrishnan, M. A. (2016). "Determination of 50% endpoint titer using a simple formula." World journal of virology **5**(2): 85-86.

Ramani, S., S. E. Crawford, S. E. Blutt and M. K. Estes (2018). "Human organoid cultures: transformative new tools for human virus studies." Curr Opin Virol **29**: 79-86.

Reed, L. J. and H. Muench (1938). "A Simple Method of Estimating Fifty Percent Endpoints." American Journal of Epidemiology **27**(3): 493-497.

Ritz, C., F. Baty, J. C. Streibig and D. Gerhard (2016). "Dose-Response Analysis Using R." PLOS ONE **10**(12): e0146021.

Roederer, M. (2015). "Parsimonious Determination of the Optimal Infectious Dose of a Pathogen for Nonhuman Primate Models." PLoS pathogens **11**(8): e1005100-e1005100.

Rouhani, S., P. Peñataro Yori, M. Paredes Olortegui, M. Siguas Salas, D. Rengifo Trigoso, D. Mondal, L. Bodhidatta, J. Platts-Mills, A. Samie, F. Kabir, A. Lima, S. Babji, C. J. Mason, A. Kalam, P. Bessong, T. Ahmed, E. Mduma, Z. A. Bhutta, I. Lima, R. Ramdass, D. Lang, A. George, A. K. M. Zaidi, G. Kang, E. Houpt, M. N. Kosek, R. F. Etiology, I. Interactions of Enteric, Malnutrition, H. the Consequences for Child and I. Development Project Network (2016). "Norovirus Infection and Acquired Immunity in 8 Countries: Results From the MAL-ED Study." Clinical infectious diseases : an official publication of the Infectious Diseases Society of America **62**(10): 1210-1217.

Santiana, M., S. Ghosh, B. A. Ho, V. Rajasekaran, W.-L. Du, Y. Mutsafi, D. A. De Jésus-Diaz, S. V. Sosnovtsev, E. A. Levenson, G. I. Parra, P. M. Takvorian, A. Cali, C. Bleck, A. N. Vlasova, L. J. Saif, J. T. Patton, P. Lopalco, A. Corcelli, K. Y. Green and N. Altan-Bonnet (2018). "Vesicle-Cloaked Virus Clusters Are Optimal Units for Inter-organismal Viral Transmission." Cell host & microbe **24**(2): 208-220.e208.

Siev, D. (2018, 03/23/2018). "Non-parametric Estimation of Median Effective Dose." Statistical Methods (STATWIs) Retrieved 05/09/2019, 2019, from https://www.aphis.usda.gov/animal_health/vet_biologics/publications/STATWI0001.pdf.

Souza, M., M. S. Azevedo, K. Jung, S. Cheetham and L. J. Saif (2008). "Pathogenesis and immune responses in gnotobiotic calves after infection with the genogroup II.4-HS66 strain of human norovirus." J Virol **82**(4): 1777-1786.

Souza, M., V. Costantini, M. S. Azevedo and L. J. Saif (2007). "A human norovirus-like particle vaccine adjuvanted with ISCOM or mLT induces cytokine and antibody responses and protection to the homologous GII.4 human norovirus in a gnotobiotic pig disease model." Vaccine **25**(50): 8448-8459.

Spackman, E., S. Malladi, A. Ssematimba and C. B. Stephens (2019). "Assessment of replicate numbers for titrating avian influenza virus using dose-response models." Journal of Veterinary Diagnostic Investigation **31**(4): 616-619.

Spackman, E., S. Malladi, A. Ssematimba and C. B. Stephens (2019). "Assessment of replicate numbers for titrating avian influenza virus using dose-response models." J Vet Diagn Invest **31**(4): 616-619.

Spouge, J. L. (1992). "Statistical analysis of sparse infection data and its implications for retroviral treatment trials in primates." Proceedings of the National Academy of Sciences of the United States of America **89**(16): 7581-7585.

Spouge, J. L. (2015). "Infectious Dose or Dilution (ID50) Server." Retrieved 3-29-2019, 2019, from https://www.ncbi.nlm.nih.gov/CBBresearch/Spouge/html_ncbi/html/id50/id50.cgi.

Sudeep, A. B., P. B. Vyas, D. Parashar and P. Shil (2019). "Differential susceptibility & replication potential of Vero E6, BHK-21, RD, A-549, C6/36 cells & Aedes aegypti mosquitoes to three strains of chikungunya virus." Indian J Med Res **149**(6): 771-777.

Sundararajan, A., M. Y. Sangster, S. Frey, R. L. Atmar, W. H. Chen, J. Ferreira, R. Bargatze, P. M. Mendelman, J. J. Treanor and D. J. Topham (2015). "Robust mucosal-homing antibody-secreting B cell responses induced by intramuscular administration of adjuvanted bivalent human norovirus-like particle vaccine." Vaccine **33**(4): 568-576.

Taube, S., A. O. Kolawole, M. Höhne, J. E. Wilkinson, S. A. Handley, J. W. Perry, L. B. Thackray, R. Akkina and C. E. Wobus (2013). "A mouse model for human norovirus." mBio **4**(4): e00450-00413.

Teunis, P. F., C. L. Moe, P. Liu, S. E. Miller, L. Lindesmith, R. S. Baric, J. Le Pendu and R. L. Calderon (2008). "Norwalk virus: how infectious is it?" J Med Virol **80**(8): 1468-1476.

Todd, K. V. and R. A. Tripp (2019). "Human Norovirus: Experimental Models of Infection." Viruses **11**(2).

Treanor, J. J., R. L. Atmar, S. E. Frey, R. Gormley, W. H. Chen, J. Ferreira, R. Goodwin, A. Borkowski, R. Clemens and P. M. Mendelman (2014). "A novel intramuscular bivalent norovirus virus-like particle vaccine candidate--reactogenicity, safety, and immunogenicity in a phase 1 trial in healthy adults." J Infect Dis **210**(11): 1763-1771.

Tu, E. T.-V., R. A. Bull, G. E. Greening, J. Hewitt, M. J. Lyon, J. A. Marshall, C. J. McIver, W. D. Rawlinson and P. A. White (2008). "Epidemics of Gastroenteritis during 2006 Were Associated with the Spread of Norovirus GII.4 Variants 2006a and 2006b." Clinical Infectious Diseases **46**(3): 413-420.

Weir, M. H., J. Mitchell, W. Flynn and J. M. Pope (2017). "Development of a microbial dose response visualization and modelling application for QMRA modelers and educators." Environmental Modelling & Software **88**: 74-83.

Weir, M. H., J. Mitchell, W. Flynn and J. M. Pope (2017). "Development of a microbial dose response visualization and modelling application for QMRA modelers and educators." Environ Model Softw **88**: 74-83.

Wobus, C. E., J. B. Cunha, M. D. Elftman and A. O. Kolawole (2016). Chapter 3.4 - Animal Models of Norovirus Infection A2 - Svensson, Lennart. Viral Gastroenteritis. U. Desselberger, H. B. Greenberg and M. K. Estes. Boston, Academic Press**:** 397-422.

Yuan, L., P. M. Jobst and M. Weiss (2017). Gnotobiotic Pigs. Gnotobiotics. T. R. Schoeb and K. A. Eaton, Academic Press**:** 349-368.

Zhao, F. R., Y. L. Xie, Z. Z. Liu, J. J. Shao, S. F. Li, Y. G. Zhang and H. Y. Chang (2017). "Lithium chloride inhibits early stages of foot-and-mouth disease virus (FMDV) replication in vitro." J Med Virol **89**(11): 2041-2046.

Table 1: HuNoV fecal shedding and diarrhea after inoculation of Gn pigs with different doses of Cin-1

Dose group	# of viral genome copies	n	Virus shedding				Diarrhea			
			(%)[a]	Mean duration days (SEM)[c,d]	AUC[c,d]	Mean onset day (SEM)	(%)[b]	Mean duration days (SEM)[c,d]	AUC[c,d]	Mean onset day (SEM)
1	2.00E+06	4	4 (100%)	2.5 (0.6)^{BC}	9506^{B}	2 (0.4)^{B}	4 (100%)	5.0 (0.3)^{A}	9.06^{A}	1.5 (0.5)^{D}
2	4.00E+05	4	4 (100%)	1.3 (0.3)^{CD}	5232^{B}	4 (1.2)^{AB}	3 (75%)	1.3 (0.9)^{ABC}	7.04^{A}	5.5 (1.2)^{ABC}
3	2.00E+05	4	4 (100%)	6.3 (0.5)^{A}	126774^{A}	1.3 (0.3)^{B}	4 (100%)	4.0 (0.9)^{AB}	9.31^{A}	2.8 (0.5)^{CD}
4	8.00E+04	6	6 (100%)	2.8 (0.5)^{B}	13495^{B}	1.5 (0.3)^{B}	6 (100%)	3.8 (1)^{AB}	7.46^{A}	3.2 (0.9)^{CD}
5	2.00E+04	3	2 (67%)	1 (0.6)^{B}	93^{B}	3.3 (2.3)^{AB}	1 (33%)	3.3 (0)^{ABC}	1.50^{B}	6.3 (1.7)^{AB}
6	3.20E+03	3	2 (67%)	1.3 (0.9)^{BCD}	2667^{B}	4 (2.1)^{AB}	3 (100%)	1.0 (0)^{BC}	3.17^{AB}	5 (2.1)^{BC}
7	8.00E+02	4	1 (25%)	0.5 (0.5)^{D}	2972^{B}	6.8 (1.3)^{A}	0 (0%)	0.0 (0)^{C}	2.75^{AB}	8 (0)^{A}

a. Gn pigs were orally inoculated with HuNoV GII.4/2003 variant (Cin-1) at 6–7 days of age. Rectal swabs were collected daily after inoculation from PID 1-7 to determine virus shedding by qRT-PCR.

b. Fecal consistency was assessed from PID 1-7 as 0, solid; 1, pasty; 2, semiliquid; 3, liquid.Pigs with daily fecal consistency scores of 2 or greater were considered to be diarrheic.

c. SEM, standard error of the mean; AUC, area under the curve.

d. Numbers in the same column followed by different capital letters (A, B, C, D) differ significantly (one-way ANOVA, $P<0.05$); while shared letters indicate no significant difference.

Table 2: Log ID50 and DD50 calculations of Cin-1

Calculations based on the % of affected Gn pigs		
Method	**Log ID_{50}**	**Log DD_{50}**
Reed-Muench		
Conventional	3.11	4.86
"skrmdb" package R	3.39	4.58
Dragstedt-Behrens *"skrmdb" package R*	3.39	4.58
Spearman-Karber		
On line tool/ *"skrmdb" package R*	3.51	4.49
Exponential *(Weir, 2017)*	3.76	4.76
Beta-Poisson *(Weir, 2017)*	3.41	4.32
Beta-Poisson *on line tool*	3.76	3.90
Calculation based on AUC		
Method	**Log ID_{50}**	**Log DD_{50}**
3PLL *drc package / AATBioquest online tool*	3.89	5.02

Table 3: Comparison of virus shedding in unvaccinated Gn pigs and humans after inoculation with the same Cin-1 inoculum

Host	Age	Cin-1 Dose	n	Virus shedding (%)	Mean duration days (range)[c]	Peak virus shedding day (PID)
Human[a]	19-48 years	5.00E+04	23	70	5.2 (2-30)	3
Human[b]	18-50 years	4.40E+03	34	76.5	-	-
Human[b]	18-50 years	4.40E+03	48	62.5	-	-
Gn pig	33-34 days	2.00E+04	3	67	1.0 (1-2)	2
Gn pig	33-34 days	8.00E+04	6	100	2.8 (2-4)	3
Gn pig	33-34 days	2.00E+05	4	100	6.3 (5-7)	4

a. Data reported by Frenck et al. 2012.
b. Date reported by Bernstein et al. 2015.
c. Virus shedding in human stools were monitored for up to 30 days; in Gn pigs for 7 days post-inoculation.

Table 4a: Comparison of goodness of fit for the determination of best-fitting ID_{50} model for Cin-1

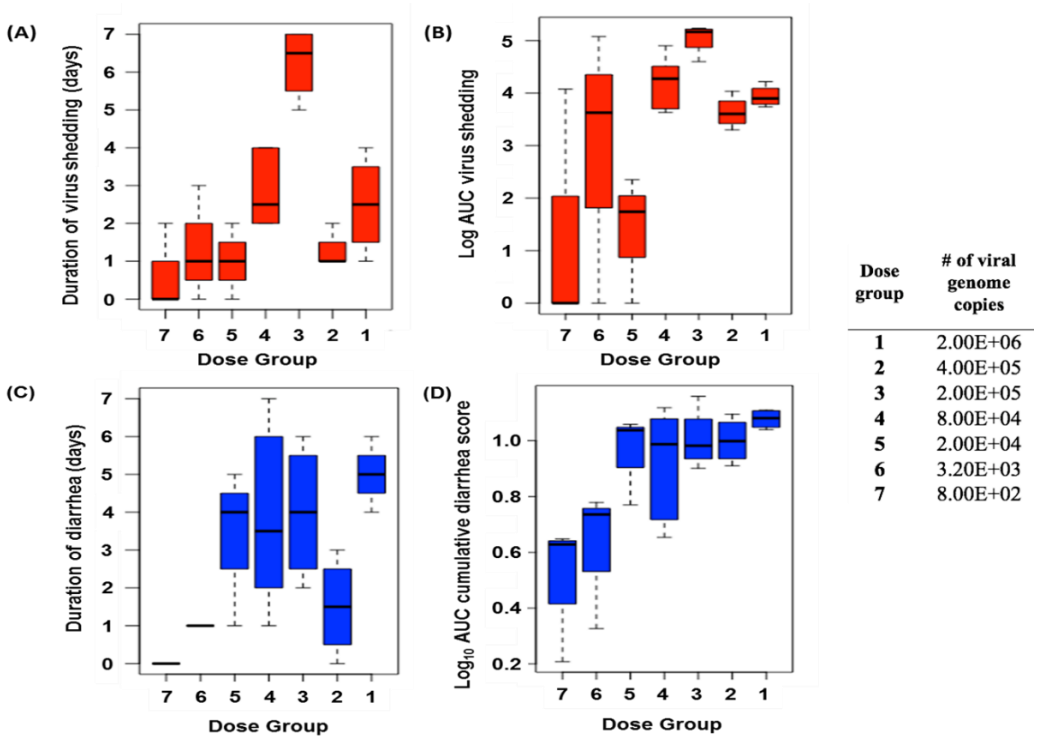
Model	Minimized Deviance	AIC	Chi-Squared p-value	Conclusion
Exponential	3.7423	5.742	0.7115	Shows a good fit to the data
Beta Poisson	1.713	5.713	0.8873	Shows a good fit to the data

Table 4b: Comparison of goodness of fit for the determination of best-fitting DD_{50} model for Cin-1

Model	Minimized Deviance	AIC	Chi-Squared p-value	Conclusion
Exponential	16.094	18.1	0.0133	Does not show a good fit to the data
Beta-Poisson	10.4989	14.5	0.0623	Shows a good fit to the data

Table 5: Comparison of Cin-1 to GII.4/2006b 092895 challenge pool used in previous studies.

Norovirus Variant	Optimal challenge dose (viral genome copy)		ID_{50} dose	Method of ID_{50} determination
GII.4/2003 Cin-1	2.0×10^5	This study	2.57×10^3	Beta-Poisson
GII.4/2006b 092895	6.43×10^5	Kocher et al 2014; Lei et al 2016	6.43×10^4	Reed-Muench



Dose group	# of viral genome copies
1	2.00E+06
2	4.00E+05
3	2.00E+05
4	8.00E+04
5	2.00E+04
6	3.20E+03
7	8.00E+02

Figure 1. Box plots showing (A) duration of virus shedding, (B) Log10 AUC of virus shedding, (C) duration of diarrhea and (D) Log10 cumulative diarrhea scores, among each dose group.

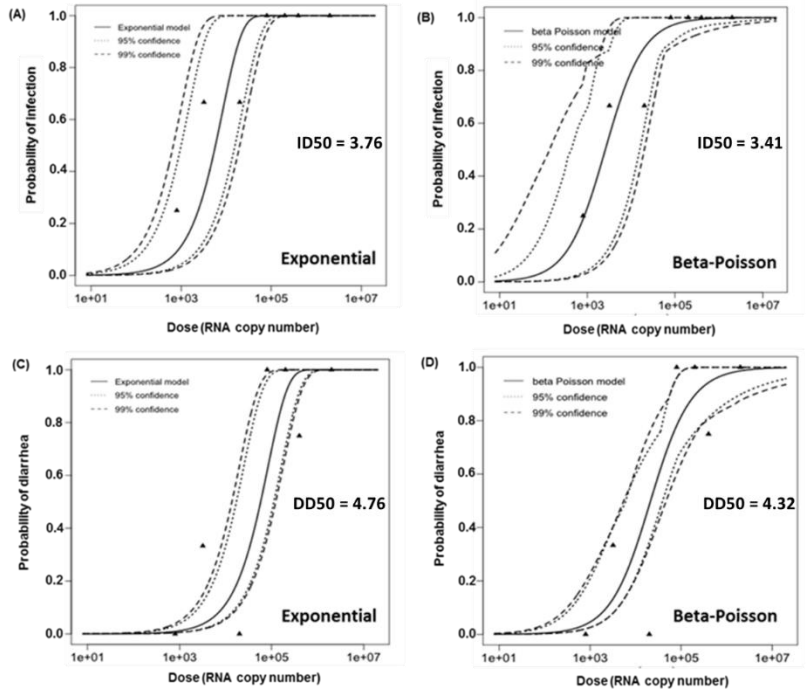


Figure 2. Dose-response curves extrapolated for the exponential model (A and C) showing the probability of infection and the probability of diarrhea, compared with the beta-Poisson model (B and D). Dotted lines show the 95% and 99% credible bands.

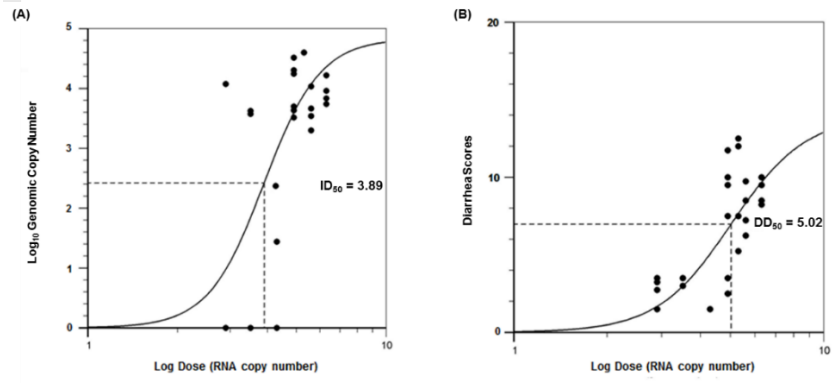


Figure 3. Estimates of (A) ID50 and (B) DD50 based on a 3PLL curve using AUC values.

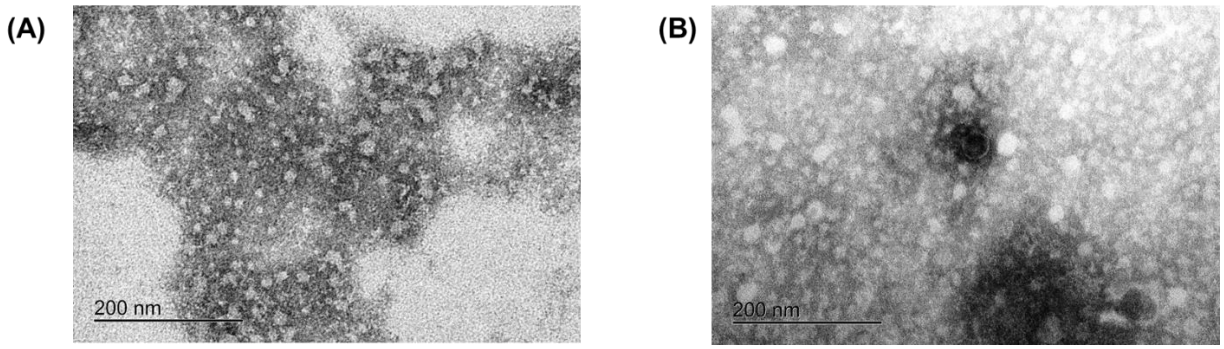


Figure 4: Electron micrograph of Cin-1 inoculum showing aggregate sizes in (A) 8 x 10^4 RNA copies and (B) 2 x 10^5 RNA copies.

Supplementary Table S1A: Parameters used for ID_{50} calculation using conventional RM method

Dose group	# of viral genome copies	Log_{10} Dose	No. of pigs shedding virus	No. of pigs not shedding virus	Shedding Accumulate	Non-shedding accumulate	Cumulative Shedding	Cumulative % of pigs shedding virus
1	2.00E+06	6.3	4	0	23	0	23/23	100
2	4.00E+05	5.6	4	0	19	0	19/19	100
3	2.00E+05	5.3	4	0	15	0	15/15	100
4	8.00E+04	4.9	6	0	11	0	11/11	100
5	2.00E+04	4.3	2	1	5	1	5/6	83.3
6	3.20E+03	3.5	2	1	3	2	3/5	60
7	8.00E+02	2.9	1	3	1	5	1/6	16.7

Supplementary Table S1B: Parameters used for DD_{50} calculation using conventional RM method

Dose group	# of viral genome copies	Log_{10} Dose	No. of pigs with diarrhea	No. of pigs without diarrhea	Diarrhea Accumulate	Non-diarrheic accumulate	Cumulative Diarrhea Rate	Cumulative % of pigs shedding virus
1	2.00E+06	6.3	4	0	18	0	18/18	100
2	4.00E+05	5.6	3	1	14	1	14/15	93.3
3	2.00E+05	5.3	4	0	11	1	11/12	91.7
4	8.00E+04	4.9	6	0	7	1	7/8	87.5
5	2.00E+04	4.3	0	3	1	4	1/5	20
6	3.20E+03	3.5	1	2	1	6	1/7	14.3
7	8.00E+02	2.9	0	4	0	10	0/10	0

Chapter 4

Parenterally-administered tetravalent VLP-based vaccine evaluated in Gn pig mode of GII.4 human norovirus infection and diarrhea

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Abstract

Human norovirus (HuNoVs) are responsible for causing more than 20% of all viral gastroenteritis among people of all age groups. There are no vaccines available. HuNoV are highly contagious and they are genetically very diverse with continuously emerging new genotypes and variants that lead to fast spreading within the human population through immune escape. Vaccine development has been limited to the use of bioengineering technologies to produce virus-like particles (VLPs) and other sub-viral particles of HuNoV. In this study, we tested the immunogenicity and protective efficacy of a novel intramuscular (IM) tetravalent VLP (VP1 of GI.1, GII.3, GII.4, GII.17) vaccine candidate in the gnotobiotic (Gn) pig model of HuNoV infection and disease. Three doses of the VLP vaccine with an alum adjuvant were administered to Gn pigs at 5 days of age (PID 0), PID 10 and PID 21. At PID 28, subsets pigs were challenged with one of three doses of GII.4/2003 Cin-1 HuNoV (2×10^5 ,8×10^4, or 2×10^4 genome copies), and monitored for 7 days for HuNoV infection and diarrhea. The IM VLP vaccine did not confer any protection against Cin-1-induced infection or diarrhea even when pigs were challenged with the lowest Cin-1 dose, although it was able to induce strong genotype-specific IgA and IgG responses in the serum of vaccinated pigs. Hemagglutination inhibition (HAI) antibody titers were detected at both pre-challenge and post-challenge. VLP-specific IFN-γ producing CD4+ and CD8+ T cells were observed to be elevated among vaccinated pigs at post-challenge day (PCD) 7 in the spleen and blood but not in the ileum. These results indicate that the IM tetravalent VLP vaccine was highly immunogenic, but the presence of high levels of immune effectors induced by the vaccine were not sufficient for protecting the Gn pigs from Cin-1 challenge. This study demonstrated the lack of cross-variant protection of HuNoV vaccine due to immune evasion because GII.4 VLP in the vaccine is derived

from the Sydney 2012 strain, a different variant from the GII.4/2003 Cin-1, the Farmington Hills strain. There are 23 amino acid substitutions in the major receptor binding domain (P2) of the capsid protein VP1 between the two variants. This finding has important implications in HuNoV vaccine development. With the highly variable antigenic diversity observed among emerging and re-emerging variants of HuNoV, vaccines have to include multiple variants and have to be routinely updated in order to ensure protection among the population.

1. Introduction

Human noroviruses (HuNoV) are the leading causative agents of acute gastroenteritis (AGE) among all age groups (Hall, Glass *et al*. 2016, Mattison, Cardemil *et al*. 2018). HuNoV are responsible for over 20% of all AGE epidemics annually (Ahmed, Hall *et al*. 2014). In the US, HuNoV-associated gastroenteritis accounts for ~21 million cases, ~2 million hospital visits, ~71,000 hospitalizations and ~800 deaths, with a vast majority (90%) of deaths occurring among the elderly (≥65 years of age) (Aliabadi, Lopman *et al*. 2015). GII.4 HuNoVs are responsible for >80% of all HuNoV-associated GE and is the most diverse of NoV genotypes (Chhabra, de Graaf *et al*. 2019). Other genotypes including GI.1, GII.3, and GII.17 are also known to cause HuNoV epidemics among different ranges of the world (Boon, Mahar *et al*. 2011, van Beek, de Graaf *et al*. 2018), with GII.17 predominating in Asia in more recent years (de Graaf, van Beek *et al*. 2015).

NoVs are transmitted through a fecal-oral route and epidemics often occur in healthcare facilities, restaurants, and schools among other settings where people are in close proximity of one another (CDC 2011, Brennan, Cavallo *et al*. 2018, Free, Buss *et al*. 2019). A vaccine against HuNoV disease is urgently needed. However, with cellular tropisms for HuNoVs only recently being identified (Estes, Ettayebi *et al*. 2019, Green, Kaufman *et al*. 2019), and the lack of an effective cell culture system, the development of cell-culture based vaccines are still out of reach at this time. Due to this reason, development of candidate norovirus (NoV) vaccines have been based on the generation of virus-like particles (VLPs) taking advantage of various recombinant protein expression systems, which has paved the path for the development of the first generation of HuNoV VLP vaccine (Jiang, Wang *et al*. 1992, Ramirez, Wahid *et al*. 2012, Tamminen, Huhti *et al*. 2012, Tan and Jiang 2012, Tan and Jiang 2019).

The aims of this study were: (i) to evaluate the protective efficacy, and (ii) to determine the immunogenic potential of an intramuscular (IM) administered tetravalent VLP vaccine composed of GI.1, GII.3, GII.4 and GII.17 HuNoV VP1 derived VLPs and aluminum hydroxide adjuvant in the Gn pig challenge model of GII.4 infection and diarrhea. At the time this study was carried out, the optimal Cin-1 challenge dose had not been established. In order to avoid a high virus challenge dose that overwhelmed the vaccine induced immunity, we challenged Gn pigs with three different Cin-1 doses, based on previous human challenge studies (Frenck, Bernstein *et al*. 2012, Bernstein, Atmar *et al*. 2015).

2. Materials and Methods

2.1 Vaccine

The vaccine mixture was formulated using equal concentrations (50 μg each) of GI.1, GII.3, GII.4 and GII.17 VLPs (total concentration of 200 μg of VLP) combined with 600 μg Aluminum hydrogel [$Al(OH)_3$] adjuvant. The vaccine was stored at 4 °C until administration.

Each of the purified VLPs were used as the detector antigen in ELISA for the detection of serum and intestinal genotype-specific IgA and IgG antibody responses (Liu, Wen *et al*. 2013) and, as the stimulating antigen in the intracellular IFN-γ staining and flow cytometry assay (Yuan, Wen *et al*. 2008, Wen, Tin *et al*. 2014). GI.1 and GII.4 VLPs were also used in the hemagglutination inhibition assay (HAI) assay.

2.2 Challenge virus inoculum

A pool of human stool specimens containing GII.4 strain, Cin-1 (Hu/GII.4/Cin-1/2003/US), a 2002 Farmington Hills-like variant, was collected by Xi Jiang's laboratory at Cincinnati Children's

Hospital Medical Center collected from a man who developed a 3-day illness characterized by diarrhea, vomiting, nausea, abdominal cramps, and fever in 2003. This sample underwent extensive quality control prior to being used as a challenge pool in various human studies (Frenck, Bernstein *et al*. 2012, Bernstein, Atmar *et al*. 2015). The HuNoV concentration of the challenge pool was quantified by using RT-qPCR and contained approximately 2 x 10^6 viral genomic RNA copies/mL of stool. The challenge pool was stored at <-70°C in individual 1 mL aliquots until the day of Gn pig inoculation.

2.3 Gnotobiotic pigs and treatments

Near-term pigs (Large white cross breed) were derived by aseptic hysterectomy and maintained in sterile isolator units as described previously (Yuan, Jobst *et al*. 2017). Rectal swabs collected from pigs were cultured on blood agar plates (Hardy Diagnostics) and in Thioglycollate media (Hardy Diagnostics) 3 days after derivation and repeated once a week until the end of the study in order to determine the sterility status of the isolators that the pigs were housed in. Pigs were fed commercial ultra-high temperature-treated sterile cow milk throughout the study until post inoculation day (PID) 21, after which they were switched over to Similac® (Abbott Laboratories) baby formula until the end of the study. A total of 23 pigs were assigned to two groups (VLP Vaccine and Control). Pigs were randomly divided into the groups upon derivation regardless of gender and body weight. One group of pigs received the tetravalent VLP vaccine starting at 5 days of age or PID 0, followed by two booster doses at PID10 and PID21. The pigs in control group received the adjuvant only at the same time points. The timing of 3 injections in Gn pigs are established in a previous study (Kocher, Bui *et al*. 2014), based on the time needed to prime and

boost immune responses in Gn pigs. At PID 28 or post-challenge day (PCD) 0, pigs in each group were further split into 3 groups each based on the virus challenge dose they received (Table 1). Serum was collected at PID 28, and PCD 7 for the detection of pre-challenge and post challenge VLP-specific IgA and IgG antibody responses and for GII.4-specific hemagglutination inhibition assay.

Prior to inoculation with HuNoV, 4ml of 200 mM sodium bicarbonate was given orally 15 min to neutralize stomach acids. The Cin-1 inoculum was mixed with 5 ml of Diluent #5 (minimal essential medium with 1 % penicillin-streptomycin and 1 % HEPES) on the day of inoculation. All pigs received oral doses of Cin-1 ranging from 2 x 10^4 to 2 × 10^5 genomic RNA copies at PID 28 / post-challenge day (PCD) 0 (Table 1). Clinical signs and fecal virus shedding were monitored daily until euthanasia. All pigs were euthanized 7 days post inoculation. At euthanasia, small and large intestinal contents (SIC and LIC) were collected from all pigs and processed, as described (Parreno, Hodgins *et al*. 1999), for the detection of intestinal antibody responses by ELISA. Ileum, spleen and blood were collected, and mononuclear cells (MNCs) were isolated from them for the detection of effector T cell responses by flow cytometry as described (Yuan, Wen *et al*. 2008, Kocher, Bui *et al*. 2014). All experiments involving the use of Gn pigs were approved by the Institutional Animal Care and Use Committee at Virginia Tech (IACUC protocol: 17-110-CVM). All experimental procedures were carried out in compliance with federal and university regulations.

2.4 HBGA-typing of Gn pigs by immunofluorescence assay

Prior to vaccination, all Gn pigs were screened for their HBGA type by immunofluorescence assay. Epithelial cells collected from the cheeks were prepared as described previously (Bui, Kocher *et*

al. 2013). HBGA phenotypes were detected from these cells using fluorescent labeled antibodies described elsewhere (Lei, Ryu *et al*. 2016). All slides were prepared with VECTASHIELD® Antifade Mounting Medium with 4,6-diamidino-2-phenylindole (DAPI) which was used as a nuclear counterstain (Vector Laboratories) for fluorescent microscopy. Based on this screening technique, HBGA type A^- and H^- pigs were excluded from this study because A^- or H^- pigs are less susceptible to NoV infection than A^+ or H^+ pigs (Cheetham, Souza *et al*. 2007, Bui, Kocher *et al*. 2013).

2.5 Detection of fecal consistency and HuNoV shedding by RT-qPCR

Fecal consistency and virus shedding were monitored at 24-hour time points at the same time daily, after HuNoV inoculation at PID 0, by rectal swab sampling. The following scoring system was used to assess fecal consistency: 0, solid; 1, pasty; 2, semiliquid; 3, liquid. Pigs with fecal consistency scores of 2 or greater were considered to be diarrheic.

Presence of HuNoV genomes in rectal swab samples collected after inoculation with different doses of Cin-1 were evaluated using RT-qPCR protocol as described previously (Lei, Ryu *et al*. 2016). Briefly, once collected, swabs, were swirled in 1 ml PBS to release the feces and centrifuged at 10000g for 5 min. 250 µl of the supernatant collected was mixed with 750 µl TRIzol (Invitrogen) following manufacturer's instructions for viral RNA extraction. The extracted RNA was then dissolved in 40 µl autoclaved dd-H_2O. Five microliters of RNA were used in 20 µl RT-qPCR reaction with a SensiFAST Probe No-ROX One-Step Kit (Bioline) to detect HuNoV genomes. Primers COG2F, COG2R, and probe RING2 were used with cycling conditions as described in a previous study (Kageyama, Kojima *et al*. 2003). A standard curve was generated using COG2 amplicon-containing cDNA expression plasmids in serially diluted tenfold from 2 x 10^6 to 2

genomic RNA copies. Amplification was performed on CFX96 Real-Time System (Bio-Rad), and data were collected and analyzed with Bio-Rad CFX Manager 2.0.

2.6 ELISA for the detection of serum IgA and IgG VLP-specific antibody responses

ELISA was carried out on heat-inactivated serum samples to identify the presence of genotype-specific IgA and IgG responses as previously described (Graham, Jiang *et al*. 1994). Briefly, 96-well microtiter plates (Falcon) were coated with purified VLPs at 0.32 µg/mL diluted in PBST + 2% non-fat dry milk (pH 7.4) and incubated overnight at 4 °C. The plates were then washed with washing buffer (PBS pH 7.4 + 0.05% Tween 20) three times and blocked with 300 µL/well of blocking buffer (PBS pH 7.4 + 5% non-fat dry milk) for two hours at room temperature. After the plates were washed two times with washing buffer and 100 µL of serially diluted test serum (1:2 to 1:65536) were added to each well and incubated for one hour at 37 °C. After washing the plates three times, 100 µL of HRP-conjugated anti-pig IgG or IgA diluted 1:5000 in PBST + 2% non-fat dry milk (pH 7.4) was added. The plates were then incubated for 1 hour at 37°C. After washing, the plates three times, 100 µL of SureBlue™ (Seracare)was added and the plate was incubated for ten minutes room temperature, in the dark for the color to develop. The development of color was inhibited by 100 µL of TMB stop solution (Seracare) and the absorbance was measured at 450 nm.

2.7 Hemagglutination inhibition assay

Hemagglutination inhibition assay was performed as described previously (Czako, Atmar *et al*. 2012). Briefly, commercially available (BioIVT) human type O erythrocytes (RBCs) were collected from healthy adult volunteers in Alsever buffer, and washed three times with PBS without Ca_2 and Mg_2. After each wash step, the blood samples were centrifuged for 10 min at 3000 RPM at 4°C and the supernatant discarded. The pellet was resuspended in 50 mL of PBS. In the

last wash step, the cells were centrifuged 10 min at 4500 RPM and then the pellet was stored at 4°C until used. The RBC working suspension was prepared using 0.75 ml of RBC pellet in 100 ml of saline buffer pH 6.2. In a V-bottomed 96-well microtiter plate, serial dilutions of the VLPs were mixed with the 10 μL RBC working solution to ensure that VLP was titrated to 4 hemagglutination units (~20 ng of GI.1 or GII.4 VLPs. Same VLPs as in the vaccine). In a separate V-bottomed 96-well microtiter plates, two-fold dilutions of the serum were made starting with an undiluted sample. GI.1 or GII.4 VLP was then added at 0.32ug/mL followed by 50ul/well of RBC working suspension was added and incubated for 2 hours at 4°C. The controls of this assay were: A) Positive Control (known serum with inhibition titer) with 3.2 μg/mL of GI.1 or GII.4 VLP, B) Negative Control (known samples with no inhibition titer) with 3.2 μg/mL of GI.1 or GII.4 VLP. C) No VLP. D) No serum. The HAI titer was defined as the reciprocal of the highest dilution of serum that completely inhibited hemagglutination by the viral antigen. Geometric mean titers (GMTs) were calculated to summarize the hemagglutination inhibition titer on PID 28 and PCD 7 for vaccinated and control pigs.

2.8 Flow Cytometry

Mononuclear cells (MNCs) collected from the ileum, spleen and blood upon euthanasia and were diluted to a concentration of 2×10^6 cells/mL and were seeded into 12-well plates and stimulated with 12 μg/mL of GI.1, GII.3, GII.4 and GII.17 antigens, mock (unstimulated negative control) and PHA (positive control) for 17 h at 37 °C in 5% CO_2 as determined previously (Kocher, Bui *et al*. 2014). CD3+CD4+ and CD3+CD8+ cell surface marker staining and IFN-γ intracellular staining have been described in previous publications (Yuan, Ward *et al*. 1996, Yuan, Wen *et al*. 2008, Lei, Ryu *et al*. 2016, Twitchell, Tin *et al*. 2016). All samples were stored in 0.05 mL of

staining buffer and were maintained at 4 °C. A minimum of 100,000 events were acquired using a FACSAria flow cytometer (BD Biosciences, San Jose, CA, USA). Flow cytometry data were analyzed using FlowJo X software (Tree Star, Ashland, OR, USA).

2.9. Statistical Analysis

Student's t-test was used for comparisons of virus shedding and diarrhea data between the treatment groups. One-way analysis of variance (ANOVA) (General linear model) was used to compare VLP-specific IgA and IgG antibody titers between the treatment groups. Tukey-Kramer HSD was used for the comparison of different time points within the same treatment group. Two-way ANOVA, followed by a Multiple t-test, was used for comparisons of frequencies of IFN-γ producing T cells between treatment groups. ANOVA analyses were carried out using JMP 14.0 (SAS Institute, Kerry, NC, USA), and all other statistical analyses were performed using GraphPad Prism 7.0 (GraphPad Software, San Diego, CA, USA). A p value less than 0.05 was accepted to be statistically significant.

3. Results

3.1 Assessment of clinical signs and HuNoV virus shedding in vaccinated and control Gn pigs

VLP vaccinated and mock vaccinated (control) Gn pigs were challenged orally with one of the three doses of Cin-1 (Dose 1, Dose 2 or Dose 3) at PID 28 / PCD 0 and were observed daily for clinical signs and virus shedding in feces until euthanasia at PCD 7. Fecal consistency scoring system (0 – normal; 1 – pasty; 2 – semi-liquid; and 3 – liquid) was used to determine whether Gn pigs experienced NoV-associated diarrhea. A fecal consistency score of 2 or greater (≥2) were deemed to be diarrheic (Lei, Ramesh *et al*. 2016). HuNoV infection was determined by the

presence of viral RNA quantified using RT-qPCR carried out on samples isolated from rectal swabs collected from PCD 1-7. There were no differences in virus shedding and diarrhea among vaccinated and control Gn pigs over each of the challenge dose groups (Table 2). Gn pigs that received the lower challenge doses (Dose Groups 2 and 3) shed virus for an average of 2 days while Gn pigs received the higher challenge dose (Dose Group 1) shed virus for at least 6 days after virus was initially detected in their feces (Figure 1A). Pigs that were given the higher challenge dose had a greater diarrhea burden indicated by an increased duration, a faster onset of diarrhea and a longer diarrhea duration as compared to pigs that received a lower challenge dose (Table 2 and Figure 1C and 1D).

The pattern of virus shedding and diarrhea in both the vaccinated and control pig groups across the three different virus challenge doses resemble those in the ID50 and DD50 study (Table 1, Chapter 3), suggesting that the vaccine did not confer any noticeable protection against GII.4/2003 Cin-1 challenge.

3.2 IM VLP vaccine induced strong VLP-specific IgG and IgA antibodies in serum but not in the intestines of Gn pigs

The VLP vaccinated Gn pigs had significantly higher titers of serum IgA and IgG antibodies specific to each of the four VLPs as measured by ELISA using each VLP as the detector antigen on PID 28 and PCD 7 compared to control pigs irrespective of virus challenge dose (Figure 2). All four VLPs in the vaccine were able to induce equally high levels of VLP-specific IgA and IgG titers. No significant differences in IgA and IgG responses in the serum were observed before and after challenge, or among different virus challenge dose groups. This shows that high titers of

serum VLP-specific IgA and IgG antibodies induced by the vaccine were not influenced by the infection status.

Upon euthanasia at PCD 7, SIC and LIC were collected from pigs to determine the presence of VLP-specific IgA and IgG intestinal antibodies. Antibody titers in vaccinated and control pigs were equally low. Interestingly, 1 out of 7 vaccinated pigs in Dose Group 1 had high VLP-specific IgA titers in LIC, and higher IgG in its SIC and LIC. However, this pig had similar overall severity of virus shedding and diarrhea compared to other pigs in the same group who did not develop intestinal antibody responses.

3.3 HAI antibody titers in Gn pig serum

Surrogate assays such as HBGA blocking assay, and HAI assay have been routinely used in place of a virus neutralization assay for functional NoV antibody detection (Czako, Atmar *et al*. 2012, Garaicoechea, Aguilar *et al*. 2015, Atmar, Ettayebi *et al*. 2019). In this study, HAI assay was used to assess the ability of the VLP vaccine to induce GI.1 and GII.4 VLP-specific functional antibodies in serum (Figure 3). High titers of GI.1-specific HAI antibodies were present in the serum of all VLP vaccinated pigs at both PID 28 and PCD7 regardless challenge dose group (Figure 3A), suggesting the induction of HAI antibody responses by the VLP vaccine. In control pigs, GI.1-specific HAI titers were only detected at PCD 7 among the two higher challenge dose groups (Figure 3A), suggesting that the Cin-1 infection primed for the cross-genogroup HAI antibody responses. GII.4-specific HAI antibody responses were also detected in all VLP vaccinated pigs at both PID 28 and PCD7 regardless challenge dose group (Figure 3B). However, it is unexpected that control pigs had similar titers of GII.4-specific HAI titers as the vaccinated pigs at PID 28 in two challenge dose groups (Groups 1 and 3).

An increase in the GI.1 and GII.4 HAI titers were observed post-challenge among all vaccinated and control pigs regardless the challenge dose group, suggesting that Cin-1 infection either primed (in control pigs) or boosted (in vaccinated pigs) HAI antibody responses.

3.4 VLP-specific IFN-γ producing CD4 and CD8 T cells were detected among all Gn pigs in systemic lymphoid tissues at PCD 7.

Upon euthanasia at PCD 7, MNCs isolated from ileum, spleen and peripheral blood were stimulated with VLPs derived from HuNoV GI.1, GII.3, GII.4 and GII.17, and stained with antibodies specific to CD3, CD4, CD8 and IFN-γ for the quantification of IFN-γ producing T cells. The IM VLP vaccine primed IFN-γ producing T cell responses specific to all 4 genotypes of HuNoV antigens. Among the pigs that were challenged with the highest virus dose (Dose 1), higher frequencies of GII.4 and GII.17 specific IFN-γ producing CD4 and CD8 T cells were present in spleen and blood of the vaccinated pigs compared to the control pigs (Figure 4). However, the level of intestinal (ileum) T cell responses were similar between pigs in VLP vaccinated and control groups. In the pigs challenged with the two lower virus doses, overall higher frequencies of IFN-γ producing $CD4^+$ and $CD8^+$ T cell responses were detected in the spleen and blood of vaccinated pigs than the control pigs (data not shown). These data suggest that the VLP vaccine primed for low level systemic effector T cell responses. However, the systemic effector T cells were not protective against HuNoV infection in Gn pigs.

4. Discussion

In this study, Gn pigs were administered with three doses of the VLP vaccine containing equal concentrations of GI.1, GII.3, GII.4, and GII.17 formulated with $Al(OH)_3$ adjuvant, or adjuvant

only (Control) and then challenged with 2 x 10^4, 8 x 10^4 or 2 x 10^5 genomic RNA copies of a Cin-1 strain of HuNoV at PID 28. Protection conferred by the vaccine was evaluated based on fecal virus shedding and fecal consistency data collected daily from PCD0 to PCD7. The serum and intestinal VLP genotype-specific IgA and IgG antibody responses induced by the vaccine were measured by ELISA, and effector T cell responses to individual VLP genotypes were measured by flow cytometry. Although the vaccine induced significant systemic antibody and IFN-γ producing T cell immune responses specific to every VLP genotype within the vaccine, the vaccine failed to induce intestinal antibody and T cell responses, and did not confer any noticeable protection against virus shedding or diarrhea when challenged with any one of the three doses of Cin-1 in Gn pigs. These results concur with previous findings in naïve mice (Tamminen, Malm *et al*. 2016), which showed that only intranasal, not IM immunization with GII.4 VLPs induced mucosal IgA antibodies capable of blocking the VLP binding to the HBGA receptors. In orally pre-exposed individuals, such as most adult humans, IM vaccine may be able to induce anamnestic intestinal IgA responses (Sundararajan, Sangster *et al*. 2015). However, in naïve hosts such as Gn pigs (and human infants), the IM vaccine could not induce sufficient intestinal immune responses to confer any protection. In a previous study in Gn pigs, VLP derived from GII.4 VA387 variant when given intranasally with MPL and chitosan as mucosal adjuvants, conferred 60% protection rate against diarrhea upon challenge with a GII.4/2006b variant (Kocher, Bui *et al*. 2014). These data suggest that mucosal vaccination routes and mucosal adjuvants may be more effective against HuNoV infection than IM route for VLP vaccine in Gn pigs (Heinimäki, Malm *et al*. 2018).

A serum HAI titer of 40 has previously been correlated with protection in human volunteers challenged with Norwalk virus (Czako, Atmar *et al*. 2012). However, the GII.4-specific serum HAI titers of 40 and above observed in Gn pigs did not correlate with protection against Cin-1.

Further studies are needed to fully understand the reasons for the discrepancy between the strong immunogenicity and the lack of protection of the VLP vaccine. A likely reason for the ineffectiveness of the VLP vaccine against the Cin-1 challenge is the lack of cross protection between different genogroups, genotypes and even variants of HuNoV. Although a previously conducted study did demonstrate cross-variant protection induced by intranasal VLP and P particle vaccines in Gn pigs (Kocher, Bui *et al*. 2014). The many different HuNoV variants in the environment indicate an ever-changing capsid composition that allows for HuNoV immune evasion (Smith and Smith 2019). The GII.4 VLP in the tetravalent vaccine was derived from a Sydney strain (emerged in 2012), a different variant from the GII.4/2003 Cin-1, the Farmington Hills strain which was prevalent in 2002. With 10 years between circulation, there is enough antigenicity difference between the two variants to allow Cin-1 to escape neutralization by the antibodies induced by the Sydney strain VLPs in the vaccine (Motoya, Nagasawa *et al*. 2017). Emergence of new GII.4 strains have been attributed to the changes in the blockade epitopes, which allows the virus escape from protective herd immunity (Lindesmith, Beltramello *et al*. 2012, Lindesmith, Costantini *et al*. 2013).

A total of 35 substitutions (93% identity) were observed when comparing the major capsid protein amino acid sequence alignment of Cin-1 2003 (JQ965810) and Sydney 2012 (JX459908). The S domain of the major capsid protein contained 2 substitutions, P1-1 domain contained 3 substitutions, the P2 domain contained 23 substitutions, and the P1-2 domain contained 4 substitutions (Table 3). Among the GII.4 blockade epitopes (Lindesmith, Costantini *et al*. 2013), a total of 11 substitutions were observed (5 substitutions in epitope A, 1 substitution in epitope B, 1 substitution in epitope C, 2 substitutions in epitope D, and 2 substitution in epitope E; Table 4).

Pairwise sequence alignment of blockade amino acid sequences of Cin-1 and Sydney 2012 strains revealed an 80.3% sequence similarity between the two variants.

We were not able to measure the HBGA blocking antibody titers in this study, a known correlate of protection, however, it could be predicted to see a good level of HBGA blocking titers against the VLP derived from the Sydney strain (or any other 3 genotype VLPs) but lower or no HBGA blocking titers against the VLP derived from Cin-1 in the sera of the vaccinated pigs.

In Gn pig studies, the gnotobiotic condition of pigs exclude confounding factors that can influence HuNoV infectivity and diarrhea, such as gut microbiome (Lei, Twitchell *et al*. 2019). It was shown that colonization with human gut microbiome enhanced HuNoV replication and diarrhea severity. In the present study, 6 out of 14 vaccinated pigs from the different challenge dose groups were contaminated by bacteria during the immunization experiment, but all 9 control pigs remained clean. The contaminating bacteria may have negatively affected the protective effect, if any, induced by the VLP vaccine.

To avoid a high virus challenge dose that may overpower the vaccine induced immunity, we tested three different challenge doses. The lowest challenge dose 2×10^4 genomic RNA copies only infected 67% of both vaccinated and control pigs, indicating that this challenge dose was too low. The challenge dose 2×10^5 genomic RNA copy numbers determined as being the optimal dose in our concurrent study (described in Chapter 3) was confirmed in this study to induce infection and diarrhea in 100% of the control pigs. After challenge with this dose of Cin-1, the VLP vaccinated pigs had same incidence, duration and titers of virus shedding and same incidence, duration and severity of diarrhea as the control pigs, clearly demonstrating the lack of protection.

In conclusion, the IM VLP vaccine was highly immunogenic but was not able to provide any protection against virus shedding and diarrhea induced by a GII.4/2003 Cin-1 variant HuNoV. Although it has been suggested based on *in silico* analysis (Motoya, Nagasawa *et al*. 2017) and *in vitro* antibody and VLP binding experiments (Mallory, Lindesmith *et al*. 2019) that antigenic diversity of variants within GII.4 genotype led to evasion of herd immunity and resulted in the repeated pandemics of HuNoV gastroenteritis, our study provides the very first *in vivo* experimental evidence for the total lack of cross-variant protection between the Sydney and Farmington Hills GII.4 strains due to immune evasion. This finding has important implications in HuNoV vaccine development. It could be foreseen that with the amount of antigenic diversity observed among emerging and re-emerging variants of HuNoV, vaccines would have to include multiple variants and have to be routinely updated in order to ensure sustained protection among the population. It remains to be seen whether this vaccine can confer protection from GII.4/2012 Sydney variant infection and diarrhea.

Author Contributions:

Conceptualization, L.Y., Validation, A.R. and L.Y.; Formal Analysis, A.R.; Investigation, A.R., J.M., E.G., S.L., E.T., T.B., and L.Y.; Resources, L.Y.; Writing – Original Draft Preparation, A.R., and L.Y.; Writing – Review & Editing, A.R., J.M., E.G., S.L., E.T., T.B., and L.Y.; Visualization, A.R., and L.Y.; Supervision, L.Y.; Project Administration, A.R. and L.Y.; Funding Acquisition, L.Y.

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Conflicts of Interest:

The authors declare that there are no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Ethical Approval:

All animal experiments were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee at Virginia Tech (IACUC protocol: 17-110-CVM).

References

Ahmed, S. M., A. J. Hall, A. E. Robinson, L. Verhoef, P. Premkumar, U. D. Parashar, M. Koopmans and B. A. Lopman (2014). "Global prevalence of norovirus in cases of gastroenteritis: a systematic review and meta-analysis." Lancet Infect Dis **14**(8): 725-730.

Aliabadi, N., B. A. Lopman, U. D. Parashar and A. J. Hall (2015). "Progress toward norovirus vaccines: considerations for further development and implementation in potential target populations." Expert Rev Vaccines **14**(9): 1241-1253.

Atmar, R. L., K. Ettayebi, B. V. Ayyar, F. H. Neill, R. P. Braun, S. Ramani and M. K. Estes (2019). "Comparison of Microneutralization and Histo-blood Group Antigen-Blocking Assays for Functional Norovirus Antibody Detection." J Infect Dis.

Bernstein, D. I., R. L. Atmar, G. M. Lyon, J. J. Treanor, W. H. Chen, X. Jiang, J. Vinje, N. Gregoricus, R. W. Frenck, Jr., C. L. Moe, M. S. Al-Ibrahim, J. Barrett, J. Ferreira, M. K. Estes, D. Y. Graham, R. Goodwin, A. Borkowski, R. Clemens and P. M. Mendelman (2015). "Norovirus vaccine against experimental human GII.4 virus illness: a challenge study in healthy adults." J Infect Dis **211**(6): 870-878.

Bernstein, D. I., R. L. Atmar, G. M. Lyon, J. J. Treanor, W. H. Chen, X. Jiang, J. Vinjé, N. Gregoricus, R. W. Frenck, Jr., C. L. Moe, M. S. Al-Ibrahim, J. Barrett, J. Ferreira, M. K. Estes, D. Y. Graham, R. Goodwin, A. Borkowski, R. Clemens and P. M. Mendelman (2015). "Norovirus vaccine against experimental human GII.4 virus illness: a challenge study in healthy adults." The Journal of infectious diseases **211**(6): 870-878.

Boon, D., J. E. Mahar, E. J. Abente, C. D. Kirkwood, R. H. Purcell, A. Z. Kapikian, K. Y. Green and K. Bok (2011). "Comparative evolution of GII.3 and GII.4 norovirus over a 31-year period." Journal of virology **85**(17): 8656-8666.

Brennan, J., S. J. Cavallo, K. Garman, K. Lewis, D. J. Irving, C. Moore, L. Thomas, J. Hill, R. Villegas, J. F. Norman, J. R. Dunn, W. Schaffner and T. F. Jones (2018). "Notes from the Field: Multiple Modes of Transmission During a Thanksgiving Day Norovirus Outbreak - Tennessee, 2017." MMWR Morb Mortal Wkly Rep **67**(46): 1300-1301.

Bui, T., J. Kocher, Y. Li, K. Wen, G. Li, F. Liu, X. Yang, T. LeRoith, M. Tan, M. Xia, W. Zhong, X. Jiang and L. Yuan (2013). "Median infectious dose of human norovirus GII.4 in gnotobiotic pigs is decreased by simvastatin treatment and increased by age." J Gen Virol **94**(Pt 9): 2005-2016.

CDC (2011). "Updated norovirus outbreak management and disease prevention guidelines." MMWR Recomm Rep **60**(Rr-3): 1-18.

Cheetham, S., M. Souza, R. McGregor, T. Meulia, Q. Wang and L. J. Saif (2007). "Binding Patterns of Human Norovirus-Like Particles to Buccal and Intestinal Tissues of Gnotobiotic Pigs in Relation to A/H Histo-Blood Group Antigen Expression." Journal of Virology **81**(7): 3535-3544.

Chhabra, P., M. de Graaf, G. I. Parra, M. C. Chan, K. Green, V. Martella, Q. Wang, P. A. White, K. Katayama, H. Vennema, M. P. G. Koopmans and J. Vinje (2019). "Updated classification of norovirus genogroups and genotypes." J Gen Virol **100**(10): 1393-1406.

Czako, R., R. L. Atmar, A. R. Opekun, M. A. Gilger, D. Y. Graham and M. K. Estes (2012). "Serum hemagglutination inhibition activity correlates with protection from gastroenteritis in persons infected with Norwalk virus." Clin Vaccine Immunol **19**(2): 284-287.

de Graaf, M., J. van Beek, H. Vennema, A. T. Podkolzin, J. Hewitt, F. Bucardo, K. Templeton, J. Mans, J. Nordgren, G. Reuter, M. Lynch, L. D. Rasmussen, N. Iritani, M. C. Chan, V. Martella, K. Ambert-Balay, J. Vinje, P. A. White and M. P. Koopmans (2015). "Emergence of a novel GII.17 norovirus - End of the GII.4 era?" Euro Surveill **20**(26).

Estes, M. K., K. Ettayebi, V. R. Tenge, K. Murakami, U. Karandikar, S.-C. Lin, B. V. Ayyar, N. W. Cortes-Penfield, K. Haga, F. H. Neill, A. R. Opekun, J. R. Broughman, X.-L. Zeng, S. E. Blutt, S. E. Crawford, S. Ramani, D. Y. Graham and R. L. Atmar (2019). "Human Norovirus Cultivation in Nontransformed Stem Cell-Derived Human Intestinal Enteroid Cultures: Success and Challenges." Viruses **11**(7): 638.

Free, R. J., B. F. Buss, S. Koirala, M. Ulses, A. Carlson, B. Loeck and T. Safranek (2019). "Successive Norovirus Outbreaks at an Event Center - Nebraska, October-November, 2017." MMWR Morb Mortal Wkly Rep **68**(28): 627-630.

Frenck, R., D. I. Bernstein, M. Xia, P. Huang, W. Zhong, S. Parker, M. Dickey, M. McNeal and X. Jiang (2012). "Predicting susceptibility to norovirus GII.4 by use of a challenge model involving humans." J Infect Dis **206**(9): 1386-1393.

Garaicoechea, L., A. Aguilar, G. I. Parra, M. Bok, S. V. Sosnovtsev, G. Canziani, K. Y. Green, K. Bok and V. Parreno (2015). "Llama nanoantibodies with therapeutic potential against human norovirus diarrhea." PLoS One **10**(8): e0133665.

Graham, D. Y., X. Jiang, T. Tanaka, A. R. Opekun, H. P. Madore and M. K. Estes (1994). "Norwalk Virus Infection of Volunteers: New Insights Based on Improved Assays." The Journal of Infectious Diseases **170**(1): 34-43.

Green, K., S. Kaufman, B. Nagata, N. Chaimongkol, D. Kim, E. Levenson, C. Aburto, A. Berhele-Yardley, J. Johnson, A. Ferreira, I. Moore and S. Sosnovtsev (2019). Human norovirus targets epithelial cells in the small intestine. The 7th International Calicivirus Conference.

Hall, A. J., R. I. Glass and U. D. Parashar (2016). "New insights into the global burden of noroviruses and opportunities for prevention." Expert Rev Vaccines: 1-3.

Heinimäki, S., M. Malm, T. Vesikari and V. Blazevic (2018). "Parenterally Administered Norovirus GII.4 Virus-Like Particle Vaccine Formulated with Aluminum Hydroxide or Monophosphoryl Lipid A Adjuvants Induces Systemic but Not Mucosal Immune Responses in Mice." Journal of Immunology Research **2018**: 8.

Jiang, X., M. Wang, D. Y. Graham and M. K. Estes (1992). "Expression, self-assembly, and antigenicity of the Norwalk virus capsid protein." Journal of virology **66**(11): 6527-6532.

Kageyama, T., S. Kojima, M. Shinohara, K. Uchida, S. Fukushi, F. B. Hoshino, N. Takeda and K. Katayama (2003). "Broadly reactive and highly sensitive assay for Norwalk-like viruses based on real-time quantitative reverse transcription-PCR." J Clin Microbiol **41**(4): 1548-1557.

Kocher, J., T. Bui, E. Giri-Rachman, K. Wen, G. Li, X. Yang, F. Liu, M. Tan, M. Xia, W. Zhong, X. Jiang and L. Yuan (2014). "Intranasal P particle vaccine provided partial cross-variant protection against human GII.4 norovirus diarrhea in gnotobiotic pigs." J Virol **88**(17): 9728-9743.

Lei, S., A. Ramesh, E. Twitchell, K. Wen, T. Bui, M. Weiss, X. Yang, J. Kocher, G. Li, E. Giri-Rachman, N. V. Trang, X. Jiang, E. P. Ryan and L. Yuan (2016). "High Protective Efficacy of Probiotics and Rice Bran against Human Norovirus Infection and Diarrhea in Gnotobiotic Pigs." Front Microbiol **7**: 1699.

Lei, S., J. Ryu, K. Wen, E. Twitchell, T. Bui, A. Ramesh, M. Weiss, G. Li, H. Samuel, S. Clark-Deener, X. Jiang, K. Lee and L. Yuan (2016). "Increased and prolonged human norovirus infection in RAG2/IL2RG deficient gnotobiotic pigs with severe combined immunodeficiency." Sci Rep **6**: 25222.

Lei, S., E. L. Twitchell, A. K. Ramesh, T. Bui, E. Majette, C. M. Tin, R. Avery, G. Arango-Argoty, L. Zhang, S. Becker-Dreps, M. A. Azcarate-Peril, X. Jiang and L. Yuan (2019). "Enhanced GII.4

human norovirus infection in gnotobiotic pigs transplanted with a human gut microbiota." Journal of General Virology **100**(11): 1530-1540.

Lindesmith, L. C., M. Beltramello, E. F. Donaldson, D. Corti, J. Swanstrom, K. Debbink, A. Lanzavecchia and R. S. Baric (2012). "Immunogenetic mechanisms driving norovirus GII.4 antigenic variation." PLoS Pathog **8**(5): e1002705.

Lindesmith, L. C., V. Costantini, J. Swanstrom, K. Debbink, E. F. Donaldson, J. Vinje and R. S. Baric (2013). "Emergence of a norovirus GII.4 strain correlates with changes in evolving blockade epitopes." J Virol **87**(5): 2803-2813.

Lindesmith, L. C., V. Costantini, J. Swanstrom, K. Debbink, E. F. Donaldson, J. Vinjé and R. S. Baric (2013). "Emergence of a norovirus GII.4 strain correlates with changes in evolving blockade epitopes." Journal of virology **87**(5): 2803-2813.

Liu, F., K. Wen, G. Li, X. Yang, J. Kocher, T. Bui, D. Jones, K. Pelzer, S. Clark-Deener and L. Yuan (2013). "Dual Functions of Lactobacillus acidophilus NCFM at The Intermediate Dose In Protection Against Rotavirus Diarrhea In Gnotobiotic Pigs Vaccinated With A Human Rotavirus Vaccine." J Pediatr Gastroenterol Nutr.

Mallory, M. L., L. C. Lindesmith, R. L. Graham and R. S. Baric (2019). "GII.4 Human Norovirus: Surveying the Antigenic Landscape." Viruses **11**(2).

Mattison, C. P., C. V. Cardemil and A. J. Hall (2018). "Progress on norovirus vaccine research: public health considerations and future directions." Expert review of vaccines **17**(9): 773-784.

Motoya, T., K. Nagasawa, Y. Matsushima, N. Nagata, A. Ryo, T. Sekizuka, A. Yamashita, M. Kuroda, Y. Morita, Y. Suzuki, N. Sasaki, K. Katayama and H. Kimura (2017). "Molecular Evolution of the VP1 Gene in Human Norovirus GII.4 Variants in 1974-2015." Frontiers in microbiology **8**: 2399-2399.

Parreno, V., D. C. Hodgins, L. de Arriba, S. Y. Kang, L. Yuan, L. A. Ward, T. L. To and L. J. Saif (1999). "Serum and intestinal isotype antibody responses to Wa human rotavirus in gnotobiotic pigs are modulated by maternal antibodies." J Gen Virol **80 (Pt 6)**: 1417-1428.

Ramirez, K., R. Wahid, C. Richardson, R. F. Bargatze, S. S. El-Kamary, M. B. Sztein and M. F. Pasetti (2012). "Intranasal vaccination with an adjuvanted Norwalk virus-like particle vaccine elicits antigen-specific B memory responses in human adult volunteers." Clin Immunol **144**(2): 98-108.

Smith, H. Q. and T. J. Smith (2019). "The Dynamic Capsid Structures of the Noroviruses." Viruses **11**(3): 235.

Sundararajan, A., M. Y. Sangster, S. Frey, R. L. Atmar, W. H. Chen, J. Ferreira, R. Bargatze, P. M. Mendelman, J. J. Treanor and D. J. Topham (2015). "Robust mucosal-homing antibody-secreting B cell responses induced by intramuscular administration of adjuvanted bivalent human norovirus-like particle vaccine." Vaccine **33**(4): 568-576.

Tamminen, K., L. Huhti, T. Koho, S. Lappalainen, V. P. Hytönen, T. Vesikari and V. Blazevic (2012). "A comparison of immunogenicity of norovirus GII-4 virus-like particles and P-particles." Immunology **135**(1): 89-99.

Tamminen, K., M. Malm, T. Vesikari and V. Blazevic (2016). "Mucosal Antibodies Induced by Intranasal but Not Intramuscular Immunization Block Norovirus GII.4 Virus-Like Particle Receptor Binding." Viral Immunol **29**(5): 315-319.

Tan, M. and X. Jiang (2012). "Norovirus P particle: a subviral nanoparticle for vaccine development against norovirus, rotavirus and influenza virus." Nanomedicine (London, England) **7**(6): 889-897.

Tan, M. and X. Jiang (2019). "Norovirus Capsid Protein-Derived Nanoparticles and Polymers as Versatile Platforms for Antigen Presentation and Vaccine Development." Pharmaceutics **11**(9): 472.

Twitchell, E. L., C. Tin, K. Wen, H. Zhang, S. Becker-Dreps, M. A. Azcarate-Peril, S. Vilchez, G. Li, A. Ramesh, M. Weiss, S. Lei, T. Bui, X. Yang, S. Schultz-Cherry and L. Yuan (2016). "Modeling human enteric dysbiosis and rotavirus immunity in gnotobiotic pigs." Gut pathogens **8**: 51-51.

van Beek, J., M. de Graaf, H. Al-Hello, D. J. Allen, K. Ambert-Balay, N. Botteldoorn, M. Brytting, J. Buesa, M. Cabrerizo, M. Chan, F. Cloak, I. Di Bartolo, S. Guix, J. Hewitt, N. Iritani, M. Jin, R. Johne, I. Lederer, J. Mans, V. Martella, L. Maunula, G. McAllister, S. Niendorf, H. G. Niesters, A. T. Podkolzin, M. Poljsak-Prijatelj, L. D. Rasmussen, G. Reuter, G. Tuite, A. Kroneman, H. Vennema, M. P. G. Koopmans and NoroNet (2018). "Molecular surveillance of norovirus, 2005-16: an epidemiological analysis of data collected from the NoroNet network." Lancet Infect Dis.

Wen, K., C. Tin, H. Wang, X. Yang, G. Li, E. Giri-Rachman, J. Kocher, T. Bui, S. Clark-Deener and L. Yuan (2014). "Probiotic Lactobacillus rhamnosus GG Enhanced Th1 Cellular Immunity but Did Not Affect Antibody Responses in a Human Gut Microbiota Transplanted Neonatal Gnotobiotic Pig Model." PLOS ONE **9**(4): e94504.

Yuan, L., P. M. Jobst and M. Weiss (2017). Gnotobiotic Pigs. Gnotobiotics. T. R. Schoeb and K. A. Eaton, Academic Press**:** 349-368.

Yuan, L., L. A. Ward, B. I. Rosen, T. L. To and L. J. Saif (1996). "Systematic and intestinal antibody-secreting cell responses and correlates of protective immunity to human rotavirus in a gnotobiotic pig model of disease." **70**(5): 3075-3083.

Yuan, L., K. Wen, M. S. Azevedo, A. M. Gonzalez, W. Zhang and L. J. Saif (2008). "Virus-specific intestinal IFN-gamma producing T cell responses induced by human rotavirus infection and vaccines are correlated with protection against rotavirus diarrhea in gnotobiotic pigs." Vaccine **26**(26): 3322-3331.

Table 1. Assignment of treatment groups for gnotobiotic (Gn) pigs

Virus Dose Group	Challenge Virus Dose	Vaccine Group	Number of Pigs
1	$2x10^5$ Genomic RNA Copies	Control	4
		VLP Vaccine	7
2	$8x10^4$ Genomic RNA Copies	Control	2
		VLP Vaccine	4
3	$2x10^4$ Genomic RNA Copies	Control	3
		VLP Vaccine	3

Table 2a. HuNoV fecal shedding and diarrhea data after inoculation with 2 x 10^5 Genomic RNA copies of Cin-1

			Virus Shedding					Diarrhea			
Dose	**Group**	**n**	**Shedding (%)**	**Mean onset days (SEM)**	**Mean days with shedding**	**Geometric mean peak titre shed (RNA copies/g of feces)**	**AUC**	**Diarrhea (%)**	**Mean onset days (SEM)**	**Mean days with Diarrhea**	**Mean cumulative score**
2 x 10^5 RNA Copies	VLP Vaccine	7	100 (7/7)	2 (0.31)	6.0 (0.31)	4.53E+04	136094 (16669)	100 (7/7)	2.29 (0.47)	3.29 (0.52)	10.1 (0.95)
	Control	4	100 (4/4)	1.25 (0.25)	6.25 (0.48)	4.12E+04	127063 (29653)	100 (4/4)	2.75 (0.48)	4 (0.91)	13.0 (1.8)

Table 2b. HuNoV fecal shedding and diarrhea data after inoculation with 8 x 10^4 Genomic RNA copies of Cin-1

			Virus Shedding					Diarrhea			
Dose	**Group**	**n**	**Shedding (%)**	**Mean onset days (SEM)**	**Mean days with shedding**	**Geometric mean peak titer shed (RNA copies/g of feces)**	**AUC**	**Diarrhea (%)**	**Mean onset days (SEM)**	**Mean days with Diarrhea**	**Mean cumulative score**
8 x 10^4 RNA Copies	VLP Vaccine	4	100 (4/4)	1.75 (0.48)	2.0 (0.41)	3.77E+03	4478 (1272)	75 (3/4)	5.75 (0.75)	1.75 (0.85)	7.13 (2.9)
	Control	2	100 (2/2)	1 (0)	2 (0)	5.45E+03	4151 (863)	100 (2/2)	6 (0.0)	2.5 (0.35)	7.5 (0)

Table 2c. HuNoV fecal shedding and diarrhea data after inoculation with 2 x 10^4 Genomic RNA copies of Cin-1

			Virus Shedding					Diarrhea			
Dose	**Group**	**n**	**Shedding (%)**	**Mean onset days (SEM)**	**Mean days with shedding**	**Geometric mean peak titer shed (RNA copies/g of feces)**	**AUC**	**Diarrhea (%)**	**Mean onset days (SEM)**	**Mean days with Diarrhea**	**Mean cumulative score**
2 x 10^4 RNA Copies	VLP Vaccine	3	66 (2/3)	5 (2)	3 (1)	1.09E+02	155 (41)	100 (3/3)	1 (0)	4 (1)	11.5 (1.76)
	Control	3	66 (2/3)	3 (2)	2 (0)	1.07E+02	140 (70)	100 (3/3)	2.3 (0.33)	4 (1)	11.0 (2.0)

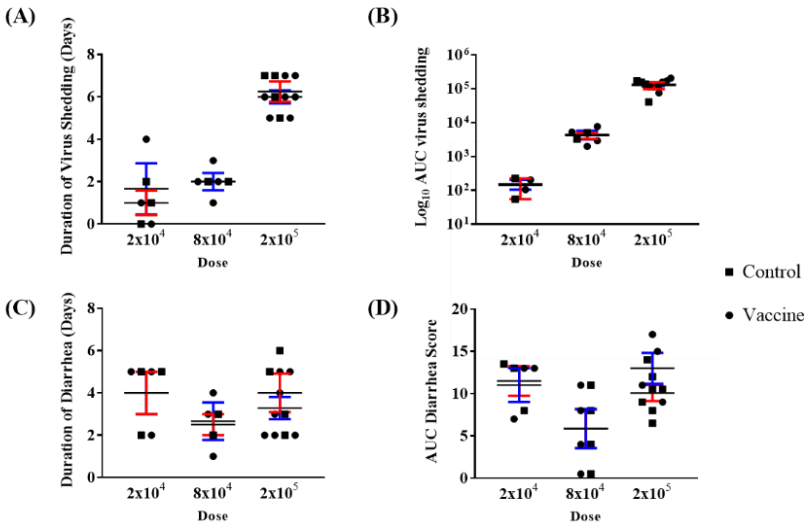

Figure 1. Virus shedding and diarrhea in vaccinated and control pigs. Daily virus shedding and diarrhea were monitored from PCD 0 – PCD 7 with (A) duration of virus shedding, (B) cumulative virus shedding shown as AUC, (C) duration of diarrhea and (D) cumulative diarrhea scores, of vaccinated and control Gn pigs among three virus challenge dose groups. There were no significant differences between the vaccine and control groups for any of the data.

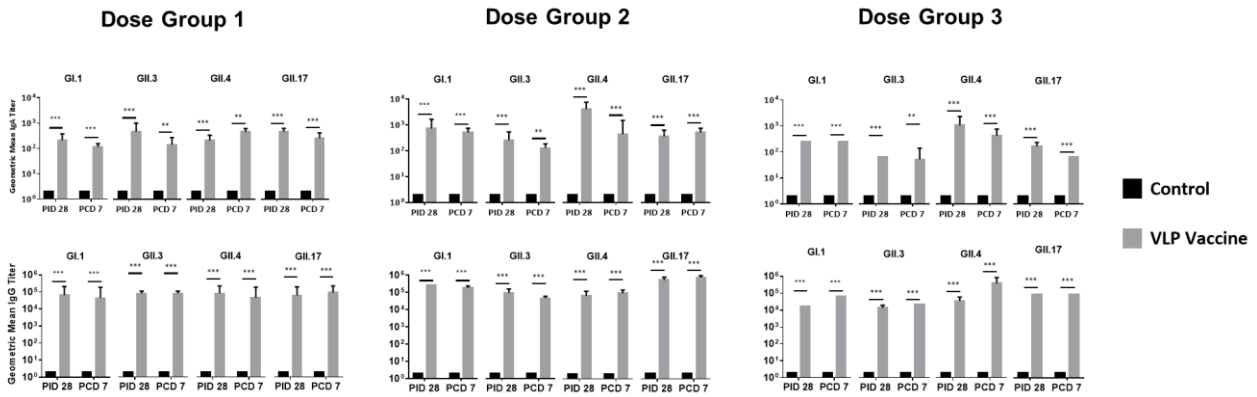

Figure 2. VLP-specific IgA (top) and IgG (bottom) serum antibodies against GI.1, GII.3, GII.4 and GII.17 HuNoV at PID 28 and PCD 7 in vaccinated and control Gn pigs among three virus challenge dose groups. Student T-test was used to compare antibody titers between the vaccine and control groups on each time point. ** $p<0.01$; ***$p<0.001$).

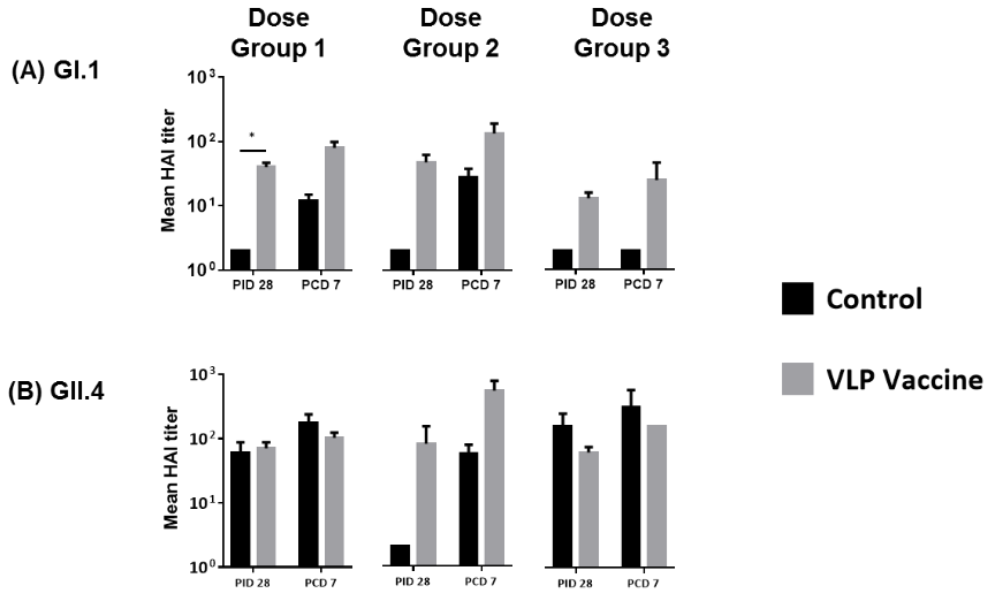

Figure 3. Serum HAI titers against GI.1 (A) and GII.4 (B) HuNoV VLP at PID 28 and PCD 7 in vaccinated and control Gn pigs among three virus challenge dose groups. ANOVA was used to compare HAI titers. There were no significant differences between the vaccine and control groups at any time point.

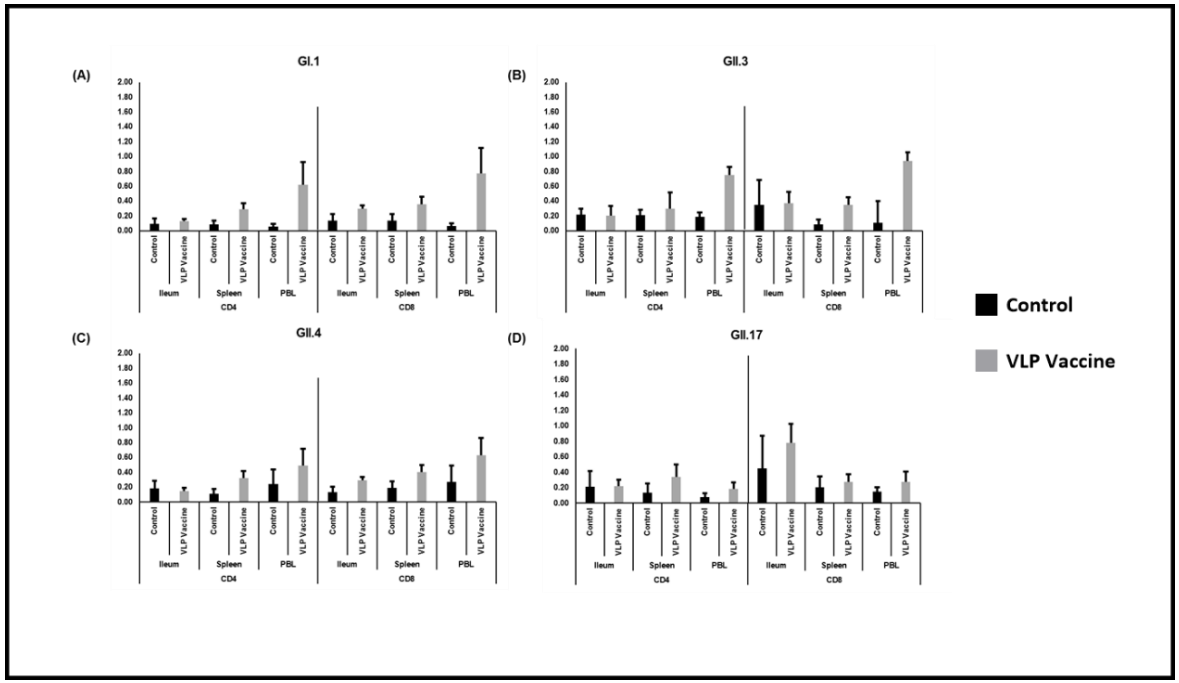

Figure 4. IFN-γ producing CD4+ and CD8+ T-cell responses in the intestinal (ileum) and systemic (spleen and peripheral blood lymphocyte, PBL) lymphoid tissues of VLP vaccinated (n=7) and Mock (n=4) vaccinated Gn pigs. Pigs were challenged with 2 x 10^5 genomic RNA copies at PID 28 and euthanized at PCD 7. Frequencies were subtracted by unstimulated background numbers. Data presented are mean frequencies of IFN-γ producing T-cells. Error bars are standard error of means.

Table 3. Amino acid sequence substitutions within S, P1-1, P1-2 and P2 domains observed between Cin-1 2003 and Sydney 2012 strains

Domain	Amino acid substitution (total)	Amino acid substitution within one group	Amino acid substitution between different group	Amino acid substitution which potentially influence the protein folding	Amino acid location
S	2	1	1	-	50–225
P1-1	3	3	-	-	226–278
P2	23	17	6	-	279–405
P1-2	4	3	1	-	406–520

Table 4. Observed amino acid substitutions among identified GII.4 blockade domains

VLP	Epitope															
	A						B		C		D			E		
	294	296	297	298	368	372	333	382	340	376	393	394	395	407	412	413
Cin-1 (2003)	A	T	H	N	N	N	M	K	G	E	N	G	T	S	T	G
Sydney (2012)	**T**	**S**	**R**	N	**E**	**D**	**V**	K	**T**	E	**G**	**T**	T	S	**N**	**T**

Chapter 5

General discussion and future directions

Ashwin Ramesh

Vaccination is one of the most successful medical practices that has played a significant role in positively impacting our longevity (Rappuoli, Pizza *et al*. 2014, Kim and Jang 2017). Novel vaccines that enter the pipeline require stringent testing and repeated quality control in order to ensure their safety, immunogenicity and efficacy before they are rolled out to the targeted population (Crowcroft and Klein 2018). Diarrhea is responsible for 9% of childhood deaths globally (Liu, Oza *et al*. 2015), a rate which has been only recently reduced due to the introduction of rotavirus (RV) vaccines as part of national immunization programs (Burnett, Jonesteller *et al*. 2017). The impact that gastroenteritis (GE) diseases have on communities especially in developing countries is evidenced by the steady burden of outbreaks and their associated mortalities (Graves 2013). Lower vaccine effectiveness against RV infection and disease in resource-limited settings has been identified to be multi-factorial (Clarke and Desselberger 2015).

The two main concepts discussed in my thesis include: (1) To determine whether intramuscularly (IM) administered vaccines are effective in priming the immune system against human-RV and human norovirus (HuNoV) infection and disease using a gnotobiotic (Gn) pig model, and (2) To determine the median infectious dose (ID_{50}) and median diarrhea dose (DD_{50}) of HuNoV GII.4 Cin-1 variant in order to establish Gn pigs as an effective animal model to study HuNoV infections, and to assist with the standardization of dose-response models used to determine an optimal infectious dose for the accurate evaluation of vaccines and therapeutics for use in future studies.

Currently, a P2-VP8* RV vaccine is in its advanced (Phase II) stages of clinical trials and is the front runner of non-replicating RV vaccines (NRRV) for vaccine licensure (Groome, Koen *et al*. 2017). Pre-clinical trials of this vaccine have been carried out in Gn pig models and have shown to be effective in protection against RV diarrhea before progressing towards human clinical trials

(Wen, Wen *et al*. 2014). As part of this thesis, we first explored the IM route of vaccine administration to test a novel nanoparticle RV vaccine in order to determine its immunogenicity and protective efficacy against RV infection and disease using the Gn pig model. This vaccine was developed based on the concept that NoV P particles (a 24-mer nanoparticle) can tolerate large sequence insertions within their P domain surface loop in order to display foreign epitopes (Tan and Jiang 2012). This vaccine can easily be synthesized and expressed using *E. coli* expression vectors. Three IM doses of this vaccine primed the Gn pig immune system and ensured the production of sufficient adaptive immune responses to provide strong protection against RV infection and diarrhea. Protection was indicated by the significant reduction in the mean duration of diarrhea, virus shedding in feces, and significantly lower fecal cumulative consistency scores in post-challenge day (PCD) 1-7 among vaccinated pigs compared to the mock immunized controls. (Ramesh, Mao *et al*. 2019). While this study only evaluated the protective efficacy against RV, further studies need to be carried out to determine whether the P particles are able to retain their immunogenicity (Kocher, Bui *et al*. 2014) against HuNoV infection and disease. Our study provided the preclinical evaluation data needed for IND application by the company, Anhui Zhifei Longcom Biopharmaceutical Co., Ltd., China, to start human clinical trials of this vaccine and helped to generate a strong case for P particle-based vaccines (Tan and Jiang 2019) encouraging further research to be carried out in this field for the development of P particle-based vaccines.

GII.4 HuNoVs belong to the most diverse genotype of NoV strains and are responsible for over 80% of all NoV-related GE in the world today. Immune responses to NoV infections have been attributed to drive the selection of emerging pandemic strains of NoV through both antigenic and genetic drifts and shifts (White 2014). Gn pigs have previously been used to determine the ID_{50} of GII.4/2006b HuNoV strain (Bui, Kocher *et al*. 2013). In Chapter 3 we estimated the Log_{10} ID_{50}

and DD_{50} to be between 3.11 to 3.76, and 3.37 to 4.87 RNA copies, respectively. Furthermore, of the different routinely used dose-response models for quantitative microbial risk-assessment, we identified that the beta-Poisson model to be the best-fitting model for determining both the ID_{50} and DD_{50}. By comparing the ID_{50} values of GII.4/2003 to GII.4/2006b strain, the GII.4/2006b variant required 35-fold higher genomic copy numbers in order to establish infection among 50% of the 33-34-day old Gn pigs. The GII.4/2003 strain is a Farmington Hills variant which was responsible for causing around 64% of cruise ship outbreaks and 45% of the land-based outbreaks, most of which occurred in long-term care facilities, schools, and restaurants, in the US in 2002 (Isakbaeva, Widdowson *et al*. 2005, Bull, Tu *et al*. 2006, Johnston, Qiu *et al*. 2007). Standardizing dose-response data from different circulating variants will help expedite HuNoV vaccine development.

VLP vaccine have been the main focus of subunit-based vaccines since they are able to replicate the morphological confirmation of HuNoVs. The advantages of using VLP vaccines as candidates is that they can easily be synthesized using different expression systems (Herbst-Kralovetz, Mason *et al*. 2010) and large amounts of VLPs can be produced very quickly to target new emerging strains. In Chapter 4, we evaluated immunogenicity and protective efficacy of a novel tetravalent VLP vaccine containing GI.1, GII.3, GII.4 and GII.17 VLPs in the Gn pig model of GII.4 infection and diarrhea. The vaccine was highly immunogenic, and was able to induce very strong VLP-specific IgA and IgG antibody responses against all 4 genotypes as well as HAI antibodies in the serum and primed for VLP-specific IFN-γ producing CD4+ and CD8+ T cell responses in the spleen and blood. However, the abundance of immune effectors did not confer any protection against the Cin-1 variant, likely due to the antigenic difference between the GII.4 variant in the vaccine and the challenge GII.4 variant. It remains to be seen if the vaccine is capable of conferring

protection when challenged with the same GII.4 variant. Findings from this study suggests that for any HuNoV VLP vaccine to confer broad protection, it has to include all major currently circulating genogroups, genotypes and multiple variants with in the genotypes.

In summary, these studies demonstrate the benefits of Gn pigs as a large animal model that is capable of recapitulating both the RV and GII.4 HuNoV infection and disease. It is very exciting times for the prospect of HuNoV research. With recent identification of cellular tropisms, easily-reproducible cell culture models will be the main focus for researchers. Research efforts focusing on the mechanisms of NoV infection, pathogenesis and immunity using cell culture and animal models will answer many questions including the one raised in Chapter 4 regarding immune evasion. In order to be able to truly test the immunogenic potential of novel vaccines and their ability to confer protection among a completely naïve population, Gn pigs provide the solution. It has to be recognized that having these powerful research tools is truly a privilege and it needs to be used more often to create significant groundbreaking advancements in enteric viral research.

References

Bui, T., J. Kocher, Y. Li, K. Wen, G. Li, F. Liu, X. Yang, T. LeRoith, M. Tan, M. Xia, W. Zhong, X. Jiang and L. Yuan (2013). "Median infectious dose of human norovirus GII.4 in gnotobiotic pigs is decreased by simvastatin treatment and increased by age." J Gen Virol **94**(Pt 9): 2005-2016.

Bull, R. A., E. T. V. Tu, C. J. McIver, W. D. Rawlinson and P. A. White (2006). "Emergence of a New Norovirus Genotype II.4 Variant Associated with Global Outbreaks of Gastroenteritis." Journal of Clinical Microbiology **44**(2): 327-333.

Burnett, E., C. L. Jonesteller, J. E. Tate, C. Yen and U. D. Parashar (2017). "Global Impact of Rotavirus Vaccination on Childhood Hospitalizations and Mortality From Diarrhea." The Journal of infectious diseases **215**(11): 1666-1672.

Clarke, E. and U. Desselberger (2015). "Correlates of protection against human rotavirus disease and the factors influencing protection in low-income settings." Mucosal immunology **8**(1): 1-17.

Crowcroft, N. S. and N. P. Klein (2018). "A framework for research on vaccine effectiveness." Vaccine **36**(48): 7286-7293.

Graves, N. S. (2013). "Acute gastroenteritis." Primary care **40**(3): 727-741.

Groome, M. J., A. Koen, A. Fix, N. Page, L. Jose, S. A. Madhi, M. McNeal, L. Dally, I. Cho, M. Power, J. Flores and S. Cryz (2017). "Safety and immunogenicity of a parenteral P2-VP8-P[8] subunit rotavirus vaccine in toddlers and infants in South Africa: a randomised, double-blind, placebo-controlled trial." The Lancet Infectious Diseases **17**(8): 843-853.

Herbst-Kralovetz, M., H. S. Mason and Q. Chen (2010). "Norwalk virus-like particles as vaccines." Expert review of vaccines **9**(3): 299-307.

Isakbaeva, E. T., M.-A. Widdowson, R. S. Beard, S. N. Bulens, J. Mullins, S. S. Monroe, J. Bresee, P. Sassano, E. H. Cramer and R. I. Glass (2005). "Norovirus transmission on cruise ship." Emerging infectious diseases **11**(1): 154-158.

Johnston, C. P., H. Qiu, J. R. Ticehurst, C. Dickson, P. Rosenbaum, P. Lawson, A. B. Stokes, C. J. Lowenstein, M. Kaminsky, S. E. Cosgrove, K. Y. Green and T. M. Perl (2007). "Outbreak Management and Implications of a Nosocomial Norovirus Outbreak." Clinical Infectious Diseases **45**(5): 534-540.

Kim, S.-H. and Y.-S. Jang (2017). "The development of mucosal vaccines for both mucosal and systemic immune induction and the roles played by adjuvants." Clinical and experimental vaccine research **6**(1): 15-21.

Kocher, J., T. Bui, E. Giri-Rachman, K. Wen, G. Li, X. Yang, F. Liu, M. Tan, M. Xia, W. Zhong, X. Jiang and L. Yuan (2014). "Intranasal P particle vaccine provided partial cross-variant protection against human GII.4 norovirus diarrhea in gnotobiotic pigs." J Virol **88**(17): 9728-9743.

Liu, L., S. Oza, D. Hogan, J. Perin, I. Rudan, J. E. Lawn, S. Cousens, C. Mathers and R. E. Black (2015). "Global, regional, and national causes of child mortality in 2000–13, with projections to inform post-2015 priorities: an updated systematic analysis." The Lancet **385**(9966): 430-440.

Ramesh, A., J. Mao, S. Lei, E. Twitchell, A. Shiraz, X. Jiang, M. Tan, Yuan and Lijuan (2019). "Parenterally Administered P24-VP8* Nanoparticle Vaccine Conferred Strong Protection against Rotavirus Diarrhea and Virus Shedding in Gnotobiotic Pigs." Vaccines **7**(4): E177.

Rappuoli, R., M. Pizza, G. Del Giudice and E. De Gregorio (2014). "Vaccines, new opportunities for a new society." Proceedings of the National Academy of Sciences of the United States of America **111**(34): 12288-12293.

Tan, M. and X. Jiang (2012). "Norovirus P particle: a subviral nanoparticle for vaccine development against norovirus, rotavirus and influenza virus." Nanomedicine (Lond) **7**(6): 889-897.

Tan, M. and X. Jiang (2019). "Norovirus Capsid Protein-Derived Nanoparticles and Polymers as Versatile Platforms for Antigen Presentation and Vaccine Development." Pharmaceutics **11**(9): 472.

Wen, X., K. Wen, D. Cao, G. Li, R. W. Jones, J. Li, S. Szu, Y. Hoshino and L. Yuan (2014). "Inclusion of a universal tetanus toxoid CD4(+) T cell epitope P2 significantly enhanced the immunogenicity of recombinant rotavirus ΔVP8* subunit parenteral vaccines." Vaccine **32**(35): 4420-4427.

White, P. A. (2014). "Evolution of norovirus." Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases **20**(8): 741-745.