Study of Infection, Immunity, Vaccine and Therapeutics Using Gnotobiotic Pig Models for Human Enteric Viruses

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Dissertation submitted to the Faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

In

Biomedical and Veterinary Sciences

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March 20th, 2015

Blacksburg, VA

Keywords: Gnotobiotic pig model, Human Enterovirus 71, Human Rotavirus, Infection and Immunity, Vaccine and Therapeutics Evaluation

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ABSTRACT

With the absence of gut microbiota, gnotobiotic (Gn) pigs are a unique animal model for studying infection and immunity, and evaluating vaccine and therapeutics for human enteric pathogens. Here, we demonstrate Gn pigs as effective large animal models for human enteric viruses, through evaluating human enterovirus 71 (EV71) infection and immunity, and vaccine and therapeutics for human rotavirus (HRV). Gn pigs could be infected via oral or oronasal route, the natural route of infection. Infected pigs developed clinical signs including fever, neurological and respiratory signs, similar to those seen in human patients. Fecal shedding up to 18 days post infection and virus distribution in intestinal, respiratory and central nervous system tissues were observed. Strong mucosal and systemic T cell responses (IFN-γ producing CD4+ and CD8+ T cells) and systemic B cell responses (serum neutralizing antibodies) were also detected. The study demonstrates a novel large animal model for EV71 to investigate viral pathogenesis, immunity, and to evaluate vaccine and antiviral drugs. Using the well-established Gn pig model for HRV, the adjuvant and therapeutic effects of prebiotics rice bran (RB) and probiotics were evaluated. RB alone or RB plus probiotic *Lactobacillus rhamnosus* GG (LGG) and probiotic *E. coli* Nissle 1917 (EcN), were shown to protect against rotavirus diarrhea (80%-100% reduction in the incidence rate) significantly and display strong immune - stimulatory

effects on the immunogenicity of an oral attenuated HRV (AttHRV) vaccine. Mechanisms for the adjuvant effect include stimulating the production of intestinal and systemic IFN-γ producing T cells and promoting mucosal IgA antibody responses. The mechanisms for reducing rotavirus diarrhea include promoting LGG and EcN growth and colonization and host gut health, and maintaining gut integrity and permeability during rotavirus infection. We showed that RB plus LGG and EcN is a highly effective therapeutic regimen against HRV diarrhea. Together, these results indicated that Gn pigs may serve as an excellent animal model for the study of infection, immunity, vaccine and therapeutics for human enteric viruses.

Dedicated to my parents Zizhong Yang and Cuie Huang,

sisters Qun Yang, Ruennian Yang and Xiaojiao Yang for their love and support

ACKNOWLEDGEMENTS

First of all, I would like to express my sincere gratitude to my advisor Dr. Lijuan Yuan for agreeing to take me in her lab as a graduate student, mentoring me through her guidance, support, encouragement, and inspiration. Her broad knowledge and expertise in the Gn pig model for enteric viruses, viral immunology and stable funding have provided me an environment which made my dissertation research possible. I am also thankful for her patience, understanding and assistance during tough times I went through in my personal and professional life. Her advice, and financial, technical and emotional support played an indispensable role for my maturation as a scientist and person. I really appreciate her genuine interests in helping me succeed in every aspect of my life. I am very fortunate to have such a great mentor and advisor and I am forever grateful for her roles in my present and future scientific career.

I would also like to thank all current and former Yuan Lab members, including but not limited to: Dr. Guohua Li, Dr. Ke Wen, Tammy Bui, Mariah Weiss, Dr. Jacob Kocher, Dr. Fangning Liu, Christine Tin, and Dr. Haifeng Wang, Dr. Erica Twitchell, Shaohua Lei and Ashwin Ramesh, for their help regarding my research projects. Their knowledge, participation and contributions are essential for the successful completion of my research projects.

Special thanks also go to the TRACCS staff Pete Jobst, Andrea Pulliam, Shannon Viers, Kimberly Allen and Veterinarians Drs. Marlice Vonck and Nicole Linstrom for their excellent animal care.

Drs. Kevin Pelzer and Sherrie Clark-Deener are also greatly appreciated for performing sow surgeries during hysterectomy. Melissa Makris has always been very helpful in flow cytometry.

I would like to thank my advisory committee members Drs. Lijuan Yuan, Liwu Li, Elankumaran Subbiah and Tanya LeRoith and former committee member Dr. Bernard S. Jortner, for always being available, helpful and giving constructive comments, suggestions and advice during my committee meetings and keeping me on track of my research project. Your immense knowledge and expertise, and willingness to help me succeed not only in my research, but also future career, has made my graduate education experience much more fulfilling than it would otherwise be. I couldn't ask for a better advisory committee.

I sincerely thank all the departmental faculty and staff, especially Dr. Roger Avery, Dr. S. Ansar Ahmed, and Becky Jones, for their efforts and support in providing an incredibly enriched and fulfilling environment during the whole process of graduate education experience.

Also important for the completion of my dissertation research are our excellent collaborators Dr. Yuan Qian, Laboratory of Virology, Capital Institute of Pediatrics, 2 Yabao Road, Chaoyang District, Beijing 100020, China; and Dr. Elizabeth Ryan, Department of Environmental and Radiological Health Sciences, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, Colorado, USA. They not only participated in the project

development, but also provided reagents, advice on the projects and contributed to the writing and editing of manuscripts.

Last but not the least, I would like to acknowledge the funding agencies Bill and Melinda Gates Foundation Grand Challenge Exploration in Global Health, WA, National Center of Complementary and Alternative Medicine, National Institutes of Health, Bethesda, MD, and Fralin Funds, Fralin Life Science Institute, Virginia Tech, VA for providing financial support.

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List of Abbreviations

Alpha-1 antitrypsin (A1AT)

Antibody-secreting cells (ASC)

Attenuated human rotavirus (AttHRV)

Cell culture immunofluorescence assay (CCIF)

Central nervous system (CNS)

Colony forming units (CFU)

Cytopathic effects (CPE)

Cytotoxic T lymphocytes (CTL)

Dendritic cells (DCs)

***E. coli* Nissle 1917 strain (EcN)**

Enzyme-linked immunosorbent assay (ELISA)

Fluorescence forming units (FFU)

Gnotobiotic (Gn)

Hematoxylin and eosin (H&E)

Histo-blood group antigens (HBGAs)

Human enterovirus 71 (EV71)

Human gut microbiota (HGM)

Human hand, foot and mouth disease (HFMD)

Human rotavirus (HRV)

Immunoglobulin secreting cells (IgSC)

Intraepithelial lymphocytes (IEL)

***Lactobacillus acidophilus* (LA)**

***Lactobacillus rhamnosus* GG (LGG)**

Large intestinal contents (LIC)

Median infectious dose (ID50)

Mesenteric lymph nodes (MLN)

Mononuclear cells (MNCs)

Multiplicity of infection (MOI)

Peripheral blood mononuclear cells (PBMCs)

Post inoculation day (PID)

Post-challenge day (PCD)

Post-feeding day (PFD)

Post-partum day (PPD)

P-selectin glycoprotein ligand-1 (PSGL-1)

Reverse transcription polymerase chain reaction (RT-PCR)

Rice bran (RB)

Scavenger receptor class B, member 2 (SCARB2)

Small intestinal contents (SIC)

Specific pathogen free (SPF)

T helper (Th)

Virulent HRV (VirHRV)

Virus like particles (VLP)

Chapter 1 Literature Review

Gnotobiotic Pig Models for Studying Human Enteric Viral Pathogenesis, Immune Responses, and for Vaccine and Therapeutics Evaluation

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Part of this chapter has been published in *British Journal of Virology*, 2014;1(3): 87-91.

1.1 GNOTOBIOTIC PIGS AS A BIOMEDICAL MODEL

1.1.1 Gn pigs

Anatomy, physiology and genetics

Pigs (*Sus scrofa*) are animals in the *Sus* genus of the *Suidae* family. They diverged from humans about 100 million years ago. Their domestication started about 9000 years ago in Asia and Europe. Currently there are about 720 species or breeds of outbred and inbred pigs, mainly in China and Europe. Their size ranges from miniature pig breeds (e.g. Gottingen, Hanford, Yucatan, Sinclair, Yucatan and Yucatan micro breeds, Banna miniature inbred lines) to large farm breeds (e.g. Yorkshire, Landrace, Hampshire, Duroc, Berkshire and Pie´train) that weigh several hundred pounds(1, 2). The farm and miniature pig breeds differ mainly in their growth rate and size at sexual maturation, whereas they should be similar on the molecular, anatomic, physiological level. All breeds reach sexual maturation at 4-6 months of age, growing from 1-2 kg at birth to 100kg at 4 months of age for farm pig breeds, and from 0.5-1 kg to 7-20 kg for the miniature pig breeds. The sizes of most organs in the miniature pigs at sexual maturation are comparable to those of adult humans. The anatomy and physiology of pigs are very similar to humans, and make them suitable for surgery and biomedical research, particularly for the cardiovascular, digestive, integumentary and urinary systems (2).

Complete pig genome sequence and analysis have been published for a domestic pig breed (Duroc) (3), a miniature pig breed (Wuzhishan) (4) and a Hungary fatty pig (5). Generally speaking, the pig and human genome have high genetic sequence homology, and similar number of genes and evolution rate. The total length of the pig genome is 2.8 gigabase in size, with 21,640 protein

coding genes, 380 pseudogenes, 2965 ncRNAs, 197,675 gene exons and 26,487 gene transcripts. By using reciprocal best BLAST analysis of the miniature pig genome (4), 16,564 orthologous genes were identified out of total 20,326 genes between pig and humans, sharing an average identity of 81.4% with their human counterparts. Interestingly, a large number of pig protein variants also have the same changes as those seen in humans, which increase risk in multifactorial traits, including obesity (ADRB3, SDC3) and diabetes (PPP1RA, SLC30A8, ZNF615) or are shown to result in relatively mild phenotypes (for example, dyslexia: KIAA0319) or late-onset diseases such as Parkinson's disease (LRRK2, SNCA) and Alzheimer's disease (TUBD1, BLMH, CEP192, PLAU) (3). These similarities indicated that pigs are very similar to humans on the genetic level and could be excellent model for human genetics and gene-related diseases, either in naturally occurring or genetically modified pig models. Due to their domestication, pigs have a highly similar evolution rate to that of humans, with synonymous substitutions per synonymous sites d(s) value of 0.160 and 0.138 to 0.201 for the pigs and humans, as well as similar levels of the purifying selection pressure in pigs and humans, as indicated by the observed ratio of the rate of nonsynonymous substitutions to the rate of synonymous substitutions (dN/dS ratio) of 0.144 for pigs and 0.163 for humans, respectively. Immunity - related genes also show similar evolution rates between pigs and humans: 17% and 18% immunity related genes showed accelerated evolution for the pigs and humans, respectively. However, ~1% of genes were found to have been gained or lost in pigs compared to the human genome (4). Specifically, 245 genes were gained and 270 genes were lost in pigs. These genes and functions should be taken into consideration when modeling human diseases and evaluating therapeutics drugs. For example, PROZ, a gene coding for the protein Z (an important protein for the formation of blood clots and thrombosis), is absent in pigs. Additionally, genes associated with immunity and olfaction showed expansion in pigs (3).

For example, at least 39 type I interferon genes have been identified in pigs, which is about twice the number identified in humans. Pigs have a large number of functional olfactory genes. About 1,301 porcine olfactory receptor genes and 343 partial olfactory receptor genes have been identified, reflecting the strong reliance of pigs on their smell when scavenging for food.

Pig as a biomedical model

Animal models are useful to biomedical research because they help us understand the progression of human diseases, develop and test therapeutics for diseases, and to evaluate the pharmacodynamics and toxic effects of drugs. Therefore, ideal animal models should be able to mimic human molecular, cellular and physiological functions and disease symptoms, survive long enough for disease progression and symptoms, provide adequate biological samples, produce large progeny, and can be easily accessible to a large group of researchers. Traditionally, due to their accessibility, low housing requirement, and easy manipulation for experimental use, fruit flies, *Caenorhabditis elegens*, zebra fish and rodents have been widely used as biomedical models for human health and diseases. They have been extremely valuable for the studying of normal structure and function of the human host, modeling various diseases and efficacy and toxicity of drugs. However, their limitations with modeling human diseases are becoming more obvious, given the differences in the level of the genetics, anatomy, physiology and immune systems. Therefore, alternative large animal models that are more closely related to humans are needed to bridge the gap. Non-human primates are more closely related to humans than any other animal species. However, the accessibility of these models is significantly limited by the high costs and ethical concerns.

The porcine genome has a size of 2.6- 2.9 gb, with 18 chromosomes and X and Y chromosomes. The overall genome structure and number and functions of genes are highly analogous to that of humans. Pig breeds worldwide are diverse, reflecting the different domestication and selective breeding process of the pig in different regions (3). Overall, these characteristics make the pig an excellent model for humans on an individual and population level. Pigs are also highly similar to humans in the anatomy and physiology of organs and tissues in most systems, including the cardiovascular system, integument system, gastrointestinal system and urinary system. The similarity and usefulness of major systems of pigs to model human diseases have been reviewed in comparison to other animals (including rodent, dog, and non-human primate)(2, 6). On the immune system level, pigs have also been shown to share up to 80 % homology to that of humans whereas mice showed about 10% to humans in all immune parameters analyzed (7). Pigs have a full set of innate and adaptive immune systems with most proteins sharing structural and functional similarities to their human counterpart. Studies have described the cluster of differentiation (CD) molecules, cell surface proteins that allow for identification and characterization of immune cell populations, and all the immune cell populations identified in human and mice are found in pigs (8). In addition, functional orthologs for the cytokines and corresponding cells for the Th1/Th2/Th17/Treg paradigm described in mice (9) have been described in pigs (10-14). In contrast to mice, pigs are similar to humans in terms of the response of porcine macrophages to lipopolysaccharide (LPS) and IFN γ stimulation (no nitric oxide response) (15), TLR7 and 9 expressions on plasmacytoid dendritic cells (pDCs) (16), and sensitivity to endotoxin and development of hyperthermia (17), the high percentage of neutrophils in peripheral blood (50-70%) and presence of direct orthologs for the chemo-attractant IL8 (17). Given these similarities,

pigs have been used to model human diseases of various systems and organs in biomedical research, preclinical pharmacological and safety testing, as well as surgical models and procedures.

Pigs used in biomedical research vary widely in terms of their sources, sizes and genetic background. Based on the size, pig models include the farm pig model and the miniature pig models. Based on the sources, breeding and housing environment, major types of pig models include conventional, specific pathogen free and Gn pig models. Based on the experimental procedures, pig models include naturally occurring models, experimentally induced models and genetically modified pig models.

Gnotobiotic (Gn) pigs

Gnotobiotic pigs are pigs with known forms of microbial life, a term that could mean germ free or association with any known number of live organisms. The word gnotobiotic, stems from Greek gnoto (well known) and biota (all forms of life). Due to its sterile housing environment and the lack of maternal antibodies through colostrum/milk, Gn pigs differ from their conventional counterparts in some aspects of gastrointestinal morphology, physiology and mucosal immune system. Gn pigs have thinner intestinal walls (less cellular and hydrated, and with reduced connective tissues, such as lamina propria and the associated small blood and lymphatic vessels)(18), and narrower and more pointy villi with a greater proportion of mature enterocytes containing possibly higher amounts of enzymes such as peptidases or disaccharidases (due to slow mitosis s and migration of crypt cells to the tip of villi and desquamation of the mucosal epithelial cells) (18, 19). A study comparing the lamina propria lymphocytes between Gn pigs and

conventional pigs using immunohistological staining have found that rapid development of lamina propria lymphocytes occurred in the early postnatal period (up to 40 days) in conventional pigs, and Gn pigs at 49 days of age had a similar distribution pattern and number of CD2+, CD4+, and CD8+ T lymphocyte subsets to 5 day old conventional pigs and very low number of Ig+ cells, suggesting the importance of gut microbial and nutritional antigens in stimulating mucosal immune system (20). A lower amount of serum gamma-globulins (21) and similar distribution and characteristics of macrophages but with few reaction centers and immunologically competent cells in the lymphatic tissues (19) have also been noted in Gn pigs. Despite the less well-developed immune systems in Gn pigs, they are fully competent and respond to endogenous and exogenous antigen stimulation similar to conventional pigs (19).

These differences in the grastrointestinal morphology, physiology and immune system between Gn and conventional pigs are primarily due to the lack of gut microbiota. The enormous amount of species and genes, and structural and functional diversity of gut microbiota have profound impacts on health and diseases of the host (22-46). The study of intestinal microbiota is still in its early stages, which focuses mainly on the description on its composition over time, physiological location and changes that occur under different physiological conditions, such as stress, diet and infectious diseases. Further studies will be needed to characterize the functions of the intestinal microbiota and their effects on the host health and diseases. Advances in this area will be important for applications of Gn pigs in biomedical research, in terms of model development and data interpretation.

Gn *pigs in biomedical research*

Gnotobiotic pigs have been used in biomedical research since the 1960s (19). The procedures of Gn pig production, housing, rearing, and microbiological monitoring were well established in the early 1960's (47-49). Gn piglets are derived near term via hysterectomy. Gn pigs of various breeds can be kept in the isolators for 6-8 weeks or longer depending on isolator size (50). Without the interference from gut microbes, Gn pigs are particularly suited for studying a variety of major topics relevant to medicine and biology, including infectious diseases (especially enteric pathogens) (51-87), gut microbiota (88-101), immunology (54, 57, 90, 92, 93, 100-117), xenotransplant (118-120), nutrition and metabolisms (95, 121-123), and toxicology (100, 124-128). With pigs increasingly being accepted as the preferred large animal model, and Gn pigs being widely adopted in biomedical research (due to the advantages of Gn pigs being recognized, reduced costs and improved infrastructure and reagents), these topics and disease models using Gn pigs are expected to become more extensive.

1.1.2 Gn pigs as enteric virus infection model

Gnotobiotic pigs are particularly suited to model human enteric virus infections, given its similarity to humans in anatomy, gastrointestinal physiology and the immune system. There is also a lack of maternal antibodies, confounding enteric pathogens and gut microbiota, providing an immunological naïve background for the study of human and enteric pathogen interactions. In fact, pigs are the natural reservoirs for a number of human enteric pathogens, such as *Salmonella enterica* serovar Typhimurium (129), Hepatitis E virus(130), enteroviruses (131, 132), and possibly norovirus (133) and sapovirus (134). The value of the Gn pigs for the study of enteric

pathogens has been recognized since early development of the Gn pig model for biomedical research (19). Gn pigs have been used to study a variety of bacterial, viral and fungal pathogens of human and animal origins, such as human rotavirus (HRV) (59, 60, 76, 135-137), human norovirus (51, 58, 105, 138, 139), porcine rotavirus (140-142), porcine norovirus (133), porcine enterovirus (132, 143), porcine endemic diarrhea diseases virus (52), porcine teschovirus (53), porcine circovirus (54, 55), porcine enteric calicivirus (63, 74), bovine rotavirus (136, 144), *Salmonella Typhimurium* (64, 145), *Escherichia coli* (146, 147), *Clostridium difficile* (148-150), *Shigella sonnei* (151) and *Candida albicans* (152). Overall, these models demonstrated that human pathogen-infected Gn pigs show shedding pattern, pathogenesis, histopathologic lesions and clinical signs that are similar to those seen in humans, although for some human enteric viruses, such as HRV and human norovirus, serial passages in Gn pigs (135) or immune-compromised Gn pigs (138, 139) are needed to produce efficient infection, replications, and same magnitude and severity of diseases seen in their human counterparts.

Currently, there are well-established Gn pig models of two major human enteric viruses, HRV (mainly G1P1A[8] group A HRV) and human norovirus (mainly GII.4 variants). These two human enteric viruses are most prevalent and responsible for the majority of acute viral gastroenteritis and deaths in young children worldwide (153, 154). Both models have been fundamental in our understanding of the infection, pathogenesis, immune responses, and evaluation of vaccines and therapeutics for these two human enteric pathogens. Here these two models are discussed in detail below.

Gnotobiotic pig model of HRV infection and diarrhea

Rotaviruses are generally considered to be host specific. However, under experimental conditions, VirHRV infections have been reported in neonatal mice (155), Gn calves (156) and pigs (137). Among these animal models of HRV, Gn pigs have proven to be a more useful model for the study of HRV infection and disease in humans. Gn pig model of HRV is unique in that they have a prolonged susceptibility to rotavirus-induced diseases for up to 8 weeks of age (157).

Several initial attempts in the 70's and 80's to establish a Gn pig model for HRV infection and disease has limited success (136, 137, 158). Gn pigs were intra-nasally inoculated at 2-28 days of age with 1-2ml human fecal filtrates (diluted 30% w/v) from young children suffering from acute gastroenteritis and fecal filtrates containing serially passaged HRV from Gn pigs (137). The results showed that fecal virus shedding and seroconversion were detected in inoculated Gn pigs. However, no clinical signs, such as diarrhea were observed during 3-4 weeks monitoring periods after infection. Histopathological lesions were not examined, although there were reports on lesions seen in VirHRV infected young children, such as loss of absorptive enterocytes, villus atrophy, hyperplasia of intestinal crypts and repair of damaged intestinal epithelium in duodenum, jejunum, and ileum (159-161). Nevertheless, this model demonstrated that HRV and serially passaged HRV in pigs could infect and replicate in Gn pigs. A later study using Gn pigs produced similar results, with successful HRV infection, but still no diarrheal diseases developed (136).

The Gn pig model of HRV infection and diarrhea was successfully established in 1996 (135) and this model has been used in over 70 published studies to date. These detailed studies on the

infection, diseases and pathogenesis were for a Gn pig model of VirHRV Wa strain (G1P1A [8]) which was previously passaged in Gn pigs 16 times (135). In this model, Gn pigs were infected readily with the pig-adapted HRV with median infectious dose (ID_{50}) of < 1 fluorescence forming units (FFU) as determined by viral shedding in fecal samples post infection. Double layered and triple layered rotavirus particles were also detected in intestinal contents by immune electron microscopy. Virus shedding could be detected at 13 hours post infection and peaked at up to 10^7 FFU. The virus infected mainly small intestinal tissues, particularly jejunum and ileum. A large amounts of virus was detected in the small intestinal tissues at 13 hours post infection and could be detected in gastric, colonic, rectal tissues and mesenteric lymph nodes (MLN) at 24 hour post infection. Virus was again restricted to small intestinal tissues at 48 -96 hours post infection.

The disease and pathological lesions in this Gn pig model were also found to be similar to VirHRV infections seen in humans, including diarrhea, loss of mature absorptive intestinal cells, villus atrophy and hyperplasia of organized gut associated lymphoid tissues (GALT) (159-161). Diarrhea was detected in 100% of Gn pigs infected with sufficient doses of VirHRV, starting from post-inoculation day (PID) 1 through PID 5. Macroscopic and microscopic changes were restricted to the small intestine and MLN. Macroscopic changes include distended small intestines containing opaque yellowish fluid with thin walls and enlarged MLN at 48-96 hours post infections. The loss of normal mature absorptive enterocytes and villus atrophy were most prominent at 48-72 hours post infection, with hyperplasia of organized gut associated lymphoid tissues (Peyer's patches and MLN) most evident at 72-96 hours post infection. Transient increase in the number of intra-epithelial cells was also noted at 72-96 hours post infection. By PID 7, macroscopic and microscopic changes disappeared and the morphology of small intestine returned to normal. For

pathogenesis, it was shown that the onset of diarrhea correlated with the presence of rotavirus in the small intestine, although villus atrophy-induced malabsorptive diarrhea was the main cause of diarrhea during rotavirus infection. Such finding point to other mechanisms of rotavirus diarrhea, such as NSP4 protein as a viral toxin, immune responses (cytokines and activated T cells) and stimulation of enteric nervous system. Another study using Gn pigs (60) also replicated the findings in HRV infected patient including virus replication and shedding in feces, upper respiratory systems, and development of viremia (162). Together, these results demonstrated that Gn pigs are indeed a unique and excellent animal model for studying the infection, diseases and pathogenesis of HRV.

Gnotobiotic pig model of human norovirus infection

Another major human enteric virus that has been studied using the Gn pig model is human norovirus, a member of the *Caliciviridae* family. Similar to HRV, human norovirus is an enteric pathogen with high public health significance, being a major cause of acute gastroenteritis affecting people of all ages and responsible for ~200,000 deaths in young children worldwide. Unlike HRV, there is no vaccine or antiviral drug available and their development is limited by the lack of cell culture systems and effective small animal models. Currently, development of suitable animal models for human noroviruses is a major area of research in the norovirus research community. A number of animal species have been shown to be infected with human norovirus, such as immunodeficient mice (163), Gn calves (164), Gn pigs (58, 165) and non-human primates (166, 167). A great deal of data has been generated using surrogate models in mice using murine norovirus and non-human primates with Tulane virus, a rhesus monkey Calicivirus. The usefulness

of these models are limited by the significant differences in clinical signs (lack of diarrhea and vomiting), pathogenesis (replication in macrophage and dendritic cells versus enterocytes) and viral receptors (sialic acid in mice versus histo-blood group antigens [HBGAs]) (168).

Pigs resemble humans on the level of gastrointestinal anatomy, physiology, immune system, as well as similar expression of norovirus receptors HBGAs with expression of A and H antigens on mucosal surfaces (165). Porcine noroviruses were also detected in swine populations worldwide and through genetic analysis, porcine norovirus is found to be highly related to human norovirus and is infectious in Gn pigs (133). Furthermore, antibodies to human norovirus have been detected in swine under nature conditions (169). These data suggest that Gn pigs could be infected by human noroviruses and a large animal model for the study of human norovirus infection and diseases is possible.

The reproduction of human norovirus infection and diseases in experimentally infected Gn pigs have been reported using genogroup II (GII) human norovirus strains, especially GII.4, the most common genotype in humans (51, 58, 138, 139, 169, 170). Importantly, virus shedding, seroconversion, viremia, diarrhea, and intestinal lesions that are comparable to those seen in humans were observed in infected Gn pigs. The infectivity of norovirus in a host is significantly restricted by the HBGAs and secretor profiles of the host. The binding patterns of HBGAs and HBGA antigen types are typed in these Gn pigs in order to match the infectivity of the norovirus inoculum and the HBGAs expression profiles of an individual host. In one study (139), HGBA A+ or H+ Gn pigs were orally infected with human norovirus GII.4 2006b variant (non-pig adapted)

at a certain dose range (up to $2.74x10^5$ RNA copies), and 100% of inoculated pigs shed virus and showed diarrhea (139). In the same study, the ID_{50} of this virus was determined to be $2.74x10^3$ and $6.43x10^4$ viral RNA copies in neonatal (4–5 days of age) and older (33–34 days of age) Gn pigs, respectively. These results suggest that with the appropriate norovirus strain and HBGAs expression, a highly reliable Gn pig model of human norovirus infection and disease can be established. Several studies have used the Gn pig GII.4 human norovirus challenge model to evaluate the efficacy of vaccines (51, 171). These studies have demonstrated Gn pigs as a top animal model choice for studying human norovirus infection and disease.

As discussed above, Gn pigs are ideal model for the study of human enteric virus infections and diseases. Gn pig models of HRV and human norovirus have been established, and contributed to our understanding of pathogenesis, immunity, and vaccine and therapeutic development for these viruses. Undoubtedly, these robust models will continue to be useful in our further studies of these areas in the future. In particular, more studies of human noroviruses using Gn pigs can be expected, given the limited animal models and lack of vaccines for this important enteric virus. The effects of gut microbiota and its bacterial components on the infectivity and diseases of human enteric viruses are profound and are being increasingly recognized (172-174). Due to its lack of gut microbiota, Gn pigs are particularly good animal models to study microbial interactions. The impact of single bacterial species or the entire human gut microbiota can be studied in the Gn pig model (89-92, 175-177). This area represents an emerging and exciting research topic in infectious disease. Additionally, Gn pigs can be used to study other human enteric viruses, such as EV71, human enterovirus 68, and human astrovirus following the success of HRV and noroviruses.

1.1.3 Gn pig model for studying immune responses to enteric viruses

The immune system, particularly the gut mucosal immune system, of pigs is highly similar to that of humans (178, 179). Although the mucosal immune system is in a less activated state in Gn pigs, due to the absence of stimulation by gut microbiota, their mucosal immune system is immuno-competent and responds normally to antigen stimulation (180). One of the unique and advantageous properties of studying immune responses to enteric viruses in Gn pigs is the lack of confounding gut microbial and environmental antigen stimulations, which allows for specific and more accurate study of immune responses to a specific enteric virus pathogen. Both innate (59, 90, 103, 105, 181-185) and adaptive immune responses (102, 104, 109, 157, 184, 186-188) to enteric viruses have been studied in Gn pigs.

Common experimental techniques used to characterize immune responses in Gn pigs include: enzyme-linked immunosorbent assay (ELISA) (for antibody and cytokine responses) (92, 186, 189, 190), ELISPOT (for effector and memory ASC, cytokine secreting cells, and total IgSCs (59, 107, 109, 191), flow cytometry (for effector and memory T cell responses, T regulatory cells and γδ T cells) (51, 100, 102, 105, 186), virus neutralizing test (for virus neutralizing antibody responses) (54, 190), and Leukogram (for measurement of white blood cells) (54). These techniques are well established, sensitive and the required instruments are readily accessible.

The reagents for characterizing pig immune responses are not as widely available as those for mice and humans, but there are already a large number of pig specific reagents and the number is increasing rapidly. There are currently >17,000 pig specific antibodies for ELISA and 8000 pig specific antibodies for flow cytometry (192). Given that high similarity of pig immunome to their human counterparts (193), many human antibodies cross-react with pig antigens. A large number of real-time PCR primers for characterizations of pig immune responses also exist. Porcine immunome analysis of a complete porcine genome have identified >1300 immunity related genes (193). Porcine gene expression microarray covering more than 20,000 genes are commercially available from different vendors (Affymetrix and Agilent Technologies). Mapping of the pig proteome is also gaining momentum and is growing rapidly, with data on 15,000 peptides from roughly 20 tissues currently available on the pig proteome database (http://www.peptideatlas. org/). A very powerful tool that could be developed for the study of immune responses to enteric virus is the combination of annotated complete swine genome assemblies, porcine immunome (193), gene expression array and proteomic tool for global analysis and data-mining of the host immune responses to a single enteric virus infection in Gn pigs. With high throughput technologies on characterizing even rare immune cell populations in swine becoming possible, a very exciting development in the near future would be the characterization and modeling of responses in the entire immune system (on the molecular, cellular, tissue, organ, system, and whole body level) to a single enteric virus over the different infection phases in Gn pigs. Gn pigs are uniquely suited to these studies as no complex and confounding gut and environmental microbial antigen stimulation of the immune system is present in Gn pigs. Furthermore, Gn pig model allows for the characterization and modeling of responses in the entire immune system to a single enteric virus in the presence of controlled gut and environmental antigens (prebiotic, probiotics, pathogenic

bacteria and viruses, drugs, toxins, etc.) Therefore, comprehensive characterization of immune responses to enteric viruses in Gn pigs is currently feasible and is becoming more promising.

Gnotobiotic pigs are ideal for the study of immune responses to enteric viruses of both human and animal origin. The development and response of total and different subsets of γδ T cells during acute HRV infection have been studied in Gn pigs (100, 186). Significantly increased frequencies of intestinal total γδ T cells and the putatively regulatory CD2+CD8+ γδ T cell subset and decreased frequencies of the putatively pro-inflammatory CD8- subsets (CD2-CD8-, CD2+CD8-) in ileum, spleen and blood at PID 3 or 5 were detected (100). These results indicate the important functions of different γδ T cell subsets during HRV infection in pigs. The phenotypical and functional characterization of different γδ T cell subsets is possible because of the large γδ T cell population in pig, while in most other animals γδ T cell population is small, including mice. The changes in the white blood cell (WBC) counts in the Gn pigs infected with porcine circovirus 2 (PCV2) were studied by measuring the actual number of neutrophils, lymphocytes, monocytes, basophils, eosinophils, and WBC in blood. Similar to natural PCV2 infection in pigs, the occurrence of lymphopenia and neutrophilia were correlated with clinical diseases (54). An increasing number of human and animal enteric viruses are being studied in Gn pigs (54, 90, 109, 171, 177, 184, 194, 195), underlining the excellence of this model for characterizing immune responses to enteric viruses.

An important goal of evaluating immune responses to enteric viruses is the identification of correlates of protective immunity for the design of safer and more effective vaccines. Major

improvements in our understanding of immune responses and correlates of protective immunity to HRV have been made using Gn pigs. The correlates of protective immunity have been determined to be virus specific IgA antibody secreting cells (ASC) and memory B cells in the local intestinal tissue using Gn pigs (157, 196-200). VirHRV Wa strain infection in Gn pigs completely protected against rotavirus reinfection, while the AttHRV Wa strain provided an increasing degree of partial protection based on the number of doses (from one to three). By measuring virus-specific ASCs and memory B-cell responses in the intestinal (duodenum, ileum, and MLN) and systemic (spleen, peripheral blood, and bone marrow) lymphoid tissues, it was found that VirHRV infected pigs have significantly higher numbers of both virus specific ASCs and memory B cells in the intestine lamina propria than the AttHRV infected pigs (196, 199, 201). Similar results have also been reported in children naturally infected with HRV (202, 203). Cytokine and cytokine secreting cells response during HRV infection in Gn pigs were also examined (59). Pro-inflammatory cytokines (tumor necrosis factor alpha (TNF-α) and interleukin-6 [IL-6]), Th1 cytokines (IL-12 and IFN-γ), Th2 cytokines (IL-4 and IL-10), Th3 cytokines (TGF-β) were characterized. It was found that balanced Th1/Th2 cytokine responses, and a robust IFN-γ and pro-inflammatory response in the early phase of virus infection are correlated with higher protective immunity in these pigs. Virus-specific IFN-gamma producing T cells responses in intestinal tissues have also been identified as correlates of protective immunity using the Gn pig model of HRV infection and diseases (104). The study of immune responses and correlates of protective immunity for HRV infection in Gn pigs hold great implication for the development of better rotavirus vaccines for use in young children, indicating the usefulness of Gn pig model for the study of immune responses to other human enteric viruses.

Studies so far have indicated that Gn pigs have been instrumental in our understanding of innate and adaptive immune responses to human enteric viruses. As more advanced and high throughput genetic, proteomic and other immune related reagents and technologies become available for pigs, our ability to characterize the immune responses to human enteric viruses in Gn pigs will increase exponentially. However, despite the high resemblance of the pig immune system to that of humans, it is important to keep in mind the differences between these two immune systems when interpreting the results from such studies. For example, the strong anti-inflammatory function of CD2+CD8- and CD2-CD8- γδ T cell subsets and regulatory function of CD2+CD8+ γδ T cell subset during rotavirus infection in Gn pigs may not necessarily reflect those responses during natural rotavirus infection, given the significantly lower frequency of γδ T cells in human intestinal and systemic tissues (186, 204). Major differences are the inversion of lymph nodes, presence of two types of Peyer's patches, and the transfer of passive immunity through colostrum and milk because of the epitheliochorial placentation in pigs. Particular examples are the lack of α defensins (205), expanded type I interferon genes (39 genes for pig and 19 for human) and cathelicidin genes (11 for pig and 1 for human) (206), the high frequencies of γδ T cells in blood and intestine (207), and the existence of CD4+ and CD8αα+ double positive T cells outside the thymus (208) in pigs.

1.1.4 Gn pig model for evaluating enteric virus vaccines

Gnotobiotic pigs are a useful tool in the evaluation of vaccines for enteric viruses. Important insights can be obtained using Gn pigs on their safety, immunogenicity and protective efficacy. A major advantage of using Gn pigs to evaluate vaccines for enteric viruses is that the results are

more clinically predictive to vaccine immunogenicity and protective efficacy in humans, compared to the commonly used mice models.

Gnotobiotic pigs are a very popular animal model for evaluating HRV vaccines and numerous studies have been conducted on different vaccine candidate types (DNA vaccines, subunit vaccines, virus like particles (VLP) vaccines, inactivated vaccines and live attenuated vaccines) (106, 209-214), vaccination routes (oral, intranasal, intramuscular or combined) (107, 108, 111, 113, 190, 212, 214), cross-protection (76, 210, 215), immunogenicity (innate immune responses, cytokines, T cells, B cells, antibodies) (59, 102, 104, 106-108, 113, 182, 187, 189, 190, 213, 214, 216), probiotic adjuvants (LGG, *Lactobacillus acidophilus* (LA) NCFM strain and *Bifidobacterium animalis lactis* Bb12) (89, 90, 93, 98, 175, 217, 218), health status (prenatal vitamin A deficiency) (209), etc. Overall, these studies resulted in positive immune responses with protection against virus shedding and/or disease, and have contributed significantly to the successful development of two live AttHRV vaccines and understanding of factors affecting vaccine efficacies in vaccinated young children. These contributions to the HRV vaccine field highlight strengths of the Gn pig model for preclinical evaluation of the safety, immunogenicity and protective efficacy of HRV vaccines. Such wide acceptance of this model for vaccine evaluation is not surprising, considering many advantages of the Gn pig model, such as a long window of susceptibility to infection and clinical diseases (which permits for multiple doses of vaccinations and study of active and cross protective immunities), adequate sample availability (blood, rectal swabs, tissues), established immunoassays and protocols, easily controllable gut environment and resemblance in mucosal immune system structure and function of young children.

Following the successes in establishing the Gn pig models for HRV and noroviruses, the same success can be achieved for other important emerging and re-emerging enteric viruses, such as EV 71. The Gn pig model for human norovirus infection and disease has already started to be recognized as the most promising animal model (51, 58, 139, 165, 168, 170), and the top choice for evaluating human norovirus vaccine candidates (168). In fact, two types of human norovirus vaccines, VLP (51, 171) and P particle (51), have already been evaluated in Gn pigs. The results have been very encouraging so far, with moderate immune responses and partial protection against virus shedding and diarrhea been reported in Gn pigs. Intranasal P particle (derived from GII.4/ VA387/1997) vaccine with MPL and chitosan adjuvant significantly increased the numbers of activated CD4+ T cells in local intestinal and systemic tissues, IFN-γ-producing CD8+ T cells in the duodenum, Tregs in the blood, and TGF-beta-producing CD4+ CD25- FoxP3+ Tregs in the spleen after challenge with a cross-variant human norovirus (GII.4/2006b) and provided 47% protection rate against norovirus induced diarrhea in Gn pigs (51). Oral/intranasal vaccination of Gn pigs with human norovirus GII.4/HS66/2001 strain derived VLP and immunostimulating complexes (ISCOM) or mutant *E. coli* LT toxin (mLT, R192G) as mucosal adjuvants resulted in a similar protection rate against homo-variant virus challenge induced shedding and diarrhea after adjustment for the insufficient challenge dose used in the study (171). Both vaccine formulations (VLP+ ISCOM and VLP+ mLT) induced 100% seroconversion, and with VLP+mLT induced Th1/Th2 serum cytokines, whereas VLP+ISCOM induced intestinal IgM, IgA and IgG antibody-secreting cells. Further evaluations of current and new norovirus vaccine candidates in Gn pigs are underway and the number is likely to increasing considerably as Gn pig model for norovirus infection and diseases become better established.

Gnotobiotic pigs have also been utilized to evaluate parenteral vaccines for HRV (113). Three high intramuscular doses of inactivated rotavirus vaccine provided protection against rotavirus shedding, but only partial or no protection against rotavirus diarrhea. Intestinal lesions were not examined in this study, but likely not protected given no protection from diarrhea (219). Parenteral vaccines are being developed to circumvent the factors that contribute to the lower efficacies of current live attenuated oral HRV vaccines in developing countries, such as high maternal antibodies in serum and breast colostrum/milk, malnutrition, and the interfering gut microflora and viruses. Further development of such vaccines for the protection against both rotavirus shedding and diarrhea are necessary. However, the results obtained in these studies suggest that Gn pig model could be used to evaluate parenteral vaccine as well. On the other hand, Gn pigs are excellent animal model for understanding the role and mechanisms of maternal antibodies, malnutrition and potential gut bacteria or viruses on lower efficacy of current rotavirus vaccines, and evaluating novel oral rotavirus vaccines and vaccine adjuvants that can overcome these issues to confer high protective efficacy in developing countries as well (89, 90, 175, 181, 217, 218, 220).

1.1.5 Gn pig model for evaluating therapeutics for enteric viruses

Gnotobiotic pigs have been used as an animal model for testing therapeutic drugs for certain enteric viruses. For example, in Gn pigs virus shedding, diarrhea and intestinal lesions result after human norovirus infection. Rarely are all these parameters of infection and disease seen in other animal models for human norovirus, providing opportunities to evaluate the effects of therapeutics on all these parameters (221). The effect of natural human IFN-a (nhIFN-a) [300 international unit (IU)/

kg/day] on norovirus shedding and diarrhea was evaluated in Gn pigs (138). The results showed that in combination with simvastatin, oral administration of nhIFN-a reduced human norovirus virus infectivity (138). The therapeutic effects of rotavirus specific antibodies produced from chicken egg yolk (222) and cow colostrum (223), and llama single variable (VH) domain on a heavy chain (VHH) nanobodies (224) have been evaluated in Gn pigs against rotavirus shedding and diarrhea as well. VHH nanobodies, when given orally as daily milk supplementation for 9 days, have shown to confer full protection against HRV diarrhea and significantly reduce rotavirus shedding.

The effects of prebiotics on the infection, shedding and diseases caused by human enteric viruses in Gn pigs have not been evaluated previously. However, studies in other animal models have demonstrated that prebiotics (such as RB) were able to modulate both innate and adaptive immune system in the intestinal and systemic tissues (225-229). RB or its components have also been shown to have direct antiviral activity through inhibition of virus attachment and intracellular viral replication (230, 231). In addition, the ability of RB to decrease the clinical scores for diarrhea has been reported in irritable bowel syndrome (IBS) patients (232), through its anti-inflammatory and gut immune-modulatory activities. Another study showed that RB has strong antimicrobial activity against the diarrhea causing bacteria V. cholerae O139 (233), thus demonstrating its value for use in protecting against bacteria pathogen induced diarrhea. No studies on the effect of RB on rotavirus infectivity and disease has been reported previously. Given the above mentioned bioactive properties of RB, evaluation of RB on its immune-modulatory effects, protection against diseases and gut health during enteric virus infections in Gn pig model are warranted.

Lactobacillus rhamnosus GG has been shown to protect against rotavirus diarrhea in human clinical trials (234-239) as well as protect against HRV shedding and diarrhea (the percentage of pigs developing diarrhea, the mean duration of diarrhea, and the mean cumulative fecal scores) in Gn pigs (88, 90, 91, 217, 240). Multiple mechanisms for the LGG protection of HRV shedding and diarrhea have been identified (88, 90, 91, 194, 235, 241, 242). LGG has been shown to modulate gut immune system through increasing virus-specific intestinal IgA antibody secreting cells (217), promoting maturation of antigen presenting cells and anti-inflammatory (TLR2 and 4 down-regulation) response (90), and reducing effects of inflammatory cytokines TNF-α and IFN-γ on intestinal epithelial cells via inhibition of TNF-α induced nuclear factor (NF)-κB signaling (243). These immune-modulatory effects of LGG resulted in more resistance to rotavirus infection and replication. In Gn pigs, it was found that LGG prevented the autophagy induced by rotavirus infections, increased apoptosis and protected against intestinal injuries (194). These mechanisms have contributed to the protective effect of LGG on rotavirus shedding and diarrhea. Additional mechanisms could be due to its ability to reduce the intestinal permeability (244, 245), and to prevent intestinal injuries during HRV infection (via stabilizing adherent junction proteins alpha-catenin and beta-catenin, tight junction proteins occludin, claudin-3 and claudin-4, and leak protein claudin-2 levels in ileum epithelium, and maintaining TGF-β level in serum and increasing mucin productions in LIC) (91).

Rice bran is well known as a fermentative substrate for Lactobacilli and promotes their growth (246-254). RB as a prebiotic has been demonstrated to increase the colonization and growth of

Lactobacillus spp in conventional mice (227, 255). However, the ability of RB to promote the growth and colonization in large and Gn animals was unknown. Therefore, confirming the capacity of RB on the growth and colonization of *Lactobacillus* spp is important for using RB clinically to promote the beneficial effects of probiotics. Gn pigs are an ideal model for such studies, as the specific effects of RB on LGG growth and colonization can be examined without other confounding factors in conventional animals, such as gut microbiota and environmental factors. A very important question that can be addressed using the Gn pig model is whether RB and LGG together can have additive or synergistic effects on rotavirus infection and diarrhea and on overall and intestinal health during VirHRV infection. These studies can potentially identify an economical, safe and effective new therapeutics against HRV infections and diseases in young children.

1.2 HUMAN ENTERIC VIRUSES

Enteric viruses are an epidemiologic class of viruses that are normally transmitted by ingestion and replicate in the intestinal tract of the host. Enteric viral infections cause largely asymptomatic infections in otherwise healthy children (256). However, enteric viral infections can result in diverse symptoms ranging from fever and diarrhea, to encephalitis, even death, particularly in malnourished or immuno-deficient neonates and infants (257). They represent enormous public health issues in both developed (258) and developing countries (259). Increasing number of major epidemics are caused by new and emerging enteric viruses, such as EV71 (260) and human enterovirus 68 (261, 262), while other enteric viral diseases, such as HRV and norovirus, are still prevalent. Thus, better understanding of viral pathogenesis, immunity, as well as development of

new vaccines and therapeutics are needed for effective control and prevention of human enteric viruses.

1.3 EV71

Human enterovirus 71 is a member of the human enterovirus A species belonging to the human enterovirus genus of the *Picornaviridae* family. Based on genetic analysis, EV71 serotypes are divided into 3 genogroups: genogroup A includes the prototype BrCr strain, genogroups B and C are further divided into subgenogroups B1-B5 and C1-C5, respectively (263, 264). EV71 virions are small, non-enveloped icosahedral particles of about 30 nm, consisting of 60 protomers, each of which contain one copy of VP1-4 structural protein with VP1-3 externalized and VP4 internalized (265-268). Its positive-sense, single-stranded polyadenylated genomic RNA of approximately 7·4 kb contains 5 genomic regions 5'UTR, P1, P2, P3 and 3'UTR, and a poly-A tail. The proteins encoded by each genomic region include: P1 (four structural proteins VP1-4), P2 (non-structural proteins 2A-2C) and P3 (non-structural proteins 3A-3D) (269, 270).

Tropisms of EV71 for cell and tissue types are mainly restricted by cellular receptors, although other host factors such as interferon responses, also impact virus infection and replication in target cells and tissues (271). Several receptors have been identified for EV71, such as the ubiquitously expressed cellular receptor, scavenger receptor B2 (SCARB2) (272) and heparan sulfate (273), white blood cell-specific human P-selectin glycoprotein ligand-1 (PSGL-1) (274), sialic-acid-linked glycan (275) and Annexin II (276) in respiratory and gastrointestinal tracts, and dendritic-cell-specific intercellular adhesion-molecule-3-grabbing non-integrin (CD209) on dendritic cells

in lymphoid tissues (277). After EV71 binds to its receptors, it is internalized into the cytoplasm. As a positive sense RNA virus, the genome is translated directly as a messenger RNA into a large polypeptide, which is then processed by viral and host proteases into individual structural and non-structural proteins. Viral genome is replicated by the error-prone RNA-dependent RNA polymerase (3Dpol) within a vesicle membrane structure (viral replication complex), resulting in a rapidly evolving and mutating virus with one or two bases in every genome copying event (266, 278). Mature and infectious viral particles are then released through cell lysis after packaging of viral RNA in assembled viral capsid in the cytoplasm (266).

EV71 is transmitted mainly through the fecal-oral route, but can also be transmitted via vesicular fluids, oral secretions, contaminated surfaces or fomites and respiratory droplets (266). Initial virus replication takes place in the oropharyngeal cavity (tonsils) and small bowel (Peyer's patches) and regional lymph nodes (deep cervical and mesenteric lymph nodes), which results in viremia (266). Most EV71 infections are controlled at this stage, with no symptoms induced. However, in some cases, EV71 can disseminate to other tissues such as central nervous system (CNS), lung, heart, skin, liver, spleen, bone marrow and pancreas, coinciding with clinical symptoms onset. Axonal retrograde transport through cranial and peripheral nerves has been reported as the route for neural invasion by EV71 in infected mice and fatal human cases by assessment of the distribution of virus and inflammation (279-281). EV71 can also infect leukocytes by binding to its receptors PSGL-1 and DC-SIGN (274, 282) and disseminate to the CNS through infected immune cells (283). Virus shedding in infected children between 1 month and 5 years can persist up to 2 weeks in throat and 7-11 weeks in stool (284).

Human enterovirus 71 causes human hand, foot and mouth disease (HFMD) in human infants and young children. The symptoms include fever, rash, diarrhea and vomiting and vesicular lesions on hands, feet, and oral mucosa (285). While most EV71 infections are asymptomatic or cause only mild and self-limiting HFMD, complicated HFMD cases with neurological symptoms, such as aseptic meningitis (headache, vomiting, fever, and stiffness of the neck), acute flaccid paralysis(limb paralysis), and rhombencephalitis (myoclonus with tremor, ataxia or both; myoclonus with cranial-nerve involvement, including ocular disturbances in some patients (nystagmus, strabismus, or gaze paresis) and bulbar palsy in one (dysphagia, dysarthria, dysphonia, and facial weakness). The combination of myoclonus followed by the rapid onset of respiratory distress, cyanosis, poor peripheral perfusion, shock, lethargy, drowsiness, coma, loss of the doll's eye reflex, and apnea, as well as pulmonary edema (respiratory distress, tachypnea, tachycardia, frothy sputum, and rapidly progressing, patchy, diffuse pulmonary infiltrates and congestion on a chest film) and hemorrhages (bleeding during tracheal aspiration),which have been associated with most deaths from EV71 infection, have been frequently documented during major outbreaks (285-288).

Despite the fact that EV71 can infect and cause injury in other tissues, such as vesicles in skin, pathological examinations during EV71 infection have been mostly focused on the CNS and lung tissues, due to their association of severe neurological and respiratory symptoms in young children. CNS inflammation and histopathological changes such as perivascular cuffs, variable edema, neuronophagia, and microglia nodules, mainly occur in the gray matter of the spinal cord and

medulla oblongata, as well as hypothalamus, subthalamic and dentate nuclei, and focal in the motor cortex of the cerebrum (280, 288-291). The inflammatory cells consist primarily of neutrophils, CD68-positive macrophage/microglial cells, and a few CD8-positive lymphocytes (280, 288, 292). Pulmonary edema and focal hemorrhages are main features of the lung lesions, the cause of which is unclear, with possible contributing factors including neurogenic pulmonary edema, cardiac dysfunction, increased vascular permeability, and cytokine storm (266).

Both innate and adaptive immune responses during EV71 infections have been characterized (277, 287, 293-317). These studies contributed significantly to our understanding of the role of immune responses in the viral pathogenesis, clinical responses, molecular mechanisms of EV71 modulating host immune responses, and protective immunity. Innate immune responses have been shown to play a dual role during the EV71 infections and development of severe diseases. Significantly higher IL-6 levels were found in the EV71 patients with pulmonary edema than those without (318). Administration of IFN α/β neutralizing antibody in mice resulted in exacerbated illness and increased tissue viral loads; the survival rate of mice upon EV71 challenge improved when type 1 IFN responses were restored (318-320). IFN-γ has been associated with protection against virus neuronal invasion and neurological lesions and symptoms (321-323). However, treatment with IFN-γ 3 days after intracranial infection with EV71 in mice also induced pulmonary edema and exacerbated pulmonary abnormalities in EV71 infected mice, suggesting that pro-inflammatory response is required for the development of pulmonary symptoms during EV71 infection (324). These protective and detrimental effects of host innate response against EV71 highlight the importance of a balanced innate immune response during EV71 infection.

Adaptive immune responses, including T cell and B cell responses, are important mechanisms of host defense against viral pathogens, and development of immunological memory in protecting against future infections. During EV71 infection, T cell immunity has been found to be closely correlated with clinical outcome of EV71 infections, with lower T cell cytokines generally noted in severe cases (316, 317). Upon stimulation with VP1 peptides, T cells derived from EV71 infected patients proliferated and produced significantly higher IL-2 and IFN γ by CD4+ T cells, indicating a strong Th1 type versus Th2 type (IL-4 and IL-10) subsets response is induced during EV71 infection (325). IFN-γ producing CD4+ T cells against EV71 VP2 were found to be the predominant T cell responses compared to VP1, 3 and 4 during acute infections (302). Another study also identified that IFN-γ producing CD4+ and CD8+ responses during acute EV71 infections in young children, mainly RNA-dependent RNA polymerase (RdRp) specific CD4+ T cell responses (299). While there are differences regarding which viral proteins are the main target for inducing IFN-γ T cell response, together these studies suggest that IFN-γ producing CD4+ and CD8+ T cell responses are induced during EV71 infection. The relationship between IFN-γ producing CD4+ and CD8+ T cell responses and viral infections and clinical outcomes during EV71 infections require further characterization.

The importance of antibody - mediated protection against EV71 infection has been demonstrated in both animal models and humans (300, 326-334). Intravenous immunoglobulin containing high titers of virus-neutralizing antibody cleared virus more rapidly when injected in children and pre-epidemic serum neutralizing antibody titer is found to be inversely correlated with morbidity and

mortality in young children during an endemic. During acute EV71 infection, strong virus-specific IgM and IgG ASC responses in the first week of illness correlate with throat viral load, duration of fever, serum neutralizing antibody responses (293). These results have also been replicated in animal models. B cell deficient mice are susceptible to EV71 infection, whereas wild type and T cell deficient mice are resistant to EV71 infection (329). Importantly, tissue virus loads could be reduced in B cell deficient mice by adoptive transfer of virus-specific antibodies produced in T cell deficient mice, indicating that T cell independent virus-specific IgM and IgG antibody responses play a major role in protecting against EV71 infection (329). Interestingly, virus neutralizing antibody titer does not correlate with the severity of EV71 disease. For example, EV71 patients with CNS and pulmonary edema symptoms had significantly lower Th1 cytokine IFN-γ and proinflammatory cytokines IL-6, TNF-α, IL-1β, and macrophage inflammatory protein-1α responses compared to cases with CNS but not edema and uncomplicated cases, with no difference in the level of neutralizing antibody titers between these groups were found (316). This observation concurred with the findings of another study that no difference in the titer and dynamics of serum neutralizing antibodies between mild, CNS cases and CNS plus neurogenic pulmonary edema cases (331). In all three severity groups, serum neutralizing antibody levels significantly increased on one day after onset of illness and peaked at 6 days after onset of illness with the majority of cases (98.3%) have a titer less than 512. Therefore, serum neutralizing antibody is one of multiple protective mechanisms against EV71 infection and disease.

With the eradication of poliovirus in most parts of the world, EV71 is currently the most important neuro-virulent infection with over a million cases of HFMD and hundreds of deaths in infants and young children annually. The highest incidence and mortality rates were observed in infants

between 6-23 months of age (286, 335). However, there are currently no anti-viral therapies or vaccines available for EV71. An effective vaccine and antiviral drugs are urgently needed to reduce EV71-induced morbidity and mortality. The pathogenesis of EV71 is mostly unknown though, increasing the difficulty of preventative and therapeutic drug development.

A major problem commonly encountered in this endeavor is the lack of suitable animal models. Current mice and monkey models for EV71 are not suitable for efficient and cost effective study of viral pathogenesis and evaluation of vaccines and therapeutics. Non-human primate (336-341) and mouse models (281, 321, 333, 342-355) have been developed. However, there are significant limitations with both types of animal models. For the primate models, the older ages of the monkeys used in most studies limit the effectiveness of pathogenic and immune response modeling of severe EV71 infections in human infants. These primate models were also mainly established using alternative inoculation routes other than its natural oral or nasal route (339, 356-359). In addition, non-human primate models are significantly limited by their economic cost and ethical concerns. Mice are the species most frequently used to establish animal models for the study of EV71 infection. While many studies on vaccines, antiviral drugs and pathogenesis were conducted in mice (300, 319, 320, 333, 342, 344, 360-372), no mouse models have replicated the respiratory symptoms and lung lesions, such as pulmonary hemorrhage and edema, by using any inoculation route (373, 374). In addition, mice older than two weeks have not been successfully infected by EV71 so far, despite using immune-deficient mice (352) and EV71 receptor transgenic mice (375). In mice, EV71 disease is not correlated with the expression pattern of SCARB2, consistent with previous results that SCARB2 function as a receptor in humans, but not in mice. Furthermore, EV71 is myotropic in mice versus neurotropic in humans (345). The lack of infection in older mice

prevents the study of protective immunity induced by vaccine using these models. Thus, alternative animal model species are needed to fill this gap, in order to improve our understanding of virology, immunity, as well as to accelerate the development of new vaccines and therapeutics for EV71.

Previous studies in Gn pigs of rotavirus and norovirus infections by others and our laboratory have shown that neonatal Gn pigs can be successfully used to model human enteric viral infections (98, 104, 135, 171, 187). The Gn pig model has been used extensively in previous studies in rotavirus vaccines and immunomodulation of probiotics, and has been widely recognized in the rotavirus research field as the best animal model for evaluation of HRV vaccines (93, 100, 103, 104, 108, 177, 187, 216, 376, 377). Furthermore, several receptors have been identified for EV71, including PSGL-1 (274), SCARB2 (272), and sialylated glycans (275). Conserved and functionally homologous proteins for these receptors have been described in pigs (378, 379). Therefore, the development of a neonatal Gn pig model for EV71 infection, disease, and immunity for the preclinical evaluation of vaccines and therapeutics, as well as elucidating the underlying mechanisms of pathogenesis is warranted.

1.4 RICE BRAN

Rice bran is a globally accessible, abundant and underutilized agricultural byproduct. RB has a distinct stoichiometry of bioactive compounds, phytochemicals, and minerals (380). RB or its components have been shown to have bioactive functions such as the prevention and treatment of chronic diseases, growth of beneficial intestinal microbes, induction of mucosal and systemic

immune responses, and protection against enteric pathogens (226, 227, 229, 255). Thus, this agricultural byproduct represents a promising and practical diet-based solution for increasing the innate resistance against enteric pathogens that cause diarrhea. In addition, because of its immune stimulatory functions, it can be potentially used as an adjuvant for vaccines against enteric pathogen infections. The stimulatory effects of RB or RB extracts on the innate immune system have been well characterized using *in vitro* and *in vivo* models (225, 226, 228, 381-390) and human clinical trials (232, 391). Dendritic cells (DCs) play essential role in modulating both innate and adaptive immune response. In vitro treatment of human monocyte-derived DCs for 24 hours with arabinoxylan RB (MGN-3/Biobran) at different concentrations (5-20 microg/ml) dose-dependently increased their the maturation and activation (up-regulation of the surface expression of CD83 and CD86), production of pro-inflammatory and immune-regulatory cytokines (IL-1β, IL-6, IL-10, TNF-α, IL-12p40) and their ability to stimulate CD4+ proliferation and production of cytokines, IFN-γ, IL-10, IL-17 (390). These findings suggest that MGN-3 could be used as adjuvant for DCs and vaccines against infection diseases, such as HRV. MGN at 2g per day in the form of MGN-3 granule powder also increased NK cytotoxicity and the level of circulating myeloid DCs in multiple myeloma patients (391). NK cell activities were also increased by MGN-3 in mice (392). Glycoprotein fraction from rice bran (GFRB) also increased the production of NO in RAW 264.7 murine macrophage cells, up to approximately 10-fold compared to the normal control at 100mug/mL concentration, and at 50μg/mL GFRB increased the production of TNF-α, IL-1β, IL-6, and IL-10 as well (383). Another study confirmed this results using macrophage cell line U937, murine macrophage cell line RAW264.7, and murine peritoneal macrophages (P-M phi) treated with MGN-3, with increased attachment and phagocytosis of yeast by macrophages (388). MGN-3 enhanced the phagocytosis of *E. coli* by human neutrophils and monocytes and

increased their oxidative burst and induction of cytokines (TNF-α, IL-6, IL-8 and IL-10) in a dose-dependent manner starting at 1 μg/ml (386). These results suggest that RB or its extracts could be used to enhance innate immune system and increase host resistance to pathogen infections as well as potential vaccine adjuvant to DC-based vaccines.

The effects of RB on total and antigen-specific adaptive immune responses have also been studied (226-229, 381, 382, 385). RB oil enhanced T and B lymphocyte proliferation, production of Th1 cytokines (IL-2, IFN-γ, and TNF-α by lymphocytes, and reduced Th2 cytokines (both serum- and lymphocyte-derived IL- 4), as well as the level of serum IgE and IgG1 (229). MGN-3 also increased the levels of Th1 cytokines in human multiple myeloma patients (391). Importantly, γ-oryzanol significantly promoted the development of antibody responses in rats stimulated with sheep red blood cells (226). Total local (feces) and systemic (serum) IgA levels and IgA expression on Peyer's patch B cells were also enhanced in mice fed a 10% RB diet, suggesting that RB promoted both mucosal and systemic B cell development (227). These studies demonstrated the immunostimulatory effects of RB on multiple components of the immune system.

Given the RB-mediated protection against bacterial pathogens (231, 382, 385, 393, 394), and the stimulatory effects on both the innate and adaptive immune systems, it represents a promising natural food product for modulating mucosal immunity and protecting against diarrhea from major enteric pathogens such as HRV. Despite their strong immune-stimulatory effects on both innate and adaptive immune systems, no previous studies have examined their adjuvant effects for any vaccine, and total and virus-specific T and B cell responses during virus infection *in vivo*. The

varied protective efficacy of HRV vaccines (90%-29%) leaves millions of infections and ~450,000 deaths in young children in developing countries. Using RB to enhance the host innate resistance to HRV infection and diarrhea and to increase the immunogenicity and protective efficacy of oral HRV vaccines can be a very promising approach.

1.5 CONCLUDING REMARKS

Pigs are increasingly being used to study various human diseases. Given their similarities to humans in terms of genetics, physiology, intestinal anatomy, and immune system, pigs represent a particularly good animal species for the modeling of human enteric pathogen infections and immune responses (7). The sterile surgical derivation and Gn status of the pigs allow studies of the infection, disease and immune responses caused by a specific enteric pathogen in the absence of interfering maternal antibodies, other maternal immune regulators, and intestinal and environmental microbes. These are the basis for us to successfully establish the pig models for HRV, human norovirus and EV71 infection and disease using neonatal Gn pigs (51, 135, 139, 201, 395). Gn pigs are excellent model for the study of viral pathogenesis. Due to the similarities of pigs and humans as well as the lack of confounding commensal microbes that are present in all specific pathogen free (SPF) animals, Gn pigs can be used to identify the pathological changes directly as the result of a particular virus infection. Virus replication, spread and the resulting tissue damages and clinical signs can be more accurately recapitulated compared to the SPF animal models. The mechanisms of the host responses and the pathology associated with the virus infection can also be more clearly identified. Thus, Gn pigs are ideal for the identification of causative agents of viral diseases and mechanisms of viral pathogenesis.

The results from Gn pig models are generally comparable to SPF conventional pig models, even though some discrepancies do exist. The presence of certain microbial species in the intestinal environment may impact the infectivity of viruses. We found that HRV replicates to higher titers in Gn pigs colonized with LA *and L. reuteri* compared to germ-free pigs (396, 397). Several other studies have also shown that gut microbiota enhanced the replication or virus entry of enteric viruses and their pathogenesis (173, 398, 399) and that elimination of microbiota delayed rotavirus infection and significantly reduced rotavirus infectivity in mice (399). On the other hand, certain species of the normal gut microbes can be protective against virus-induced tissue damages and clinical signs. LGG has been found to reduce HRV diarrhea in Gn pigs by protecting against virus induced tissue injury in the intestine (400, 401).

Discrepancies between the Gn pigs and SPF animal models mainly result from the lack of gut microbiota. It is well known that gut microbiota interact with host immune system and modulate their responses to viral infections. Such discrepancies can be minimized by establishing human gut microbiota (HGM) transplanted Gn pig models (402). Similar to rotavirus infection of the *Lactobacillus* spp monoassociated Gn pigs, HGM Gn pigs shed higher titer of the virus after HRV inoculation compared to the germ-free pigs (402). Because gut microbiota play significant roles in shaping the neonatal immune system and host susceptibility to enteric pathogens (403), HGM transplanted Gn pigs may be a better animal model for certain human infectious diseases than germ-free and SPF animal models. HGM transplanted Gn pigs can be used to introduce the effects of gut microbiota on the host immune responses to viruses and vaccines. Using HGM Gn pigs,

our previous study has shown that transplanted human gut bacteria can modulate host immune responses to HRV infection and vaccination (404).

In conclusion, given the advantages of Gn pig models, it is expected that Gn pigs will be used more widely for the study of viral pathogenesis and immune responses that are specific to a virus. Similarly, Gn pigs can also be used to study pathogenesis or immune responses to other types of stimulus, be it microbial, chemical, or physical. These models can be applied to evaluate vaccines and therapeutics, bridging the gap between mice small animal models and human clinical trials. Currently, the applications of Gn pigs in modeling human diseases are limited by the cost and relative lack of research infrastructure and reagents. However, these limitations are becoming less prominent as the scientific community is increasingly interested in Gn pigs and how these models can become more commonly used in biomedical researches.

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Chapter 2

2.1 Neonatal Gn pig model of EV71 infection and immune responses

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Published in the *Emerging Microbes & Infections* (2014) 3, e35; doi:10.1038/emi.2014.35.

2.2 ABSTRACT

2.2.1 Abstract

Vaccine development and pathogenesis study for EV71 are significantly limited by the lack of suitable animal models. Here we report the development of a novel neonatal Gn pig model using a non-pig-adapted neuro-virulent EV71 strain (BJ110, C4 genotype). Porcine small intestinal epithelial cells, peripheral blood mononuclear cells, and neural cells were infected *in vitro*. Oral and combined oral-nasal infection of 5-day-old neonatal Gn pigs with a dose of $5x10^8$ fluorescence forming units resulted in a shedding period up to 18 days post infection, with viral titers peaked at 2.22×10^8 viral RNA copies/ml in fecal swab samples. Viral capsid proteins were detected in enterocytes of small intestines on post infection day 7 and 14. Additionally, viral RNA was also detected in intestinal and extra-intestinal tissues, including central nervous system, lung and cardiac muscle. The infected neonatal Gn pigs developed fever, forelimb weakness, rapid breathing, as well as some hand, foot and mouth disease symptoms. Flow cytometry analysis demonstrated increased frequencies of both CD4+ and CD8+ IFN-γ producing T cells in the brain and the blood on post infection day 14, but decreased frequencies in the lung. Furthermore, high titers of serum virus neutralizing antibodies were generated in both orally and combined oral-nasally infected pigs on post infection day 7, 14, 21, and 28. Together, these results demonstrated that neonatal Gn pigs represent a novel animal model for evaluating vaccines for EV71 and understanding the viral pathogenesis and immunity.

2.2.2 Keywords

Human Enterovirus 71, Animal model, Neonatal Gn pigs, Vaccine evaluation, Pathogenesis, Adaptive immune responses

2.3 INTRODUCTION

Human Enterovirus 71 (EV71) is a small, non-enveloped and positive-sense, single-stranded RNA virus. It is a member of the human enterovirus A species belonging to the *Picornaviridae* family. It causes HFMD, which is frequently associated with severe and sometimes fatal neurological and respiratory diseases. Most EV71 infections are asymptomatic or cause only mild and self-limiting HFMD symptoms such as fever, diarrhea, skin rash, herpangina, and vomiting. However, complicated cases with neurological symptoms, such as cerebellar ataxia, poliomyelitis-like syndrome and acute flaccid paralysis, as well as pulmonary edema and hemorrhages (which have been associated with most deaths from EV71 infection), have been frequently documented during major outbreaks (1-3). With the eradication of poliovirus in most parts of the world, EV71 is currently the most important neuro-virulent. EV71 infections result in over a million cases of HFMD and hundreds of deaths in infants and young children annually, with the highest incidence and mortality rates observed between 6-23 months of age (1, 4). However, there are currently no anti-viral therapies or vaccines available for EV71. An effective vaccine and antiviral drugs are urgently needed to reduce EV71-induced morbidity and mortality. The pathogenesis of EV71 is mostly unknown though, increasing the difficulty of preventative and therapeutic drug development.

Non-human primate and mouse models are commonly used to study EV71 infection. However, there are significant limitations with both types of animal models. For the primate models, the ages of the monkeys used in most studies tend to be older than the age range (6 months to 3 years) of infants during which most severe and fatal EV71 infections occur, limiting the effectiveness of pathogenic and immune response modeling of severe EV71 infections in human infants. These primate models were also mainly established using alternative inoculation routes other than oral or nasal inoculation, which are the natural infection routes for EV71 (5-9). In addition, economic and ethical issues associated with primate models greatly limit their utility. Mice are the species most frequently used to establish animal models for the study of EV71 infection. While many studies on vaccines, antiviral drugs and pathogenesis were conducted in mice, no mouse models have replicated the respiratory symptoms and lung lesions, such as pulmonary hemorrhage and edema, by using any inoculation route (10, 11). In addition, mice older than two weeks have not been successfully infected by EV71 so far, despite using immune-deficient mice (12) and EV71 receptor transgenic mice (13). The lack of infection in older mice prevents the study of protective immunity induced by vaccine using these models. Therefore, better animal models are needed for testing vaccines and evaluating therapeutic approaches for EV71 infection and diseases.

Pigs have been widely used to study a variety of human diseases, due to their similarities to humans in terms of anatomy, physiology, genetics and immune systems (14). It has been shown that, out of many immune system parameters evaluated, less than 10% of the murine immune system is similar to the human immune system, compared with more than 80% of the porcine immune system (14). This presents pigs as better models for evaluation of human infectious diseases, immune responses and vaccine development than mice. Previous studies in Gn pigs of

rotavirus and norovirus infections by our laboratory and others have shown that neonatal Gn pigs can be successfully used to model human enteric viral infections (15-19). Furthermore, several receptors have been identified for EV71, including PSGL-1 (20), SCARB2 (21), and sialylated glycans (22). Conserved and functionally homologous proteins for these receptors have been described in pigs (23, 24). Thus, we hypothesized that neonatal Gn pigs may be susceptible to EV71 infection and can provide a good animal model.

In this study, we infected porcine intestinal epithelial cell line (IPEC-J2), peripheral blood mononuclear cells (PBMCs) and neural cells, as well as 5-day-old neonatal Gn pigs with a recently isolated C4 genotype neuro-virulent EV71 strain BJ110 through oral or combined oral-nasal routes. Clinical signs, virus shedding, virus tissue distribution, histopathology and IFN-γ producing T cell responses as well as serum neutralizing antibody titers were studied to establish the neonatal Gn pigs as a novel animal model for EV71 infection.

2.4 MATERIALS AND METHODS

2. 4.1 Cell cultures

Vero cells (ATCC# CCL-81) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 2% fetal bovine serum (FBS), 1% penicillin and 1% streptomycin according to the vendor's instructions. The IPEC-J2 cell line was a generous gift from Dr. Anthony Blikslager (North Carolina State University, Raleigh, NC) and was previously cultured in our laboratory (25). PBMCs were isolated from neonatal pigs as previously described (26) and were cultured in the same medium as Vero cells. Pig neural cells were isolated from neonatal pig brain using neural tissue dissociation kit (Miltenyi Biotec. Cat. No.130-092-628) and were cultured in Neurobasal-A

medium (Gibco, Cat. No. 10888), supplemented by 2% B27 and 1% 0.5 mM glutamine. Cultures of PBMCs and neural cells were identified by morphology via light microscopy.

2.4.2 Virus inoculum preparation

EV71 BJ110 strain (or s110 strain) was isolated from a young male patient severely affected with EV71-induced neurological symptoms in Beijing, China in 2008 (12). The 3rd passage of the BJ110 strain in Vero cells was used in the current study. The virus has not undergone any passages in pigs. EV71-infected Vero cells were infected at 0.1 multiplicity of infection (MOI) and cultured for 3 days at 37°C with 5% CO_2. After two freeze-thawing cycles at -20 °C, cell debris was removed by centrifugation at 2,100 rpm for 10 min at 4°C. Supernatant was collected, concentrated and semi-purified by ultracentrifugation (28,000 rpm for 4 h at 4°C using SW28 rotor in Beckman Coulter Optima-L90K ultracentrifuge) through 35% sucrose cushion before storage at -80°C. Virus titers were determined by cell culture immunofluorescence (CCIF) assay. Immediately before inoculation, virus was diluted in DMEM with 1% penicillin and 1% streptomycin to appropriate concentration.

2.4.3 Cell culture immunofluorescence assay (CCIF)

Monolayers of Vero cell culture in 96 well plates were infected with 10 fold serially diluted virus inoculum or processed rectal swab samples and incubated at 37°C and 5% CO_2 for 18 h. After washing three times with phosphate-buffered saline (PBS, pH8.0), EV71 infected cells were fixed and permeabilized with 80% acetone for 10 min at room temperature and air dried. Then, after washing, 50 µl of mouse anti-EV71 antibody (Abcam, ab36367, diluted 1: 1000 with PBS plus

1% BSA (Bovine Serum Albumin) were added into each well and incubated for 1h at 37°C. Followed by washing, the plates were incubated with 50 µl of goat anti-mouse IgG1 antibody labeled with fluorescein isothiocyanate (FITC) (Sigma Aldrich, Cat. No F0257) for 1 h at 37°C. Finally, the plates were mounted with glycerol and examined under a fluorescence microscope (Nikon Eclipse TS100). The number of fluorescent cells in each well was recorded and the virus titer was reported as FFU/ml. The protocol for assessing the infectivity of EV71 in pig primary cell cultures was the same as described above, except that porcine cell cultures were used in place of Vero cell cultures.

2.4.4 RT-PCR and Taqman® real time PCR

Reverse transcription polymerase chain reaction (RT-PCR) was used to identify the EV71 virus and also to detect virus shedding in rectal swab samples. Primer EV71-1 (5'ATAATAGCAYTRGCGGCAGCCCA3') was used in Dr. Qian's lab previously and primer EVVP1-R (5'AGCTGTGCTATGTGAATTAGGAA3') was described in a previous publication (27). Reverse transcription was completed in a Bio-Rad MyCycler^{TM} thermal cycler at 55 °C for 60 min. PCR cycling conditions were 95°C for 3 min for initial denaturation and 35 cycles of 95°C for 20s, 55°C for 20s and 68°C for 20s, and final elongation at 68°C for 7 min. RT-PCR products (317bp) were analyzed in 1% gel and then purified and sequenced. Obtained sequences were then compared with the VP1 sequence of the BJ110 strain in GenBank (Accession No. HM002486.1).

A two-step Taqman® real time PCR was used to quantify the EV71 RNA copies in rectal swab and tissue samples from inoculated neonatal Gn pigs. A primer pair (EV71VP14F:

5’GGAGATAGC GTGAGCAGAGC 3’and EV71VP1 4R: 5’ACAGCGTGTCTCAATCATGC3’) and a Taqman® probe ([6-FAM]-TCACTCACGCTCTACCAGCACCCA-BHQ1) specific for EV71 BJ110 strain were designed using OligoPerfect™ Designer from Life Technologies based on the VP1 gene sequence of EV71 BJ110 strain and were ordered from the same company. The reverse transcription step was the same as for RT-PCR described above, except for the primers. Taqman® real time PCR was conducted in a Bio-Rad iQ5^{TM} real time PCR machine. The 25 µl total reaction volume consisted of 12.5 µl of 2x Sensimix buffer (Bioline. Cat. No. QT725-02), 1 µl of EV71VP14F (10 µM), 1 µl of EV71VP14R (10 µM), 0.5 µl of Taqman Probe (10 µM), 2.5 µl of cDNA Template and 7.5 µl of ddH2O. PCR conditions were 1 cycle of 95°C for 10 min; 40 cycles of 95°C for 10 s and 60 °C for 60 s with real time detection at the end of each cycle. To quantify the EV71 RNA copies, a linear standard curve was also generated during each assay using serial dilutions of EV71 DNA standard by adjusting to a concentration gradient of 1×10^8 copies/µl to 1×10^0 copies/µl. The detection limitation was 100 copies.

2.4.5 Infection of neonatal Gn pigs

Near-term pigs (Large White cross breed) were derived by hysterectomy and maintained in germ-free isolator units as described (28). In the *in vivo* study, specific doses of EV71 BJ110 strain viral inoculums diluted in Diluent #5 (MEM, 1% Penicillin and Streptomycin, 1% HEPES) were given to 5-day-old Gn piglets through oral (O) or combined oral-nasal (O/N) route to test the infectivity of EV71 in neonatal Gn pigs (Table 1). Control pigs were given an equal amount of Diluent #5. Sterilized microchips (IPTT-300, BioMedic Data Systems, Inc. Seaford, DE) were implanted

subcutaneously behind the ear of all pigs to measure body temperature. Clinical signs and body temperature were observed twice daily until euthanasia. Rectal swabs were taken daily for detection of virus shedding. Upon euthanasia, organs and tissues were examined for gross lesions and various tissues were taken for histopathology, immunohistochemistry, RNA isolation, and cell isolation for *in vitro* cell culture and flow cytometry analysis. Blood was sampled from the jugular vein of each pig weekly for monitoring of serum neutralizing antibody titers. Rectal swabs were taken weekly to monitor sterility with blood agar plates and thioglycollate media. All animal protocols were reviewed and approved by Institutional Animal Care and Use Committee of Virginia Polytechnic Institute and State University.

2.4.6 Histopathology

Tissues harvested from euthanized pigs were immediately immersion-fixed in 3.7% paraformaldehyde (MP Biomedicals, Cat No. 2150146.5) for 24 h at room temperature. Fixed tissues were trimmed, paraffin embedded, sectioned, deparaffinized, rehydrated and stained with hematoxylin and eosin (H&E). Additionally, fixed small intestinal tissues were also resin embedded and stained with toluidine blue. Resulting sections were examined under a light microscope.

2.4.7 Immunohistochemistry

Unstained tissue slides from the histopathology study were used for immunohistochemistry. The same primary and secondary antibodies were used as in the CCIF assay described above. Briefly,

deparaffinized and rehydrated slides were digested with IHC proteinase K for 20 min at room temperature. After washing twice in Tris Buffered Saline (TBS)-0.1% Triton X-100 for 5 min, slides were blocked with 10% normal goat serum in TBS-1% BSA for 1 h at room temperature. Then primary mouse anti-EV71 monoclonal antibody (diluted 1:1000 in TBS-1%BSA) was added and incubated overnight at 4^{o} C. After washing twice in TBS-Triton X 100 for 5 min, FITC-conjugated secondary goat anti-mouse IgG (diluted 1:128 in TBS-1% BSA) was added and incubated for 2 h at room temperature. All incubation steps were conducted in a humidified chamber. After counterstaining in PI for 30 min at room temperature, slides were then mounted with VectorShield mounting medium and examined under a fluorescent microscope.

2.4.8 Flow cytometry

Frequencies of IFN-γ producing CD3+CD4+ and CD3+CD8+ T cells among CD3+ lymphocytes in various tissues (ileum, spleen, blood, lung and brain) were determined by intracellular staining and flow cytometry. Sample collection, processing, data collection and analysis for flow cytometry were conducted as described in a previous publication (16). Mononuclear cells (MNCs) from lung and brain were isolated using the same procedure as for the spleen (26). MNCs were stimulated *in vitro* with semi-purified EV71 antigen (10µg/ml) or mock-stimulated for 17 h before being subjected to intracellular staining (16).

2.4.9 Viral neutralization assay

Virus neutralization assay was performed according to a previously described protocol with modification (29). Briefly, Vero cells were cultured in 96 well plates for 4 days in cell culture medium. Serially diluted serum samples (heat inactivated) were mixed 1:1 with fixed virus dilution (100 FFU/50 µl) and incubated at 37 °C for 1 h. Prior to infection, medium was discarded and each well was washed with PBS once. Then a 100 µl serum/virus mixture was added to each well. Duplicate wells were infected for each serum dilution. After incubation at 37 °C for 24h, plates were fixed with 80% acetone at room temperature for exactly 10 min inside a chemical hood. After washing the plates with PBS+0.05% Tween 20 (pH7.4) once for 2 min, mouse anti-EV71 VP1 monoclonal antibody (Abcam ab36367; 1:1000 dilution in PBS+1% BSA) and goat polyclonal secondary antibody to mouse IgG - H&L (horseradish peroxidase (HRP)) (Abcam, ab6789, 1: 1000 in PBS+ 1%BSA) were then added and incubated for 1 h at 37 °C sequentially. After incubation with each antibody, the plates were washed 3 times with PBS-0.05%Tween 20. Subsequently, 100 µl /well aminoethylcarbazole (AEC) solution (Sigma-Aldrich Inc., AEC101-1KT) was added and incubated at room temperature for 15 to 30 min depending on color development. AEC solution was then aspirated off, and PBS (200 µl/well) was added to stop the reaction. Plates were examined under a light microscope. The cytoplasm, but not the nucleus, of an infected cell is stained with red color. The highest dilution at which complete neutralization (0 red staining cells in the well) achieved was recorded as the serum EV71 neutralizing titer.

2.4.10 Statistical analysis

Kruskal-Wallis test was performed for comparing body temperature, IFN-γ producing CD4+ and CD8+ T cell frequencies and virus neutralizing antibody titers between EV71 inoculated and control groups using SAS 9.3 software. $P<0.05$ were considered statistically significant.

2.5 RESULTS

2.5.1 Identification of EV71 BJ110 strain virus inoculum

EV71 was identified by RT-PCR, Cytopathic effects (CPE) and CCIF (Figure 1a I-IV). The size of the RT-PCR product was 314bp and its sequence matches with VP1 gene sequence of the EV71 BJ110 strain (Genbank: HM002486.1) (data not shown). CPE characteristic of EV71 were seen in Vero cells, including rounding, aggregation, detaching and apoptosis, which typically began to appear 24 h after infection at 1 MOI. EV71 viruses were further detected by anti-EV71 capsid protein VP1 specific monoclonal antibody in CCIF (Figure 1a IV). Based on these results, we confirmed that the virus stock was EV71 BJ110 strain.

2.5.2 EV71 infects porcine cell cultures *in vitro*

The infectivity of EV71 was tested using porcine intestinal epithelial cells (IPEC-J2), PBMCs, and neural cells. Human EV71 BJ110 strain infects all three porcine cell cultures (Figure 1b). Consistent with most positive-strand RNA viruses, virus replication and assembly were detected in the cytoplasm, as indicated by exclusive cytoplasmic fluorescence. Although all three porcine cell cultures were susceptible to EV71 BJ110 strain infection, difference in the efficiency of intracellular viral replication and assembly was observed among these cell types, as shown by the

strongest immunofluorescence detected in IPEC-J2, intermediate immunofluorescence in PBMCs and weakest immunofluorescence in neural cells. However, no significant CPE were observed in any of these porcine cell cultures.

To assess the efficiency of EV71 replication in IPEC-J2 cells, viral growth curves in both IPEC-J2 and positive control Vero cells were measured using Taqman® real time PCR. The viral RNA titers in cell culture medium at different time points after infection at 0.1, 1 and 10 MOI are shown in Figure 1c. The extracellular viral titer for Vero cells peaked at 60 h post infection at 1 and 10 MOI. However, Vero cells infected at 1 MOI reached the highest viral titer of approximately $1x10^8$ RNA copies/ml. Extracellular virus titers for the IPEC-J2 cells peaked at 48 h post infection at 1 and 10 MOI. After infection at 0.1 MOI, the virus titer peak was delayed to 72 h. Similar to the result observed for Vero cells, the highest extracellular viral titer of $1.1x10^5$ RNA copies/ml was seen for the IPEC-J2 cells when infected at 1 MOI.

2.5.3 Virus shedding and tissue distribution in infected neonatal Gn pigs

Viral shedding was detected by RT-PCR or Taqman® real time PCR. For both oral and combined oral-nasal infection groups, virus shedding was detected (Figure 2a and b, Table 1). When detected by RT-PCR, virus shedding was only detectable from PID 5 for the orally infected pigs. Consistent with RT-PCR results, viral titers peaked between PID 5 and PID 8 for the orally infected pigs as determined by Taqman® real time PCR. For the combined oral-nasally infected pigs, virus titer peaks ranged from PID 1 and PID 4, which are generally lower compared to peaks seen in the orally infected pigs. Both groups started shedding virus from PID 1, and lasted until PID 18 and

PID 12 (ranging from 2.16 x 10^3 to 2.22 x 10^8 RNA copies/ml) for the orally and the combined oral-nasally infected pigs, respectively. No viral RNA was detected in the control group.

Virus tissue distribution and titer at different time points after infection were determined by Taqman® real time PCR and immunohistochemistry. For the combined oral-nasally infected pigs, viral capsid proteins were detected in the cytoplasm of enterocytes of the small intestine in the combined oral-nasally infected pigs on PID 7 and PID 14 (Figure 2c). Additionally, high virus titer was detected in many tissues on both PID 7 and 14, including small intestine, central nervous system and lung in the infected pigs, whereas no viral RNA was detected in the control pigs (Figure 2d). Antigen presence and tissue viral titers were not determined for the orally infected pigs due to the lack of tissues samples.

2.5.4 Clinical signs in pigs mimic human patients

After infection of neonatal Gn pigs with EV71 BJ110 strain, clinical signs were monitored twice daily from PID 0 until euthanasia, ranging from PID 7 to PID 28. Body temperatures in the combined oral-nasally infected group were significantly higher than the mock control group and the orally infected group on PID 4, 5 and 6 (Figure 3a). There was no significant difference in body temperature between the orally infected group and the mock control group at any time points (Figure 3a). As seen in human patients, fever (body temperature ≥40 °C) and lethargy were two of the most common clinical manifestations observed for the combined oral-nasally infected pigs (Figure 3a and Table 2). Occasionally, vesicles were present in the snout of the infected pigs (Figure 3d). Neurological signs included limb weakness (particularly forelimb weakness),

diminished reflexes, ataxia, myoclonic jerk, convulsions and in some pigs, involuntary movements of the mouth, facial muscles, ears and irritability (Figure 3b). Respiratory signs, including fast and deep breathing (tachypnea and hyperpnea), open mouth breathing and peculiar laying position, were observed in 5 out of 8 pigs (62.5%) infected with EV71 BJ110 strain. These clinical signs were more frequently observed in the combined oral-nasally infected pigs than the orally infected pigs. However, none of the infected pigs with neurological and respiratory signs progressed to cardiopulmonary failure or death during the entire study period. No clinical signs were observed in the mock infected pigs. In addition, the sterility of the Gn pigs was monitored weekly by plating rectal swab samples on blood agar plates and thioglycollate media. All bacterial cultures were negative for all the gnotobioitic pigs used in this study, thus excluding the possibility that clinical signs were caused by extraneous microbial intestinal infections.

2.5.5 Pathology in neonatal Gn pigs infected with EV71

Grossly, multifocal mottling with petechial hemorrhages and swelling were observed in the lungs (Figure 3e and f) in 2 of 8 infected pigs (one orally inoculated pig on PID 29, one oral-nasally inoculated pig on PID 21). No gross lesions were observed in any other tissues, except for hemorrhages and atrophy of mesenteric lymph nodes.

Histopathological changes in neonatal Gn pigs infected with EV71 were only observed in the lungs, small intestine (particularly in ileum), and mesenteric lymph nodes (Figure 4). Peribronchial and alveolar hemorrhage and edema were present along with infiltration of lymphocytes, prominent peribronchiolar lymphoid tissue, and thickening of alveolar septae. Occasional

neutrophils were observed in the bronchi. In addition, hemosiderin laden macrophages were seen in the alveolar space. Hemorrhage and infiltration of lymphocytes were also seen in the pleura. In the small intestine, one prominent feature in the EV71 infected pigs is the increased size and number of Peyer's patches and significantly larger amount of immune cells in the lamina propria. Scattered eosinophils were seen in the mucosa of the duodenum, jejunum and ileum, as well as vacuolated lymphocytes were noted in the Peyer's patches of the ileum. No severe lesions in the intestinal epithelium was present on the time points (7, 14, 21 and 28 days after infection) examined (Figure S1). Prominent hemorrhage, numerous hemosiderin-laden macrophages in the walls of sinuses, reduction of lymphoid tissue and infiltration of eosinophils were observed in the mesenteric lymph nodes (data not shown). No lesions were observed in any part of the central nervous system, including cerebral cortex, cerebellum, the brainstem and spinal cord. In addition, there were no microscopic lesions observed in other tissues examined, including heart, skeletal muscle, kidney, spleen, liver, and tongue.

2.5.6 Robust adaptive immune responses in EV71 infected pigs

To examine the adaptive immune responses during EV71 infection in neonatal Gn pigs, frequencies of virus-specific IFN-γ producing CD3+CD4+ and CD3+CD8+ T cells among CD3+ lymphocytes in both systemic and local tissues and serum virus neutralizing antibody titers were analyzed by using flow cytometry and microplate virus neutralization assay, respectively. Representative dot plots of CD3+CD4+ IFN-γ+ and CD3+CD8+ IFN-γ+ T lymphocytes among total CD3+ mononuclear cells are shown for blood (Figure 5a). Compared to the mock control group, the combined oral-nasal infection group had higher frequencies of the CD4+ T cell subset

in blood and brain, but lower frequencies in lung on both PID 7 and PID 14. For the CD8+ T cell subset, higher frequency was induced in ileum on PID 7 and in blood and brain on PID 14, whereas the frequency was reduced in both brain and lung on PID 7 and lung on PID 14 (Figure 5b). However, these changes in the frequencies of both T cell subsets were not statistically significant.

The results from serum virus neutralizing antibody assay showed that significantly higher neutralizing antibody titers were induced in orally inoculated pigs on PID 7, PID 14 and PID 21 compared to the baseline on PID 0 (Figure 5c). Significantly higher antibody titers were also induced in the combined oral-nasally infected pigs on PID 7 and PID 14 compared to the baseline on PID 0. However, significantly higher serum antibody titers were induced in the orally infected pigs than the combined oral-nasally infected pigs on both PID 7 and PID 14. Serum neutralizing antibody response beyond PID 21 was not determined, except in one orally infected pig which had slightly decreased titer on PID 28 compared to PID 21.

2.6 DISCUSSION

Due to the ongoing extensive and severe EV71 epidemic in Asia associated with a high morbidity and mortality rate and the lack of appropriate treatments, it is imperative that effective vaccines and therapeutic drugs be developed. Substantial progress in our understanding of the basic EV71 virology has been made, which may facilitate the intensive efforts among researchers, governments, and industries to develop vaccines and antiviral drugs against EV71. For example, the crystal structure of EV71 has recently been determined (30, 31). However, a major obstacle that still remains is the lack of a reliable working animal model for EV71 infection. In this study,

we provided clear evidence that neonatal Gn pigs can be infected both orally and oral-nasally, resulting in virus shedding patterns, neurological and respiratory signs, as well as T cell and antibody responses that mimic human diseases. Comparing the oral infection group to the combined oral-nasal infection group, the oral infection group had higher fecal virus shedding, lower body temperature (absence of fever versus fever in the combined oral-nasal infection group), higher serum neutralizing antibodies and less severe clinical signs than the combined oral-nasal infection group. Therefore, Gn pigs can be used as an alternative animal model to the currently available murine and non-human primate models.

Intestinal epithelial cells, PBMCs, and neural cells are important targets for virus infection, replication, dissemination and pathogenesis during EV71 infection. The fact that porcine intestinal IPEC-J2 cells can be infected by EV71 BJ110 strain suggests that neonatal pigs can be infected orally, as EV71 is known to resist gastric acid in the stomach (32). Based on the intensity of immunofluorescent signal and short time of infection before detection (20 h), as well as high viral RNA titer in the infected IPEC-J2 cell culture supernatant, it appears that EV71 infection in IPEC-J2 is fairly efficient. The growth curve of EV71 BJ110 strain in IPEC-J2 further shows that EV71 replicates in porcine intestinal epithelial cells effectively (Figure 1c). Immune cells play an essential role in the host defense against EV71 infection, but are also the means through which EV71 spreads and causes lesions and diseases in human patients (32, 33). Lymphopenia has been associated with EV71 induced pulmonary edema (34). In our study, porcine PBMCs were also infected by EV71. In a single experiment, porcine neural cells were infected with EV71, even though the virus replication in the neural cells was not robust. The infection of porcine neural cell culture by EV71 BJ110 strain is consistent with the detection of EV71 viruses in the brain of

human patients and infection of human neuronal cell lines by EV71 *in vitro* (35) (36). However, proinflammatory cytokine responses induced by EV71 may contribute to the neurological and pulmonary disease without viral invasion of neurons (37). Indeed, we have observed increased frequencies of CD3+CD4+ IFN-γ+ T lymphocytes in the brain on both PID7 and 14. (Figure 5b) This, alone with the low replication level of EV71 in neural cells, may explain the lack of lesions in the central nervous system of the infected neonatal Gn pigs in the current study. However, given neural cells used in the current study are different from neurons and the detection of high viral RNA level in the central nervous system (Figure 2d), we cannot rule out the possibility of productive infection of neurons by EV71 and the associated neuronal injuries being undetected. Taken together, *in vitro* infection of pig cell cultures (IPEC-J2, PBMCs and neural cells) by EV71 supported our *in vivo* results that showed neonatal Gn pigs can be infected with EV71.

In order to better represent the EV71 infection in young children less than 3 years old (38), 5 days old neonatal Gn pigs were used in our study. The results showed that long lasting and high levels of virus shedding were detected by Taqman® real time PCR in the fecal samples of both orally or combined oral-nasally infected pigs. This is consistent with our results showing more efficient EV71 infection in IPEC-J2 cell culture compared to PBMCs, suggesting that intestinal epithelial cells are the major site of viral replication during EV71 infection in neonatal Gn pigs. Consistent with the detection of high EV71 shedding titers in the fecal samples of the EV71 infected pigs in the combined oral-nasal inoculated pigs, viral capsid proteins were also detected in the enterocytes of the ileum on PID 7 and PID 14, further confirming that EV71 infection and replication in intestinal epithelial cells is efficient and persistent. In short, these results indicate that oral or combined oral-nasal infection of neonatal Gn pigs with EV71 BJ110 allows EV71 to

infect and replicate in the intestinal epithelial cells very effectively and persistently. This result is significant considering that EV71 BJ110 strain has not been passaged in pigs. It is currently unknown whether EV71 infects and circulates in pigs under natural environmental conditions. Epidemiology studies of swine populations are needed to examine the zoonotic potential of human EV71.

Similar to human patients, clinical signs observed in the neonatal Gn pigs inoculated with EV71 included typical HFMD symptoms, as well as neurological and respiratory symptoms. However, only occasional skin lesions were seen, suggesting EV71 BJ110 strain is not intensely dermotropic in pigs. Interestingly, combined oral-nasal infection tends to cause more severe and frequent neurological and respiratory signs than oral infection alone in the neonatal Gn pigs (Table 2). It may be due to the stronger systemic inflammation induced by simultaneous stimulation of mucosal immune system at multiple sites in the combined oral-nasal infection group, which is corroborated by high fever, reduced virus replication and shedding, as well as resultant lower T cell and serum neutralizing antibody responses in this group. It may also be due to the direct and local effects of virus on the lungs or virus dissemination to the CNS through the olfactory bulb.

Febrile response induced during virus infection is a major defense mechanism to rid the host of virus replication. Oral inoculation of pigs with EV71 did not induce significant body temperature increases compared to the mock-inoculated pigs, which may have resulted in the higher titers and longer periods of fecal virus shedding. Consequently, the higher viral antigen load induced higher neutralizing antibody titers in the orally inoculated pigs. However, it is not known why oral

infection by EV71 did not induce febrile responses in pigs. The small number of pigs in this group may have been a contributing factor. Further experiments with greater number of pigs in each group need to be performed to address this issue.

While no detailed mechanistic data are available, the following route for the systemic spread of EV71 viruses is likely to have occurred in the current pig model. Upon oral inoculation, EV71 viruses infect and replicate in the small intestinal epithelial cells. Then EV71 viruses reach the blood circulation, leading to viremia and fever. EV71 viruses are then spread to target organs such as lung, central nervous system, kidney, cardiac muscle and skin, through blood or lymph circulation system. Once reaching the target organ, EV71 viruses infect and replicate in the cells of the target organ, inducing inflammation or immune responses, which are responsible for the removal of the invading viruses. Viral systemic spread to different organs occurs at different time points. In particular, EV71 viruses spread to the central nervous system results in virus replication in midbrain, medulla, cervical spinal cord, and caudal cerebral cortex at 7 days post infection, and then shifted to cervical spinal cord and rostral cerebral cortex on 14 days post infection. Based on the tissue viral RNA titers (Figure 2d) in the current study, it is not known how long EV71 viruses persist in different target organs, or any additional target organs are involved. With the oral-nasal inoculation, the systemic spread of EV71 viruses presumably follows the same route, in addition to a possible spread of EV71 viruses from nerves in the nasal cavity to the central nervous system directly.

Despite the observed clinical signs and the presence of high titers of viral RNA, it is unclear why no extensive tissue damage or massive inflammation was observed in the central nervous systems and other tissues, including intestinal tissues. It could possibly be due to the lack of normal microbiota in these Gn pigs. Gut microbiota is increasingly recognized as a major contributor to the mucosal and systemic inflammation and diseases (39). In the lung, lesions such as edema and focal hemorrhage seen in 2 out 8 inoculated pigs have also been observed in fatal EV71 infections in humans (40). However, these lesions were observed in only 25% of all inoculated pigs, and absent in some pigs that displayed respiratory symptoms. Given the small number of pigs included in the current study, the possibility that these lung lesions were caused by factors other than EV71 infection alone cannot be ruled out. The presence of pulmonary hemorrhage and edema as well as the respiratory and neurological symptoms in one orally infected pig is particularly important, as these features are frequently associated with high mortality rate in young children (3), but they have only been seen in intra-cerebrally inoculated monkeys so far (41). Together, these pathological results indicate that oral or combined oral-nasal infection by neuro-virulent EV71 BJ110 in neonatal Gn pigs can cause respiratory and neurological lesions and clinical signs similar to those seen in human patients.

Besides virus replication, shedding, clinical signs and pathology, adaptive immune responses are essential parameters in the evaluation of vaccine, characterization of immunity, determination of correlates of protective immunity and immunopathogenesis study during EV71 infection. In establishing the neonatal Gn pig model, EV71-specific IFN-γ+ T cell responses and serum EV71 neutralizing antibody titers were determined at different time points of EV71 infection. IFN-γ plays a major role in host defense against virus infections. Increased EV71 neuro-virulence in IFN-

γ receptor deficient mice has also been observed (12). Another study using PBMCs from EV71 patients showed that significantly lower IFN-γ were secreted by PBMCs from patients with pulmonary edema than those without pulmonary edema after *in vitro* stimulation with EV71 (42). Consistent with these results, all infected pigs (including three pigs that showed both respiratory signs and lung lesions) in this study exhibited decreased frequencies of both CD3+CD4+ IFN-γ+ and CD3+CD8+ IFN-γ+ T cells in the lung on PID 7 and PID 14. This may have contributed to the neurological and respiratory signs observed in this study. However, as an inflammatory cytokine, IFN-γ can also cause tissue injury and lead to exacerbation of EV71 infection. The increased frequencies of both CD3+CD4+ IFN-γ+ and CD3+CD8+ IFN-γ+ T cells in the brain is also consistent with a study showing significantly increased IFN-γ levels in cerebrospinal fluid from EV71 patients with pulmonary edema compared with patients without pulmonary edema (43). Our data also showed that the frequency of IFN-γ producing T cells varies depending on time points post infection and tissues infected. These differences indicated the dynamic balance of the protective effects and immune-pathogenesis effects of IFN-γ during EV71 infection, depending on the infection time course and tissues involved, and may help explain the seemingly conflicting results regarding the role of IFN-γ during EV71 infection. Serum EV71 neutralizing antibody titers increased from PID 7, to PID 14 and peaked at PID 21 in the orally infected pigs. The same trend was observed in the combined oral-nasally infected pigs from PID 7 to PID 14, although the titer is much lower. Notably, one oral dose of EV71 BJ110 strain, without any adjuvant, induced serum neutralizing antibody titer significantly higher than those seen in human patient (44) and more importantly, comparable to those achieved by multiple high doses of current candidate vaccines with adjuvants through non-oral immunization (45, 46). Therefore, oral challenge of neonatal Gn pigs with EV71 BJ110 strain is highly immunogenic suggesting that attenuated EV71 has the

potential as an oral EV71 vaccine candidate. Such high immunogenicity also renders current Gn pig model suitable for EV71 vaccine evaluation.

By virtue of this report, the neonatal Gn pig model is one of the most comprehensively described animal models for EV71. These results showed that neonatal Gn pigs represent a suitable alternative animal model to the mice and non-human primate models for EV71. First, the observed pulmonary hemorrhage and edema (although their causality is debatable), as well as neurological and respiratory signs in the orally infected neonatal Gn pigs make this pig model a unique neonatal animal model. A recent study in 3-3.5 year old Rhesus macaques showed that only mild lesions in the central nervous system and lungs were present via oral and respiratory route inoculation (41). The results here indicate the comparable capacity of pigs to non-human primates in modeling EV71 infection in humans. Second, the use of the neuro-virulent EV71 BJ110 strain (a member of C4 genotype) is important for development of vaccines and therapeutics for the ongoing EV71 epidemic (47). Third, no previous animal models for EV71 have been established using Gn animals. Our Gn pig model of EV71 infections allow for more specifically studying viral pathogenesis, immunogenicity and efficacy of vaccines, and development of antiviral therapies. Fourth, unlike studies in mice (11, 48), the EV71 strain used in this study has not undergone host adaption in pigs, making our Gn pig model more accurately reflect the pathogenicity of EV71 in humans. Taken together, pigs are a particularly attractive alternative animal species for the development of a better animal model for EV71 infection.

In summary, our study provides *in vitro* and *in vivo* evidence that neonatal Gn pigs can be orally and oral-nasally infected by a recent, non-pig adapted neuro-virulent human EV71 BJ110 strain. Long-period shedding of high virus titer in fecal samples (up to 18 days post infection and with a peak titer of 2.22 x 10^8 RNA copies/ml) and spreading of virus to the central nervous system and respiratory system tissues from intestinal tissues were observed. Infection resulted in the development of some HFMD clinical signs, as well as neurological and respiratory signs that mimic some symptoms of the severe EV71 diseases in human infants. Strong changes in the frequencies of both CD3+CD4+ IFN-γ+ and CD3+CD8+ IFN-γ+ T cells were detected in the lungs and brain tissues on PID 7 and PID 14. High serum EV71 neutralizing antibody titers were also induced in both orally and combined oral-nasally infected pigs. Together, these results demonstrated that neonatal Gn pigs represent a promising novel animal model species for the preclinical evaluation of vaccines and antiviral drugs, as well as elucidating the underlying mechanisms of pathogenesis for EV71 infection in humans.

2.7 ACKNOWLEDGEMENTS

We thank Pete Jobst, Andrea Pulliam, Kim Allen, and Shannon Viers for their excellent animal care. We thank Melissa Makris for assistance in flow cytometry. The study was partially supported by Fralin Funds, Fralin Life Science Institute, Virginia Tech.

2.8 CONFLICT OF INTEREST

The authors declare no conflict of interests.

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Table 2.1 Summary of virus shedding in neonatal Gn pigs infected with EV71 BJ110 strain.

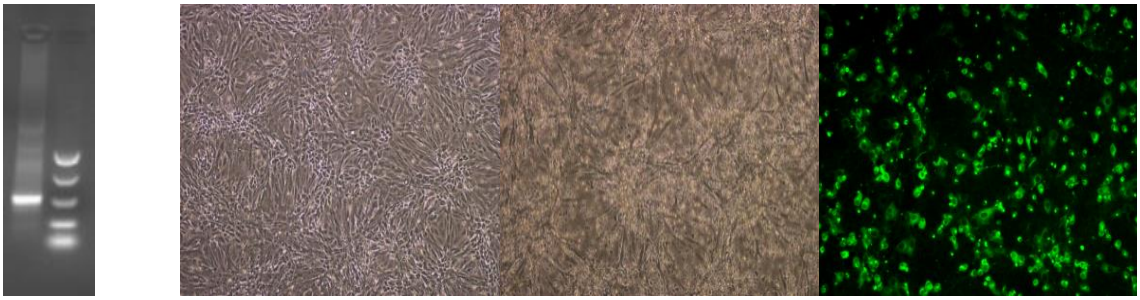
Inoculum	Inoculation route	Dosage (FFU)	Euthanasia	Number of pigs infected	Shedding period	Peak titer (RNA copies/ml)	Peak of virus shedding
EV71 BJ110	Oral-nasal	4.5×10^8-5.0×10^7	PID7/14/21	4/4	PID1-12	3.68×10^6	PID1
EV71 BJ110	Oral	5.0×10^8	PID7/14/21	4/4	PID1-18	2.22×10^8	PID6
Diluent	Oral	N/A	PID7/14/21	0/4	N/A	NA	NA

Table 2.2 Clinical signs in neonatal Gn pigs infected with EV71 BJ110 strain.

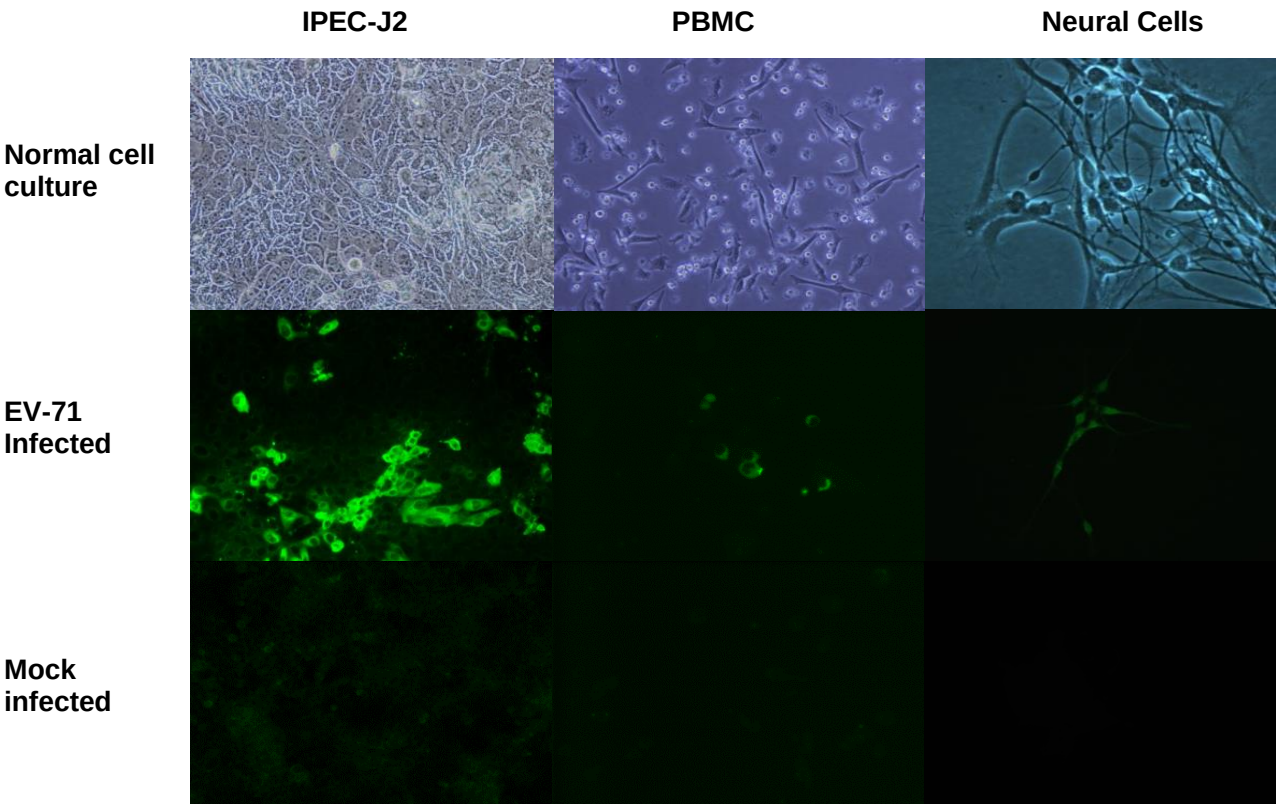
Group	PPD	Inoculation route	Dosage	Virus shedding	Fever (≥40ºC)	Diarrhea	Skin lesion	Respiratory signs	Neurological signs	Frothy mouth
EV71 BJ110	5	Oral-nasal	$4.5x10^8$ FFU/oral; $1.0x10^7$ FFU/nasal	4/4	4/4	0/4	0/4	4/4	4/4	0/4
EV71 BJ110	5	Oral	$5x10^8$ FFU/dose	4/4	0/4	0/4	1/4	2/4	2/4	1/4
Control	5	Oral	5 ml Diluent #5	0/4	0/4	0/4	0/4	1/4	0/4	0/4

a

RT-PCR **Mock-infected control** **CPE in EV71-infected cells** **Immunofluorescence**



b


IPEC-J2
PBMC
Neural Cells
Normal cell culture
EV-71 Infected
Mock infected

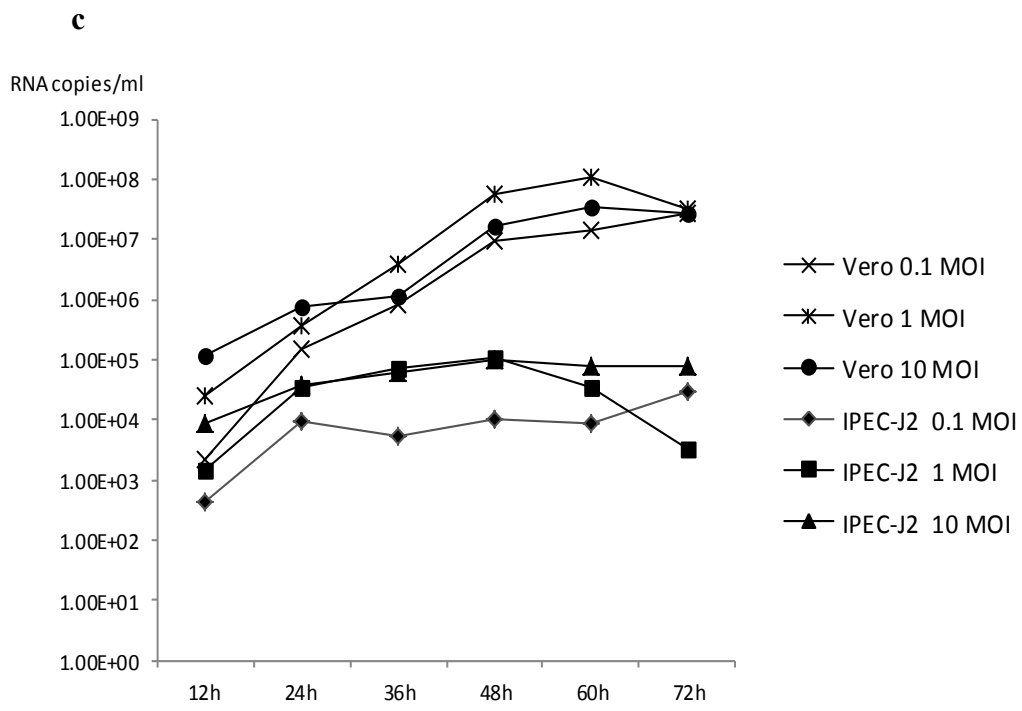
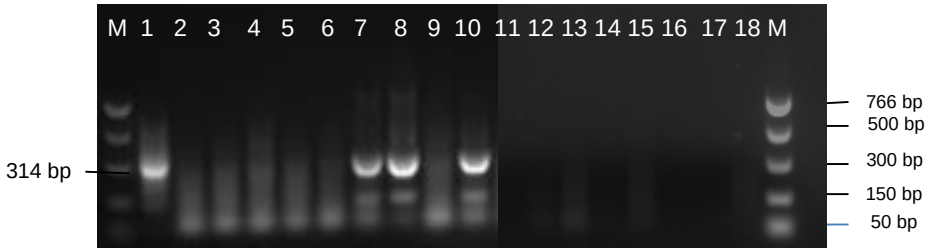
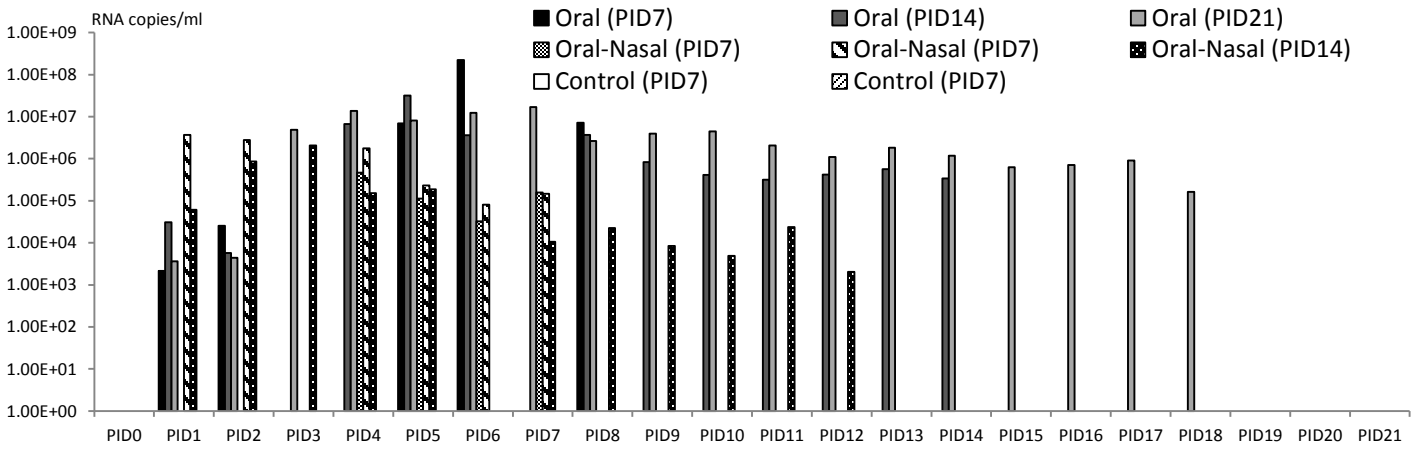


Figure 2.1 Human EV71 BJ110 strain infects and replicates in pig intestinal epithelial, PBMCs and neural cell culture *in vitro*. a. Identification of virus inoculum. (I), RT-PCR detection of EV71 BJ110 strain. The positive PCR products were purified and sequenced. The sequence shares 100% identity to the published EV71 BJ110 strain VP1 gene sequence. (II), Mock infected Vero cell culture; (III), CPE in Vero cells 72h post inoculation with EV71 BJ110 strain at 10 MOI. (IV), EV71 infected Vero cells were detected by CCIF. **b**. Porcine cell cultures can be infected by EV71 BJ110 strain. EV71 was detected by CCIF in IPEC-J2 cells, PBMCs, and neural cells. **c**. Growth curves for EV71 BJ110 strain in Vero and IPEC-J2 cells suggest that EV71 infects and replicates efficiently in IPEC-J2 cells.

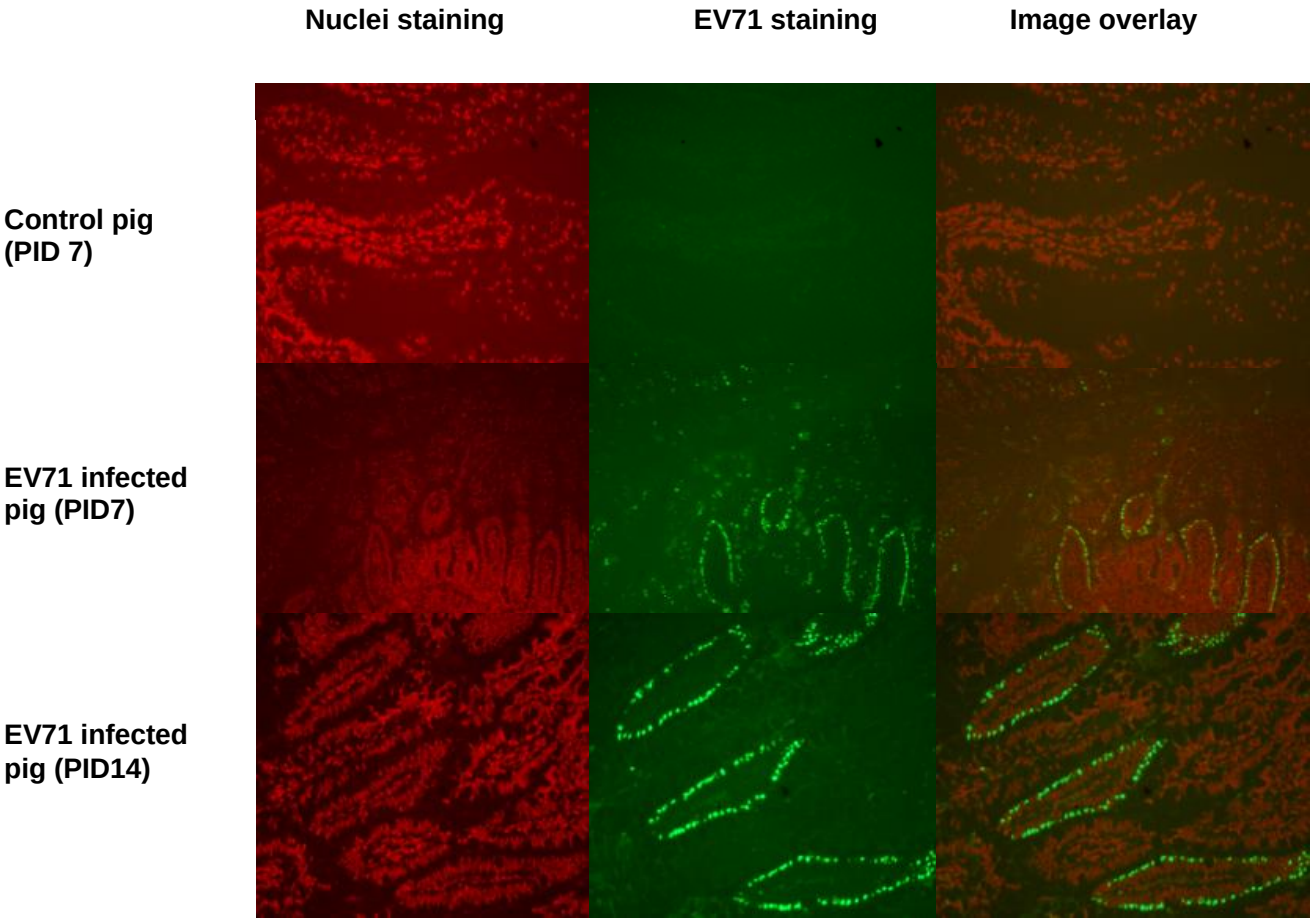
a


M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 M
314 bp
766 bp
500 bp
300 bp
150 bp
50 bp

b


RNA copies/ml
1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
Oral (PID7)
Oral (PID14)
Oral (PID21)
Oral-Nasal (PID7)
Oral-Nasal (PID7)
Oral-Nasal (PID14)
Control (PID7)
Control (PID7)
PID0
PID1
PID2
PID3
PID4
PID5
PID6
PID7
PID8
PID9
PID10
PID11
PID12
PID13
PID14
PID15
PID16
PID17
PID18
PID19
PID20
PID21

c


Nuclei staining
EV71 staining
Image overlay
Control pig (PID 7)
EV71 infected pig (PID7)
EV71 infected pig (PID14)

d

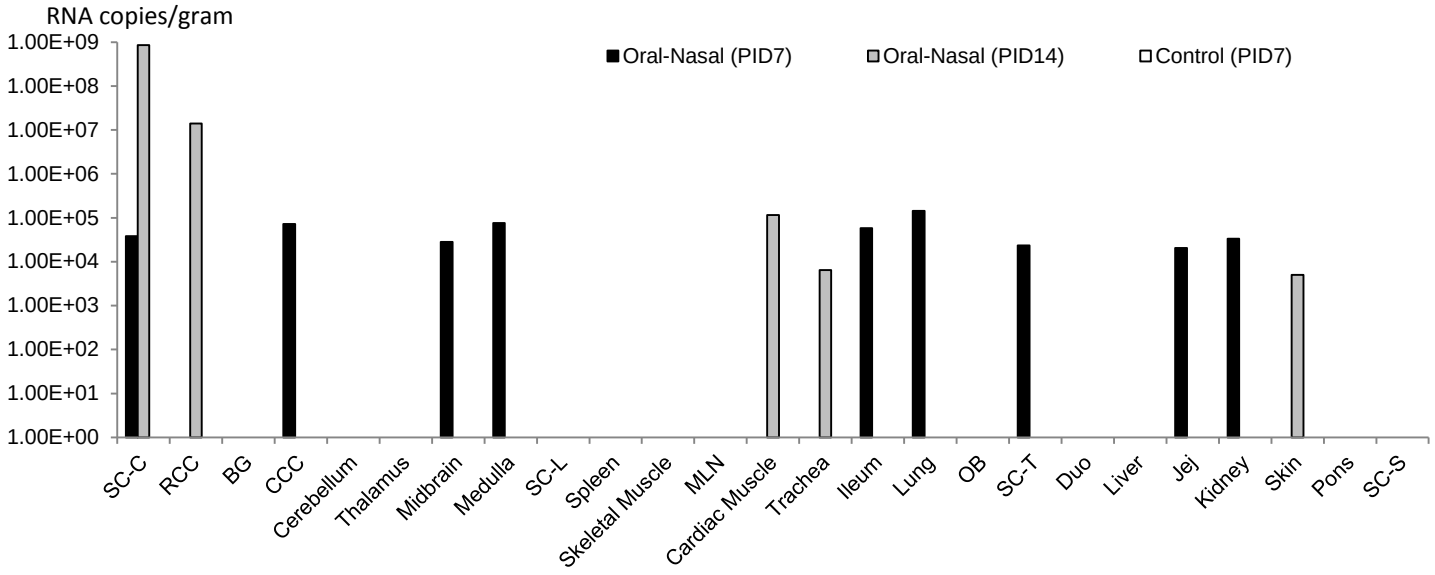
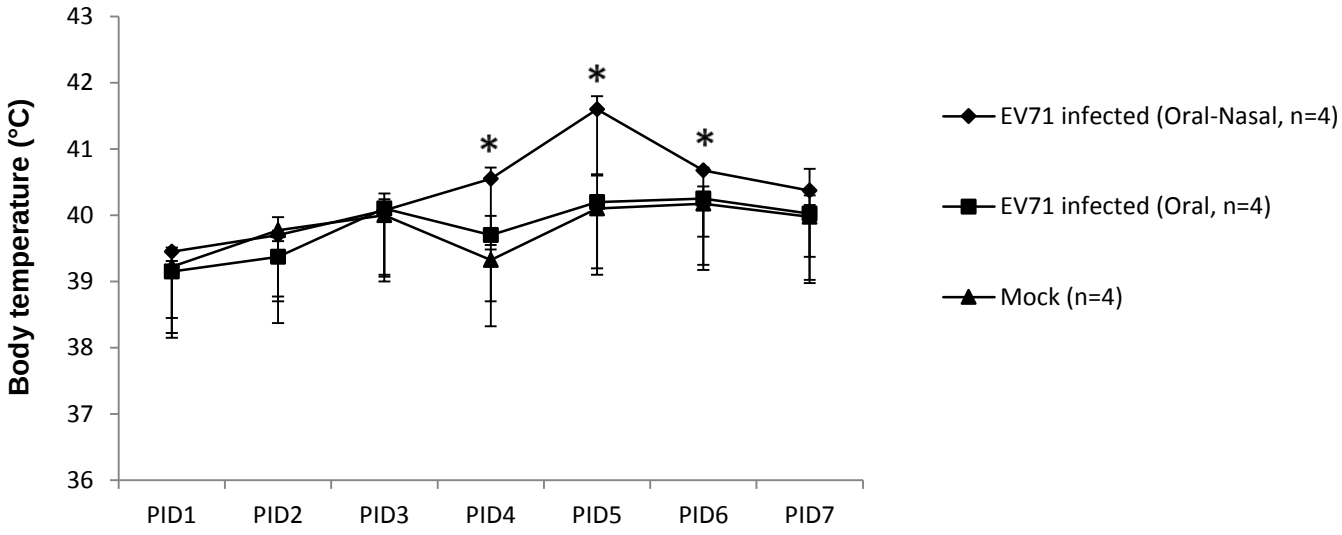


Figure 2.2 EV71 BJ110 strain fecal shedding, tissue distribution and dynamics in infected neonatal Gn pigs. a. RT-PCR detection of EV71 in rectal swab samples. Lanes 2-10: PID 0-8 from an orally infected neonatal Gn pig. Lanes 11-18: PID 1-8 of a mock infected pig. M: marker; Lane 1: positive control. **b**. Taqman® real time PCR detection of virus shedding in rectal swab samples from neonatal Gn pigs infected with EV71 BJ110 strain. Bars depict virus titers detected from individual pigs on different PIDs. Route of inoculation and the euthanasia time (in the parentheses) of the pigs are marked by the legends. Viral titers are shown as the mean of two replicates for the same sample. The negative samples are shown as blank on the bar graph. **c**. Detection of viral antigen in the ileum of Gn pigs infected with EV71 BJ110 strain through oral-nasal route at a dose of 5 x 10^8 FFU on PID 7 and PID 14 by immunofluorescence staining. Mouse anti-EV71 capsid protein VP1 monoclonal antibody (Abcam, ab36367) was used as the primary

antibody and goat anti-mouse IgG1 antibody labeled with fluorescein isothiocyanate (Sigma Aldrich, Cat. No. F 0257) was used as secondary antibody. Nuclei were stained red by propidium iodide (Invitrogen, P3566). **d**. Taqman® real time PCR detection of EV71 viral RNA in tissues of infected Gn pigs at PID 7 or PID 14. Route of inoculation and the euthanasia time (in the parentheses) of the pigs are marked by the legends. Viral titers are shown as the mean of two replicates for the same sample. The negative samples are shown as blank on the bar graph. SC-C, spinal cord-cervical; RCC, rostral cerebral cortex; BG, basal ganglia, CCC, caudal cerebral cortex; SC-L, spinal cord-lumbar; MLN, mesenteric lymph nodes; OB, olfactory bulb; SC-T, spinal cord-thoracic; Duo, duodenum; Jej, jejunum, SC-S, spinal cord-sacral. All data are representatives of at least two independent experiments.

a


Body temperature (°C)
43
42
41
40
39
38
37
36
PID1
PID2
PID3
PID4
PID5
PID6
PID7
*
*
*
EV71 infected (Oral-Nasal, n=4)
EV71 infected (Oral, n=4)
Mock (n=4)

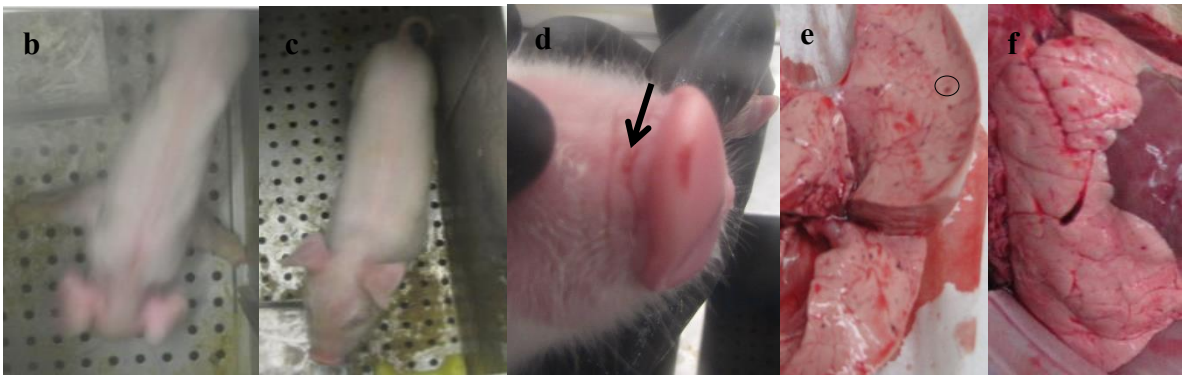


Figure 2.3 Fever, limb paralysis, vesicles and lung lesions in EV71 infected neonatal Gn pigs. **a**. Body temperature in the EV71 infected neonatal Gn pigs. Body temperature was measured through subcutaneously implanted microchips posterior to the ear. Body temperature at each time point was an average of three measurements. The normal core body temperature of pigs ranges from 38 to 40ºC, with an average of 38.8 ºC. Temperature higher than 40 ºC is considered fever. **b**. Forelimb weakness in neonatal Gn pigs infected with EV71 BJ110 strain. **c**. An age-matched mock infected control neonatal Gn pig; **d**. Vesicles (indicated by black arrow) on the snout of EV71 infected neonatal Gn pigs. **e**. Multifocal mottling with petechial hemorrhages (indicated by the circle) in the lung seen in an oral-nasally inoculated Gn pig on PID 21. **f**. Normal lung from a mock infected Gn pig. * P value < 0.05 by Kruskal-Wallis test.

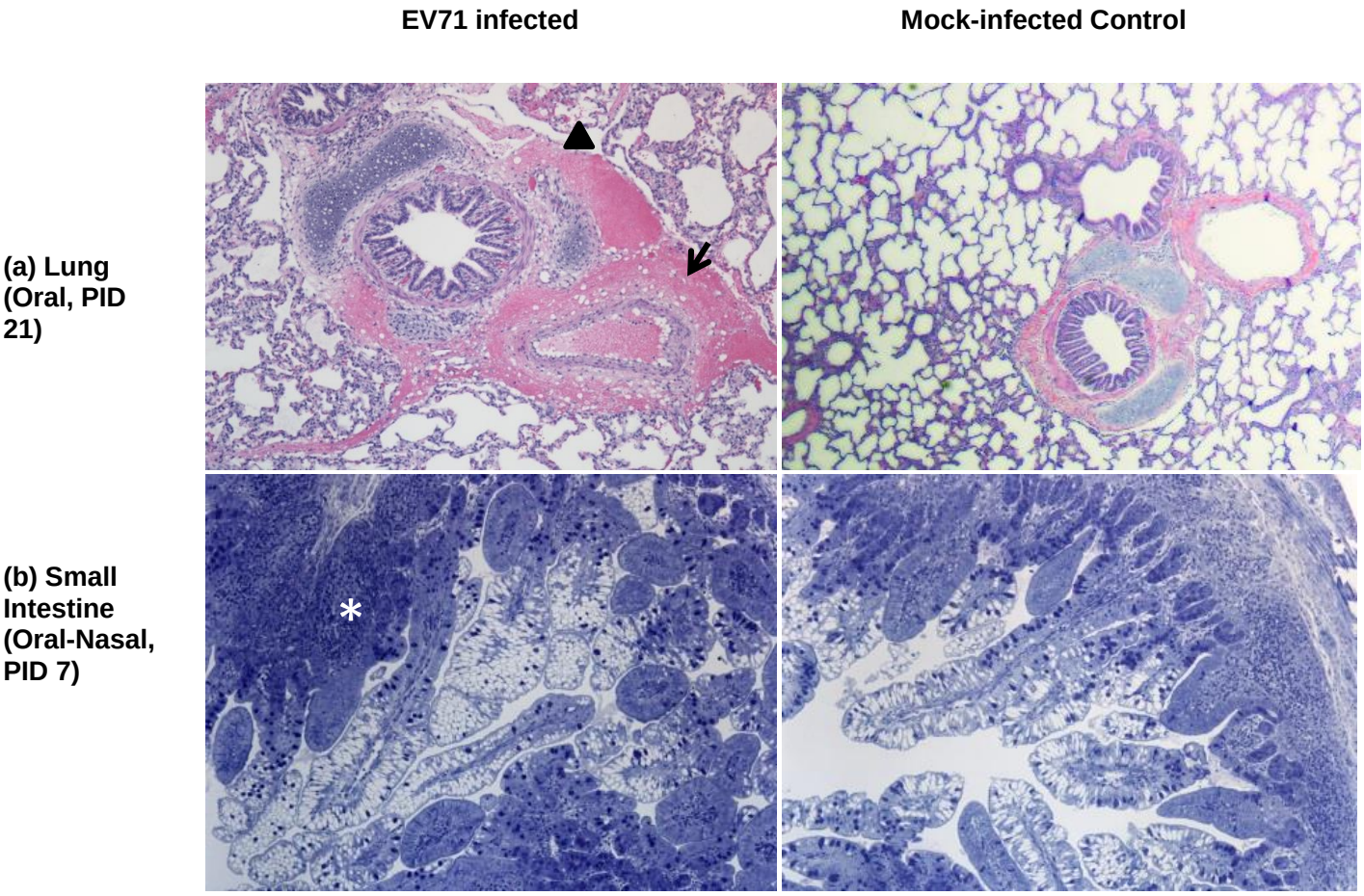
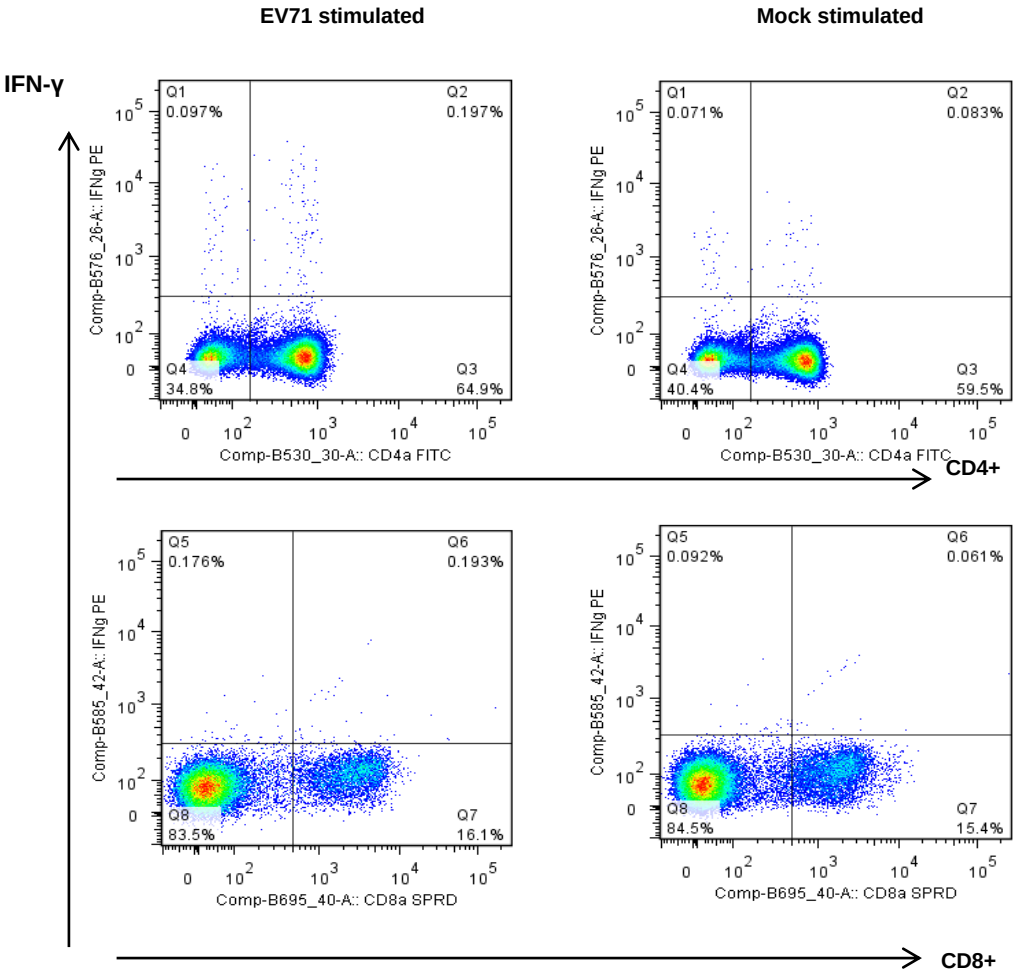
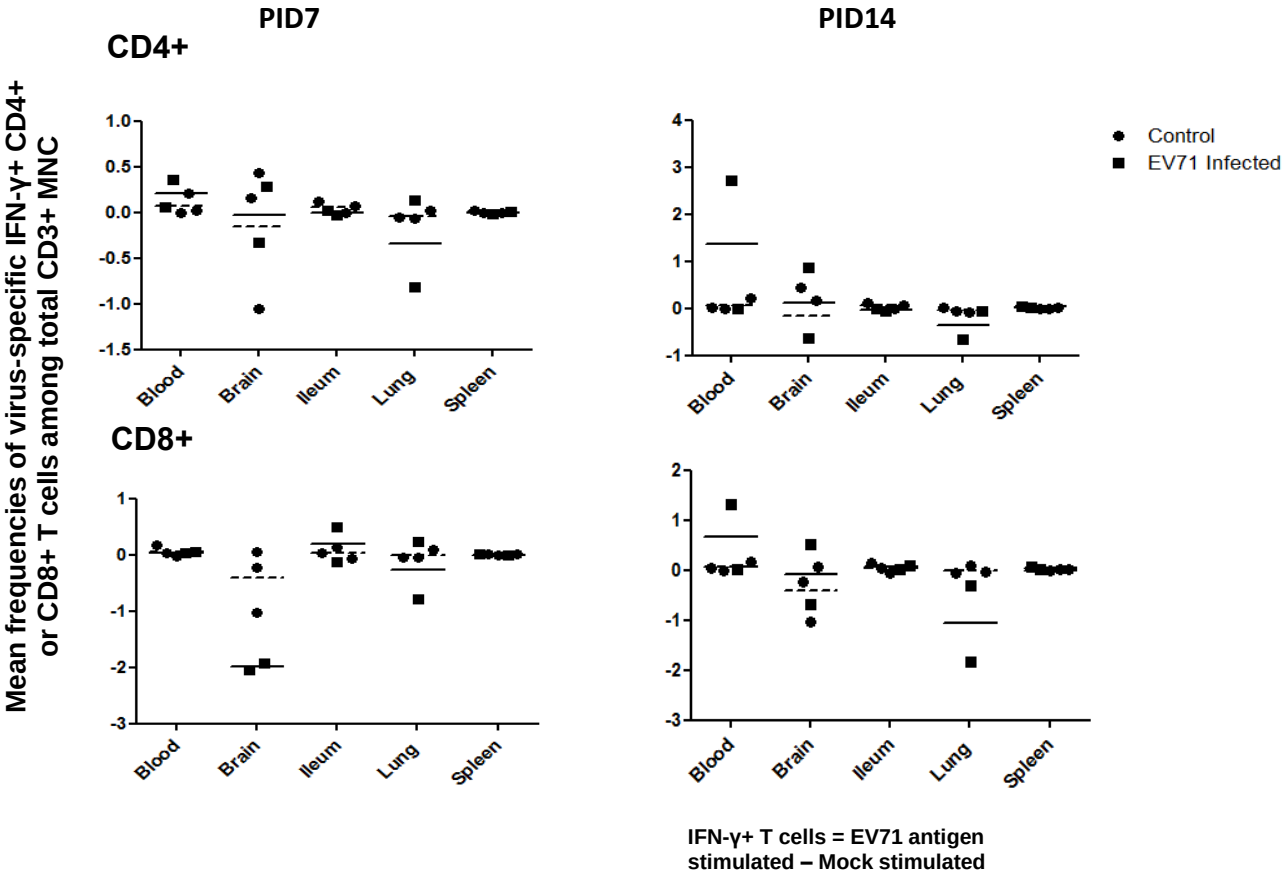


Figure 2.4 Microscopic lesions in neonatal Gn pigs infected with EV71 BJ110 strain. Upper panel shows a section of the lung of an orally infected Gn pig and a mock-infected control on PID 21. The infected pig has peribronchial and perivascular hemorrhage (indicated by the arrow). An adjacent alveolus contains scattered erythrocytes and macrophages (shown by the black triangle). Lower panel shows the small intestine of an oral-nasally infected Gn pig on PID 7 with prominent presence of immune cells in the lamina propria and significantly increased number of payer's patches (indicated by asterisk). Lung tissues were stained with H&E; Small intestinal tissues were sections of resin-embedded tissue stained with toluidine blue.

a


EV71 stimulated
Mock stimulated
IFN-γ
Q1
0.097%
Q2
0.197%
Q4
34.8%
Q3
64.9%
Comp-B576_26-A:: IFNg PE
Comp-B530_30-A:: CD4a FITC
Q1
0.071%
Q2
0.083%
Q4
40.4%
Q3
59.5%
CD4+
Q5
0.176%
Q6
0.193%
Q8
83.5%
Q7
16.1%
Comp-B585_42-A:: IFNg PE
Comp-B695_40-A:: CD8a SPRD
Q5
0.092%
Q6
0.061%
Q8
84.5%
Q7
15.4%
CD8+

b


PID7
PID14
CD4+
CD8+
Mean frequencies of virus-specific IFN-γ+ CD4+ or CD8+ T cells among total CD3+ MNC
Control
EV71 Infected
Blood
Brain
Ileum
Lung
Spleen
IFN-γ+ T cells = EV71 antigen stimulated – Mock stimulated

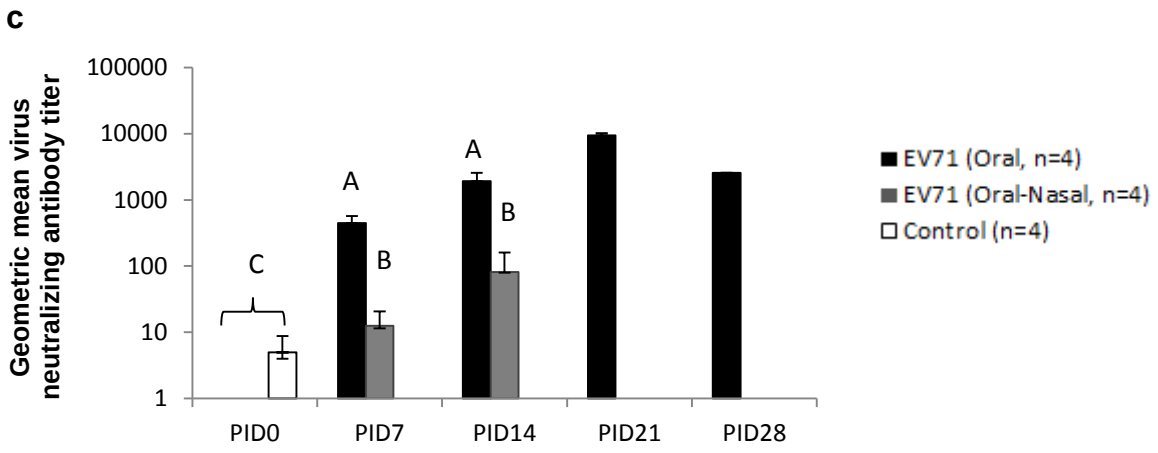


Figure 2.5 Immune responses during EV71 infection in neonatal Gn pigs. a. Representative dot plots showing the frequency of CD3+CD4+ IFN-γ+ and CD3+CD8+ IFN-γ+ T lymphocytes among total CD3+ mononuclear cells in blood. MNCs were stimulated with semi-purified whole EV71 antigen or control medium for 17h before staining. **b**. Frequency of IFN-γ producing CD3+CD4+ and CD3+CD8+ T cells among CD3+ mononuclear cells in ileum, spleen, blood, brain and lung following EV71 BJ110 infection via oral-nasal route in neonatal Gn pigs on PID 7 and PID 14. **c**. Serum neutralizing antibody response in the EV71 infected neonatal Gn pigs. * P value < 0.05 by Kruskal-Wall

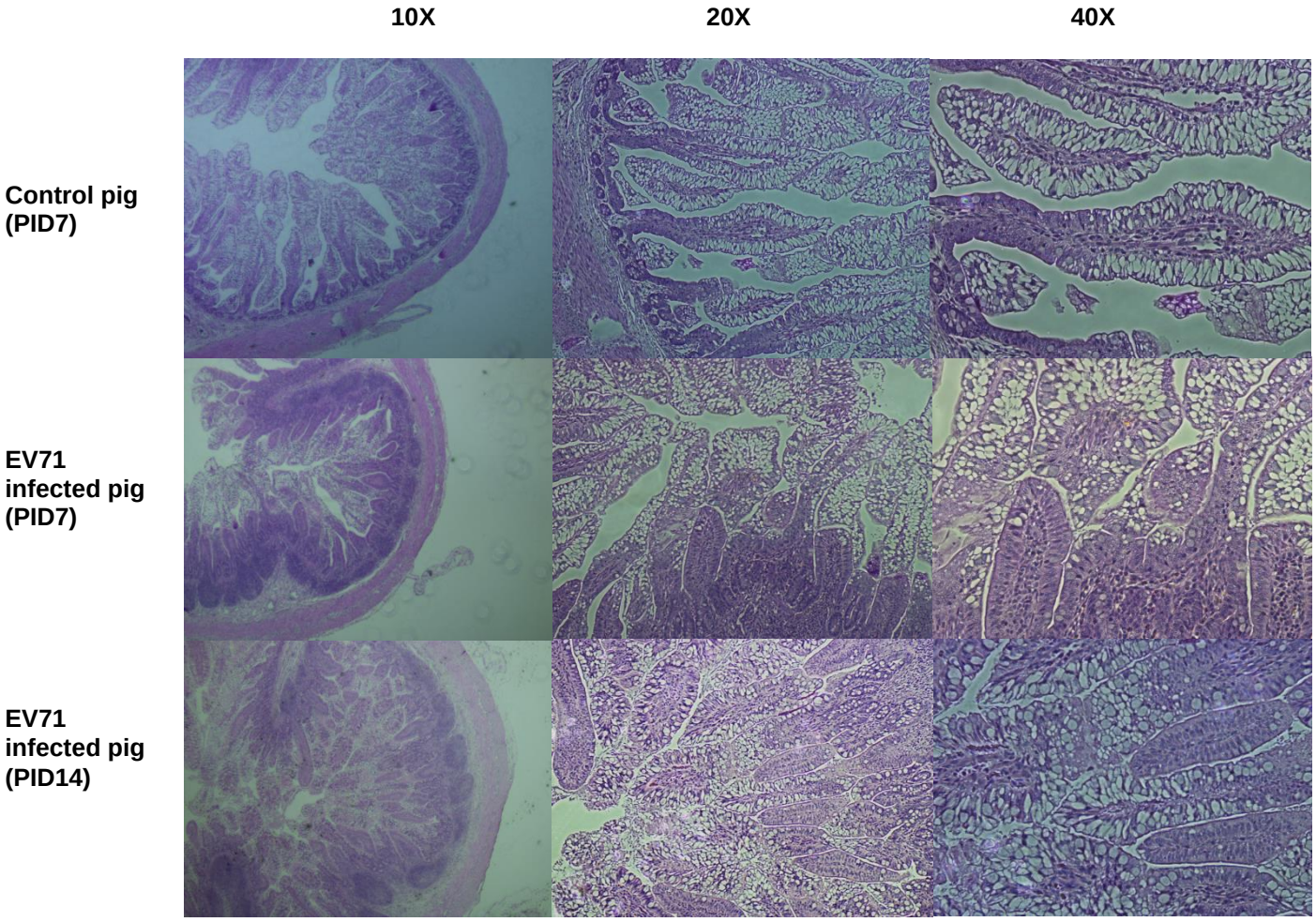


Figure S2.1 Histological sections of the ileum from the EV1 infected neonatal Gn pigs. The slides are prepared in the same way as in Figure **2c**. No observable lesions can be detected in the ileum of combined oral-nasally infected neonatal Gn pigs on both PID 7 and PID 14, despite the detection of viral capsid proteins in the enterocyte cytoplasm by immunofluorescence staining.

Chapter 3

3.1 Dietary rice bran protects against rotavirus diarrhea and promotes Th1 type immune responses to HRV vaccine in Gn pigs

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Published in *Clinical and Vaccine Immunology* (2014); 21(10):1396-403. doi: 10.1128/CVI.00210-14. Epub 2014 Jul 30.

3.2 ABSTRACT

3.2.1 Abstract

Rice bran (RB) contains a distinct stoichiometry of phytochemicals that can promote gut mucosal immune responses against enteric pathogens. Effects of RB on rotavirus diarrhea and immunogenicity of an AttHRV vaccine were evaluated in Gn pigs. Four treatment groups were included: RB plus vaccine, vaccine only, RB only, and mock. Pigs in the RB groups were fed with the amount of RB that replaces 10% of pigs' total daily calories intake from milk starting from 5 days of age until euthanasia. Pigs in the vaccine groups were orally inoculated with two doses of the AttHRV vaccine. A subset of pigs from each group was orally challenged with the homologous VirHRV on post-inoculation day 28. Diarrhea and virus shedding were monitored daily from post-challenge day 0 to 7. RB feeding significantly protected against diarrhea upon VirHRV challenge and enhanced the protective rate of the vaccine against rotavirus diarrhea. Consistent with the protection, RB significantly increased IFN-γ producing CD4+ and CD8+ T-cell responses in intestinal and systemic lymphoid tissues. Furthermore, RB also increased the number of total IgM and IgA immunoglobulin secreting cells, total serum IgM, IgG and IgA titers and HRV-specific IgA titers in intestinal contents. RB reduced the numbers of intestinal and systemic HRV-specific IgA and IgG antibody secreting cells and reduced serum HRV-specific IgA and IgG antibody titers before challenge. These results demonstrated clear beneficial effects of RB in protection against rotavirus diarrhea and stimulation of non-specific and HRV-specific immune responses, as well as its biased Th1-type adjuvant effect for the vaccine.

3.2.2 Keywords

Gnotobiotic pigs, RB, HRV, immune responses

3.3 INTRODUCTION

Rice Bran, a globally accessible, abundant and underutilized agricultural byproduct, has a distinct stoichiometry of bioactive compounds, phytochemicals, and minerals (1). It has been studied for bioactive functions such as the prevention and treatment of chronic diseases, growth of beneficial intestinal microbes, induction of mucosal and systemic immune responses, and protection against enteric pathogens (2-5). Thus, this agricultural byproduct represents a promising and practical dietary based solution for increasing innate resistance against enteric pathogens that cause diarrhea. In particular, its immune stimulatory functions can be potentially used as a vaccine adjuvant for enteric pathogen infections. Given that RB can support colonization of gut probiotics (e.g. *Lactobacilli spp*.), enhance mucosal IgA production (2), and significantly reduce the enteric burden of Salmonella infection in mice (5), continued investigation of dietary RB's mechanisms for protection against viral pathogens that cause significant global morbidity and mortality (e.g. rotavirus) is warranted.

Previous studies have shown the immune-modulatory effects of RB on both innate and adaptive immunity *in vitro* and *in vivo* (4). RB oil enhanced T and B lymphocytes proliferation, production of Th1 cytokines (IL-2, IFN-γ and TNF-α) by lymphocytes, and reduced Th2 cytokines (both serum and lymphocyte derived IL-4) as well as the level of serum IgE and IgG1 (3). MGN-

3, an arabinoxylan derived from RB, also increased levels of Th1 cytokines in human multiple myeloma patients (6). Importantly, γ-Oryzanol significantly promoted the development of antibody responses in rats stimulated with sheep red blood cells (4). Total local (feces) and systemic (serum) IgA levels, and IgA expression on Peyer's patches B cells, was also enhanced in mice fed 10% RB diet, suggesting RB promoted both mucosal and systemic B cell development (2). These studies demonstrated the immune-stimulatory effects of RB on multiple components of the immune system.

Given the RB-mediated protection against bacterial pathogens (7-9) and stimulatory effects on both the innate and adaptive immune systems, RB represents a promising natural food product for modulating mucosal immunity and protecting against diarrhea from major enteric pathogens, such as HRV. The Gn pig model of HRV infection and diarrhea has been extensively utilized to study HRV infection and vaccination (10-14). In this study, using the well-established neonatal Gn pig model, we aim to: 1) determine whether RB can reduce the susceptibility to infection and diarrhea upon VirHRV challenge; 2) examine the ability of RB to promote the development of intestinal and systemic T and B cell immune responses and improve the protective efficacy of oral rotavirus vaccine compared to the control diet.

3.4 METHODS

3.4.1 Gn pig experimental groups and treatment

Neonatal Gn pigs were derived and maintained in sterile isolators as previously described (15). Four treatment groups were included: RB + AttHRV, AttHRV only, RB only, and mock control.

Heat-stabilized, gamma radiated RB, (from the Neptune variety) provided by Anna McClung from the USDA-ARS Rice Research Center (Stuttgart, AK), was added to the Gn pigs' milk diet (Ultra-high-temperature treated cow-milk), starting at 5 days of age (PPD 5) until the end of experiment. The amounts of RB were calculated to replace 10% of the pigs' daily caloric intake from milk. AttHRV is produced from the 35^{th} passage of Wa strain (G1P1A[8]) HRV in the MA104 cell culture (16). Two oral doses of the AttHRV vaccine were given at approximately 5×10^7 FFU/dose on PPD 5 and PPD 15. Mock group received neither RB nor AttHRV vaccine. A subset of pigs from each group were orally challenged with the virulent HRV (VirHRV) Wa strain (G1P1A[8]) at a dose of approximately 1×10^5 FFU on post first inoculation day (PID) 28. The ID_{50} of the VirHRV in neonatal Gn pigs was determined as approximately 1 FFU (17). For measuring antibody titers, blood samples were collected weekly starting from PPD5 and upon euthanasia. Rectal swabs were taken daily for monitoring of virus shedding and diarrhea from post-challenge day (PCD) 0 to 7. Pigs were euthanized on PID 28 or PCD 7 and MNCs from ileum, spleen, and blood were isolated for detection of T and B cell responses using flow cytometry and Enzyme-Linked ImmunoSpot (ELISPOT) assay. All animal experiments were conducted according to the protocols approved by the Institutional Animal Care and Use Committee at Virginia Polytechnic Institute and State University.

3.4.2 Detection of virus shedding by ELISA

Rectal swabs taken daily upon VirHRV challenge were processed by washing two swabs in 8ml Diluent #5 (MEM, 1% Penicillin and Streptomycin, 1% HEPES) and then centrifuged at 2100 rpm for 15 minutes at 4 °C. Supernatants were then aliquoted and stored at -20°C. Virus antigens in the

fecal swabs were detected by ELISA (18). Rectal swabs from mock treatment Gn pigs were used as negative controls.

3.4.3 Detection of total CD3+CD4+ T helper (Th) cells, CD3+CD8+ cytotoxic T lymphocytes (CTL) cells and IFN-γ producing T cell responses by flow cytometry

Frequencies of total and IFN-γ producing CD4+ and CD8+ T cells among CD3+ mononuclear cells in ileum, intraepithelial lymphocytes (IEL), spleen, and blood were determined by intracellular staining and flow cytometry according to a previous publication (19). Data were acquired using a BD FACSAria flow cytometer and data were analyzed using FlowJo 7.22 (Tree Star, Inc.)

3.4.4 Detection of total immunoglobulin secreting cells (IgSC), HRV-specific antibody-secreting cells (ASC), total and HRV-specific serum and intestinal antibody titers

The ELISPOT assay for rotavirus-specific ASC response and data reporting were performed as previously described (16, 20, 21). The ELISPOT assay for measuring the total IgSC response and data reporting followed a previously described protocol (16). To determine the HRV-specific IgA and IgG antibody titers in serum and intestinal contents, isotype-specific ELISA was performed according to the protocol previously described (22, 23). Total IgM, IgA and IgG antibody titers in serum and intestinal contents were assessed by following an established ELISA protocol (24).

3.4.5 Statistical analysis

Kruskal-Wallis rank sum test was used for comparisons of data on virus shedding and diarrhea, Th and CTL cells, IFN-γ+ CD4+ and CD8+ T lymphocytes, total IgSC antibody secreting cells and HRV-specific ASCs. Fisher's exact test was used for comparisons on the percentages of virus shedding and diarrhea. Total and HRV-specific antibody titers in the serum were analyzed using the ANOVA-general linear model (GLM). Total and HRV-specific antibody titers in the large and small intestinal contents were analyzed using the ANOVA – Turkey test.

3.5 RESULTS

3.5.1 RB reduced HRV diarrhea but not virus shedding

The effects of RB on rotavirus infection and disease were determined by comparing the virus shedding and diarrhea parameters among the four treatment groups: RB + AttHRV; AttHRV only; RB only; and mock control. The results summarized in Table 1 showed that RB only group had significantly lower incidence (100% to 20%), shorter mean duration (5.6 to 0.2 days), and reduced severity of diarrhea (diarrhea score 14.4 to 4.4) compared to the mock control diet group. When compared to the AttHRV group, RB + AttHRV vaccine treated Gn pigs had significantly reduced incidence of diarrhea (67% to 0%), shorter mean duration (4.6 to 0 days), and reduced severity of diarrhea (diarrhea score 9.8 to 4.4). Importantly, RB only group had less diarrhea compared to the group with AttHRV vaccine alone, with reduced incidence (20 vs 67%) of diarrhea, significantly shorter mean duration (0.2 vs 4.6 days) and lower diarrhea scores (4.4 vs 9.8).

No significant difference in virus shedding was observed between RB only group and mock controls. Compared to the AttHRV vaccine group, RB + AttHRV group had increased virus

shedding (50 vs 100%), significantly earlier onset (6.0 vs 2.0), and significantly longer mean duration of virus shedding (1.3 vs 3.2 days). In addition, the RB alone group had slightly longer (not significantly) mean duration of virus shedding (6.2 vs 4.7 days) compared to the mock controls. These data suggest that RB protects against rotavirus induced diarrhea through mechanisms that are independent of affecting rotavirus replication.

3.5.2 Effect of RB on total Th and CTL development in intestinal and systemic lymphoid tissues

The frequency of total Th and CTL lymphocytes among lymphocytes in different tissues on PID 28 were determined. The results are shown in Figure 1. Compared to the Mock control group, RB only group had similar frequencies of total Th and CTL T cells among lymphocytes in both intestinal (ileum and IEL) and systemic (spleen and blood) lymphoid tissues. Similarly, there are no significant differences between AttHRV group and RB + AttHRV group, with the only exception of significantly down-regulated CTL response in IEL of the RB + AttHRV group. These results suggest that RB did not influence the development of total Th and CTL cells.

3.5.3 RB enhanced IFN-γ+ CD4+ and CD8+ T cell responses

Effector T cell responses against rotavirus is an important protective mechanism against infection. The effects of RB on effector T cells was assessed by the frequency of IFN-γ producing CD4+ and CD8+ T cell populations among total CD3+ mononuclear cells in both intestinal tissues (ileum and IEL) and systemic lymphoid tissues (spleen and blood). The results are shown in Figure 2.

Compared to the mock control group, RB only group had significantly increased frequencies of IFN-γ+CD4+ T cell populations in ileum, spleen and blood on PID 28 and IFN-γ+CD8+ T cell populations in ileum, spleen and blood on both PID28 and PCD 7. Compared to the AttHRV vaccine group, RB + AttHRV group had significantly increased frequencies of both IFN-γ+CD4+ and IFN-γ+CD8+ T cell populations in ileum, spleen and blood on PID 28 and PCD7, except for IFN-γ+CD4+ T cells in ileum and spleen on PCD7. There were no significant differences in IEL at any time point. These data demonstrate that RB has strong stimulating effects that favor Th1 type immune responses.

3.5.4 RB promoted the development of intestinal and systemic IgSC

Total immunoglobulins (Ig) in the intestinal and systemic tissues, particularly intestinal IgA, play a significant role in non-specific mucosal protection against viral infections. The number of IgM, IgA and IgG IgSCs in the ileum, spleen and blood were measured by ELISPOT and compared between RB only group and mock group (Figure 3). RB group alone showed significantly increased numbers of IgM IgSC in ileum and spleen as well as numbers of IgA IgSC in spleen and blood at PID 28. The numbers of IgA IgSC in the ileum and numbers of IgG IgSC in all tissues did not differ significantly between RB only and mock control groups. These data indicated that dietary RB intake can promote the development of intestinal and systemic IgSCs (IgM in ileum and spleen, IgA in spleen and blood).

3.5.5 RB stimulated the production of total IgM, IgA and IgG in serum

Total serum IgM, IgA and IgG antibody titers in Gn pigs fed with or without RB were determined using ELISA and the results are shown in Figure 4. On PID 21, RB only pigs had significantly higher level of IgM and IgA titers compared to the controls. Additionally, RB only pigs had significantly higher levels of serum IgM, IgA and IgG antibody titers than the controls on PID 28. Post HRV challenge, RB only pigs had significant higher level of IgA titer than the controls. In addition, RB + AttHRV pigs had significantly higher levels of IgM and IgA titers on PID 28 and IgM titers on PCD 7 than the AttHRV pigs. These data demonstrate that RB promoted the production of total serum IgM, IgA and IgG antibody titer in both naïve and vaccinated Gn pigs.

3.5.6 RB decreased the intestinal and systemic HRV-specific IgA and IgG ASC responses to AttHRV vaccination but not VirHRV challenge

HRV-specific serum IgA levels as well as numbers of intestinal IgA and IgG ASCs have been shown to be associated with the protection against rotavirus infection and diarrhea (16, 23). HRV-specific ASC responses are shown in Figure 5. Compared to the AttHRV alone group, the RB + AttHRV group has significantly lower numbers of both IgA and IgG ASC in the ileum, spleen and blood on PID 28. On PCD 7, compared to the non-vaccinated RB only and mock groups, both AttHRV and RB + AttHRV groups had significantly higher numbers of HRV-specific IgA and IgG ASC in the ileum. The two vaccinated groups also had significantly higher numbers of IgA ASC in the blood than the mock group. RB only group had significantly higher numbers of IgA ASC in the ileum and blood in comparison to the mock group. Together, these results demonstrated that RB down-regulated virus-specific IgA and IgG effector responses induced by the AttHRV vaccine at PID 28, but not memory B cell responses upon VirHRV challenge.

3.5.7 RB reduced serum HRV-specific IgA and IgG antibody responses to AttHRV

To further confirm the results that RB down-regulated HRV-specific IgA and IgG ASC response at PID 28, HRV-specific serum IgA and IgG antibody titers were determined by ELISA (Figure 6). Consistent with HRV-specific IgA ASC data, serum IgA titer is significantly lower on both PID 21 and PID 28, but with no significant difference on PCD 7 in the RB + AttHRV group in comparison to the AttHRV vaccine group. For both RB + AttHRV and AttHRV groups, HRV-specific serum IgG antibody titers were not significantly different on PID 21, PID 28 and PCD 7, although RB + AttHRV group had significantly higher IgG antibody titer on PID 10. RB only group had significantly lower virus-specific IgG antibody titer than the mock group on PCD 7.

3.5.8 RB increased HRV-specific IgA titer in the intestinal contents

Total immunoglobulins and HRV-specific antibody responses in the small intestinal contents (SIC) and large intestinal contents (LIC) were measured by ELISAs (Figure 7). RB did not significantly change the levels of total immunoglobulins (IgA, IgG and IgM) in the intestinal contents on PID 28 or PCD 7, except for the decreased total IgA titer in LIC on PID 28 compared to the control pigs on PID 28 (Fig. 7A). Important to note, HRV-specific IgA titers in both SIC and LIC of the RB + AttHRV pigs were higher at PID 28 and significantly higher at PCD 7 compared to the AttHRV pigs (Fig. 7B). These data demonstrated that RB can enhance the production of virus-specific IgA antibodies by intestinal memory B cells in the AttHRV-vaccinated pigs after VirHRV challenge, even though the numbers of virus-specific IgA ASC (Fig. 5) and the titers of virus-specific IgA antibody in serum before challenge were reduced (Fig. 6).

3.6 DISCUSSION

In this study, we examined the effects of RB supplementation on rotavirus infection and diarrhea, the total and virus-specific T and B cell responses, and isotype-specific antibody responses induced by the AttHRV vaccine using neonatal Gn pigs as a model system. We observed that 10% dietary RB supplementation to milk significantly protected against rotavirus diarrhea, but did not reduce rotavirus replication. RB also strongly promoted the development of IFN-γ producing T cells, IgM and IgA producing IgSC, total serum IgM, IgA and IgG antibody and HRV-specific intestinal IgA production, but significantly reduced HRV-specific IgA and IgG ASC in intestinal and systemic lymphoid tissues as well as HRV-specific serum IgA production at PID 28.

Rice bran alone reduced rotavirus diarrhea incidence and severity without reducing rotavirus shedding. Surprisingly, while RB and AttHRV vaccine synergistically and completely protected against rotavirus diarrhea, the protection of AttHRV vaccine against rotavirus shedding was reduced by RB. These results strongly suggest that mechanisms by which RB protects against rotavirus diarrhea is independent of rotavirus infection. The underlying mechanisms for rotavirus-induced diarrhea are currently not completely understood. The pathogenesis of rotavirus induced diarrhea has been reviewed (25, 26). Four distinct but nonexclusive mechanisms have been implicated: 1) Malabsorption due to the destruction of absorptive enterocytes in the villus, caused by rotavirus infection and increased intracellular [Ca^{2+}]; 2) NSP4 enterotoxin mediated increase in membrane permeability and tight junction disruption; 3) Increased secretion from the crypt cells and intestinal motility via stimulation of enteric nervous system by rotavirus or NSP4 enterotoxin;

and 4) Villus ischemia caused by unidentified vasoactive substances during rotavirus infection. RB could interfere with each of these four mechanisms. In fact, extracts from RB have been shown to be effective in reducing diarrhea through inhibition of the intestinal mucosal Cl^- ion secretion by intestinal epithelial cells (27, 28). This mechanism is likely to have contributed to the protective effects of RB against rotavirus induced diarrhea in the current study. It is also reported that zinc and enkephalinase inhibitors attenuate rotavirus-induced diarrhea (26). Certain RB phytochemicals might have functioned as such inhibitors. Further studies are underway to examine the effects of RB on the intestinal barrier integrity and permeability during rotavirus infection.

Both effector T and B cell responses play important roles during rotavirus infection and are associated with the protective efficacy of rotavirus vaccine against rotavirus infection and diarrhea (19, 23, 29). The significantly increased frequencies of IFN-γ producing CD4+ and CD8+ T cell responses in local (ileum) and systemic (spleen and blood) lymphoid tissues at both PID 28 and PCD 7 suggest that RB promoted the development of effector T cell responses. However, this effect was not due to the enhanced expansion of total Th and CTL cells, as RB did not significantly increase their frequencies among lymphocytes in both intestinal and lymphoid tissues.

Rice bran also significantly enhanced the development of total IgM IgSCs in ileum and spleen and IgA IgSCs in spleen and blood, and levels of total serum IgM, IgA and IgG antibodies pre- and postchallenge, as well as rotavirus-specific IgA antibody levels in intestinal contents after challenge, indicating the stimulatory effect of RB on the development of total and specific B cell responses to the AttHRV vaccine and VirHRV challenge. Similarly, a previous study showed that

the number of peripheral blood lymphocytes was significantly increased in Wistar male rats fed a 10% hemicellulose extracted from RB fiber (RBF) diet for 2 weeks (30). However, the significantly reduced numbers of rotavirus-specific IgA and IgG ASCs in both local and systemic tissues as well as the correspondingly lower levels of rotavirus-specific serum IgA and IgG antibody titers on PID 28 suggest that the immune-stimulatory effect (adjuvanticity) of RB are biased towards Th1 T cell responses before challenge and this effect is antigen-specific. Thus RB functioned as Th1 type immune response "food adjuvant" for the AttHRV vaccine. This observation is consistent with previous studies showing that RB feeding in mice up-regulated Th1 cytokines and down-regulated Th2 and antibody responses. The reduction in rotavirus-specific B cell and serum antibody response at challenge may have contributed to the increased fecal rotavirus shedding in RB + AttHRV treatment group over the AttHRV only treatment group. However, RB did not negatively affect the rotavirus-specific memory B cell responses and enhanced rotavirus-specific intestinal IgA antibody responses at PCD 7, suggesting that RB increased priming of local virus-specific B cells even under the Th1 biased condition before challenge. The molecular mechanisms and kinetics by which RB modulates T and B cell responses warrant further studies.

In summary, results from the current study demonstrated that RB significantly reduced the susceptibility to rotavirus diarrhea without reducing rotavirus shedding upon VirHRV challenge in Gn pigs compared to the control diet. Furthermore, RB promoted the development of intestinal and systemic IFN-γ producing CD4+ and CD8+ T cell responses, total IgM IgSC in ileum and spleen, total IgA IgSC in spleen and blood, as well as total serum IgM, IgA and IgG antibody production. Additionally, RB increased HRV-specific IgA titers in the intestinal contents postchallenge. RB alone also significantly increased the virus-specific IgA ASC response

postchallenge in ileum and blood. These results have significant clinical implications in the prevention and management of enteric pathogen-induced diarrhea using dietary RB in developing countries. Clinical trials should be conducted before RB is recommended for use alone and in combination with rotavirus vaccines (and other vaccines) to reduce diarrheal diseases and to improve human health.

3.7 ACKNOWLEDGEMENT

This work was supported by the Bill and Melinda Gates Foundation Grand Challenge Exploration in Global Health grant OPP1043255 E.R with G-6289 subcontract to L.Y. and a grant (R01AT004789) from the National Center of Complementary and Alternative Medicine, National Institutes of Health, Bethesda, MD to L.Y. We thank Dr. Marlice Vonck, Dr. Sherrie Clark-Deener, Pete Jobst, Andrea Pulliam, Kim Allen, and Shannon Viers for animal care. We thank Mariah Weiss for assistance in animal care and experiments, and Melissa Makris for assistance in flow cytometry. Dr. Anna McClung of the USDA-ARS Rice Research Unit (Stuttgart, AK) provided the heat stabilized RB used in this study. The AttHRV and VirHRV strains were from Dr. Linda J. Saif, The Ohio State University.

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Table 3.1 Clinical signs and rotavirus fecal shedding in Gn pigs after VirHRV challenge

Treatments	n	Clinical signs				Fecal virus shedding		
		% with diarrhea[*, a]	Mean days to onset[**]	Mean duration (days)[**]	Mean cumulative scores[**, b]	% shedding virus[*]	Mean days to onset[**]	Mean duration (days)[**]
RB+AttHRV	6	0^{C}	$8\ (0)^{A}$	$0\ (0)^{C}$	$4.4\ (1.2)^{C}$	100^{AB}	$2.0\ (0.5)^{B}$	$3.2\ (0.9)^{B}$
AttHRV only	12	67^{AB}	$4.4\ (0.8)^{B}$	$4.6\ (0.5)^{B}$	$9.8\ (1.4)^{B}$	50^{B}	$6.0\ (0.7)^{A}$	$1.3\ (0.2)^{C}$
RB only	5	20^{BC}	$7.2\ (0.8)^{AB}$	$0.2\ (0.2)^{C}$	$4.4\ (1.6)^{C}$	100^{AB}	$1.6\ (0.2)^{B}$	$6.2\ (0.2)^{A}$
Mock	9	100^{A}	$1.4\ (0.2)^{C}$	$5.6\ (0.3)^{A}$	$14.4\ (1.0)^{A}$	100^{A}	$2.0\ (0.3)^{B}$	$4.7\ (0.7)^{AB}$

[a]Pigs with daily fecal scores of ≥2 were considered diarrheic. Fecal consistency was scored as follows: 0, normal; 1, pasty; 2, semiliquid; and 3, liquid.

[b]Mean cumulative score calculation included all the pigs in each group.

[c]Standard error of the mean. In the groups where some but not all pigs had diarrhea or shedding, the onset of diarrhea or shedding for non-diarrheic/shed pigs were designated as 8 for calculating the mean days to onset.

[d]For days of diarrhea and virus shedding, if no diarrhea or virus shedding until the euthanasia day (PCD7), the duration days were recorded as 0.

*Fisher's exact test was used for comparisons. Different letters (A, B, C) indicate significant differences in protection rates among groups ($p < 0.05$), while shared letters indicate no significant difference.

**Kruskal-Wallis rank sum test was used for comparisons. Different letters (A, B, C) indicate significant differences in protection rates among groups ($p < 0.05$), while shared letters indicate no significant difference.

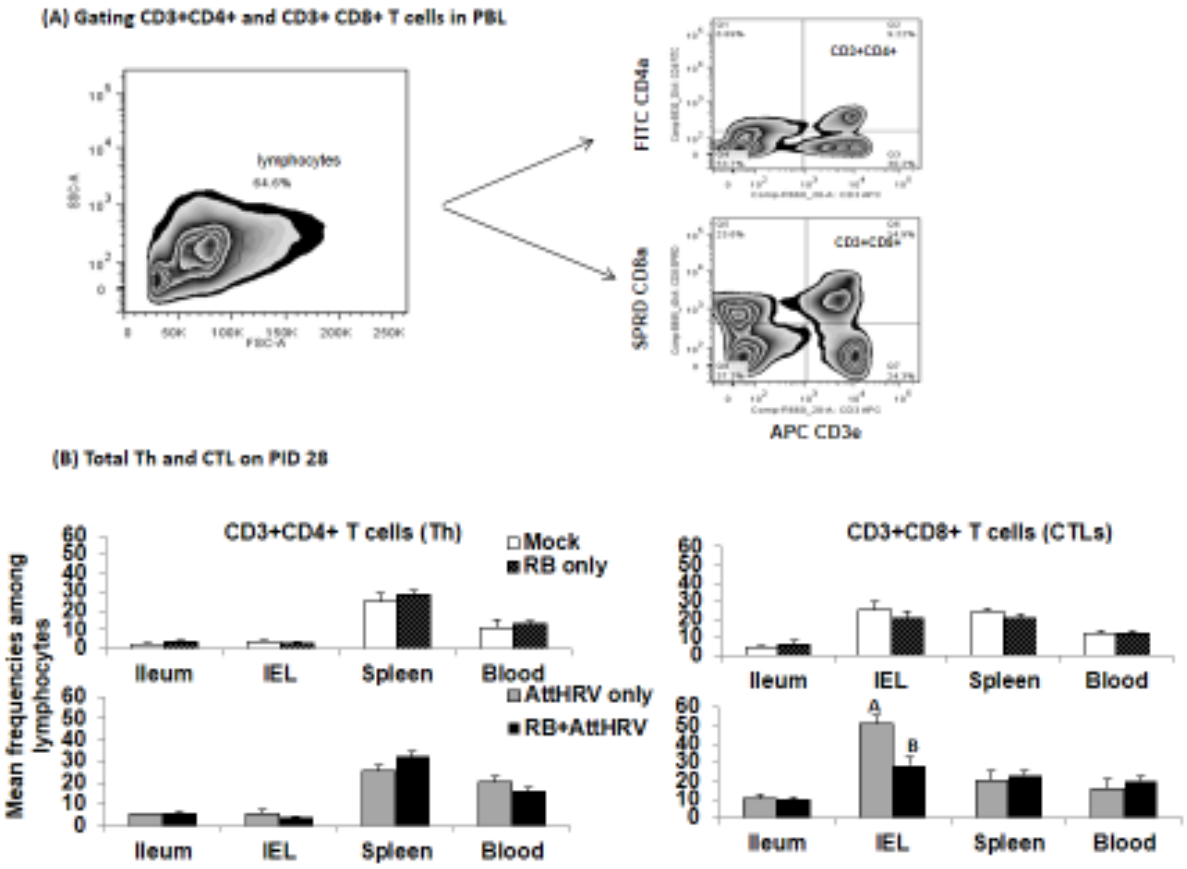

Figure 3.1 Total Th and CTL responses in control or vaccinated Gn pigs fed with or without RB supplemented diet. MNCs from Gn pigs in each treatment group euthanized on PID 28 were analyzed by flow cytometry. The MNCs were stimulated with semi-purified AttHRV antigen for 17 hrs *in vitro*. **(A).** Gating strategies for lymphocytes, CD3+CD4+ (Th) and CD3+CD8+ (CTL) cells. **(B).** Frequencies of CD3+CD4+ (Th) and CD3+CD8+ (CTL) cells among lymphocytes from each tissue were represented by frequencies of CD3+ CD4+ subset (left panel), or CD3+ CD8+ (right panel) T cell subset among lymphocytes. Numbers on the y-axis indicate the percentage of CD3+CD4+ or CD3+CD8+ T cells among lymphocytes in the respective tissues shown on the x-axis. Error bars indicate standard error of the mean. Different capital letters (A, B) indicate significant differences between groups, while shared letters indicate no significant difference (Kruskal-Wallis rank sum test, $p < 0.05$; $n = 3$-6).

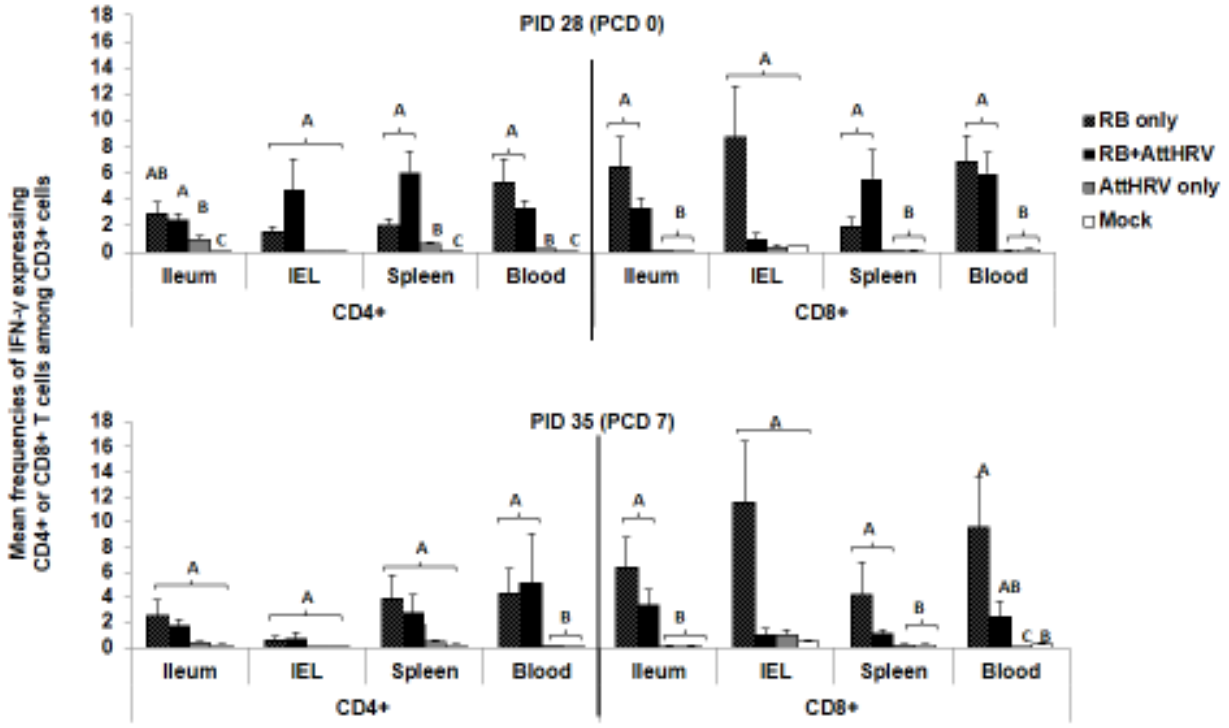

Figure 3.2 IFN-γ producing CD4+ and CD8+ T cell responses in control or vaccinated Gn pigs fed with or without RB supplemented diet. MNCs from Gn pigs in each treatment group euthanized on PID 28 (left panel) or PCD 7 (right panel) were analyzed by flow cytometry after the MNCs were stimulated with semi-purified AttHRV antigen for 17 hrs. Frequencies of IFN-γ producing T cells among total CD3+ cells from each tissue were represented by frequencies of IFN-γ+ CD4+ subset (Top panel), or IFN-γ+ CD8+ (bottom panel) T cell subset among CD3+ cells. Numbers on the y-axis indicate the percentage of IFN-γ producing CD4+ or CD8+ T cells among CD3+ cells in the respective tissues shown on the x-axis. Error bars indicate standard error of the mean. Different capital letters (A, B, C) indicate significant differences between groups ($p < 0.05$), while shared letters indicate no significant difference (Kruskal-Wallis rank sum test, $p<0.05$; n = 3-6 for PID 28 and n = 4-12 for PCD 7).

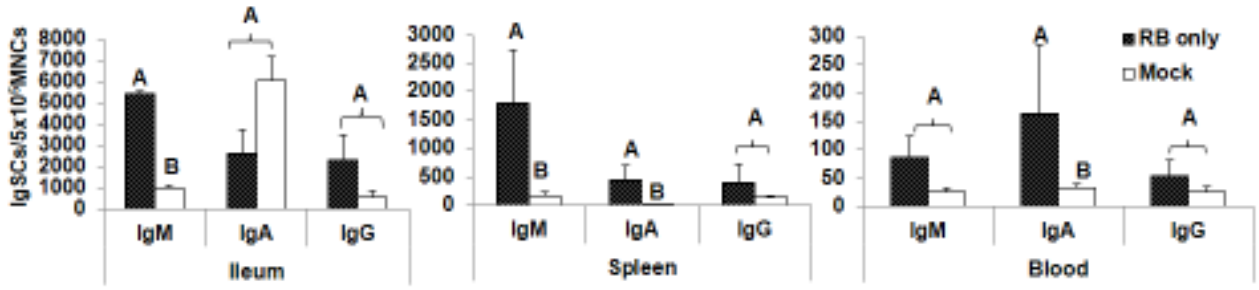

Figure 3.3 Mean numbers of total IgSCs in Gn pigs fed with or without RB supplemented diet for 28 days. MNCs from Gn pigs in RB only and mock groups euthanized on PID 28 (without AttHRV vaccine and HRV challenge), were analyzed by total IgSC ELISPOT. Numbers on the y-axis indicate the number of total IgM-, IgA-, or IgG- immunoglobulin secreting cells per 5 x 10^5 MNCs in the respective tissues shown on the x-axis. Error bars indicate standard error of the mean. Different capital letters (A, B) indicate significant differences between groups ($p < 0.05$), while shared letters indicate no significant difference (Kruskal-Wallis rank sum test, $p<0.05$; n = 3-4).

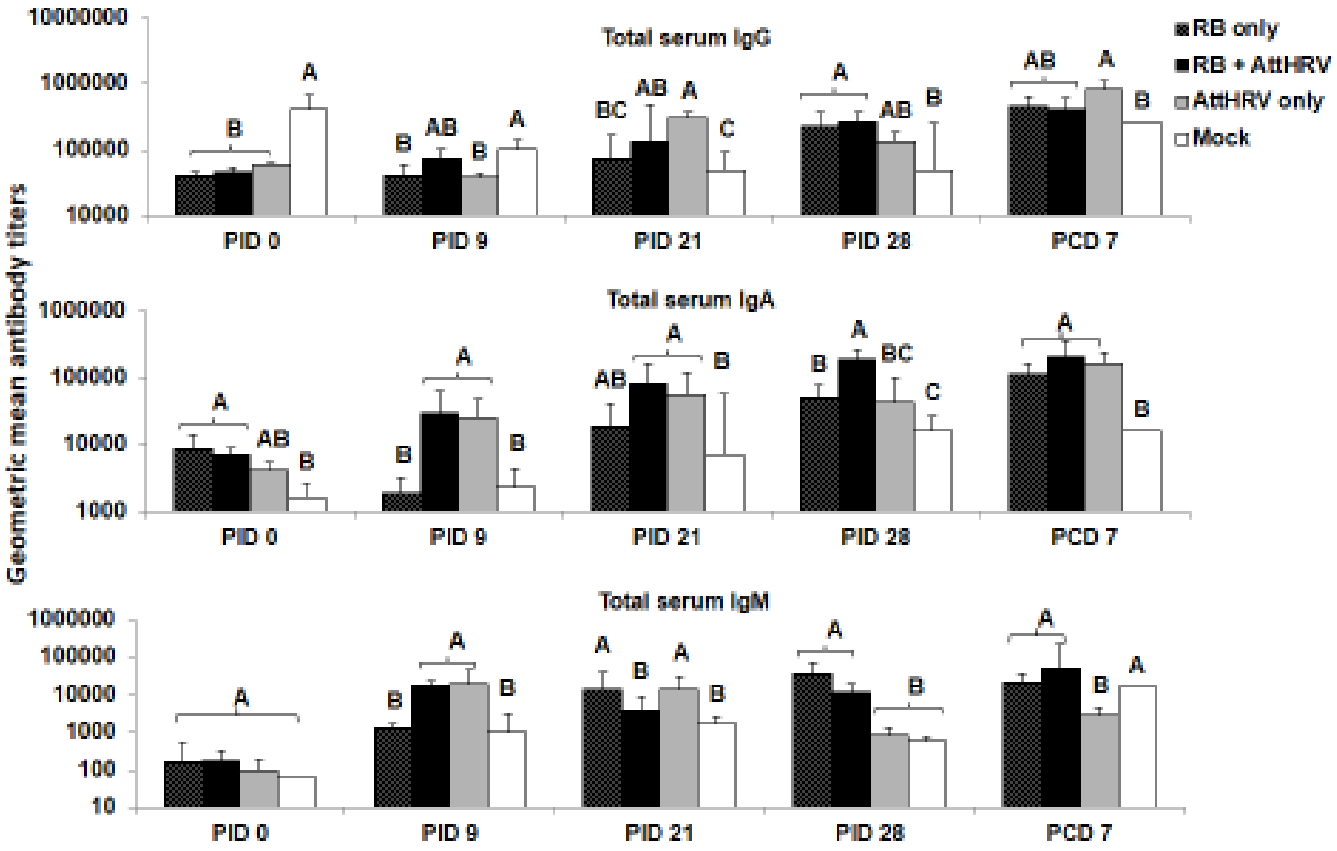

Figure 3.4 Total serum IgM, IgA and IgG antibody responses in control or vaccinated Gn pigs fed with or without RB supplemented diet. Serum antibody titers were measured by ELISA. Data on PID 0, 9, 21, 28 and PCD 7 are shown. Different capital letters (A, B, C) indicate significant difference among different treatment groups for the same time point and same isotype while shared letters indicate no significant difference (ANOVA - general linear model [GLM], $p<0.05$; n = 10-18).

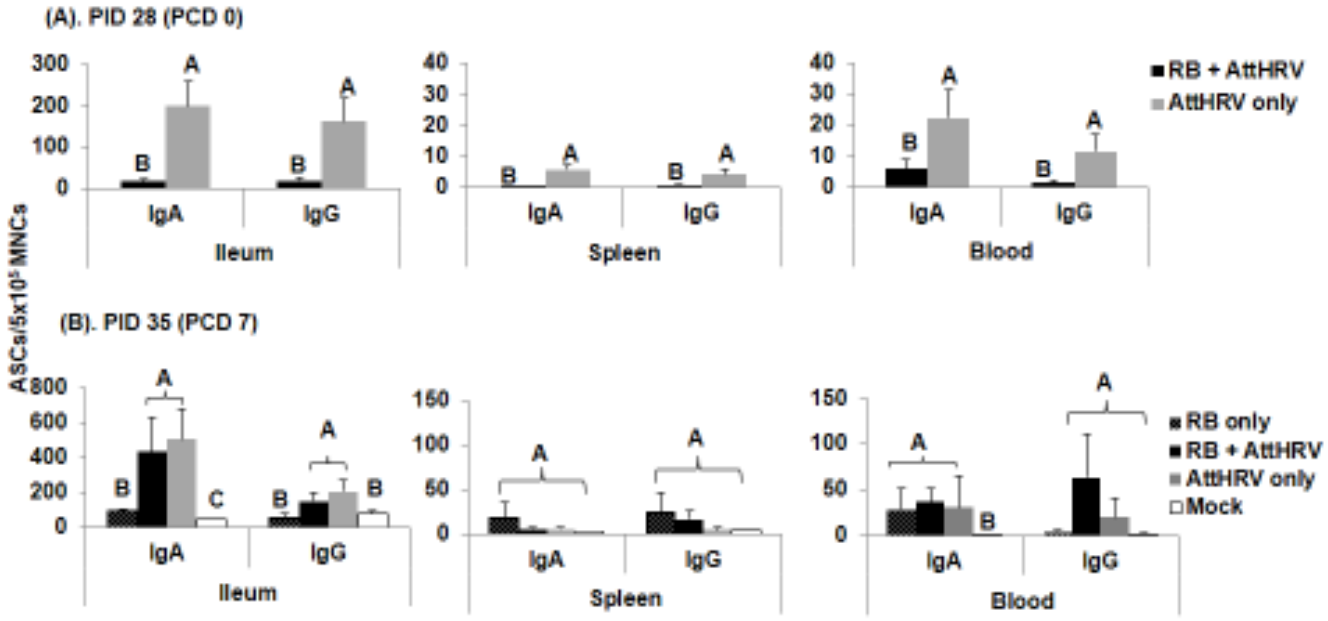

Figure 3.5 Mean numbers of HRV-specific ASCs in Gn pigs fed with or without RB supplemented diet at PID 28 and PCD 7. MNCs from Gn pigs in each treatment group euthanized on PID 28 (top figure) or PID 35/PCD 7 (bottom figure), were analyzed by HRV-specific ELISPOT. Numbers on the y-axis indicate the number of HRV-specific IgA or IgG antibody secreting cells per 5 x 10^5 MNCs in the respective tissues shown on the x-axis. HRV-specific ASC responses were not detected in any tissue for both RB only and Mock groups on PID 28, therefore they are not presented. Error bars indicate standard error of the mean. Different capital letters (A, B, C) indicate significant differences between groups, while shared letters indicate no significant difference (Kruskal-Wallis rank sum test, $p < 0.05$; $n = 4$-7).

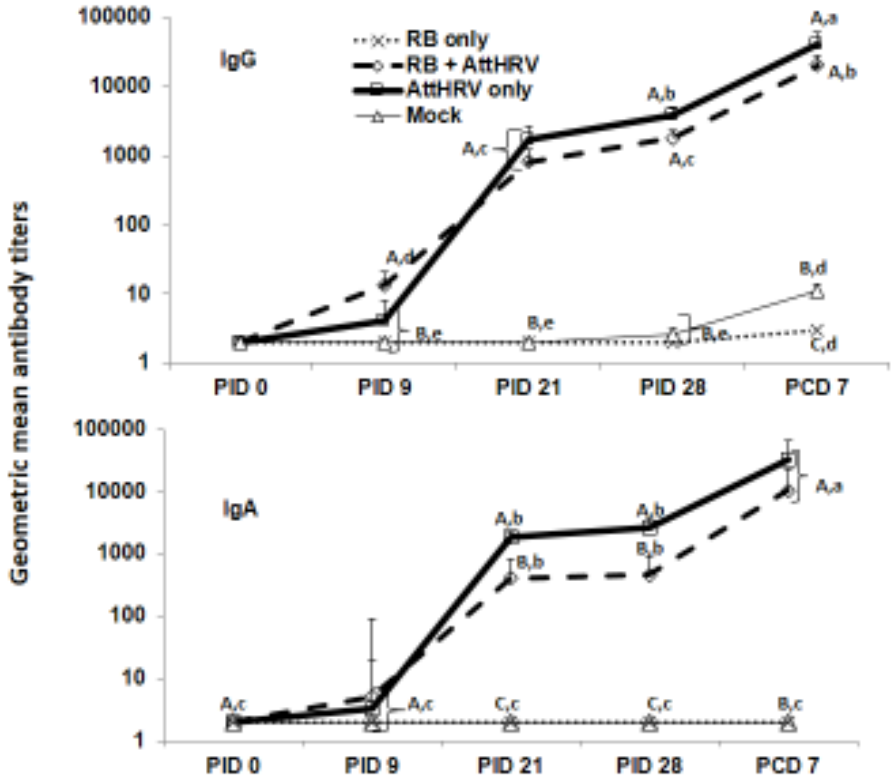

Figure 3.6 HRV-specific IgA and IgG antibody titers in serum of Gn pigs fed with or without RB supplemented diet. Antibody titers were measured by ELISA and are presented as geometric mean titers for each treatment group. Error bars indicate standard error of the mean. Different capital letters (A, B, C) indicate significant difference among different treatment groups for the same time point, while different lower case letters (a, b, c, d, e) indicate significant difference among different time points for the same treatment group. Shared uppercase or lowercase letters indicate no significant difference (ANOVA - general linear model [GLM], $p<0.05$; n = 10-18).

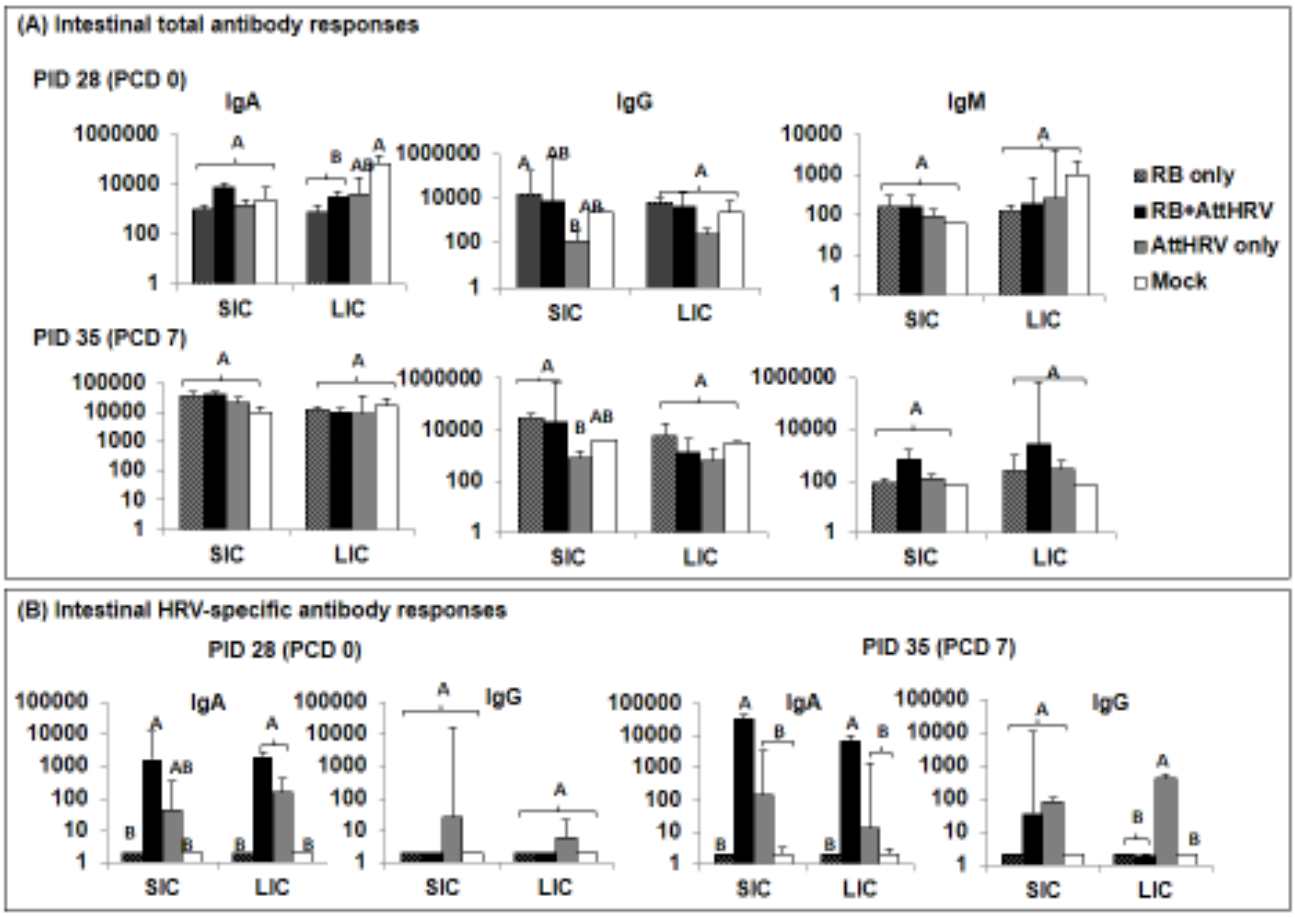

Figure 3.7 Total and HRV-specific IgA and IgG antibody titers in SIC and LIC of Gn pigs fed with or without RB supplemented diet. Antibody titers in intestinal contents were measured by ELISA and are presented as geometric mean titers for each treatment group. Error bars indicate standard error of the mean. Different capital letters (A, B) indicate significant difference among treatment groups for the same time point while shared letters indicate no significant difference (ANOVA – Turkey test, $p<0.05$; n = 3-6 for PID 28 and n = 4-12 for PCD 7).

Chapter 4

4.1 Combination of RB and probiotic LGG and EcN provides complete protection against rotavirus diarrhea via promoting gut epithelium health and enhancing resistance to perturbance in gnotobiotic pigs

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Manuscript in preparation

4.2 ABSTRACT

4.2.1 Abstract

Rice bran has been previously shown to promote the growth and colonization of *Lactobacillus spp.* and reduce the colonization by the diarrhea-causing enteric pathogen *Salmonella enterica* serovar Typhimurium in a mouse model. Our previous study indicated that RB alone can reduce HRV - induced diarrhea and enhance HRV vaccine efficacy in neonatal Gn pigs. In this study, we investigated dietary RB effects on the growth and colonization of diarrhea-reducing probiotic bacteria LGG and EcN, and the combination of RB and LGG and EcN on rotavirus diarrhea and gut permeability and health in the Gn pig. RB (mixed with the pigs' milk diet replacing 10% calorie intake) was given daily starting at post-partum day (PPD) 5 until the end of experiment. On PPD 3, 5, 7, pigs were inoculated with LGG and EcN (1:1 mixture, total 10^4 colony forming units (CFU)/dose) to initiate colonization. All the pigs were challenged with 1 x 10^5 ID_{50} of VirHRV Wa strain (G1P1A[8]) orally at PPD33. Diarrhea and virus shedding were monitored from post-challenge day (PCD) 1 to 7. RB completely prevented HRV diarrhea but did not significantly reduce HRV fecal shedding in LGG and EcN colonized pigs. RB significantly promoted intestinal colonization and growth of both the gram-positive LGG and gram-negative EcN (increased ~ 5 logs). RB also increased the pig body weight gain (1.5-fold higher) at 4 and 5 weeks of age compared to non-RB fed pigs. After HRV challenge, non-RB fed pigs had significantly increased mitotic index (due to the reparative process of the injured epithelium) and villus width (due to inflammatory cells and edema) in the ileum compared to RB fed pigs. Furthermore, RB prevented the increase of intestinal permeability induced by HRV challenge as indicated by the decreased concentration of Alpha-1 antitrypsin (A1AT), a gut inflammation and permeability marker in intestinal contents. All these effects may have contributed to the mechanisms for the protection

against rotavirus diarrhea. The combination of RB and probiotics LGG and EcN may represent a safe, effective and economically viable therapeutic approach against diarrhea-inducing enteric pathogens, such as rotavirus, norovirus, and *Salmonella enterica* serovar Typhimurium.

4.2.2 Keywords

Rice bran, LGG, HRV, diarrhea, gut permeability

4.3 INTRODUCTION

Human rotavirus is a segmented, double stranded RNA virus in the *Reoviridae* family. It is a leading cause of severe gastroenteritis among children less than 5 years old, resulting in 2 million hospitalizations and 450,000 deaths each year, mainly in developing countries (1, 2). Currently, there are two commercially available vaccines for HRV, Rotarix (RV1, GlaxoSmithKline, FDA approval in 2008) and RotaTeq (RV5, Merck, FDA approval in 2006). Both vaccines have contributed to the significantly reduced infection and diarrhea incidences in young children since their introduction. However, their efficacy is varied among different geographical locations and social-economic societies (>90% to 29%), with lower rate of protection in the developing countries (3). Next generation rotavirus vaccines, vaccine adjuvants and therapeutics will need to be developed and animal models such as the Gn pig, are essential in the evaluation of these intervention strategies. The Gn pig model of HRV infection and diarrhea has been used widely to study rotavirus infection and immunity (4, 5), vaccines (6-8) and vaccine adjuvants(9, 10) by us

and other laboratories in the rotavirus research community. Gnotobiotic pigs are considered to be the best pre-clinical animal model to study HRV infection and diarrhea, immune responses and evaluate vaccines and therapeutics.

Rice bran contains prebiotic compounds (11) and a variety of bioactive components (polyphenols, fatty acids and peptides) (12, 13) that have been shown to have protective effects against many human diseases such as cancer (14), obesity(15) and diabetes(16) and immune modulatory effects(17). Its therapeutic effects against enteric pathogen infections and diseases have also began to be studied in animal models (18-20). By feeding mice a 10% RB diet, it was shown that RB reduced the colonization and invasion of *Salmonella enterica* serovar Typhimurium into enterocytes and intestinal mucosa (20). RB was also shown to protect against enteric pathogen (human immunodeficiency virus) infection and disease through directly inhibiting enteric pathogen entry and replication (21). In another mouse study, 10% dietary RB feeding for 28 days resulted in increased production of mucosal and systemic IgA (22). The modulation of gut microbiota, such as 170- fold increase in the population of probiotic *Lactobacillus spp* and decreased colonization of mucin degrading microbes (phylum Verrucomicrobia), has been proposed as one of the possible mechanisms for RB to reduce the colonization and invasion of *Salmonella* bacteria (20). However, our previous study in Gn pigs demonstrated that dietary RB feeding (10% RB diet) for 28 days also significantly reduced HRV induced diarrhea, without decreasing HRV replication and shedding (18). The lack of microbiota in the Gn pigs indicates that the RB protective effects against HRV diarrhea can be independent from the RB prebiotics, and possibly due to its strong mucosal and systemic immune stimulatory activities (Th1 and IgA) and by promoting gut health (intestinal epithelium health, increased gut integrity and reduced gut

permeability). Therefore, RB may protect against enteric pathogen infections and diseases through multiple mechanisms, including direct anti-microbial activities, prebiotic effects, and promoting intestinal epithelial health and mucosal immune responses.

Lactobacillus rhamnosus GG is a gram-positive bacterium in the *L. rhamnosus* species that was first isolated in 1983 by Barry R. Goldin and Sherwood L. Gorbach (23). It is widely studied for treatment and prevention of gastrointestinal diseases and infections, and increasingly for extra-intestinal diseases as well, such as atopic dermatitis, allergic reactions, urogenital infections, and nasal pathogens (24). It has been shown to reduce the severity and duration of rotavirus diarrhea and persistent diarrhea in multiple clinical trials (25, 26). LGG has also been found to reduce intestinal permeability in children with irritable bowel syndrome (27). EcN is one of the best characterized probiotics. It is widely used to reduce acute and protracted diarrhea (28, 29), and has been shown to protect Gn pigs from lethal challenge by *Salmonella* Typhimurium (30). Given the above discussed effects of RB, LGG and EcN individually on rotavirus and *Salmonella* infection and diarrhea, studies to examine their combined therapeutic effects in the Gn pig model are warranted.

In this study, we hypothesized that RB can promote the growth of LGG or both LGG and EcN, enhance gut health, reduce gut permeability, and together with LGG and EcN, provide effective protection against HRV diarrhea and shedding. The objectives of this study are to evaluate: 1) the protective effects of the combined dietary RB and LGG + EcN feeding against HRV diarrhea and shedding; 2) the prebiotic effects of dietary RB on the growth and colonization of both gram-

positive LGG and gram-negative EcN probiotic strains, and 3) the effect of RB on intestinal health and permeability in LGG and EcN colonized Gn pigs.

4.4 MATERIALS AND METHODS

4.4.1 Experimental groups

Near-term pigs (Large White cross breed) were derived by hysterectomy and maintained in germ-free isolator units as described (31). Sterility checks were conducted every week by collecting rectal swabs and plating on blood agar plates and in thioglycollate media. Neonatal Gn pigs were randomly divided into four treatment groups: probiotics plus RB feeding (RB+LGG+EcN), RB only (RB only), probiotics only (LGG+EcN), and mock control (mock). Low dose LGG and EcN (10^4 CFU/dose each) was administered orally via a syringe to pigs at post-partum day (PPD) 3, 5 and 7 to initiate colonization. Heat-stabilized, gamma irradiated RB (Calrose variety) was added to the Gn pigs' milk diet (Ultra-high-temperature treated cow-milk) replacing 10% daily calories intake, starting at PPD 5 until the end of experiment. A subset of pigs from RB+LGG+EcN and LGG+EcN groups were euthanized before challenge on (PPD) 33 (n=6). The rest of the pigs were challenged orally with 10^5 FFU of the virulent Wa strain (G1P1A[8]) HRV on PPD 33 (PCD 0) and euthanized on post-challenge day 3 (PCD, n=6) or PCD 7 (n=6). The pigs were weighed weekly starting on PPD 5 until euthanasia. Rectal swabs were collected daily for monitoring of virus shedding by ELISA and CCIF from PCD 0 to 7. Rectal swabs were also collected daily for monitoring of diarrhea from PCD 0 to 7. Diarrhea scoring was conducted by different researchers independently and are defined as 0) solid, 1) pasty, 2) semi-liquid, and 3) liquid. Scores of 2 or higher are considered diarrheic. All animal experiments were conducted according to the protocols

approved by the Institutional Animal Care and Use Committee at Virginia Polytechnic Institute and State University.

4.4.2 Titration of HRV antigen and live virus shedding

To determine the effects of dietary RB and LGG and EcN feeding on the HRV shedding, rectal swabs were collected daily upon VirHRV challenge from PCD 0 to 7. Rectal swabs were processed by washing two swabs in 8ml Diluent #5 (MEM, 1% penicillin and streptomycin, 1% HEPES) and then centrifuged at 2100 rpm for 15 minutes at 4 °C. Supernatants were then aliquoted and stored at -20°C. Viral antigen and live viruses in the rectal swabs were then measured using ELISA and CCIF as previously described (32). Rectal swabs from mock treatment Gn pigs were used as negative controls.

4.4.3 LGG and EcN counting

Lactobacillus rhamnosus GG strain (ATCC 53103) and EcN (a gift from Dr. Jun Sun, Rush University, Chicago, IL) used in the current study were propagated in lactobacilli MRS broth (Weber, Hamilton, NJ) and Luria Broth (LB) media, respectively in our lab. Rectal swabs were collected on PPD 5, 10, 15, 26 and 33 for LGG and EcN counting. LGG was cultured anaerobically using BBL Gaspak jars (Fisher, Hanover Park, IL, USA) containing Anaerogen packs (BD) according to a protocol in a previous publication (33). For titration of LGG shedding, one fecal swab is washed in 4 ml 0.1% peptone water (10 fold dilution) and 10^2, 10^3, and 10^4 fold dilution series were then prepared. Then 0.1 ml of each dilution series was spread on a MRS agar plates

and incubated anaerobically in a 37 °C incubator for 24 hours. Plates with 20-200 bacteria colonies were then enumerated. The results are expressed as CFU/ml. For EcN counting, basically the same procedures were followed as for LGG, except for the replacement of 0.1% peptone water and MRS with LB media and plates were cultured aerobically (placed directly in the 37 °C incubator).

4.4.4 Ileum histopathological scoring

After euthanasia, sections of ileum were collected for histopathology examination. Samples were fixed in 4% paraformaldehyde, routinely processed for H&E staining and evaluated with light microscopy. The pathologist was blinded to identification of the animal until after microscopic analysis of all samples was complete. A histopathological scoring system for ileal samples was developed using guidance from previous publications (34-38). The mitotic index (MI) was obtained by dividing the total number of mitotic figures in 50 randomly selected crypts not associated with Peyer's patches by 50. Ten randomly selected ileal villi and crypts, not overlying Peyer's patches were measured for each sample to provide an average villus length and crypt depth. Villus length was measured from the tip of the villus to the junction with the crypt and crypt depth was measured from the junction with the villus to crypt base. Villus length to crypt depth ratio (V:C) was obtained by dividing the mean villus length by the mean crypt depth for each sample. V:C score was assigned as follows: **0** (normal), > or = 6:1; **1** (mild), 5.0-5.9:1; **2** (moderate), 4.0-4.9:1; **3** (marked), 3-3.9:1; **4** (severe), < 3:1. The mid-villus width of 10 random ileal villi, not overlying Peyer's patches were measured and averaged. The number of cells within the ileal lamina propria was given a subjective score ranging from 1+ to 4+.

4.4.5 Alpha-1 antitrypsin (A1AT) concentration measurement

After euthanasia, LIC samples were collected in cryovials and immediately frozen in liquid nitrogen until permeability analysis (measuring A1AT level) using a commercial porcine A1AT ELISA kit (BIOTANG Inc. Lexington, MA). Briefly, LIC samples were thawed. Then 0.1 ml samples were diluted in 0.2 ml sample dilution buffer, mixed and centrifuged at 2000 rpm for 15 minutes at 4 °C. Supernatant was collected and 0.1 ml of each sample was added in duplicate to wells of the plate. The ELISA was performed following exact instructions contained in the kit. OD values were measured at 405 nm within 30 minutes of adding stop solution. Final concentrations (μg/ml) were then obtained using the standard curve methods (polynomial with an order of 3).

4.4.6 Statistical Analysis

Kruskal-Wallis rank sum test was used for comparisons of virus shedding duration and titer, diarrhea duration and score, body weight change, LGG and EcN count, ileum histology score, and A1AT concentration. Fisher's exact test was used for comparisons of percentages of virus shedding and diarrhea. Statistical significance was assessed at $P<0.05$.

4.5 RESULTS

4.5.1 RB completely protected against rotavirus diarrhea in LGG and EcN colonized Gn pigs

The effects of RB on HRV induced diarrhea and virus shedding in LGG and EcN fed Gn pigs were assessed (Table 1). RB alone significantly protected against HRV diarrhea (80% protection rate) but did not reduce HRV shedding (as shown by the comparison between pigs in RB only group

and mock group). LGG+EcN alone also significantly reduced incidence of HRV diarrhea (50% protection rate), but significantly prolonged HRV shedding (as shown by the comparison between LGG+EcN group and mock group). RB+LGG+EcN completely protected against HRV diarrhea (100% protection rate), however, it did not significantly alter HRV shedding compared to the mock group. When comparing the LGG+EcN and the RB+LGG+ EcN pigs, the latter had no diarrhea and significantly reduced HRV shedding, with significantly delayed onset (1.2 versus 2.8 days), shortened mean duration (6.8 versus 5.2 days) and ~217-fold reduction in peak virus titer (1.3 x 10^5 versus 6 x 10^2 FFU/ml). The results suggest that although RB or LGG+EcN alone does not reduce virus shedding, the addition of RB to the diet of LGG+EcN colonized pigs can prevent the increase of HRV shedding in the LGG+EcN fed pigs.

4.5.2 RB significantly enhanced the growth and colonization of LGG and EcN

To determine whether dietary RB can promote the growth and colonization of probiotic bacteria LGG in Gn pigs, similar to its effects in mice (20), the titers of LGG and EcN were determined on specified time points following 3 low oral doses (10^4 CFU/dose) (Figure 1). RB significantly increased the growth of both gram-positive LGG and gram-negative EcN probiotic strains in Gn pigs. The RB fed pigs shed significantly higher counts of LGG (~ 10^4 increase) starting from post-feeding day (PFD) 7 through the entire monitored period until PFD 30. Similarly, RB also significantly enhanced the shedding of EcN (~10^5 increase) starting from PFD 2 through the entire monitored period until PFD 30. The peak titer for LGG shedding was 4 x 10^7 CFU and for EcN was 3 x 10^8 CFU. Together, these results showed that RB significantly enhanced the growth and

colonization of both LGG and EcN, with the effect on LGG growth manifested later and a slightly lower peak titer than that for EcN.

4.5.3 RB increased pig body weight gain

Growth rate is an important indication for the gut and overall health of the host. To monitor the effect of RB on the growth rate of Gn pig, weekly body weight gains were compared between the LGG+EcN and the RB+LGG+ EcN pig groups (Table 2). RB increased the weekly body weight gain in Gn pigs on the 4^{th} and 5^{th} week after RB feeding started, as shown by the mean body weight gain of 0.87 versus 0.63 kg on the 4^{th} week, and 0.80 versus 0.62 kg on the 5^{th} week for the RB+LGG+EcN versus LGG+EcN group, respectively. These results suggest that RB can promote the growth of Gn pigs after 4 weeks of RB feeding, rendering the hosts healthier and thus more resistant to HRV infections and diarrhea.

4.5.4 The combination of RB and LGG + EcN prevented epithelial damage from HRV challenge

To examine the effects of RB on the intestinal epithelium health in Gn pigs fed with LGG and EcN, H&E stained slides of the distal ileum were evaluated and scored via light microscopy. The parameters and scores for the different treatment groups at different time points are shown in Table 3 and Figure 2. Out of the all 7 parameters assessed, RB feeding significantly changed 3 parameters: mitotic index, villus width and the abundance of lamina propria cells (Figure 2). Mitosis of intestinal crypt cells is increased to replace the damaged intestinal epithelial cells following HRV infection in Gn pigs. Dietary RB maintained the level of mitotic index in LGG and

EcN colonized Gn pigs during VirHRV infection on PCD 3 and PCD 7, whereas LGG and EcN colonized Gn pigs without dietary RB feeding had significantly increased mitotic index on PCD 3 and PCD 7 compared to RB fed LGG and EcN colonized Gn pigs. Villus width is increased due to influx of immune cells and edema during rotavirus infection and inflammation. Dietary RB maintained the width of villus in ileum of LGG and EcN colonized Gn pigs during VirHRV infection on PCD 3 and PCD 7, whereas in the non- RB fed pigs, villus width increased significantly from PCD 0 to PCD 7 (Figure 3). Additionally, RB maintained the abundance of lamina propria cells in LGG and EcN colonized Gn pigs during VirHRV infection, whereas non-RB fed pigs had significantly reduced lamina propria cells on PCD 3 and PCD 7. Furthermore, RB prevented the decrease in villus length during HRV infection from PCD 0 to PCD 3 and PCD 7, although these differences are not statistically significant.

When compared at the same time point post VirHRV challenge between the two treatment groups, RB fed pigs have significantly lower mitotic index and narrower villus width, but higher or significantly higher abundance of lamina propria cells and longer villus length in the LGG and EcN colonized pigs, further supporting its protective effects against HRV induced ileum epithelial damage and inflammation, and its mucosal immune stimulatory effects. Therefore, RB not only protected against damage to intestinal epithelium, but also maintained the intestinal homeostasis (a balance of inflammation and immune response) in the face of HRV challenge.

4.5.5 RB reduced intestinal permeability during rotavirus infection

Serum level of A1AT is elevated significantly during the inflammatory response, and can be transported across the intestinal epithelial layer into intestinal lumen due to increased permeability

of the intestinal epithelial barrier. A1AT is protease resistant and highly stable in the intestinal contents. Therefore, it has been used as a marker for inflammation and intestinal permeability (39). In this study, we measured the A1AT level in LIC using ELISA (Figure 4). RB maintained the level of A1AT in LIC during VirHRV infection on PCD 3 and PCD 7, whereas in non- RB fed pigs, A1AT level increased on PCD 7. However, the changes are not statistically significant due to high variability. When compared between RB and non- RB fed Gn pigs colonized with LGG and EcN at the same time points, RB fed pigs had consistently lower levels (but not statistically significant) of A1AT in LIC on PCD 0, PCD 3 and PCD 7. Therefore, RB may help maintain the normal level of permeability in the ileum during the course of HRV infection.

4.6 DISCUSSION

In this study, using the Gn pig model of HRV infection and diarrhea, we have demonstrated that dietary RB provided complete protection against HRV diarrhea. The results also showed that dietary RB significantly enhanced the growth and colonization of both LGG and EcN in the intestine of Gn pigs, promoted body weight gain, protected against damage to intestinal epithelium while maintaining intestinal homeostasis (a balance of inflammation and immune response), and maintained intestine permeability during HRV infection in LGG and EcN colonized Gn pigs. Together, these results demonstrated that the combination of RB and LGG and EcN can provide complete protection against HRV diarrhea and pointed to its potential mechanisms.

This is the first study that has tested the therapeutic effects of combined RB and probiotic LGG and EcN against HRV diarrhea. While RB, LGG, or EcN individually can confer varying degrees

of protection against HRV diarrhea, this study showed that combining RB and the two diarrhea reducing probiotics can achieve complete protection against rotavirus diarrhea. We have previously showed that RB together with the oral AttHRV vaccine provided complete protection against HRV diarrhea in Gn pigs (18). The complete protection against rotavirus diarrhea is significant as no rotavirus vaccines or antiviral drugs have shown complete effectiveness. RB's effect on reducing diarrhea is not closely related to reducing HRV shedding. This non-specific therapeutic effect against diarrhea implies that the combination of RB and LGG and EcN could potentially be used to provide broad spectrum protection against diarrhea caused by various enteric pathogens and also diarrhea of unknown etiology. The optimal timing and dosage of RB and LGG and EcN may need to be further determined for each pathogen or type of diarrhea. Additionally, specific molecular mechanisms underlying this protection require further studies.

Despite their adjuvant effects on rotavirus vaccine and protection against rotavirus diarrhea (18, 40-43), neither RB alone nor LGG+EcN alone (Table 1) reduced HRV shedding in Gn pigs. LGG at both high dose and low dose did not reduce HRV shedding in our previously study (40). Thus, RB and LGG+EcN seem to have similar effects on HRV pathogenesis, reducing diarrhea without impacting virus replication and shedding. Interestingly, RB significantly reduced HRV shedding in the LGG and EcN colonized pigs, as shown by significantly delayed onset of shedding, mean duration days and lower peak shedding titers (Table 1). The mechanism for this phenomenon is unclear. It may possibly be due to the synergistic effects of RB with LGG and EcN. LGG is normally found in gut microbiota of human infants and young children (44). Therefore, RB, when used in humans, is likely to significantly reduce HRV shedding, in spite of its lack of such effect in germ-free pigs. LGG was found to significantly reduce rotavirus shedding in a conventional

mouse model (42). These results indicate that the combination of RB, LGG and EcN is promising to provide effective protection against HRV diarrhea and significant reduction in rotavirus shedding in conventional pigs and young children whose gut microbiota containing LGG. Further human clinical trials of this novel therapeutic combination against HRV diarrhea and shedding are warranted.

In this study, RB was demonstrated to increase the growth and colonization of both probiotic bacteria LGG and EcN up to 5 logs in Gn pigs. This result supports previous findings that RB feeding can increase the abundance of the beneficial gut bacteria *Lactobacillus spp*. in mice (22). As LGG is a gram-positive bacterium in the Firmicutes phylum and EcN is a gram-negative bacterium in the Proteobacteria phylum, these results suggest that RB can promote the growth of a variety of probiotic strains. This is not surprising given the complex composition of RB (12, 45). However, the growth rate and abundance achieved by different probiotic strains with RB may be different. It is important to take these differences into consideration when use RB and probiotics clinically.

Rice bran enhanced the growth of Gn pigs, indicating increased gut and overall health. It also promoted gut health by preventing epithelial damage (intestinal crypt cell mitosis) while maintaining the homeostasis of the mucosal immune system (maintained the number of lamina propria cells and villus width) during HRV infection in Gn pigs. During inflammation induced by HRV infection, intestinal permeability is increased, resulting in edema and diarrhea. RB components responsible for gut permeability may be a mechanism that have contributed to its

remarkable effects in preventing HRV induced diarrhea. Meanwhile, RB maintained the number of lamina propria cells, which are mainly lymphocytes (CD2+ and CD4+ T lymphocytes and sIgA secreting plasma cells) (46), suggesting its ability to stimulate the intestinal mucosal immune system during HRV infection in LGG and EcN colonized pigs. Previous studies in mice (22) and Gn pigs (18) indicated that RB alone or together with probiotic bacteria such as *Lactobacillus spp* can increase the production of mucosal and systemic total IgA by plasma cells. Thus, the increased lamina propria cells in the RB group pigs may be due to its immune-stimulatory effects on the mucosal immune system. Together, the anti-inflammatory and immune modulatory effects of RB and LGG and EcN promoted intestinal epithelial health and homeostasis, contributing to an intact intestinal barrier that is resistant to HRV diarrhea.

It is not known which components or specific compounds of RB contributed to the HRV diarrhea reducing activities. However, heat-resistant amylase, protease and hemicellulase treated rice fiber, which has significantly lower contents of protein, lipids and carbohydrates, has been shown to be able to prevent diarrhea in dextran sodium sulfate (DSS) - induced experimental colitis mouse models (11). This result suggests that dietary fiber portion of RB, such as cellulose, hemicellulose and lignin, plays an important role in decreasing diarrhea during inflammation bowel syndrome. In fact, arabinoxylan, a dietary fiber from RB, has been demonstrated to significantly decrease diarrhea score in irritable bowel syndrome adult patients through its anti-inflammatory and immune modulating activities (47). RB components promoting probiotic bacteria growth and colonization are likely to vary depending on the specific bacterial species. However, heat-resistant amylase, protease and hemicellulase -treated dietary fiber was unable to increase the shedding of *Lactobacillus spp* and *Bifidobacterium* (11), suggesting that carbohydrate or lipid components of

RB could be the main prebiotics for LGG and EcN in this study. A recent study in mice found that 10% RB oil diet significantly increased occupational ratio of *Lactobacillales* group of bacteria in the gut microbiota (48). Further studies are underway to identify the RB components that are responsible for its HRV diarrhea ameliorating properties and prebiotic properties.

Both LGG and RB are natural products, and have been demonstrated to have various health benefits and disease prevention and treatment effects in humans and animal models. LGG has been shown to be safe in all age and health groups, even in immune-compromised individuals (49). Given its natural colonization in the gastrointestinal systems, LGG has been studied extensively for its activities in treating gastrointestinal diseases and infections, such as diarrhea and enteric pathogens. However, this is the first study that showed the combined effects of RB and LGG+EcN in treating enteric pathogen infection and diseases. The results here indicated the synergistic effects of RB and LGG+EcN in preventing HRV diarrhea and reducing HRV shedding. Thus, the combination of RB and LGG+EcN could be a natural, safe and highly effective therapeutic against diarrhea and infection caused by HRV and other enteric pathogens in young children.

4.7 ACKNOWLEDGEMENT

This work was supported by the Bill and Melinda Gates Foundation Grand Challenge Exploration in Global Health grant OPP1043255 E.R with G-6289 subcontract to L.Y. and a grant (R01AT004789) from the National Center of Complementary and Alternative Medicine, National Institutes of Health, Bethesda, MD to L.Y. We thank Drs. Kelvin Pelzer, Sherrie Clark-Deener and Nicole Linstrome for their veterinary services for our Gn pigs throughout the study. We also

thank former and current TRACSS staff members Pete Jobst, Kim Allen, Andrea Pulliam, Julie Holdridge, and Sarah Grace Kenley, for their excellent animal care. Dr. Anna McClung of the USDA-ARS Rice Research Unit (Stuttgart, AK) provided the heat stabilized RB used in this study. The AttHRV and VirHRV strains were from Dr. Linda J. Saif at The Ohio State University.

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Table 4.1 RB protects against HRV diarrhea and shedding in LGG and EcN fed neonatal Gn pigs.

Treatments	N	Clinical signs				Fecal virus shedding (ELISA)			Fecal virus shedding (CCIF)
		% with diarrhea[*, a]	Mean days to onset[**]	Mean duration days[**]	Mean cumulative scores[**, b]	% shedding virus[*]	Mean days to onset[**]	Mean duration days[**]	Peak virus titer (FFU/ml)
RB+LGG+EcN	6	0^{B}	8 (0^{c})^{A}	0 (0)^{B}	6.2 (0.5)^{C}	100^{A}	2.8 (0.3)^{A}	5.2 (0.3)^{C}	$6.0x10^{2\ B}$
LGG+EcN	6	50^{B}	5.2 (1.3)^{A}	0.7 (0.3)^{B}	8.9 (0.6)^{B}	100^{A}	1.2 (0.2)^{B}	6.8 (0.2)^{A}	$1.3x10^{5\ A}$
RB only	5	20^{B}	7.2 (0.8)^{A}	0.2 (0.2)^{B}	4.4 (1.6)^{C}	100^{A}	1.6 (0.2)^{B}	6.2 (0.2)^{B}	ND
Mock	9	100^{A}	1.4 (0.2)^{B}	5.6 (0.3)^{A}	14.4 (1.0)^{A}	100^{A}	2.0 (0.3)^{AB}	4.7 (0.7)^{BC}	ND

[a]Pigs with daily fecal scores of ≥2 were considered diarrheic. Fecal consistency was scored as follows: 0, normal; 1, pasty; 2, semiliquid; and 3, liquid.

[b]Mean cumulative score calculation included all the pigs in each group.

[c]Standard error of the mean. In the groups where some but not all pigs had diarrhea or shedding, the onset of diarrhea or shedding for non-diarrheic/shed pigs were designated as 8 for calculating the mean days to onset.

[d]For days of diarrhea and virus shedding , if no diarrhea or virus shedding until the euthanasia day (Post challenge day 7), the duration days were recorded as 0.

[*]Fisher's exact test was used for comparisons. Different letters indicate significant differences in protection rates among groups ($p < 0.05$), while shared letters indicate no significant difference.

[**]Kruskal-Wallis rank sum test was used for comparisons. Different letters indicate significant differences in protection rates among groups ($p < 0.05$), while shared letters indicate no significant difference. (N=5-9) ELISA, enzyme-linked immunosorbent assay; CCIF, cell culture immunofluorescent assay; FFU, fluorescence forming unit; ND, not determined.

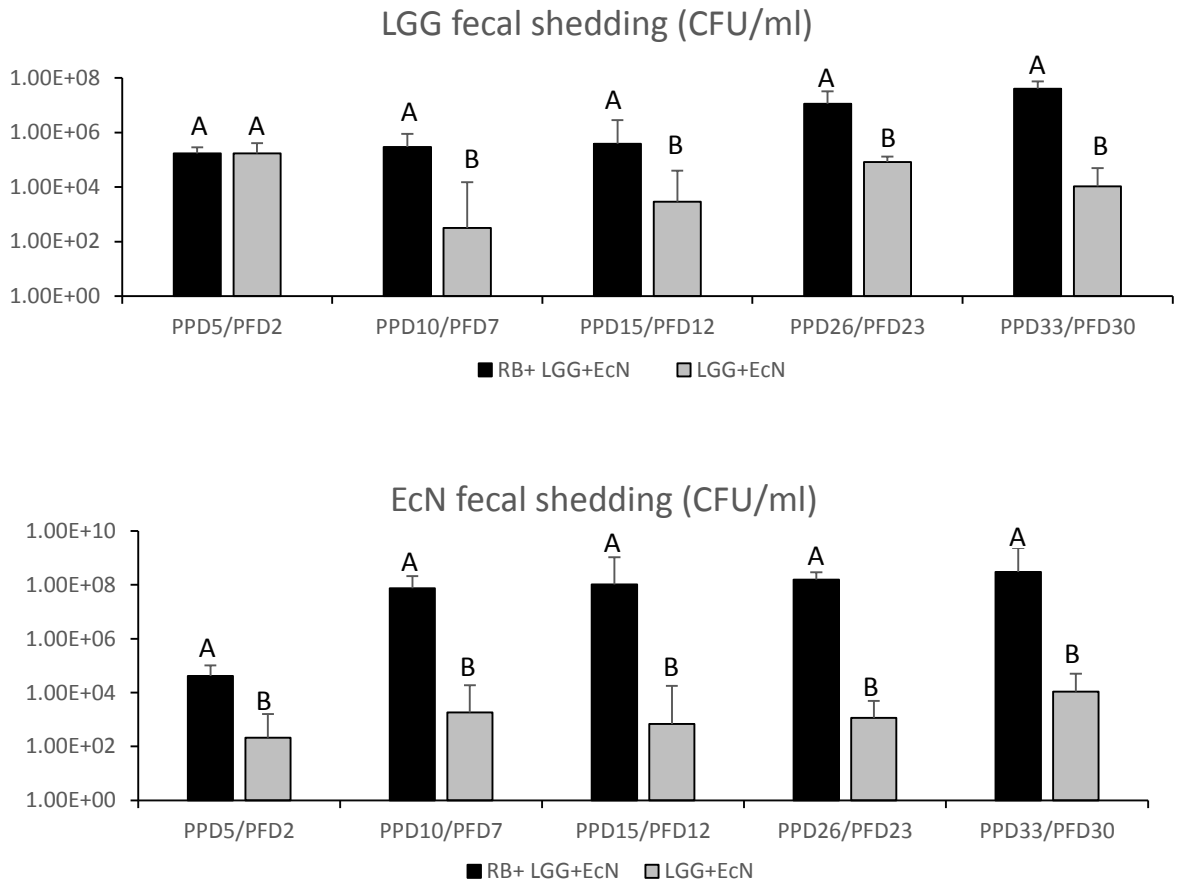

Figure 4.1 RB promotes the growth and colonization of the probiotics LGG and EcN in neonatal Gn pigs. Rectal swabs were diluted in 4 ml 10% peptone water (10 fold dilution) and additional 10 fold series dilutions from 10^2 to 10^4 were prepared and plated on LGG agar plates (LGG counting) or LB agar plates (EcN counting). The plates were incubated at 37^0C incubator for 3 days. Colonies on each plate are then counted and titers calculated. Geometric means of the counts in each group at the specified time points are presented. Error bars are standard error of mean. PPD, post-partum day; PFD, post probiotic feeding starting day. **Kruskal-Wallis rank sum test was used for comparisons. Different letters indicate significant differences in protection rates among groups ($p < 0.05$), while shared letters indicate no significant difference (n =10 -18).

Treatment group	n	PPD13-19	PPD20-26	PPD27-33 (PCD0)	PPD34-40 (PCD7)
RB+LGG+EcN	12	0.58 (0.09)^{A}	0.90 (0.08)^{A}	0.75 (0.11)^{A}	0.80 (0.08)^{A}
LGG:EcN	16	0.54 (0.06)^{A}	0.96 (0.05)^{A}	0.51 (0.05)^{B}	0.42 (0.17)^{A}

Table 4.2 RB enhanced the growth of Gn pigs from PPD27 to PPD33. RB (up to 10% of total caloric intake) was added to the Gn pigs' milk diet (ultra-high-temperature treated cow-milk) daily, starting at PPD 5 until the end of experiment. Weight gain over a specific period is calculated by subtracting the weight (in kilograms) at the beginning of a period from the weight at the end of the period. Mean weekly body weight gain of each treatment group is represented here. Number in the parenthesis is standard error of mean. Kruskal-Wallis rank sum test was used for comparisons. Different letters indicate significant differences in weight changes among groups (n=3-16; $p < 0.05$), while shared letters indicate no significant difference. PPD, post-partum day; PCD, post challenge day.

Treatments	Euthanasia day	n	MI	villus length mm	crypt depth mm	V:C	V:C score	villus width mm	lamina propria cells
RB+LGG+EcN	PCD 0	6	0.11 (0.01)	0.4 (0.02)	0.14 (0.01)	2.75 (0.14)	3.83 (0.17)	0.04 (0) ^{B}	1.58 (0.20)
	PCD 3	4	0.08 (0.01) ^{B}	0.4 (0.05)	0.13 (0.01)	2.98 (0.30)	3.5 (0.29)	0.04 (0) ^{B}	1.25 (0.25)
	PCD 7	6	0.11 (0.01) ^{B}	0.37 (0.02)	0.13 (0.01)	2.86 (0.17)	3.5 (0.22)	0.04 (0) ^{B}	1.75 (0.25) ^{A}
LGG+EcN	PCD 0	5	0.06 (0.02) ^{b}	0.37 (0.01)	0.14 (0.01)	2.73 (0.18)	3.6 (0.24)	0.05 (0) ^{Ab}	2.2 (0.20) ^{a}
	PCD 3	6	0.29(0.06) ^{Aa}	0.33(0.03)	0.14(0.00)	2.41(0.22)	4(0)	0.06(0) ^{Ab}	0.67(0.25) ^{b}
	PCD 7	6	0.30(0.02) ^{Aa}	0.30(0.03)	0.13(0.01)	2.75(0.23)	3.67(0.21)	0.07(0.01) $^{Aa.}$	0.83(0.21) ^{Bb}

Table 4.3 The effects of dietary RB on gut (ileum) health during HRV infection in Gn pigs colonized with LGG and EcN. After euthanasia, H & E stained sections of ileum from each pig were prepared and read by a veterinary pathologist blinded to the identity of

the animals. Mean values for each parameter is presented. Number in the parenthesis is standard error of mean. Kruskal-Wallis rank sum test was used for comparisons. Different upper case letters indicate significant differences among groups at the same time points; different lower case letters indicate significant differences among the time points within the same group ($p < 0.05$), while shared letters indicate no significant difference.

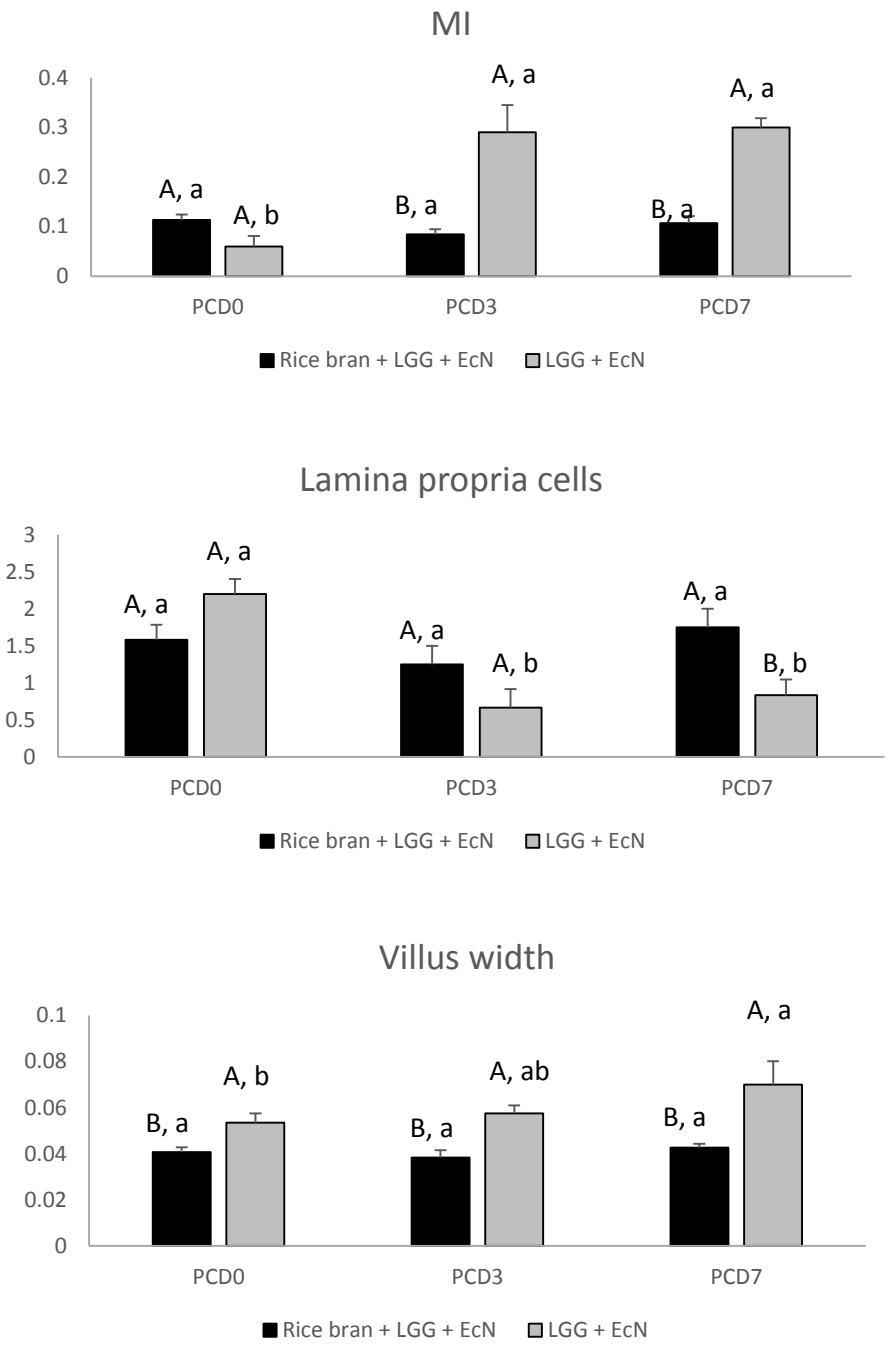

Figure 4.2 RB promoted intestinal health and maintained intestinal homeostasis in LGG and EcN colonized Gn pigs during HRV infection. Selected data from Table 3 are represented in the

figure. Kruskal-Wallis rank sum test was used for comparisons between different groups at the same time point (upper case letters) and between different time points for the same treatment group (lower case letters). Different letters indicate significant differences ($p < 0.05$), while shared letters indicate no significant difference (n= 4 - 6).

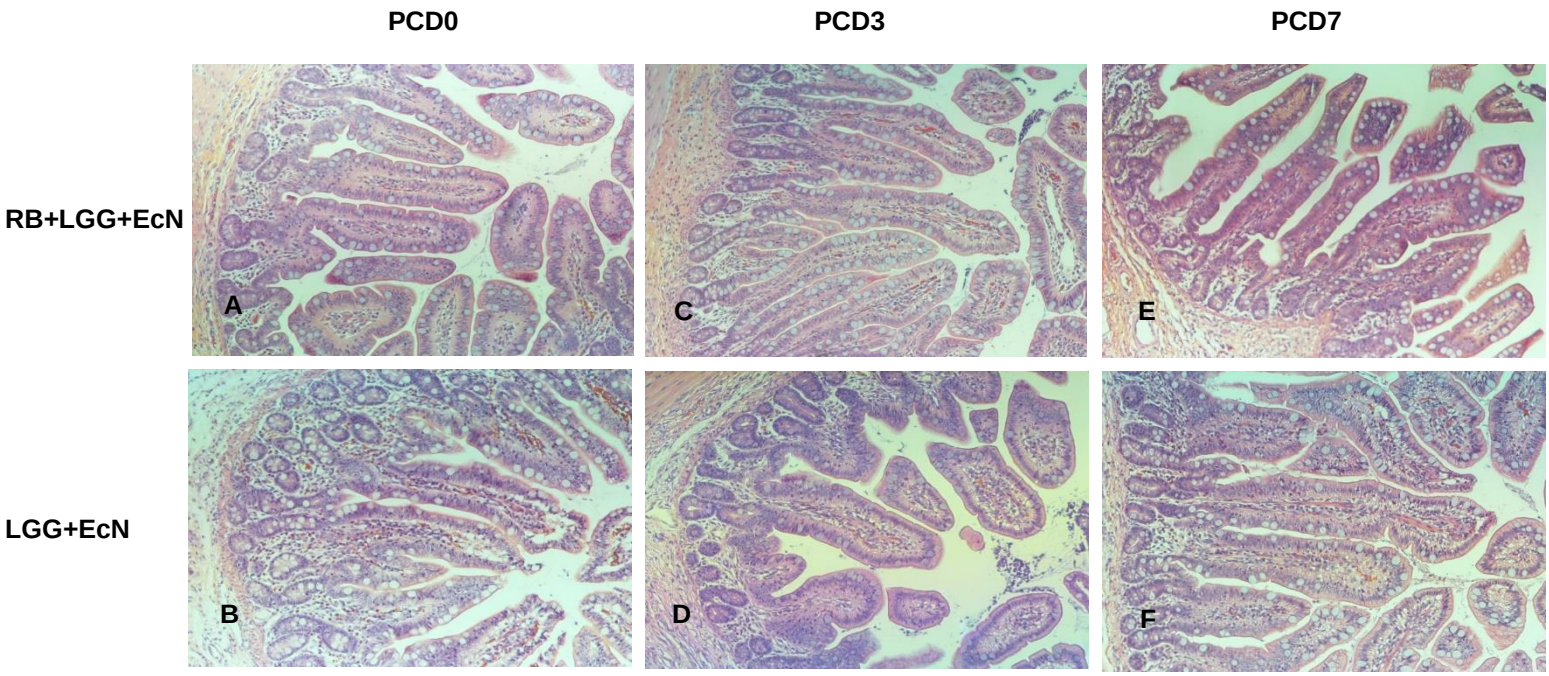

Figure 4.3 RB prevented the increase in villus width during HRV infection in Gn pigs colonized with LGG and EcN. Top panel are representative images for ileum sections of pigs in the RB+LGG+EcN group, whereas bottom panel shows the respresentative images for ileum sections of pigs in the LGG+EcN group. For each group, image for the non-infected pig on PCD0 is shown on the left (**A** and **B**), the images for the HRV infected pig on PCD3 and PCD7 are shown in the middle and on the right, respectively(**C**-**F**). On both PCD3 and PCD7, RB fed pigs have narrower or signficantly narrower villus width compared to the non- RB fed pigs, relfecting the reduced number of lanima propria immune cells and edema generated during HRV induced inflammatory responses. Images were taken at 100X magnificantion. H & E stain.

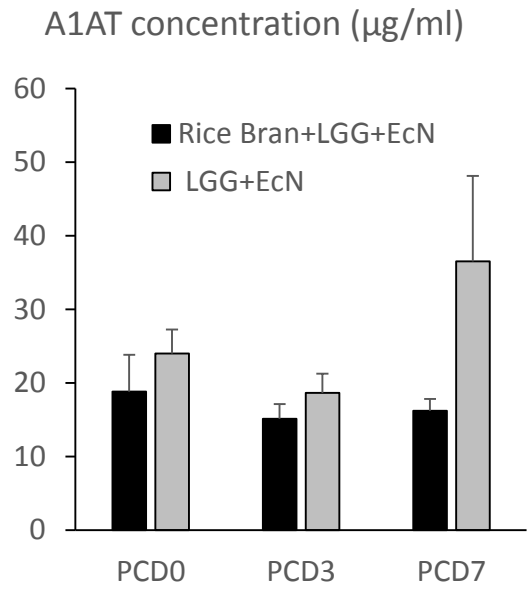

Figure 4.4 RB slightly reduced gut inflammation and permeability maker A1AT during HRV infection and diarrhea in Gn pigs colonized with LGG and EcN. LIC samples were collected in cryovials upon euthanasia and immediately frozen in liquid nitrogen until further analysis. Samples were diluted 3 fold before determination of the A1AT concentration with a commercial ELISA kit. Average value of duplicate for each sample was calculated first and then the means for all pigs in the same group at specific time point were calculated and are presented in the figure. Error bars indicate standard error of mean. Kruskal-Wallis rank sum test was used for comparisons and there were no statistically significant differences between treatment groups and among time points (n=4-6), though there was a trend for lower A1AT concentrations in the RB+LGG+EcN group.

Chapter 5

5.1 General Conclusions

5.2 GENERAL CONCLUSIONS

In the first phase of this study, we demonstrated that 5 day old neonatal Gn pigs can be infected orally or oronasally with non-pig adapted EV71 neurovirulent strain BJ110 (C4 genotype) isolated from a young child with neurological symptoms (1). Infection resulted in virus shedding pattern, clinical signs, pathology, and immune responses similar to those seen in human patients. Virus shedding in the fecal samples was detected from PID 1 to PID 18. Fever, one of the most common clinical signs in human patients, was induced in the combined oral-nasally infected pigs from PID 4 and PID 6. High titers of neutralizing serum antibodies against EV71, and strong IFN-γ producing CD4+ and CD8+ T cell responses were generated. Although no severe pathology was observed in tissues of intestines, respiratory and central nervous systems, notable respiratory and neurological signs were present in the infected neonatal Gn pigs, especially those infected though combined oral-nasal infection route. In particular, in contrast to other animal models for the EV71 infections in mice and monkeys, Gn pigs were infected through the natural route of infection, namely, oral route, by an original EV71 strain isolated from human patients and produced the clinical signs seen only in mice or monkeys infected through non-natural routes of infections (2), in immune-compromised animals (3), transgenic animals (4, 5), or using adapted virus strains (6, 7). Based on our results, we conclude that neonatal Gn pig model for EV71 represents an excellent alternative animal model to the current mice and monkey models for virus pathogenesis study and vaccine and antiviral drug development.

In the second phase of this study, we evaluated the effects of RB on the susceptibility to HRV infection and diarrhea, its immune-modulatory and adjuvant effects on the immunogenicity and protective efficacy of oral rotavirus vaccine AttHRV (8), as well as the combined therapeutic effect

of RB plus LGG and EcN for HRV infection and diarrhea and its associated mechanisms using the well-established Gn pig model for HRV infection and diarrhea. We found that RB strongly enhanced IFN- γ producing CD4+ and CD8+ T cell responses in intestinal (ileum) and systemic tissues (spleen and blood), total IgM- and IgA- ASCs in ileum, spleen and blood, total serum IgM, IgA and IgG antibody titers on PID28, HRV-specific ASCs in ileum and blood upon primary HRV challenge, and HRV-specific IgA antibody titers in small and large intestinal contents on both PID28 and PCD7. However, RB significantly decreased HRV-specific IgA and IgG ASCs in iluem, spleen and blood on PID28 and serum HRV-specific IgA and IgG antibody titers on PID21 and 28 and PCD7. As a result, RB increased the protective efficacy of oral rotavirus vaccine AttHRV against HRV diarrhea (67% vs 0% incidence rate, significantly delayed onset days 4.4 vs 8, mean duration days 4.6 vs 0, and mean cumulative score 9.8 vs 4.4), without increasing protection against HRV virus shedding. Our results also demonstrated that RB in combination with probiotics LGG and EcN completely protected against HRV diarrhea via promoting intestinal and overall health, including increased body weight gain, maintained villus width and length, crypt cell mitosis and lamina propria immune cell numbers during HRV infection. Additionally, we found that RB significantly promoted the growth and colonization of both LGG and EcN in Gn pigs. Furthermore, consistent with previous results (9-11), increased HRV replication and shedding was observed in LGG and EcN colonized Gn pigs. RB was able to prevent the increase in HRV replication and shedding in these pigs, mechanism of which is unclear, possibly due to the antiviral or gut immune stimulatory effects of RB, its components or fermentative products by LGG or EcN. Together, these results showed that RB has strong immune-stimulatory and adjuvant effects for increasing the immunogenicity and protective efficacy of HRV vaccine, and RB plus LGG and EcN is an effective therapeutic for HRV diarrhea.

In conclusion, successful development of the Gn pig model for EV71, and evaluation of RB as an oral rotavirus vaccine adjuvant as well as therapeutics for HRV diarrhea using neonatal Gn pigs further highlight the value and robustness of neonatal Gn pigs in the study of infection, immunity, vaccine and therapeutics for human enteric viruses. It is expected that neonatal Gn pigs will be more widely applied in the study of infection, pathogenesis and immunity for established and emerging human enteric viruses of significant public health importance, such as HRV, norovirus, and EV71. Despite its limitations mentioned above, neonatal Gn pig models for human enteric viruses will likely to be an invaluable tool in the development of vaccines and therapeutics for this important group of human viruses.

5.3 FUTURE DIRECTIONS

The Gn pig model for EV71 infection and immunity developed here represents a novel and alternative animal model to the current mouse and non-human primate models. This is also the only Gn animal model developed so far for this virus. The fact that neonatal Gn pigs were productively infected with the non-adapted human neuro-virulent strain BJ110 orally and oral-nasally, and displayed virus shedding and tissue distribution pattern, clinical signs (HFMD, neurological and respiratory signs), histological lesions and adaptive immune responses similar to those seen in severely infected human patients are very promising and suggest that this model could change current situation of limited animal models and significantly contribute to our understanding of viral pathogenesis and development of new vaccines and antiviral drugs. Future directions using this Gn pig model include: 1). Further infectivity (e.g. ID50 study in 5 and 33 day-old Gn pigs) and pathogenesis study of the BJ110 strain in Gn pigs; 2). Evaluation of EV71

vaccines, in particular attenuated BJ110 strain as a live vaccine candidate, and 3). Evaluation of antiviral drugs for EV71.

The immune-modulatory and adjuvant effects of RB on the immunogenicity and protective efficacy of oral rotavirus vaccines, and highly effective therapeutic effect of RB plus LGG and EcN for HRV diarrhea, are two very promising approaches to the problems of low and varied protective efficacy of current rotavirus vaccines and significant morbidity and mortality caused by HRV gastroenteritis in developing countries. Further studying and development of RB as vaccine adjuvant for vaccine against rotavirus and other enteric viruses, and RB plus LGG and EcN as therapeutics for HRV diarrhea and promoting intestinal health should be pursued. Some of the currently ongoing and future directions are: 1). Identifying the components of RB that are important for its adjuvant effects; 2). Studying the effect of RB for vaccines against other enteroviruses, such as norovirus and EV71; 3). Studying the combined adjuvant effects of RB and immune-modulatory probiotic strains such as LGG (12-15) and LA (16-19) for rotavirus vaccine; 4). Further testing and development of RB plus LGG and EcN as a broad-spectrum therapeutics against diarrhea caused by enteric pathogens and other etiologies, such as HRV, norovirus, *Salmonella*, traveler's diarrhea, irritable bowel syndrome, and inflammatory bowel diseases in the Gn pig model and human clinical trials.

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