Pathogenicity and diagnostics of non-group A porcine rotaviruses

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Pathogenicity and diagnostics of non-group A porcine rotaviruses

by

Hai Thanh Hoang

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

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The student author and the program of study committee are solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University
Ames, Iowa
2017

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DEDICATION

To

my parents, wife and daughters

for their unconditioned love and support.
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ABSTRACT

Rotavirus-associated diarrhea is a common enteric disease in piglets. Group A, B, and C rotaviruses have been implicated in US swine. While group A rotaviruses have been widely studied and attributed as a major cause of the disease, little known about group B and C rotaviruses with respect to their pathogenicity/pathogenesis as well as diagnostics and prevention/control. To address such shortfalls, three studies were carried out.

The objective of the first study was producing monoclonal antibodies (MAbs) against PoRV A, B, and C. To that goal, full-length VP6 protein gene of each serogroup was cloned from feces positive for respective virus and expressed in a baculovirus system using Bac-to-Bac cloning and expression kits. The recombinant proteins, purified in their native conditions, were used to immunize mice. A VP6-based ELISA and an indirect fluorescent antibody test using Sf9 cells expressing VP6 of PoRV A, B or C were used to screen hybridomas. The protein specificity of selected MAbs were further verified by Western immunoblot, and the isotype of each MAb was determined using a commercial murine antibody isotyping kit. Based on all these evaluations, MAb 10A11, 10B1 and 11H3, which were of IgG isotype, were selected for PoRV A and C, respectively. The MAbs specific for PoRV A and C were proven to be useful for immunohistochemical staining to detect these viruses in formalin-fixed intestinal tissues, which can aid more accurate diagnostic investigation of rotavirus-associated diarrhea.

The second study was to compare the pathogenicity of porcine rotavirus (PoRV) A, B and C individually or in combinations in immunologically naïve newborn piglets. Forty-eight one-day-old Cesarean-Derived Colostrum-Deprived (C CDC) pigs were divided into eight groups. Pigs in each group were challenged with rotaviruses that belong to individual group A, B, C or all combinations. Clinical signs including diarrhea were recorded every 12 hpi. Rectal swabs
were obtained before inoculation and then every 12 hpi. Half of the pigs in each group were euthanized at 24 hours post inoculation (hpi) and the rest at 72 hpi. Intestinal contents were collected at necropsy. The swabs and contents were tested by RT-PCR for virus shedding. Multiple parts of the intestine (duodenum, proximal jejunum, mid jejunum, distal jejunum, and ileum) were collected for histopathology. There was no remarkable difference in the occurrence and appearance of diarrhea caused by PoRV A, B, and C or combinations. The onset of PoRV B and C fecal shedding was earlier than that of PoRV A. All viruses were able to cause atrophic enteritis. Although the lesions caused by PoRV A were more prevalent in mid to distal jejunum, the antigen was found throughout of small intestine. In contrast, PoRV B and C caused atrophic enteritis through the entire small intestine with detection of corresponding viral nucleic acid or antigen, respectively. This study showed that all rotavirus groups were equally pathogenic to immunologically naïve neonates but may vary in their replication patterns in intestine.

The third study was conducted to establish an in vitro method to isolate and propagate non-group A rotaviruses since a suitable virus isolation method was not available. Two IPEC cell lines (IPEC-1 and IPEC-J2), which are epithelial cells originated from small intestines of neonate pigs, were adopted to virus isolation test. Virus isolations were attempted on fecal samples collected from neonatal piglets experimentally inoculated with PoRV B or C. By applying a roller culture technique with a high concentration of trypsin in maintenance media, a PoRV C strain designated IA/2015 (G6P[5]I5) was successfully isolated and sustained at a high titer in IPEC-1 cells, but not IPEC-2, while PRV B could not be isolated. The isolate and newly established virus isolation method can be useful for further studies of PoRV C, diagnostic assay development as well as for vaccine production.
CHAPTER 1. GENERAL INTRODUCTION

Dissertation organization

My dissertation contains an abstract, a literature review, three research chapters, a general conclusion and an acknowledgement. Chapter 1, literature review, presents the overviews of porcine rotavirus in term of classification, epidemiology, immunity, pathogenicity, clinical signs, diagnosis, and prevention. Chapter 2, 3, and 4, are research papers which have been prepared for publication. Chapter 5 provides the general conclusions of the studies.

GENERAL OVERVIEW OF PORCINE ROTAVIRUS AND ITS INFECTION IN SWINE

I. The virus

1.1 Taxonomy and classification

Porcine rotavirus (PoRV) belongs to the genus Rotavirus in the family Reoviridae. Rotaviruses (RVs) are divided into ten groups (A-J) base on the antigenicity or the sequence of VP6, one of the capsid proteins (1, 2, 3, 4). Of these groups, groups A, B, C, E, and H have been reported to cause disease in pigs (5, 6). In each group, rotaviruses are further classified into P and G serotypes and genotypes based on VP4 (protease sensitive protein) and VP7 (glycoprotein), respectively, which are outer capsid proteins (7). For serogroup A rotaviruses, serotypes are designated by neutralization assays in which viruses having less than “20-fold difference between homologous and heterologous reciprocal neutralizing antibody titers” are grouped into the same serotype (8). On the other hand, genotypes are designated by comparing virus sequences. In the case of rotavirus A (RVA), viruses sharing ≥80% nucleotide identity of VP4 are considered to be in the same P genotype while 80% nucleotide identity of VP7 is also used as cut-off for G typing (9). Because VP4 and VP7 stimulate neutralizing antibody response and the
homotypic immune response is important in protection, it is imperative to identify G and P type of each rotavirus strain. Serotypes are indicated by the number right after G or P (e.g., G1P1) whereas genotype is expressed by the number in the square bracket after G or P (e.g., G1P[1]). The G genotypes and serotypes are coherent and interrelated. Due to lack of P-type specific monoclonal antibodies, the relation between P genotypes and serotypes have not been well determined. Geographic distribution and prevalence of G and P types of porcine rotavirus A (PoRV A) is shown in Table 1. For the more precise classification of RVs in case of reassortants, a classification system which uses nucleotide sequence comparisons and phylogenetic analyses of all 11 viral genomic RNA segments has been suggested for group A rotaviruses (9, 10). The notation Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx is used for the VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6 genes, respectively, which is further explained in Table 2. Such a systemic classification using the entire 11 genome segments is not available for non-group A rotaviruses.

For porcine rotavirus B (PoRV B), limited sequence information is available. Only NSP2 (11, 12), NSP5 (12), and VP7 (12-14), and VP6 (15) have been characterized. The identified G types to date are summarized in Table 3. Although there is no consensual cut-off for G genotyping of group B porcine rotaviruses, ≥76% and 79% identity for nucleotide and amino acid sequence respectively (13) or ≥80% nucleotide identity (15) have been used as cut-off. For porcine rotavirus C (PoRV C), there have been a growing number of reports of virus molecular characterization since 2013. The G and P types identified for PoRV C to date are summarized in Table 4. For G types, the cut-off for grouping is ≥85% identity of nucleotide sequence for VP7 (16, 17). For P typing, ≥80% (18) or ≥83% (19) nucleotide identity of VP4 sequence has been used. It appeared that G6 P[5] has been the dominant genotype among PoRV C in Japan (18) and
Brazil (20), while the dominant P and G genotype combination of PoRV C in US swine is not known.

1.2 Genome and morphology

The rotavirus genome has 11 segments of double-stranded RNA which encode six structure proteins (VP1,2,3,4,6,7) and six nonstructural proteins (NSP1,2,3,4,5,6) (7), shown in Table 5. Migration patterns of rotavirus RNA segments on a gel known as RNA electropherotypes, which can be visualized by polyacrylamide electrophoresis (PAGE), are unique for each of rotavirus groups A, B, C, and D (21). There is no information about electropherotype of rotavirus group E and H which have also been detected in pigs. Because electropherotypes have not been adequate for the identification of groups or types (21), PCR-based assays or sequencing have become a common tool for grouping or typing. As such, PAGE-based electrophenotyping is no longer very useful in characterizing rotaviruses.

Regardless of groups, rotaviruses have the same morphology (22), that is a wheel-shape, non-enveloped icosahedral virion (Figure 1). The size of rotaviruses is comparable between group A, B, and C, ranging from 65 nm to 80 nm in diameter. The mature virion has three layers of the capsid making a double-shelled capsid (Figure 2). The most inner layer consists of 120 VP2 units which are organized in a T=1 symmetry and is constituted by 12 decamers. A decamer is built by five VP2 dimers (i.e., VP2A and VP2B) around a five-fold symmetry axis. The core layer acts as a cage for the viral RNA-dependent RNA polymerase (VP1), the guanylyltransferase and methylase (VP3) and the viral genome (23). The middle layer consists of 260 trimers of VP6 for group A (24), whereas that of group C contains 240 trimers of VP6 (25). There is no such information regarding VP6 of group B, D and E. VP6 has the biggest mass among the rotaviral proteins and helps to stabilize the capsid by interacting with VP7 on the top
and VP2 on the bottom. The stabilized capsid is important for virus entry as well as virus replication inside the infected cell. VP6 forms double-layered particles with VP2 which serve as a place for the initial viral gene transcription (See details under 1.3). The most outer layer comprises of 60 spikes of trimeric VP4 and 260 trimers of VP7(26), which are crucial for virus attachment and entry into target cells. There are 132 channels connecting the core with the outer layer, which is a distinctive feature of rotavirus virion.

1.3 Replication in cells

The replication of rotavirus is not fully understood. The entry of rotavirus to the cells is a multistep process, which requires VP7 and the proteolytic cleavage of VP4 into VP5 and VP8. The VP8 first binds to cell receptors such as histo-blood group antigen (27) or gangliosides containing sialic acid (28). VP5 and VP7 then interact with several co-receptors such as integrin (α2β1, αvβ3, αxβ2, α4β1) and/or heat shock cognate protein (hsc70). All the receptors may be localized in a close proximity of lipid rafts which serve as a platform for the interaction between rotavirus and cellular receptors (29).

The internalization of rotavirus has been controversial. Different rotavirus strains may have different entry pathways. The rotavirus is known to enter cells through receptor-mediated endocytosis (30). The OSU strain of PoRV A was shown to enter MA-104 cells, African green monkey kidney cell line, by receptor-mediated endocytosis (30). The coordination of VP4 and VP7 in receptor binding helps the virus disrupt the endosomal membrane to be released to the cytoplasm. In this process, due to low intracellular calcium concentration, the outer layer of the virus is removed to form double-layered particles (DLP) of VP6 and VP2. Besides receptor-mediated endocytosis, it has also been demonstrated that the virus may enter cells by direct penetration because some lysosomotropic agents (ammonium chloride, chloquine, methylamine,
amantadine and dansylcadaverine) and endocytosis inhibitor (sodium azide and dinitrophenol), which have been known to interfere with receptor-mediated endocytosis pathway of viruses, could not prevent virus entry to the cells (31, 32).

Rotaviruses are known to carry their own replicating complexes which bind to the inner side of VP2. The replicating complexes contain VP1 (RNA-dependent RNA polymerase) and VP3 (guanylyltransferase and methylase). The viral positive-sense single-stranded RNAs, which are capped and non-polyadenylated, are transcribed in DLP then released into cytoplasm through channels. The transcribed RNAs are then used for either viral protein production (i.e., translation) or virus genome replication.

Interactions of NSP2 and NSP5 result in the formation of cytoplasmic inclusion bodies named viroplasm that attracts VP1, VP2, VP3, and VP6. In the viroplasm, the assembly of these structure proteins forms DLPs. The initial assembly complexes are formed by the binding of VP2 and VP1/VP3. Positive-sense RNAs are then incorporated into these complexes with the help of NSP2 for negative-sense RNA synthesis and formation of double-stranded RNAs. The virion cores are formed as a result of the assembly of 12 initial assembly complexes, which are covered by VP6 to form new DLPs. Then, DLPs move to endoplasmic reticulum to get outer layer (VP4 and VP7) with help of NSP4. NSP4, which binds to VP7 to make NSP4-VP7 complexes in endoplasmic reticulum membrane, have an affinity to VP6(33). Virions were released from the infected cells by smooth vesicles through the apical side of intestinal cells (34) and cell lysis (35, 36).

1.4 Physicochemical properties

Some of the physical and chemical properties of rotavirus have been studied by others. Due to the characteristic triple layered structure without envelope, rotavirus is resilient to
solvents (ether, chloroform), refrigerants (e.g., Genetron®), detergents, repeated freeze-thawing, and sonication (37). All group A, B, and C rotaviruses are, however, sensitive to a high temperature, but their thermostability varies among strains. In one study, the infectivity of a simian rotavirus A (SA11 strain) in MA-104 cells was decreased by 80% and 99% after placing at 50°C for 5 and 30 minutes, respectively, as determined by plaque assay (37). In another study (38), the strain SA11 and FH4232 (human rotavirus A) were sensitive to a higher temperature (4°C for 22 days, 37°C for 7 hours to 14 days, 56°C for 15 minutes, 80°C and 100°C for 1 minute). Although the infectivity of Gottfried strain (PoRV A) was reported to be stable at 56°C for 30 minutes (39). Murine rotavirus B in the intestinal filtrate which was treated at 56°C for 30 minutes could not cause diarrhea in suckling rats (40). The infectivity of PoRV C (Cowden strain) in MA-104 cells was reduced to 1% when incubated at 56°C for 30 minutes (39).

The pH stability of rotavirus A has been studied by numerous investigators. According to Estes, et al (37), the infectivity of the SA11 strain was reduced to 70% and 60% of the original titer when placing for 1 hour at pH 3.5 and 10, respectively. However, Meng (38) stated that the infectivity of the SA11 and FH4232 strains was not changed when they were treated in pH 3 to 11 at 37°C for 1 hour. This difference appears to be due to the methods used to measure virus infectivity as immunofluorescence test which was used in the latter study is more sensitive than plaque assay which was used in the former study. Nevertheless, Terrett (39) showed that the OSU strain lost 27% and 0% of the original titer when treated at pH 3 and pH 5 for 30 minutes at 37°C, respectively, while Gottfried strain lost 17% and 11% of the original titer, respectively, under the identical conditions. Overall, these data suggest that RVA is relatively stable in a wide range of pH although strain variation is apparent. Murine rotavirus B in intestinal filtrates could not cause diarrhea in suckling rat after being treated at pH 3 for 30 minutes at 37°C (40). Porcine
rotavirus C (Cowden strain) lost 16% and 17% of its original titer when treated at pH 3 and pH 5 for 30 minutes at 37°C, respectively while NB strain lost 34% and 0%, respectively, under the same condition (39).

The density of rotavirus A, B, and C are similar, ranging from 1.36 to 1.38 g/ml. Rotaviruses can be seen in the form of single-layered, double-layered or triple-layered particles, which can be separated by a rate-zonal centrifugation method using cesium chloride or sucrose gradient due to the difference in density. For RVA, double-layered particles are sedimented at 380S to 400S on a sucrose gradient cushion and have a density of 1.38 g/ml in CsCl gradient whereas triple-layered particles are sedimented at 520S to 530S on the sucrose gradient cushion and have a density of 1.36 g/ml in CsCl gradient (7). Group B human rotavirus (HRV) has the density of 1.373 g/ml and 1.435 g/ml for double- and single-layered particles, respectively (41). PoRV C has the density of 1.369 g/ml and 1.387 g/ml for double- and single-layered particles, respectively (22).

II. Pathogenesis

The understanding of rotavirus pathogenesis is primarily based on studies of RVA. After being ingested, rotavirus targets mature, non-dividing enterocytes of villi (generally tip of the villus) in the small intestine, leading to diarrhea. Malabsorption is widely accepted as a cause for diarrhea (21). The outcome of virus replication in intestinal epithelial cells is cell lysis leading to villous blunting and atrophy. Such changes result in a large amount of undigested and unabsorbed particles in the lumen of small intestine, which are osmotically active and eventually travel to the colon. When the colon cannot absorb or hold water any longer, diarrhea due to osmosis starts. In addition, affected pigs that developed diarrhea have shown some defective functional changes in small intestinal epithelial cells, such as decreasing in the activity of
digestive enzymes (e.g., sucrase, lactase, maltase) and Na⁺K⁺ATPase which is crucial to transport nutrients into cells and increasing the activity of thymidine kinase (42). All these changes occur due to damage in small intestinal epithelia and contribute to malabsorption.

It has been reported that diarrhea can be developed before the presence of pathological changes in the small intestine (43, 44), suggesting that other factors or mechanisms may also contribute to diarrhea. One of them is production of NSP4 which is considered as an enterotoxin of rotavirus. During rotaviral replication, expressed NSP4 causes an increase of intracellular calcium concentration ([Ca²⁺]i) (45, 46). When it is released from infected cells, NSP4 can act as paracrine to adjacent uninfected cells by stimulating a phospholipase C-inositol 1,3,5-triphosphate (PLC-IP₃) cascade, resulting in a surge of [Ca²⁺]i. The high [Ca²⁺]i depresses the expression of disaccharidases, leading to suboptimal digestion of disaccharides in the intestine, which eventually contributes to malabsorption. High [Ca²⁺]i also disrupts the microvillar cytoskeleton network and the tight junctions which leads to increased paracellular permeability and activates Cl⁻ transporter, causing increasing secretion of cryptic cells. NSP4 or serotonin secreted from enterochromatin cells are thought to stimulate enteric nervous system which attributes to increasing intestinal secretion and mobility (47). These events contribute to the development of diarrhea.

The severity of disease in pigs varies depending on age, immune status, and insulting rotavirus strain and serogroup. There is conflicting information on age resistance of pigs to rotavirus A. One study (48) reported that the rotavirus-associated disease was most common in 1- to 4-week-old piglets in which diarrhea was severe and villous atrophy was prominent while diarrhea in 1- to 3-day-old pigs was mild. The authors postulated that such a difference may have been attributed to the difference in the level of materially derived immunity between the 2
groups. The authors speculated that the maternal antibody in the older pigs had been decreased to a lower level than the neonatal pigs at the time of exposure to rotavirus. In contrast, another study of experimental rotavirus infection in 3 week-old pigs (49) showed that the pigs had mild diarrhea and lesions in small intestine. Furthermore, another investigators could not prove the age resistance of pigs to rotavirus as the infectivity scores measured by immunoperoxidase testing on intestine segments of neonatal pigs and 2- to 12-week-old pigs were not significantly different (50). Reasons for the observed discrepancy may have been differences in the immune status of pigs used and/or viral strain used. Nonetheless, older pigs are generally believed to manage rotavirus infection and disease better than neonatal pigs because: a) the older pigs have fewer receptors on the surface of enterocyte (51); b) the enterocyte turnover rate in the older pigs is 2-3 times quicker than that in neonatal pigs (52) and; c) the ability of the colon to absorb water is higher in older pigs than neonates.

While the small intestine is the main replication site of rotaviruses, RVA antigen and RNA were detected in non-intestinal tissues of infected pigs, such as mesenteric lymph nodes, liver, lung, and choroid plexus (53). Especially, a high amount of the viral antigen and RNA was detected in mesenteric lymph nodes with microscopic lesions such as necrosis and depletion of lymphoid cells, suggesting that rotavirus could replicate in mesenteric lymph nodes. However, such a speculation cannot be confirmed because virus isolation attempts were not reported. In the other positive tissues, the amount of antigen was low and the amount of RNA was similar to that in blood, indicating that rotavirus may be passively transported to these tissues due to viremia. Viremia was detectable at 1 day post inoculation (dpi) and last until 14 dpi. Transient viremia from 1 to 7 dpi in inoculated pigs has also been reported by other investigators (54). These investigators proposed hypothetical mechanisms by which rotavirus enters the blood stream. One,
the virus may use antigen-presenting cells as transporters. Two, the virus may pass through cell barriers because NSP4 may increase the blood flow through villi, leading leakage or transport the virus into blood circulation. Three, the virus may go through the basement membrane when the intestinal epithelial cells are detached. Nonetheless, the clinical and biological importance of rotaviral viremia and non-intestinal replication are largely unknown.

Strain difference in the pathogenicity has been observed. Two group A porcine rotavirus strains designated OSU and SDSU had shown similar clinical signs, changes in body weight gain, and virus excretion patterns in experimentally infected pigs (55). The microscopic lesions in the small intestine induced by the 2 strains were also similar although villous atrophy and villous fusion caused by the SDSU strain at 3 and 7 dpi were more severe than those by the OSU strain. It is unknown though whether these two strains are similar at the molecular level because the sequence of SDSU strain is not available. In other report (56), two PoRV A isolates (4F and 4S) were obtained from the same diarrheic pig but showed different in-vitro growing characteristics in MA-104 cells. The isolate 4F grew more slowly and created smaller plaques than the isolate 4S. Molecularly, the isolate 4F had gene 4 (VP4) that migrated faster than that of the isolate 4S in electrophoresis. When gnotobiotic pigs were inoculated with these isolates for continuous pig passages ranging from 1 to 5, the isolate 4F caused diarrhea but not the isolate 4S. Sequence comparison revealed that the two isolate shared 99.2% and 99.8% amino acid identity for VP6 and NSP1(NS53) respectively, but shared 67.3% and 70.6% nucleotide and amino acid identity, respectively, for VP4 (57). Such a strain difference in the pathogenicity has also been demonstrated with a bovine rotavirus (58).

Several rotavirus proteins are considered as viral virulence factors. VP4, VP3, VP7, NSP1, NSP2, and NSP4 have been reported to contribute to the virus pathogenicity (59). Among
these viral proteins, NSP4 as enterotoxin is strongly believed to be a virulent factor because it has been reported to play a role in causing diarrhea particularly in a murine model. It appears that NSP4 can cause diarrhea in inoculated mice in a dose-dependent fashion. In one study (60), 0.1 and 1.0 nmol of NSP4 caused diarrhea in 60% and 100% of the inoculated mice at 6 to 7 days of age, respectively. In 8- to 9-day-old mice, 0.1, 1.0, and 5.0 nmol of NSP4 caused diarrhea in 0%, 60%, and 80% of mice, respectively. Another study demonstrated that mutations in NSP4 altered the pathogenicity of virus (61). The virulent OSU (OSU-v) strain of PoRV A (i.e., wild type) caused diarrhea in 89% of the inoculated mice whereas an attenuated OSU (OSU-a) rotavirus strain with six mutations in NSP4 did not cause diarrhea in mice. Furthermore, 5µg of recombinant OSU-v NSP4 caused diarrhea in 56% of the inoculated mice whereas the same dose of OSU-a NSP4 caused diarrhea in only 16% of the inoculated mice.

Although a review article (62) states that group A and C rotaviruses are more virulent than group B rotavirus because they infect more cells causing more severe villous atrophy and diarrhea, comparison of the pathogenicity of PoRV A, B and/or C has not been reported in refereed publications. Information on the pathogenicity of rotavirus E and H is not available as rotavirus E was reported only in England and rotavirus H has been identified recently.

III. Clinical manifestations

The incubation time varies by various factors but generally ranges from 18 to 96 hours after exposure in piglets. Common clinical signs observed in piglets infected with rotavirus are anorexia, depression, diarrhea, and vomiting. Occasionally infected piglets also show elevated body temperature (21). Diarrhea in affected animals is characterized as follows: “Feces were watery to creamy consistency and white, yellow, or brown, similar to that observed in transmissible gastroenteritis (TGE).”(48). The severity of diarrhea induced solely by rotavirus is
mild and lasts for 1 to 3 days. Dehydration is also mild (48, 63). While morbidity is about 20% (21) or 50-80% (64), the mortality among diarrheic dehydrated piglets is up to 15-20% (21, 48). Rotavirus infection is generally subclinical in pigs older than 3 months of age although diarrhea and vomiting can be sometimes observed in older pigs (64).

Clinical signs of rotavirus infection in experimentally inoculated pigs have been more severe than those in naturally infected ones. The reason may be that most of experimental studies used gnotobiotic or cesarean-derived, colostrum-deprived (CDCD) pigs which lack maternal immunity. Under experimental conditions, neonatal piglets (1- to 5-day old) develop much more severe diarrhea, lasting for 4-8 days, followed by dehydration as compared to older pigs (7- to 21-day old). The mortality rate in piglets with dehydration can reach up to 50–100% (65, 63, 43) while no or low mortality can be observed in the older pigs (66, 67, 49).

Co-infections with other enteric pathogens are not uncommon in pigs infected with rotavirus in the field. *Escherichia coli, Isospora suis, Clostridium difficile,* and porcine reproductive and respiratory syndrome virus (PRRSV) have been identified in sucking piglets infected with rotavirus (68, 69). In pigs with concurrent infections, clinical signs have been more severe in terms of the severity and duration of diarrhea, degree of dehydration, and mortality rate (70, 71). In addition, concurrent infections with rotaviruses of different groups have been reported. (72). However, clinical significance of infection with multiple groups of rotavirus for the disease and pathology has not been studied.

Grossly, affected pigs have thin-walled small intestine containing yellowish or gray fluid, which is similar to gross lesions observed in pigs affected by TGE (48). A typical microscopic lesion in pigs infected with porcine rotavirus group A (48, 73),(74), group B (75) or group C (44) is villous atrophy. Blunted and shortened villi, which are only 1/10 to 1/4 long compared to the
normal ones, are covered with cuboidal and squamous epithelial cells. Subsequently, the villi fuse with adjacent ones (76). The crypts are deepened due to hyperplasia of crypt cells. For group A rotaviruses, lesions are mostly found in jejunum and ileum, which have also been corroborated by experimental infection studies (48, 49, 77). A review (59) stated that lesions in pigs infected with a rotavirus B or C can be present throughout the small intestine.

**IV. Immunity**

There have not been many studies on pig immunity against porcine rotavirus. Almost all of the studies conducted in pigs used human rotaviruses; hence observations may not exactly mirror porcine rotavirus immunity. In particular, information regarding the innate immunity against rotaviruses in pigs is sparse.

**4.1 Innate Immunity**

Plasmacytoid dendritic cells collected from pigs inoculated with a HRV have shown to produce a high amount of interferon alpha (IFN-α) found in serum, which was independent of the virus doses (78). In the same study, a lower amount of interleukin-12 (IL-12) and a minimal amount of IL-10, tumor necrosis factor-alpha (TNF-α), and IL-6 were also found in serum. In another study, the severity of HRV-associated clinical signs was reduced by enhancing activity of plasmacytoid dendritic cells, natural killer cells, and IL-12 response (79). IFN-α and IFN-γ have been shown *in vitro* that they can inhibit HRV A (Wa strain) infection in Caco-2 cells, reducing its infectivity to 10% and 1%, respectively, in the cell measured by immunoperoxidase staining. However, IFN-γ, not IFN-α, was shown to inhibit the virus entry to Caco-2 cells, suggesting that IFN-γ and IFN-α have different mechanisms to inhibit the virus infection (80). Nonetheless, the antiviral role of IFN-γ against rotavirus *in vivo* has not been clear in a mouse and pig model (78, 81).
It has been reported that rotaviruses can modulate the innate immunity. Type I IFN production can be inhibited by viral NSP1 protein as NSP1 has shown to degrade IFN regulatory factor 3, 5, 7 (82, 83) and inhibit NF-κB activity (84) by stimulating the degradation of β-TrCP (85). In addition, the antiviral state which is induced by IFN can be inhibited by rotavirus because STAT1 (signal transducer and activator of transcription) phosphorylation and STAT1/STAT2 nuclear translocation have been shown to be inhibited by rotavirus infection (86, 87).

4.2 Adaptive immunity

Both passive and adaptive immunity plays an important role in protection against rotaviruses. Neonatal pigs can have maternally derived passive immune protection from the dams through colostrum or milk that contain mainly IgG and IgA, respectively. According to Ward (88), piglet receiving colostrum containing rotavirus-specific IgG whose concentration is approximately a half of those in naturally sucking piglets could mitigate the clinical signs by rotavirus infection and had fast recovery. Similarly, gnotobiotic piglets that received immune serum intraperitoneally (18 to 26 mg of IgG per 1 ml) or colostrum and milk have a significant lower rate of diarrhea and lower level of virus shedding than those in control groups (89).

To assess the role of B cells and CD8+ T cells in anti-rotavirus immunity, genetically-modified pigs were used (90). The pigs were manipulated to have CD8 cells depleted and/or to be defective in immunoglobulin heavy encoding gene (HCKO), which hinders B-cell development. These pigs along with wild-type pigs were first vaccinated with an attenuated HRVA Wa strain and at 28 days later were challenged with a virulent strain of the same virus. The HCKO defective pigs had significantly longer virus shedding than did the wild-type pigs. Furthermore, HCKO defective/CD8 depleted pigs had significantly longer duration of diarrhea
and virus shedding than did the wild-type pigs. These results suggest that both B cells and CD8+ T cells are important in anti-rotavirus immunity.

In piglets infected with the PoRV A Gottfried strain, IgM antibody secreting cells (ASCs) were detected in mesenteric lymph node (MLN) at 3 dpi and the number of these cells in MLN and spleen researched to the peak at 7 dpi. IgA ASCs were first detected at 7 dpi in MLN, spleen, and lamina propria of duodenum and ileum. The peak numbers of these cells were found at 14 and 21 dpi in duodenum lamina propria. IgG ASCs were first detected at 7 dpi in MLN, spleen, and lamina propria of duodenum and ileum and peaked at 14 dpi in spleen and duodenum lamina propria. At 14 and 21 dpi, the number of IgA ASCs were higher than that of IgG and IgM ASCs in MLN and lamina propria of duodenum and ileum. In another pig study with SB1A strain of PoRV A, the numbers of IgA and IgG ASCs in the intestinal lamina propria were 21- and 1.4-fold, respectively, higher than those in the spleen (91). When pigs were orally inoculated with a HRV A isolate, a positive correlation between acquired protection and the level of lymphocyte proliferation as well as the number of cells secreting IgA in the small intestine was observed, suggesting that intestinal immune response is crucial to anti-rotavirus protective immunity. According to Azevedo (92), both intestinal IgA and serum IgA were correlated with protection against virus shedding and diarrhea, suggesting that rotavirus-specific IgA antibody is a good indicator of protective immunity.

It has shown that VP4- and VP7-specific antibody can protect pigs from virus challenge (93) because VP4 and VP7 (94) contain neutralizing epitopes (95). Pigs recovered from prior rotavirus infection are totally protected from subsequent challenge with a homotypic, but not heterotypic, rotavirus (93). The cross-protection between serogroups or serotypes is trivial if not none. However, it has been reported that repeated infection or vaccination even with the same
strain can broaden the degree of cross-protection between serotypes (96, 97). It is speculated that neutralizing epitopes in VP4 are independent from those in VP7 (98) and some may be cross-reactive (99). Thus, humoral immune response against these epitopes can be boosted after repeated infection or vaccination. Nonetheless, the determinant for heterotypic protection is still unclear as the neutralizing epitope(s) that are in common of serotypes or genotypes have not been identified.

After infection or vaccination, pigs have shown to produce VP6-specific antibody to a significantly higher level than those against VP4, NSP4, NSP2, and VP7. Although anti-VP6 antibody has not shown to neutralize the virus or protect neonate piglets (92, 100), it was reported to be protective in mice (101-106). Furthermore, anti-VP6 IgA has shown to have the “intracellular neutralization” ability in vitro by interfering with viral transcription, which is not apparent in traditional neutralization tests (107, 108). Since not many studies have been conducted to assess the role of VP6 antibody-mediated immunity in protecting pigs, its anti-rotavirus ability remains to be further studied.

Because NSP4 of rotavirus has shown an important role in the viral pathogenesis as discussed above, the role of anti-NPS4 immune response in protection against rotavirus has been of great interest. When infected with PoRV A (109), piglets had a significant higher titer of anti-NSP4 IgG than anti-NSP4 IgA in serum. However, these antibodies could not protect these piglets from subsequent challenge with the viruses with different G (VP7) and P (VP4) but mostly identical NSP4 three weeks after the primary infection, suggesting that NSP4 antibody may not play an important role in anti-rotavirus protective immunity. Another study (110) showed a positive correlation between NSP4 antibody and protection when piglets were challenged and re-challenged with the same virulent HRV isolate. It is, however, difficult to
extrapolate the exact role of NSP4 antibody in protection from this study as the role of antibody against other viral proteins in the protection could not be ruled out due to the fact that the same virus was used in both primary and secondary challenges. Nonetheless, in a mouse study, NSP4 antiserum demonstrated its ability to protect mice from developing diarrhea (111). Therefore, it remains to be further investigated to clarify the role of anti-NSP4 antibody in protection against rotavirus.

V. Diagnosis and Diagnostics

5.1 Sampling and diagnosis

Rotavirus-associated diarrhea can be preliminarily diagnosed by disease history of the farm, pig age (nursing, post-weaning), clinical signs, and gross lesions. Common clinical signs and gross lesions which can be observed with rotavirus infection are described above. Subsequently, samples, such as feces, intestinal contents, or small intestinal tissues, should be collected for laboratory testing such as nucleic acid detection methods (PCRs), histopathology, and antigen detection methods (i.e., immunohistochemistry, immunofluorescence). It is best to collect samples within 24 hours after diarrhea is seen because the rotavirus shedding generally reaches the peak at this time (21).

Diarrhea in piglets can be attributed to many microbial agents, environmental and nutritional factors. The differential list for infectious diseases in nursing pigs (i.e., pre-weaning piglet diarrhea) should include *Escherichia coli, Clostridium difficile, Clostridium perfringens* type A and C, *Enterococcus durans, Salmonella* spp, *Isospora* (coccidia), *Cryptosporidia parvum*, porcine epidemic diarrhea virus (PEDV), and transmissible gastroenteritis virus (TGEV), and PRRSV. The differential list for infectious diseases in weaning pigs (i.e., post-weaning diarrhea) include: *E. coli, Salmonella* spp., *Brachyspira* spp. *Lawsonia intracellularis*, Coccidia,
roundworms, whipworms, TGEV, PEDV, PRRSV, porcine circovirus.

(https://vetmed.iastate.edu/sites/default/files/VDL/pdf/porcinepathologyguides.pdf)

5.2 Laboratory methods

Various conventional and advanced laboratory methods have been developed and used to detect the presence of virus and virus-specific antibodies in clinical specimens. Historically, isolation of rotavirus has been difficult and very laborious. Because of that nature, while visualization of virus presence in feces was commonly used for rotaviruses like many other enteric viral pathogens, nucleic acid-based tests have become a common tool recent days.

5.2.1 Virus detection

Because rotavirus is shed in feces with a high amount, up to $10^9$ particles per ml (74) and the morphology is unique, electron microscopy (EM) is applicable to visualize the presence of rotavirus in feces or intestinal contents (112). Although it is laborious and expensive and requires experienced personnel for sample preparation and to operate the instrument, EM is considered a quick test. Serogroups of rotaviruses can be differentiated by immune EM using serogroup-specific antibodies (i.e., anti-VP6 antibody for each serogroups/types).

Rotavirus isolation by cell culture has been challenging. From pigs, rotavirus was first isolated in 1976 (113). In this instance, diarrheic feces were first inoculated in 2- to 28-day-old gnotobiotic pigs. Subsequently, the feces containing the virus were inoculated in primary calf kidney and pig kidney cells. The cytopathic effect (CPE) and virus-specific immunofluorescent staining were observed in the first passage of cell culture. However, this result was not reproducible in subsequent passages, indicating that this culture system was not suitable or optimized for propagating rotavirus. In 1977, several cell culture based virus isolation attempts were made for bovine (114), porcine (115) and human (116) rotaviruses. In those studies, a roller
culture technique and/or incorporation of proteolytic enzyme (i.e. trypsin, pancreatin) into cell culture media were used with successful isolation of group A rotaviruses. Numerous kidney epithelial cells, such as Madin-Darby bovine kidney (MDBK), calf kidney cells, rhesus monkey kidney epithelial cells (LLC-MK2), and primary pig kidney cells, were found to be permissive to rotaviruses. Since then, African green monkey cell line MA-104 was adopted and has been widely used for rotavirus isolation, particularly for RVA (117,118, 119, 120, 121,122).

Pretreatment of inoculums with a proteolytic enzyme or adding the enzyme to a culture medium and use of roller culture have been vital conditions for the successful virus isolation.

Successful isolation of non-group A porcine rotaviruses are rare. To date, isolation of only one PoRV B has been reported by Japanese scientists (123), but there is no further information of this virus isolate. For group C porcine rotavirus, all the reported work used one virus strain, Cowden (124, 125). This virus strain was first isolated using primary pig kidney cells cultured in media supplemented with pancreatin in roller tubes; however, details of materials and conditions are lacking. The viral infectivity, which was assessed by immunofluorescence microscopy of inoculated cells, increased intensely after six passages. Subsequently, the virus was successfully adapted to grow in MA-104 cells in roller culture, not a stationary culture. Although this protocol for isolating PoRV C was available, no more PoRV C isolates have been made since the Cowden strain was isolated. Furthermore, the method is complicated by the necessity of numerous blind passages in two different cells for primary isolation and adaptation. It is necessary to identify more sensitive cell culture systems for non-group A porcine rotavirus isolation. Recently, non-transformed intestinal IPEC-1 and IPEC-J2 cells which were originated in small intestine of 1-day old piglets have been used in studying enteric viral pathogens such as TGEV and PEDV (126), and PoRV A (127). Thus, IPEC-1 and
IPEC-J2 cells might be candidate cell lines to be evaluated in attempting to isolate non-group A rotaviruses.

5.2.2 Antigen detection

Historically, immunofluorescence test has been commonly used to detect rotavirus A antigens in frozen intestinal tissue sections (128) and has also applied to detect rotavirus B and C in paraffin-embedded intestinal tissues (44, 75). The sample quality has been an issue as intestinal tissues can be easily decomposed. As monoclonal antibodies were developed and became available (129, 130), an immunohistochemical staining method was developed to detect RVA antigens in formalin-fixed and paraffin-embedded tissues (131) and has become a standard tissue assay to demonstrate the presence of rotaviruses, particularly for rotavirus A. However, such a virus-specific monoclonal antibody for non-group A rotaviruses is lacking.

Some antigen-capturing ELISAs (ACEs) using a microtiter plate had been developed to detect rotavirus antigen of rotavirus A (132, 133), B (134), and C (135) in feces under experimental conditions. However, only group A rotavirus-specific ACEs is commercially available, mostly for use in human medicine (136). Also, commercial latex agglutination kits have been available to detect rotavirus A antigens in feces (136). Recently commercial kits for veterinary use have also been made available in the US and/or other countries, such as Pig Rotavirus Group Specific Antigen ELISA Kit-LS-10367 (LSBio, Seattle, WA) and Pig Rotavirus Antigen ELISA Kit (Flarebio Biotech LLC, College Park, MD). However, the performances of these kits have not been evaluated by independent investigators.

Although infections with non-group A porcine rotaviruses in young pigs are frequent, reagents specific for these viruses, such as monospecific antibodies, are lacking, which hinders the development of laboratory methods such as immunohistochemistry (IHC) or ACE.
5.2.3 Viral genome detection

While RNA electrophenotyping was commonly used to characterize rotaviruses, Northern blot techniques using virus-specific hybridization probes were used to detect rotaviral genomic RNA or mRNAs in clinical specimens (137, 138, 139). Subsequently, polymerase chain reaction (PCR)-based assays have been widely used to detect the presence of rotavirus RNA in clinical specimens including feces. These assays have also been useful for rapid genogrouping and genotyping (i.e., G or P types) for rotavirus A (140, 141, 142), B (143,13), and C (143, 16). Recently, a real-time RT-PCR panel which can detect the nucleic acid of PoRV A, B and C has been developed (72) and adapted by many veterinary diagnostic laboratories for the rotavirus detection as it could provide simultaneous detection of 3 serogroups and a high-throughput capability. In addition, the real-time RT-PCR assay has also been used in a quantitative manner to estimate the level of rotavirus in a specimen, which aids in “feedback” practice to mitigate the disease or boost the herd immunity against selected strains of rotaviruses.

The availability of viral sequences in public domains (e.g., GenBank®) and advances in technology allows laboratories to utilize sequencing for VP4, VP6 and/or VP7 genes for genotyping and molecular characterization of rotaviruses.

5.2.4 Antibody detection

Since rotaviruses are common in swine herds, detecting anti-rotavirus serum antibody has little diagnostic value. Nevertheless, the status of the infection in the pigs can be assessed by determining the isotype of the antibodies. The recent or active infection is indicated by detecting high titer of IgM or IgA. Evidence of previous exposure and vaccination status in a population
can also be assessed by serology. In some cases, neutralizing antibodies have been measured using fluorescent focus reduction assay (144) or plaque reduction assay (145) to assess possible antiviral immune status against PoRV A.

Several ELISAs were developed to isotype the antibody against human rotaviruses A in pig sera, which were used in research (92,144,146). In addition, an ELISA was developed to detect antibodies against PoRV A (147). An ELISA detecting PoRV C antibody has been described (148). No commercial ELISA to detect antibodies of PoRV B or PoRV C is available.

VI. Epidemiology

The presence of rotaviruses has been widely detected in pig producing regions worldwide. The economic lost caused by rotavirus infection is considered moderate (149) even though rotaviruses do not cause as severe disease as does TGEV or PEDV. Yet, cost analysis of rotavirus-associated enteric disease in the swine industry has not been performed.

Rotaviruses are believed to be ubiquitous in swine herds because the virus is shed with a high amount in feces and their resistance to various environment conditions. The prevalence of group A porcine rotavirus ranges from 2.5 % to 78.7%, while those of group B and C porcine rotaviruses are up to 25.9% and 78.0%, respectively. The variability of prevalence is attributed to differences in geographic locations, ages of pigs tested, tests that were used, and sampling scheme (i.e., random fecal samples from general populations or diarrheic fecal samples) as shown in Table 6.

Pigs are frequently found to be infected with multi-groups of rotaviruses, which makes the prophylaxis more challenging. According to unpublished data from the Iowa State University Veterinary Diagnostic Laboratory, PoRV A tend to be prevalent in 3- to 6-week-old piglets (post-weaning ages), whereas PoRV B have been detected equally in all ages although more than
50% of rotavirus B positives were in 3 weeks or older pigs, and PoRV C have been most frequently detected in neonates and suckling piglets (pre-weaning). This pattern is similar to the results of a diagnostic laboratory-based survey study by the University of Minnesota Veterinary Diagnostic Laboratory which tested a large number of samples (n=7508) by PCR-based assay (72). As the frequency of various rotavirus serogroups observed among diarrheic pigs shows a tendency of different age distribution, it could be speculated that the pathogenicity or pathogenesis of rotavirus A, B, and C may differ in suckling piglets and older/adult pigs. However, no experimental data supporting this speculation is available.

Because a high level of rotavirus can be shed in feces from affected pigs, the fecal-to-oral route is widely accepted as the main transmission pathway of rotavirus among pigs as well as between other species. Because of this nature, most of experimental rotavirus challenge studies have used the oral route to inoculate pigs with feces containing the virus (65, 42, 63, 150, 151, 152, 75, 49, 77, 43, 54, 153, 154, 155). At barns, pigs are believed to be exposed to rotavirus via pig-to-pig contact or from contaminated environment such as floor. The facts that rotaviruses can be detected in feces from asymptomatic pigs (156, 157, 158, 159) or pregnant sows 5 days before to 14 days after farrowing (160) makes it rather difficult to intervene the virus transmission at a barn or on herd. Furthermore, since rotaviruses can maintain their infectivity in environmental water for several days at 20°C (161), rotavirus-contaminated water may also be a source of virus transmission.

Besides the fecal-to-oral route transmission, aerosol transmission has been postulated. It was reported that rotavirus can infect mice through aerosol and the antigen was detected in mouse lungs by antigen-capture ELISA up to 8 days after infection (162). In the Mid-Pacific island, HRV caused diarrhea and was transmitted quickly among peoples, raising a concern that
the virus may be transmitted through respiratory route (163). In another study (54), gnotobiotic piglets intranasally inoculated with an attenuated HRV under experimental conditions could shed the virus in nasal secretions. And the piglets also shed virus nasally when they were experimental inoculated with a virulent HRV isolate orally, intranasally or via gavage. Thus, air or aerosol spreading is a potential transmission route of rotavirus although this transmission mode has not been proven in pigs.

Rodents and insects have been proposed as potential vectors for transmission of rotaviruses. Rotavirus A was detected by ACE in the feces from mice and rat trapped in a pig farm (164), suggesting that mice could play a role in rotavirus transmission. However, it was not determined whether the detected virus was a porcine rotavirus. In another study, 1.1% of rodents trapped in two cities in China were positive for rotavirus. A half of the positive samples had rotaviruses with the same P and G type as those detected in human populations in the cities based on sequence analysis (165). In addition, flies were demonstrated to be able to carry a simian rotavirus A (SA11 strain) on their legs, potentially serving as mechanical vector (166). Besides rodents and insects, wild boars have been proposed to be involved in rotavirus transmission to domestic swine as many group A rotaviruses detected by PCR assay in feral swine share the same P and G types of group A rotaviruses found in domestic swine in the same geographic regions as determined by sequence analysis (167). There is no information about the transmission of PoRV through feed contamination or boar semen.

Pigs are the natural host of porcine rotaviruses. However, cross-species transmission has been documented. Either whole genomes or some gene segments of PoRVs have also been sporadically detected in sick humans, cattle and horses (168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180). A porcine rotavirus-like rotavirus isolated from a clinically ill cow
could cause diarrhea in both piglets and calves for up to 14 dpi after experimental exposure (181), indicating that rotavirus can cross the species barrier. Cross-species transmission has also been made with bovine rotaviruses (182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192). There have been a growing number of reports describing children patients (less than 5 years old) infected with a PoRV-like (170, 172, 173, 174, 177) or bovine rotavirus-like rotavirus (185, 191), suggesting that rotavirus is a potential zoonotic agent.

In feces, PoRV A was reported to be able to maintain its infectivity for 32 months at 10°C (193) while HRV A could maintain its infectivity for 2.5 months at 30°C (194), suggesting that rotavirus survives better at a low temperature. The HRV A (Wa strain) was shown to survive longer in non-porous materials (e.g., glass, stainless steel, plastic) than in porous materials (e.g., cloth, poster card, paper), indicating that a low humidity may be detrimental to rotavirus infectivity (195). Overall, rotaviruses can survive longer in a lower temperature and high humidity environment. Isolation of rotavirus A from dust in the nursery which had not been used to house pig for 3 months has been reported, suggesting that infectious rotavirus can be remained in dust for a long time (196). While all these findings confirm that rotavirus is sturdy in the environment, the stability of rotavirus in natural water bodies may be different. A study reported (161) that the time taken to diminish 10^3 PFU of the simian rotavirus SA11 strain at 20°C was 2-3 days in estuarine water but 3 to >14 days in fresh water; however, the same observation could not made between two water types when sampling was made one year later, suggesting that the survival of rotavirus was independent of salinity. The authors postulated that seasonal changes may affect in the virus survival in natural waters but did not speculate any specific parameters including temperature. In another study (197), a HRV can stay for up to 64 days in filtered river water without a change in the titer, measured by plaque assay, at both 4°C and 20°C. In raw
unfiltered river water, the viral titer was decreased by almost $10^2$ and $10^3$ at 4°C and 20°C, respectively, after 15 days. The investigators speculated that there may have been an antiviral effect from the microorganisms present in the raw unfiltered water because the virus lost 99% of its original titer after 10 days in that water. Yet, this postulation remains to be confirmed.

VII. Prevention and control

7.1 Management

Since rotaviruses are ubiquitous and sturdy, it is difficult to eradicate it from pig farms. In addition, a high titer of rotavirus is shed in feces from affected pigs which can overcome the maternally derived lactogenic passive immunity of piglets if they are exposed to the virus (198). Nonetheless, the risk for exposure and the disease severity can be reduced by strictly following biosecurity procedures as well as good management practices. The virus load can be reduced by implementing a good sanitation protocol. Rooms need to be cleaned and disinfected well between uses. The floor of rooms should be constructed for minimal fecal accumulation and efficient cleaning. Also, the shorter farrowing interval has been recommended to minimize the fecal accumulation and infection of latest farrowed litters (199). It is also recommended to keep the room at a warm and stable ambient temperature (35°C) to reduce the mortality of nursing piglets by rotavirus infection (200). Interestingly, a field-based study (201) showed that herds using all-in-all-out (AIAO) practice had a significantly higher rate of rotavirus infection as compared to herds using continuous flow. However, the herbs using AIAO also implemented an early weaning strategy. Thus, it is not clear that AIAO practice was the cause of higher rotavirus detection rate. Thus, the role of AIAO in rotavirus prevention and control is still questionable.
7.2 Medication and nutritional intervention

There is no specific treatment for rotavirus infection. All therapies and management practices are supportive and to alleviate adverse effects of the disease. Antibiotics can be used to control co-infected bacterial agents or secondary bacterial infections in rotavirus-infected pigs. Dehydration and weight loss due to rotavirus-associated diarrhea can be lessened by using an electrolyte solution containing glucose-glycine (202), although supplying water with electrolytes to pigs while refraining from feed has been commonly practice on farms when diarrhea is noticed. In children, it has been reported that feeding of transforming growth factor to diarrheic piglets helps their jejunum villi recovered quicker from rotavirus-associated damages (203). Animal plasma protein has been shown to be useful and better than soy protein to speed up the recovery process and interfere with the virus infection because it contains growth factors and virus-binding proteins (204). Soy isoflavones, including genistein, can inhibit rotavirus replication in vitro (205,206); however, the effect in vivo is not known.

Probiotics (i.e., beneficial bacteria) can be a good supporter. Pigs fed with Lactobacillus rhamnosus had milder diarrhea than those not fed (207). Lactobacillus reuteri showed its ability to prevent rotavirus from infecting MA-104 cells in vitro (208). Lactobacillus acidophilus has also been reported to act as an adjuvant to improve the efficacy of an attenuated rotavirus vaccine since pigs immunized with the vaccine supplemented with L. acidophilus had a higher number of intestinal IFN-γ-producing CD8+ T cells and intestinal anti-RV IgA and IgG secreting cells, and higher serum anti-RV IgA titers compared to those received the vaccine without L. acidophilus (209).
Vitamin A is believed to play an important role in the immune response to rotavirus or vaccines because gnotobiotic pigs with vitamin A deficiency showed weak innate or adaptive immune responses against HRV (210) or HRV vaccine (211, 212).

7.3 Oral passive immunization, vaccination and feedback

Chicken egg yolk antibodies (IgY) have been studied for rotavirus intervention. It has been reported that oral feeding of anti-RVA IgY can prevent or minimize diarrhea caused by rotavirus in gnotobiotic piglets (213), newborn calves (214), and mice (215). The protection mechanism of IgY may be inhibition of the virus binding to cell surfaces (216), preventing intercellular spread, immobilization of the microbes by agglutination, and/or neutralization of rotaviral enterotoxin (217, 218, 219).

Vaccination has been practiced to protect piglets against rotavirus. Except PoRV A, there is no commercial vaccine for PoRV B or C. Vaccines have been used with two objectives. The first is to build lactogenic immunity in sows which can be passed onto newborn piglets. When pregnant or lactating sows were vaccinated with an attenuated PoRV A G5P[7], the titers of secreted IgA and IgG in the milk were increased, giving piglets born to those sows a partial protection against subsequent challenge such as delaying the onset of disease and shortening the duration of diarrhea and virus shedding. However, the efficacy of the commercial vaccines in the field is still questionable because rotavirus-associated diarrhea of piglets occurred in herds using vaccines (220). The current belief is that suboptimal efficacy of the vaccines may be attributed to antigenic mismatch between the vaccine strains and insulting viral strains as for VP7 (i.e., G types) and VP4 (i.e., P types). The second objective of vaccination is to induce active immunity in piglets. It has been demonstrated that inactivated rotavirus vaccines could not provide the protection to vaccinated pigs because it could not induce mucosal immunity in the intestine (221).
On the other hand, an attenuated live virus vaccine has shown to protect CDCD piglets from a challenge with homologous viruses under experimental conditions (221). However, the efficacy of the attenuated live virus vaccine may not be clear under field conditions (222) as the maternal antibody in nursing piglets has shown to neutralize the vaccine virus itself which was orally administered so that the vaccine virus could not replicate (89). A possible solution for this problem may be that the vaccine should be administrated orally multiple times.

Since vaccines are not highly effective for PoRV A and are not available for PoRV B and C, an intentional exposure to a biological material containing rotavirus (also known as ‘feedback’) has been used in pig farms. Fecal material or homogenized small intestine tissue from acutely affected piglets commonly serves as inoculum and given orally to animals. However, there is no information in literature relating standard procedures for preparing and administrating the feedback material and evaluating the efficacy of feedback. Microbiological safety is of great concern since unwanted microorganisms can be concurrently given along with rotavirus due to the nature of feedback materials. Strict biocontainment practice is required to prevent unintended transmission of rotavirus to animals or neighboring farms.

**VIII. Conclusion**

Rotavirus is common in pig farms causing diarrhea. Besides rotavirus A, rotavirus B and C have been detected frequently in diarrhea piglets. However, a few studies have been done specifically on non-group A rotaviruses for various reasons, resulting in a knowledge gap for the pathogenesis and immunobiology of the viruses and also lack of tools and specific reagents for better diagnosis, prevention and disease control. Much of these lacking have been attributed to the inability or difficulty to isolate non-group A rotaviruses, which requires a great attention for future development. Application of molecular biological techniques, including reverse genetics,
may hold a hope for some aspects, expecting a continuous difficulty in obtaining isolates of non-
group A rotaviruses through conventional virus isolation technique.
References


### Table 1. Geographic distribution and estimated prevalence (in the parenthesis) of G and P types of porcine rotavirus group A

<table>
<thead>
<tr>
<th>Country</th>
<th>Study year</th>
<th>G types</th>
<th>P types</th>
<th>Predominant G/P types</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belgium</td>
<td>2014</td>
<td>G4 (29.4%) G5 (29.4%) G9 (23.5%) G3 (5.9%)</td>
<td>P[7] (29.4%) P[6] (23.5%) P[23] (23.5%) P[13] (11.8%)</td>
<td>G4 P[6] (17.6%)</td>
<td>(224)</td>
</tr>
<tr>
<td>Canada</td>
<td>2005-2006</td>
<td>G4 (55%) G5 (18%) G2 (9%) G9 (9%) G11 (9%)</td>
<td>P[6] (64%) P[13] (15.3%) P[27] (15.3%)</td>
<td>G4 P[6] (45%)</td>
<td>(225)</td>
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<td></td>
<td>2011-2012</td>
<td>G2 (59%) G11 (22%) G5 (20%)</td>
<td>P[13] (64%) P[27] (23%) P[34] (13%) P[6] (10%)</td>
<td>G2 P[13]</td>
<td>(157)</td>
</tr>
<tr>
<td>England</td>
<td>2009-2010</td>
<td>G4 (29.8%) G5 (27.5%) G2 (6.7%)</td>
<td>P[32] (55.2%) P[7] (28%) P[6] (11%)</td>
<td>G4 P[32] (18.6%) G5 P[32] (13.5%)</td>
<td>(226)</td>
</tr>
<tr>
<td>Korea</td>
<td>2006-2007</td>
<td>G5 (70%) G8 (17%) G9 (9%)</td>
<td>P[7] (93%) P[23] (2%) P[1] (1%)</td>
<td>G5 P[7] (64%)</td>
<td>(227)</td>
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<td>Japan</td>
<td>2000-2002</td>
<td>G9 (33%) G5 (18%) G3 (11%) G4, G1</td>
<td>P[6] (28%) P[23] (28%) P[7] (17%)</td>
<td>G9 P<a href="17%25">23</a></td>
<td>(228)</td>
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<tr>
<td>USA</td>
<td>2004-2012</td>
<td>G9 (78.2%) G4 (8.6%) G11 (8.6%)</td>
<td>P[7] (13%) P<a href="78.2%25">13</a></td>
<td>G9 P[13] (60.9%)</td>
<td>(231)</td>
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*The number of samples collected for testing when provided.*
### Table 2. Genotypes of rotavirus A (updated from reference 233)

<table>
<thead>
<tr>
<th>Name of genotypes</th>
<th>Gene product</th>
<th>Cut-off percent nucleotide identity</th>
<th>Number of genotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>G (Glycosylated)</td>
<td>VP7</td>
<td>80%</td>
<td>28G</td>
</tr>
<tr>
<td>P (Protease sensitive)</td>
<td>VP4</td>
<td>80%</td>
<td>39P</td>
</tr>
<tr>
<td>I (Inner capsid)</td>
<td>VP6</td>
<td>85%</td>
<td>21I</td>
</tr>
<tr>
<td>R (RNA-dependent RNA) polymerase</td>
<td>VP1</td>
<td>83%</td>
<td>14R</td>
</tr>
<tr>
<td>C (Core protein)</td>
<td>VP2</td>
<td>84%</td>
<td>14C</td>
</tr>
<tr>
<td>M (Methyltransferase)</td>
<td>VP3</td>
<td>81%</td>
<td>13M</td>
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<tr>
<td>I (Interferon Antagonist)</td>
<td>NSP1</td>
<td>79%</td>
<td>24A</td>
</tr>
<tr>
<td>N (NTPase)</td>
<td>NSP2</td>
<td>85%</td>
<td>14N</td>
</tr>
<tr>
<td>T (Translation enhancer)</td>
<td>NSP3</td>
<td>85%</td>
<td>16T</td>
</tr>
<tr>
<td>E (Enterotoxin)</td>
<td>NSP4</td>
<td>85%</td>
<td>21E</td>
</tr>
<tr>
<td>H (pHosphoprotein)</td>
<td>NSP5</td>
<td>91%</td>
<td>16H</td>
</tr>
</tbody>
</table>
Table 3. Geographic distribution and estimated prevalence (in the parenthesis) of G and P types of porcine rotavirus group B

<table>
<thead>
<tr>
<th>Country</th>
<th>Study year</th>
<th>G types</th>
<th>P types</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>India</td>
<td>2016</td>
<td>G7 (33%)</td>
<td>N/A*</td>
<td>(12)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>G19 (17%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>G20 (33%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>G21 (17%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Japan</td>
<td>2000-2007</td>
<td>G3 (21%)</td>
<td>N/A</td>
<td>(13)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>G4 (2.7%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>G5 (76.3%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>USA</td>
<td>2009</td>
<td>G6, G8, G10, G11, G12, G14, G16, G17, G18, G20</td>
<td>N/A</td>
<td>(14)</td>
</tr>
<tr>
<td>Brazil</td>
<td>2016</td>
<td>G14</td>
<td>N/A</td>
<td>(234)</td>
</tr>
</tbody>
</table>

* N/A = not available
Table 4. Geographic distribution and estimated prevalence (in the parenthesis) of G and P types of porcine rotavirus group C

<table>
<thead>
<tr>
<th>Country</th>
<th>Study year</th>
<th>G types</th>
<th>P types</th>
<th>Predominant G/P</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belgium</td>
<td></td>
<td>G1 (8.3%), G6 (66.7%)</td>
<td>N/A</td>
<td>N/A*</td>
<td>(224)</td>
</tr>
<tr>
<td>Czech</td>
<td>2010-2013</td>
<td>G6 (28.5%)</td>
<td>P[6],[8] (40%), P[2] (20%)</td>
<td>N/A</td>
<td>(235)</td>
</tr>
<tr>
<td></td>
<td>n=7 for G types</td>
<td>n=5 for P types</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>n=26 for G types</td>
<td>n=9 for p types</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>n=22 for G type</td>
<td>n=21 for P type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>USA</td>
<td>2009-2011</td>
<td>G6 (70%)</td>
<td>N/A</td>
<td>N/A</td>
<td>(17)</td>
</tr>
<tr>
<td></td>
<td>n=70</td>
<td>G5 (17.2%), G1 (11.4%), G9 (1.4%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2004,2011,2012</td>
<td>G6 (28%)</td>
<td>N/A</td>
<td>N/A</td>
<td>(236)</td>
</tr>
<tr>
<td></td>
<td>n=25</td>
<td>G4 (28%), G1 (20%), G2,3,5 (8%)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*N/A = not available
**Table 5.** Information of genome fragments and their corresponding proteins adapted to bovine rotavirus RF strain (G6P6[1]). (233)

<table>
<thead>
<tr>
<th>Genome segment</th>
<th>Size (bp)</th>
<th>Encoded protein</th>
<th>Size (kDa)</th>
<th>Location in virion</th>
<th>Molecules/virion</th>
<th>Functions*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3302</td>
<td>VP1</td>
<td>125</td>
<td>Core</td>
<td>12</td>
<td>RdRp; ssRNA binding; complex with VP3</td>
</tr>
<tr>
<td>2</td>
<td>2687</td>
<td>VP2</td>
<td>94</td>
<td>Core</td>
<td>120</td>
<td>Core shell; RNA binding; required for RdRp activity</td>
</tr>
<tr>
<td>3</td>
<td>2592</td>
<td>VP3</td>
<td>88</td>
<td>Core</td>
<td>12</td>
<td>Guanyllytransferase; methyltransferase; 2,5 - phosphodiesterase; ssRNA binding; complex with VP1</td>
</tr>
<tr>
<td>4</td>
<td>2362</td>
<td>VP4</td>
<td>86</td>
<td>Outer layer</td>
<td>180</td>
<td>Homotrimer; P type neutralization antigen;</td>
</tr>
<tr>
<td>5</td>
<td>1581</td>
<td>NSP1</td>
<td>58</td>
<td>Nonstructural</td>
<td></td>
<td>Interferon antagonist; E3 ligase; RNA binding</td>
</tr>
<tr>
<td>6</td>
<td>1356</td>
<td>VP6</td>
<td>44</td>
<td>Middle layer</td>
<td>780</td>
<td>Homotrimer, species determinant; protection (intracellular neutralization); required for transcription</td>
</tr>
<tr>
<td>7</td>
<td>1062</td>
<td>VP7</td>
<td>37</td>
<td>Outer layer</td>
<td>780</td>
<td>Homotrimer; glycoprotein; G type neutralization antigen; Ca2+ dependent</td>
</tr>
<tr>
<td>8</td>
<td>1059</td>
<td>NSP2</td>
<td>36</td>
<td>Nonstructural</td>
<td></td>
<td>Octamer; binds RNA, NTPase; NDP kinase; helix destabilizing; essential for viroplasm formation</td>
</tr>
<tr>
<td>9</td>
<td>1074</td>
<td>NSP3</td>
<td>34</td>
<td>Nonstructural</td>
<td></td>
<td>Dimer; binds to: 3 terminus of viral ss (+) RNA, cellular eIF4G, Hsp90; displaces PABP; inhibits host protein translation</td>
</tr>
<tr>
<td>10</td>
<td>751</td>
<td>NSP4</td>
<td>Nonstructural</td>
<td></td>
<td></td>
<td>RER transmembrane glycoprotein; viroporin; intracellular receptor for DLPs; interacts with viroplasms and autophagy pathway; modulates intracellular Ca2+ and RNA replication; enterotoxin (secreted); virulence</td>
</tr>
<tr>
<td>11</td>
<td>666</td>
<td>NSP5</td>
<td>21</td>
<td>Nonstructural</td>
<td></td>
<td>Dimer; phospho- and O-glycosylated protein; RNA binding; kinase; essential for viroplasm formation; interaction with VP2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NSP6</td>
<td>12</td>
<td>Nonstructural</td>
<td></td>
<td>Interaction with NSP5, localized in viroplasm</td>
</tr>
</tbody>
</table>

*Abbreviations:
- RdRp: RNA-dependent RNA polymerase
- ssRNA: single-stranded RNA
- eIF4G: Eukaryotic translation initiation factor 4 G
- Hsp: Heat shock protein
- PABP: Poly(A)-binding protein
- RER: Rough endoplasmic reticulum
- DLP: Double-layered particle
<table>
<thead>
<tr>
<th>Country</th>
<th>Assay used</th>
<th>Pig ages</th>
<th># of positive/total</th>
<th>% positive for respective type</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>USA</td>
<td>PCR</td>
<td>1-3 days</td>
<td>779/954</td>
<td>17% (133/779)</td>
<td>(72)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4-20 days</td>
<td>1580/2144</td>
<td>26.7% (422/1580)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>21-55 days</td>
<td>2354/2538</td>
<td>21.8% (513/2534)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>&gt;55 days</td>
<td>989/1207</td>
<td>19.8% (196/989)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PCR</td>
<td>Nursing and weaned</td>
<td>35/371</td>
<td>7% (26/371)</td>
<td>(231)</td>
</tr>
<tr>
<td></td>
<td>PCR</td>
<td>Nursing (&lt;21 d)</td>
<td>56/70</td>
<td>15.7% (11/70)</td>
<td>(14)</td>
</tr>
<tr>
<td></td>
<td>PAGE</td>
<td>Weaned (21-55 d)</td>
<td>78/81</td>
<td>2.5% (2/81)</td>
<td>(237)</td>
</tr>
<tr>
<td></td>
<td>PCR</td>
<td>Weaned (&gt;55 d)</td>
<td>22/22</td>
<td>18.2% (4/22)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PAGE</td>
<td>Nursing</td>
<td>68/146</td>
<td>76.4% (52/68)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PCR</td>
<td>Weaned</td>
<td>22/146</td>
<td>40.9% (9/22)</td>
<td>(17)</td>
</tr>
<tr>
<td></td>
<td>PCR</td>
<td>≤3 days</td>
<td>78% (442/568)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4-20days</td>
<td>65% (466/722)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>21-55days</td>
<td>6% (84/1447)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>&gt;55days</td>
<td>14% (61/443)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PCR</td>
<td>Mostly &lt;3 weeks</td>
<td>74/380</td>
<td>19.5% (74/380)</td>
<td>(236)</td>
</tr>
<tr>
<td>Vietnam</td>
<td>PCR</td>
<td>Total</td>
<td>178/730</td>
<td>24.38% (178/730)</td>
<td>(238)</td>
</tr>
<tr>
<td>East Africa</td>
<td>PCR</td>
<td>Total</td>
<td>117/446</td>
<td>78.7%</td>
<td>(239)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nursing</td>
<td>32.9%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Grower</td>
<td>5.8%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Canada</td>
<td>PCR</td>
<td>Finisher (normal feces)</td>
<td>48/98</td>
<td>49% (48/98)</td>
<td>(157)</td>
</tr>
<tr>
<td>Korea</td>
<td>PCR</td>
<td>Diarrheic fecal</td>
<td>182/475</td>
<td>38.3% (182/475)</td>
<td>(227)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>36/137</td>
<td>26.2% (36/137)</td>
<td>(240)</td>
<td></td>
</tr>
</tbody>
</table>
# Table 6 (continued)

<table>
<thead>
<tr>
<th>Country</th>
<th>Assay used</th>
<th>Pig ages</th>
<th># of positive/total</th>
<th>% positive for respective type</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>Ireland</td>
<td>PCR</td>
<td>Weaned and grower, asymptomatic</td>
<td>19/292</td>
<td>6.5% (19/292)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PCR</td>
<td>Weaned and grower, asymptomatic</td>
<td>13/292</td>
<td>4.4% (13/292)</td>
<td></td>
</tr>
<tr>
<td>India</td>
<td>PAGE</td>
<td>Diarrheic feces</td>
<td>9/35</td>
<td>25.71% (9/35)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PCR</td>
<td>Feces</td>
<td>9/187</td>
<td>4.8% (9/187)</td>
<td></td>
</tr>
<tr>
<td>Italy</td>
<td>PCR</td>
<td>Diarrheic feces</td>
<td>54/188</td>
<td>28.7% (54/188)</td>
<td></td>
</tr>
<tr>
<td>Japan</td>
<td>PCR</td>
<td>&lt;21 days</td>
<td>104/155</td>
<td>67.1% (104/155)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PCR</td>
<td>&gt;21 days</td>
<td>51/155</td>
<td>32.9% (51/155)</td>
<td></td>
</tr>
<tr>
<td>Brazil</td>
<td>PAGE</td>
<td>Diarrhea</td>
<td>190/735</td>
<td>25.9% (190/735)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EIA</td>
<td>&lt;60 days, Diarrhea</td>
<td>117/451</td>
<td>25.94% (117/451)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ELISA</td>
<td>Not diarrhea</td>
<td>45/299</td>
<td>15.05% (45/299)</td>
<td></td>
</tr>
<tr>
<td>New Zealand</td>
<td>PAGE</td>
<td>Diarrhea</td>
<td>62/302</td>
<td>20.5% (62/302)</td>
<td></td>
</tr>
<tr>
<td>Czech</td>
<td>PCR</td>
<td>Total</td>
<td>75/293</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nursing</td>
<td></td>
<td>22.3% (21/94)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4-8 weeks</td>
<td></td>
<td>35% (7/20)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>8-12 weeks</td>
<td></td>
<td>34.5% (10/29)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sows</td>
<td></td>
<td>12.5% (9/72)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Boars</td>
<td></td>
<td>0% (0/5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Finisher</td>
<td></td>
<td>38.4% (28/73)</td>
<td></td>
</tr>
</tbody>
</table>
Figure 1. Microphotograph of PoRVC particles in feces visualized by negative-staining electron microscopy
Figure 2. Architectural features of rotavirus virion. (A) PAGE gel showing, 11 dsRNA segments comprising the rotavirus genome. The gene segments are numbered on the left and the proteins they encode are indicated on the right. (B) Cryo-EM reconstruction of the rotavirus triple-layered particle. The spike proteins VP4 is colored in orange and the outermost VP7 layer in yellow. (C) A cutaway view of the rotavirus TLP showing the inner VP6 (blue) and VP2 (green) layers and the transcriptional enzymes (shown in red) anchored to the VP2 layer at the five-fold axes. (D) Schematic depiction of genome organization in rotavirus. The genome segments are represented as inverted conical spirals surrounding the transcription enzymes (shown as red balls) inside the VP2 layer in green. (E and F) Model from Cryo-EM reconstruction of transcribing DLPs. The endogenous transcription results in the simultaneous release of the transcribed mRNA from channels located at the five-fold vertex of the icosahedral DLP. Source: (246), with permission of the author.
Figure 3. Replication cycle of rotavirus. Source: (7), with permission of the publisher.
CHAPTER 2. DEVELOPMENT AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES SPECIFIC FOR PORCINE ROTAVIRUS A, B, AND C

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2Department of Veterinary Diagnostic and Production Animal Medicine, College of Veterinary Medicine, Iowa State University, Ames, IA, USA

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Abstract

Rotavirus-associated diarrhea is a common enteric disease in piglets. Group A, B and C rotaviruses have been commonly implicated in US swine while group H was recognized recently. To date, isolates and genomic information of porcine rotavirus (PoRV) B and C are limited due to difficulty in virus isolation. As a result, laboratory tools specific for PoRV B and C are sparse. The study objective was producing monoclonal antibodies (MAbs) against PoRV A, B and C. To that goal, the whole VP6 gene of rotavirus of each group was cloned from feces positive for each group and expressed in a baculovirus system using Bac-to-Bac cloning and expression kit. The recombinant proteins, purified in native conditions, were then used to immunize mice. An ELISA using recombinant VP6 protein of PoRV A, B or C and IFA test using Sf9 cells expressing VP6 protein of PoRV A, B or C were used to screen hybridomas. The resultant MAbs were verified for their protein specificity by Western immunoblot and isotyped. Based on the evaluation, MAb 10A11, 10B1 and 11H3, all of which were of IgG, were selected for PoRV A, B and C, respectively. Of three MAb10A11 and 11H3 were successfully used in immunohistochemical staining to detect PoRV A and C, respectively, in formalin-fixed paraffin-
embedded tissues collected from experimentally infected pigs. In conclusion, murine MAbs specific for VP6 protein of PoRV A, B and C were successfully produced, which can be useful reagents for research of PoRV A, B and C laboratory diagnosis of PoRV A and C.

I. Introduction

Porcine rotavirus, which belongs to the genus *Rotavirus* in the family *Reoviridae*, is an important swine viral enteric pathogen causing diarrhea in piglets. Rotavirus genome has 11 segments of double-stranded RNA, approximately 18.5 kb in size, which encode 6 non-structure proteins (NSP1, 2, 3, 4, 5, 6) and 6 structure proteins (VP1, 2, 3, 4, 6, 7). The virions have three layers making a double capsid structure. The outermost layer comprises of VP4 (86 kDa) and VP7 (37 kDa) proteins which are necessary for virus entry and are important in protective immunity as these proteins contain neutralization epitopes (1, 2). The middle layer is the VP6 protein (44kDa) which is the most abundant and conserved structural protein and highly immunogenic. The core comprises scaffolding VP2 proteins, RNA-dependent-RNA polymerase VP1, and the guanylyltransferase and methylase VP3 (3).

Based on the antigenicity and sequence of VP6 protein/gene, rotaviruses are divided into 10 groups, A through J (4,5,6,7). Of these, group A, B, and C rotaviruses have demonstrated their ability to cause disease in pigs (8,9). The nucleic acid of group H rotavirus has been detected by next-generation sequencing in feces from a diarrheic pig without any other rotaviruses; however, Koch’s postulates have not been fulfilled yet. Although diagnostic laboratory data suggest that porcine rotavirus (PoRV) C has been detected more commonly in neonatal and suckling piglets (<21 days of age) compared to PoRV A and B (10), most of the studies of porcine rotavirus have been done on PoRV A.
Because genomic sequences of PoRV B and C have been sparse, laboratory tools specific for PoRV B and C are limited except PCRs (11,12,10). Disadvantages of PCR are: a) inability to distinguish the infectious virus from inactive form; b) inability to detect agents that have mutation(s) in the primer binding sites; and c) negative impact on its performance by inhibitors in feces (13). Antigen or antibody-based methods, such as enzyme-linked immunosorbent assay (ELISA), immunohistochemistry (IHC), and immunofluorescence microscopy, could be good alternatives and/or supplements. Thus, the goal of this study was to produce monoclonal antibodies (MAbs) specific for PoRV A, B, and C which can be used in diagnostics and research.

II. Materials and Methods

2.1 Study design

The VP6 gene of each of PoRV A (ISU2010035768), B (ISU2011025309A), and C (ISU2010025060P) was cloned and expressed in a baculovirus system. The recombinant VP6 proteins were then purified and used to immunize mice. Two weeks after the last immunization, sera were collected from the mice and tested by ELISA to confirm the immune response to the given antigen in each mouse. Mice with the highest optical density (OD) value to be euthanized for murine spleens, and the splenocytes were subsequently collected and used for cell fusions. After 1 to 2 weeks, all hybridoma supernatants were screened by ELISA using each of the recombinant VP6 proteins as antigen, and the positive ones were further tested by indirect fluorescent antibody (IFA) assay. Monoclonal antibodies positive by both ELISA and IFA test were then selected for further evaluation. The specificity of the selected MAbs for respective VP6 protein was confirmed by Western immunoblot (WIB). Isotypes of the MAbs were determined. VP6-specific MAbs of IgG isotype with the strongest reactivity on ELISA, IFA assay and WIB for each rotavirus group were then selected and evaluated for their utility in
immunohistochemical staining of formalin-fixed paraffin-embedded tissues for specific detection of PoRV A, B or C.

2.2 Sequencing VP6 of porcine rotavirus B

Since VP6 sequences of group B porcine rotaviruses were not available in public domains at the time of starting the study, the PoRVB strain employed for the study was sequenced by using full-length amplification of cDNAs (FLAC) method (14).

Fecal samples containing PoRV B (ISU2011025309A) from previous studied (15) were used to clone PoRV B VP6 gene. PoRV B RNA was extracted from the fecal samples using Trizol (Thermo Fisher Scientific, Walham, Massachusetts) by following manufacturer’s instruction. The single-stranded viral RNA was precipitated by mixing with 4M LiCl (Sigma-Aldrich, St. Louis, Missouri) at the 1:1 ratio and incubating for 16 hours at 4°C followed by a centrifugation at 16000 x g for 30 minutes. The double-stranded (ds) RNA from the supernatant was purified by using MinElute gel extraction kit (Qiagen, Valencia, California) (16). The ds RNA was electrophoresed on a 10% polyacrylamide gel in a large gel format for 16-20 hours at 100 volts (V) (17). The gel then was stained with 1:10000 diluted SYBR Gold (Thermo Fisher Scientific) for 30 minutes. The individual bands visualized under ultraviolet light were cut out and applied in SnakeSkinR tubing, 10kDa (Thermo Fisher Scientific) for electrophoresis at 150V for 30 minutes to collect RNA fragment from the gel. The RNAs were then precipitated by ethanol.

The full-length amplification of VP6 was done based on the protocol described somewhere (18). Briefly, 200ng of rotavirus RNA was ligated to 250ng PC3-T7 loop primer (5’-p–GGATCCCGGGAATTCCGTAATACGACTCACTATATATTATTTTATAGTGAGTCGTATTA–OH-3’) (16) using T4 RNA ligase (Promega, Madison, Wisconsin) supplemented with 10%
DMSO (Sigma-Aldrich) and 20% PEG (Sigma-Aldrich) at 37°C for overnight. Then, the primer ligated RNAs were purified by using MinElute gel extraction kit and denatured at 98°C for 2 minutes with 1M betaine (Sigma-Aldrich) and 2.5% (v/v) DMSO, then chilled on ice for 5 minutes to prevent the RNA going back to the original conformation. The cDNAs were synthesized using SuperScript™ III first-strand synthesis system for RT-PCR (Thermo Fisher Scientific) by following manufacturer’s instructions. The products were incubated with 0.1M NaOH at 70°C for 20 minutes to degrade RNA and then with 0.1M Tris-HCl (pH 7.5) and 0.1M HCl at 68°C for 1 hour and 65°C for 2 hours to neutralize NaOH.

The synthesized cDNAs were purified by using MinElute gel extraction kit by following manufacturer’s instructions and then used for PCR with Accuprime™ Taq polymerase (Thermo Fisher Scientific) and 1.25µM PC2 primer (18). The running condition was 72°C for 2 minutes, 94°C for 2 minutes (initial dissociation), 35 cycles of 94°C for 25 seconds (increasing 1second per cycles), 65°C for 30 second and 68°C for 5 minutes, and final extension at 68°C for 10 minutes. PCR products were analyzed by 1% agarose gel electrophoresis and purified by MinElute gel extraction kit, then subjected for TA cloning using pGEM®-T vector (Promega). The plasmids were sequenced with M13 primers to determine the cloned sequence.

2.3 Cloning and expression of PoRV A, B, and C VP6 genes

The cloning and expression of VP6 genes of PoRV A, B, and C were done with a Bac-to-Bac® cloning and expression kit (Invitrogen, A11098) as per the manufacturer’s instruction. Briefly, the full-length VP6 gene of each of PoRV A, B, and C and the corresponding coding regions were amplified by a PCR method using primers shown in Table 1, which were designed by PrimerSelect (DNASTAR, Madison, WI), and AccuPrime™ Pfx polymerase (Thermo Fisher Scientific, Waltham, MA). Each PCR product then was cloned in a pFastBac™ TOPO® vector
(Thermo Fisher Scientific). Next, the plasmids with VP6 gene of PoRV A, B or C, which were intact and had the correct direction, were transformed to MAX Efficiency™ DH10Bac™ competent E. coli cells (Thermo Fisher Scientific) by following manufacturer’s recommended procedure. Subsequently, the bacmids were extracted using PureLink® HiPure DNA isolation kit (Thermo Fisher Scientific) by following the manufacturer’s user manual. The bacmids, 500ng/µl in TE buffer (10mM Tris, 1mM EDTA, pH8) were transfected into Sf9 cells (Thermo Fisher Scientific) using Cellfectin® (Thermo Fisher Scientific) by following manufacturer’s recommended procedure. After 96-hour incubation, the media containing PoRV A, B or C VP6 recombinant baculovirus were collected and were designated passage 1. After being propagated to passage 2 and tested for the titers by plaque assay, each of the recombinant baculoviruses was used to infect Sf9 cells prepared in Sf-900 II SFM media (Thermo Fisher Scientific) at 10 multiplicity of infection (moi) and the inoculated Sf9 cells were incubated for 48 hours. The cell supernatants were collected for protein purification.

2.4 Purification of expressed recombinant VP6 proteins

The recombinant VP6 proteins of PoRV A, B, and C were purified under native conditions using the Ni-NTA Purification System (Thermo Fisher Scientific) in which urea was not used in attempt to preserve the native structure of VP6 protein. In brief, VP6 protein expressing Sf9 cells in two 192cm² cell culture flasks (Thermo Fisher Scientific) for each rotavirus group were lysed in 8ml of 1X native purification buffer (50mM NaH2PO4 and 0.5M NaCl; pH 8.0) added with leupeptin (Sigma Aldrich, St. Louis, MO) at the rate of 0.5 µg/ml by freeze-and-thawing three times and then passed through 18-gauge needles. The lysates were centrifuged at 8000 x g for 30 minutes for clarification. The resulting supernatants were poured into Ni-NTA gel columns and retained for one hour with the columns being rotated. Then the
gels were washed four times by flowing the native purification buffer containing 20 mM imidazole (pH 8.0) through the columns. Finally, the proteins were eluted by flowing 8ml of the native buffer containing 250 mM imidazole (pH 8.0) through each column. Imidazole was then removed from each protein preparation by centrifuging each eluate in Amicon® Ultra-15, 30 kDa (Millipore, Billerica, MA) at 4000 x g for 30 minutes.

For the VP6 protein of PoRV B, mice screening results demonstrated that the 6X His tag had to be removed for immunization purpose; hence it was cut using AcTEV™ Protease (Thermo Fisher Scientific) and removed from the final VP6 protein as suggested in the manufacturer’s user manual.

2.5 Mouse immunization and cell fusion

All of pertinent procedures were done at the Hybridoma Facility of Iowa State University. Three specific-pathogen-free 4- to 6-week old BALB/c mice each were immunized twice with a 2-week interval by subcutaneous injection of each of the VP6 proteins (20 µg per mouse) mixed with incomplete Freund’s adjuvants (MP Biomedicals, Santa Ana, CA) at the 1:1 ratio. Sera were collected from all immunized mice via mandibular vein 2 weeks after the second immunization and were evaluated by ELISA as described below. Mice were euthanized for sterile splenectomy at 5 days after the third time injection without any adjuvant via intraperitoneal route. Splenocytes were aseptically harvested from each collected murine spleen for cell fusion. Sp2/O-Ag14 murine myeloma cells (ATCC, Manassas, VA) were prepared as fusion partner at the rate of 4 x 10⁷ and washed three times by Dulbecco’s Modified Eagle’s Medium (Sigma) without fetal bovine serum. The Sp2/O cells were pelleted by centrifugation and mixed with pelleted mouse splenocytes at the 1:1 ratio. Fusion of two cell types was enforced in the presence 1ml of polyethylene glycol (Roche). Next, 10ml of warm DMEM was
added dropwise and after 10-minute incubation, the mixture was centrifuged at 100 x g for 10 minutes. The resulting pellet was suspended in the 75ml DMEM containing 1X of HAT (100µM hypoxanthine, 0.4µM aminopterin, 16µM thymidine; Sigma) supplemented with 10% horse serum (Atlanta Biologicals, Flowery Branch, GA) and antibiotics containing 100 units/ml penicillin and 100µg/ml streptomycin (Sigma-Aldrich), and dispensed into cell-culture grade, flat-bottomed 96-well polystyrene plates and incubated at 37°C for 1 to 2 weeks. When 90-100% confluency of the cells was made, the supernatants were collected for screening by ELISA. The selected hybridomas were transferred to 24-well plates for expansion. Subsequently, when the cells reached full confluence in the wells, the supernatants were collected for screening by ELISA for the second time. The selected hybridomas were serially scaled up to larger cell cultureware (i.e., 25cm² flasks and then 75cm² flasks) and eventually stored frozen in the liquid nitrogen.

2.6 ELISA

To prepare antigens, MaxiSorp® flat-bottom 96-well plates (Nunc, 442404) were coated with each VP6 (50ng/well) in a commercially available phosphate buffer (pH 7.2) containing 0.1M sodium phosphate and 0.15M sodium chloride (Thermo Fisher Scientific) overnight at 4°C. The plates then were washed five times with a wash buffer (phosphate-buffered saline with 0.1% Tween 20, pH7.2), blocked with 300µl/well of 1% bovine serum albumin (Jackson ImmunoResearch Laboratories, West Grove, PA) for 2 hours at ambient temperature and dried for 2 hours at 37°C. The primary antibodies (i.e., mouse serums or hybridoma supernatant; 50µl/well) were added to the plates and incubated for 30 minutes at 37°C in a humidified chamber. After washing three times with the wash buffer, the plates were incubated with anti-mouse IgG secondary antibody labeled with peroxidase (Seracare, Milford, MA) for 30 minutes
at 37°C. After three times washing, the plates were incubated with a TMB substrate (100µl/well) (Seracare, Milford, MA) at room temperature (20-22°C) in the dark. Colorimetric reactions were stopped by adding 0.1M H₂SO₄ stop solution (100µl/well). The plates were then read by a microplate ELISA reader and OD of each hybridoma supernatant was determined at 450nm wavelength. The hybridomas with OD>0.5 were considered positive for the target VP6 and were selected for further testing.

2.7 Indirect fluorescent antibody assay

To prepare antigens, Sf9 cells were seeded in 96-well cell culture plates at the rate of 3x10⁵ cells/well. One hour later, the cells were inoculated with recombinant baculovirus containing VP6 gene of PoRV A, B, or C at the rate of 1 moi. At 48 hours after infection, the cells were fixed by 80% cold acetone aqueous solution for 15 minutes at -20°C.

Hybridoma supernatants which were positive for the respective VP6 by ELISA were applied to each well in the plates (50µl/well). The plates were incubated at 37°C for 1 hour in a humidified chamber. After washing three times with phosphate-buffered saline (PBS, pH7.2), optimally diluted goat anti-mouse IgM+IgA+IgM (H+L) polyclonal antibody conjugated with fluorescein isothiocyanate (Seracare, Milford, MA) was dispensed into each plate (100 µl/well) and incubated for 30 minutes at 37°C under the humidified condition. The plates were then washed three times with PBS and examined under a fluorescent microscope for specific fluorescence.

The finally selected MAbs specific for PoRV A and C VP6 were further evaluated on MA-104 and IPEC-1 infected with PoRV A and PoRV C, respectively by IFA test in the identical manner described above.
2.8 Western immunoblot (WIB)

Western immunoblotting was performed to confirm the production of the VP6 proteins as well as the specific recognition of MAbs for the VP6 proteins (19). The purified 6xHis-VP6 proteins were processed through SDS-PAGE and transferred to nitrocellulose (NC) membranes (Bio-Rad Laboratories, Hercules, CA). Each membrane was blocked with 20ml of 5% (W/V) non-fat dried milk at room temperature for 1 hour at room temperature. For confirming the production of the VP6, the membranes were incubated with 10ml of 1:10000 diluted 6X His tag monoclonal antibody (Thermo Fisher Scientific) for 1 hour. While for the specific recognition of MAbs for the VP6 proteins, the membranes were incubated with 10ml of hybridoma cell culture supernatant containing MAb that was positive for PoRV A, B or C by ELISA and IFA for 1 hour at room temperature. After washing three times with Tris-buffered saline (pH 7.4) with 0.5% Tween 20 (TBST), the membranes were incubated anti-mouse IgG labeled with peroxidase (Seracare, Milford, MA) for 1 hour at room temperature. Finally, each membrane was washed three times with TBST and immersed in 5 ml of TMB substrate (Seracare). The colorimetric reactions were stopped by washing two times with distilled water.

2.9 Isotyping of monoclonal antibodies

Monoclonal antibodies in all selected hybridoma supernatants were isotyped using IsoStrip™ Mouse Monoclonal Antibody Isotyping Kit (Roche, Basel, Switzerland) by following the manufacturer’s instructions.

2.10 Immunohistochemistry (IHC)

To evaluate the diagnostic utility of MAbs produced, the selected MAbs were applied to immunohistochemical staining of formalin-fixed paraffin-embedded intestinal tissues for rotaviruses. While anti-PoRV A VP6 MAb was used in forms of hybridoma cell culture
supernatants, MAbs for VP6 of PoRV B and C were purified by Melon™ gel Monoclonal IgG purification kit (Thermo Fisher Scientific, 45214) to reduce the background issue and increase the concentration before being used in IHC based on preliminary testing results.

Intestinal tissues used to evaluate the MAbs were collected from piglets experimentally infected with PoRV A, B or C in a previous study conducted in our laboratory (15). After being formalin-fixed and paraffin embedded, the tissues were cut into 4-μm sections and fixed into glass slides at 55°C for 30 minutes. Subsequently, the IHC processes were carried out by Leica Bond III autostainer (Leica Biosystems, Buffalo Grove, IL) following the standard protocol from Iowa State University Veterinary Diagnostic Laboratory (SOP #9.369).

III. Results

3.1 Sequence analysis of PoRV B 2011025309A

Sequencing demonstrated that VP6 gene (full-length) of PoRV B (ISU2011025309A) is 1269 nucleotides long and has one open reading frame of 1176 nucleotides which encodes 391 amino acids. This sequence has the same length as VP6 genes of other porcine rotaviruses. Sequence comparison showed 66-90% similarity with VP6 genes of other group B porcine rotaviruses which became available in the public domain during the study and 68-78% similarity with rotavirus B in other species (human, bovine).

3.2 Protein expression

VP6 protein of PoRV A, B or C was successfully expressed from each of the 3 constructed recombinant baculoviruses in Sf9 cells although the level of protein expression from each construct differed. All 3 purified proteins were approximately 43-44 kDa in size on 12% SDS-PAGE followed by Western immunoblot using anti-6X His tag antibody as illustrated in Figure 1.
3.3 Screening hybridoma supernatant by ELISA

Two weeks after fusion, supernatants of hybridomas in 96 well plates were collected and screened for production of antibody against VP6 proteins of PoRV A, B and C by ELISA. Twenty-positive hybridoma clones were selected and subcultured to 24-well plates for expansion. After 2 weeks, supernatants of those hybridomas were screened again by ELISA to make sure that the hybridomas were stable in antibody secretion. Finally, two hybridoma clones each for PoRV A, B and C VP6 which had the highest OD were selected and designated 10A11 (group A), 12C10 (group A), 10B1 (group B), 10F7 (group B), 10B5 (group C), and 11H3 (group C).

3.4 Characterization of monoclonal antibodies

The six selected hybridoma clones were shown to produce antibody specific for the intended target, i.e., VP6 protein of PoRV A, B or C without any cross reactivity when tested by IFA assay against Sf9 cells inoculated with the recombinant baculovirus containing the respective VP6 gene as illustrated in Figure 2. Moreover, the MAb 10A11 and 11H3 specifically recognized PoRV A and C, respectively, which were propagated in MA-104 and IPEC-1 cells, respectively as showed in Figure 3.

All six MAbs except one (10F7) were of IgG isotype (Table 2). On WIB, all the MAbs specifically recognized the corresponding recombinant VP6 proteins of 43-44 kDa in size as shown in Figure 4. Furthermore, the MAb 10B1 reacted with native VP6 protein from a lysate of virus material prepared from PoRV B-positive feces (unshown data).

3.5 Application of monoclonal antibodies in IHC for porcine rotaviruses

Anti-PoRV A VP6 MAb 10A11, in the form of the hybridoma culture supernatant, could specifically detect the presence of PoRV A in small intestinal tissues collected from neonatal
piglets experimentally infected with PoRV A (Figure 5 left panel). The specific reactivity of the MAb for PoRV A remained up to 1:10000 dilution.

Initial IHC attempts with anti-PoRV C VP6 MAb 11H3 in the form of the hybridoma culture supernatant was unsuccessful. After purification and concentration, the MAb was able to specifically recognize the presence of PoRV C in intestinal tissues collected from experimentally infected pigs as shown in Figure 5 (right panel).

In contrast to the MAbs specific for VP6 of PoRV A and C, the anti-PoRV B VP6 MAb 10B1 and 10F7 did not work in IHC to detect the presence of PoRV B in intestinal tissues from experimentally infected pigs despite of applying purification/concentration of hybridoma supernatants and trying different protocols for antigen retrieving methods (enzyme versus heat), different buffers for heat retrieving method, and different running temperature conditions.

IV. Discussion

The VP6 protein of rotavirus is an abundant and highly immunogenic rotaviral protein among viral structural proteins and is most commonly used as target for rotavirus diagnostics (20). It has also been used as the basis for (sero)grouping of rotavirus (3). That was the rational for developing VP6-specific monoclonal antibodies for each of porcine rotavirus A, B, and C.

The development of MAb against VP6 of PoRV B had a couple of challenges. First, there was no VP6 sequence for PoRV B available in public domains when this study was initiated. The sequence was achieved by using full-length amplification of cDNA technique, which was developed by (14), to sequence genomes of double-stranded RNA viruses. While this method itself should have been straightforward and easily reproducible, a problem was encountered in this study because the protocol had to be used on the RNA of PoRV B extracted from feces. Some RNA fragments, such as NSP3, NSP1, and VP1, seemed to be more preferably ligated
with PC3-T7 loop primers than the rest including VP6; hence, VP6 sequences could not be achieved. This problem was resolved with separating the RNA fragments by PAGE, excising individual bands from the gel, and eluting each RNA fragment by electrophoresis in a dialysis tube. The eluted RNAs were then precipitated and used for ligation. This method is newly attempted for the first time in this study and should be applicable in sequencing other viruses with ds RNA genomes. Second, attempts to get a hybridoma cell clone that secret MAbs specific to VP6 of PoRV B were unsuccessful while the clones positive to 6X His tag were dominant. The reason could be the 6X His tag interfere with biological activity of protein (21). This problem was resolved by cleaving of 6X His tag from the recombinant PoRV VP6 protein before immunizing mice with the protein.

Unfortunately, none of the MAbs against PoRV B worked in IHC although they were functional in the other tests such as ELISA, IFA assay, and WIB. Thus, the development of PoRV B MAb that can be used in IHC is remained to be further explored. Moreover, it should be noted that the monoclonal antibodies for PoRV A and C produced in this study were not evaluated on other strains within each serogroup using a large numbers of clinical cases. Therefore, it remains to be assessed how broadly each MAb can recognize various rotavirus strains circulating in swine herds, knowing a high degree of genetic variability among rotaviruses within each group although VP6 is known to be antigenically conserved for each group.

No MAb against PoRV C is commercially available although PoRV C appears to be common in pre-weaning piglets compared to PoRV A or B based on results of PCR-based assessment at diagnostic laboratories (10). In this sense, MAb 11H3 would be a valuable reagent for diagnostic and research purpose. Although PCR-based assays are being used most commonly to detect PoRV in fecal materials from diarrheic pigs at many veterinary diagnostic laboratories,
PCR testing has limitations: a) the diagnostic sensitivity of PCR can be interfered by inhibitors commonly found in feces or the mutations commonly found in RNA viruses; b) PCR cannot differentiate infectious viruses from inactivate ones; and c) it is not uncommon to detect more than one rotavirus groups when testing feces. Thus, the accuracy of rotavirus diagnostic related to the causative role of PoRV in enteric disease can be enhanced by employing an assay detecting virus in tissues with lesions, such as cryosection fluorescent antibody test and IHC when monospecific antibodies for the virus are available. Since rotaviruses are ubiquitous in swine herds, detecting rotavirus antibody may have little diagnostic value. However, serology assays such as IFA assay and indirect or blocking ELISA which can assess isotype of the antibodies might still be valuable in identify the status of the rotavirus infection/vaccination in swine populations. Thus, the recombinant VP6 proteins and MAbs achieved in this study can be the crucial reagents for developing assays for rotavirus antigen and antibodies, which are warranted to be further studied in the future.

In conclusion, recombinant VP6 proteins of PoRV A, B, and C and murine monoclonal antibodies (IgG isotype) specific for VP6 proteins of PoRV A, B, and C were successfully developed, which can be used for research and diagnostic purposes. In particular, the availability of the antibodies 10A11 and 11H3, which are specific to PoRV group A and C, respectively and can be used in IHC, may be useful to study the pathogenesis of rotaviruses in pigs.
References


### Table 1. Primers used for amplifying full length and coding regions of VP6 of porcine rotavirus A, B, and C

<table>
<thead>
<tr>
<th>Primers</th>
<th>Forward</th>
<th>Reverse</th>
<th>References</th>
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<tbody>
<tr>
<td>VP6- A</td>
<td>GGCTTTTAAACGAAGTCTTC</td>
<td>GGTCACATCCTCTCACTA</td>
<td>(22)</td>
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<tr>
<td>VP6- B</td>
<td>GGTATTAATTAGTCCAATCAGGGTTGC</td>
<td>CTATTTTTTTTCATCCTCTCGAAGCA</td>
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</tr>
<tr>
<td>VP6- C</td>
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<td>AGCCACATAGTTCACATTTTC</td>
<td>(23)</td>
</tr>
<tr>
<td>VP6- A coding</td>
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<td>VP6- B coding</td>
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<td>ATGGACGTGCTATTTTTCCATTG</td>
<td>CATCACCATTCTCTCAGGGA</td>
<td>This study</td>
</tr>
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</table>
Table 2. Characteristics of anti-porcine rotavirus (PoRV) VP6 monoclonal antibodies produced

<table>
<thead>
<tr>
<th>Hybridoma ID</th>
<th>Isotype</th>
<th>Serogroup specificity</th>
<th>Immunofluorescence test on MA-104 cell infected with PoRV A</th>
<th>Immunohistochemistry on small intestine from pigs inoculated with PoRV A</th>
<th>PoRV B</th>
<th>PoRV C</th>
</tr>
</thead>
<tbody>
<tr>
<td>10A11</td>
<td>IgG</td>
<td>PoRV A</td>
<td>+</td>
<td>+</td>
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<td>–</td>
</tr>
<tr>
<td>12C10</td>
<td>IgG</td>
<td>PoRV A</td>
<td>N/D*</td>
<td>N/D</td>
<td>N/D</td>
<td>N/D</td>
</tr>
<tr>
<td>10B1</td>
<td>IgG</td>
<td>PoRV B</td>
<td>N/A§</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>10F7</td>
<td>IgM</td>
<td>PoRV B</td>
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<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>10B5</td>
<td>IgG</td>
<td>PoRV C</td>
<td>N/D</td>
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<td>N/D</td>
<td>N/D</td>
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<tr>
<td>11H3</td>
<td>IgG</td>
<td>PoRV C</td>
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<td>+</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

*N/D = not determined
§N/A = not applicable
Figure 1. Confirmation of VP6 protein expression in Sf9 cells inoculated with recombinant baculovirus constructed to contain VP6 gene of porcine rotavirus A (lane A), B (lane B) and C (lane 3) by Western immunoblot using anti-6XHis tag monoclonal antibody.
Figure 2. Specific reactivity of monoclonal antibody 10A11, 10B1 and 11F3 with recombinant VP6 protein expressed in Sf9 cells infected with a recombinant baculovirus constructed to contain VP6 of porcine rotavirus A (panel A), B (panel B), or C (panel C) or wild type (D), respectively, as determined by indirect fluorescent antibody assay
Figure 3. Specific reactivity of anti-VP6 monoclonal antibodies 10A11 and 11F3 with porcine rotavirus A (left panel) and C (right panel) propagated in MA-104 and IPEC-1 cells, respectively, as determined by indirect fluorescent antibody assay.
Figure 4. Specific recognition of recombinant VP6 protein of porcine rotavirus A, B and C by monoclonal antibody 10A11 (A), 10B1 (B) and 11F3 (C), respectively, as determined by Western immunoblot technique.
Figure 5. Immunohistochemical staining of enterocytes infected with porcine rotavirus A (left panel) and C (right panel) in small intestine by VP6-specific monoclonal antibody 10A11 and 11F3, respectively.
CHAPTER 3. COMPARATIVE PATHOGENICITY OF PORCINE ROTAVIRUS A, B AND C IN NEONATAL PIGS

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Abstract

Rotavirus, which has been classified in ten groups (A to J), is an important cause of diarrheal disease in human and animals. In pigs, rotavirus A, B and C are common groups identified and cause enteric disease mostly in young pigs. Historically porcine rotavirus (PoRV) A has been the main cause of post-weaning diarrhea. Recently neonatal diarrhea associated with PoRV C infection has been increasingly diagnosed while PoRV B has been identified in all ages, suggesting that rotavirus groups vary in the pathogenicity in neonatal pigs. The following study was conducted to compare the pathogenicity of PoRV A, B, and C individually or in combinations in naïve newborn piglets. Forty-eight one-day-old cesarean-derived colostrum-deprived (CDDC) pigs were divided into eight treatment groups. Pigs in each group were challenged with PoRV that belong to individual group A, B, C or all combinations of group A, B and C. Clinical signs were recorded every 12 hours post inoculation (hpi). Rectal swabs were obtained before inoculation and then every 12 hpi. Half of the pigs in each group were euthanized at 24 hpi and the rest were at 72 hpi. Intestinal contents were collected at necropsy. The swabs and contents were tested by RT-PCR assay for virus shedding. Multiple parts of the
intestine (i.e., duodenum, jejunum and ileum) were collected for histopathology. All rotaviruses used caused diarrheal disease in neonatal piglets regardless of their groups. There was no remarkable difference in the severity of diarrhea caused by rotavirus A, B, and C or combinations although the onset of PoRV B and C shedding was earlier than PoRV A. All the rotavirus inocula were able to cause atrophic enteritis. Although the lesion caused by PoRV A was more prevalent in mid to distal jejunum, the viral antigen was found throughout the small intestine. Porcine rotavirus B and C caused diffuse villous atrophic change throughout the small intestine which was corresponded with the positive viral nucleic acid and antigen detection, respectively. In conclusion, all rotavirus groups are equally pathogenic to immunologically naïve neonates but may vary in their replication patterns in intestine.

I. Introduction

Porcine rotavirus, a non-enveloped and icosahedral RNA virus, belongs to the genus *Rotavirus* in the family *Reoviridae*. The rotavirus genome has 11 double-stranded RNA segments with varying size which encode 6 nonstructural proteins (NSP1, 2, 3, 4, 5, and 6) and 6 structural proteins (VP1, 2, 3, 4, 6, and 7). The virion has three protein layers making double capsid structure. The most outer layer comprises of VP4 and VP7 which are necessary for virus entry. The middle layer is made by VP6, a most abundant structure protein. The core comprises scaffolding VP2 proteins, RNA-dependent-RNA polymerase VP1, and the guanylyltransferase and methylase VP3 (1).

Rotaviruses are divided into 10 groups (A-J) base on antigenicity and sequence of VP6 (2-5). Of these, rotavirus A, B, C, E, and H have been implicated in enteric disease of pigs (6, 7). Porcine rotavirus (PoRV) A, B, C, and H have been found in US swine populations (7, 8).
All rotaviruses are known to primarily target mature, non-dividing enterocytes on the tips of villi in the small intestine (1). The viral replication in enterocytes results in the cell lysis and consequently causes villous blunting and atrophy. The age of pigs (9) and rotavirus strains (10) have been attributed for different degrees of villous atrophy severity. Clinically, rotavirus infection in pigs is manifested by diarrhea and dehydration (11), which causes a moderate economic loss in the swine industry (12). Rotavirus-associated diarrhea is attributed to malabsorption as well as increased secretion due to enterocyte destructions, viral enterotoxin (NSP4) and stimulation of the enteric nervous system (1).

Based on rotavirus PCR test results on case submissions to veterinary diagnostic laboratories, PoRV A has been most prevalent in 21- to 42-day-old pigs (i.e., post-weaning), whereas PoRV B have been found equally in all ages although more than 50% of rotavirus B positives were in pigs of 3 weeks of age or older, and PoRV C have been frequently found in neonates and suckling piglets (i.e., pre-weaning). From the observed difference in age distribution of rotavirus groups, it was hypothesized that PoRV A, B and C may differ in their ability to cause disease in neonatal pigs. The following comparative pathogenicity study was conducted to address it. The specific objective of the study was to compare the clinical signs, viral shedding, and intestinal destruction in pigs infected with PoRV A, B, and C singularly and in all combinations including co-infecting a subset of pigs with all three groups simultaneously.

II. Materials and Methods

2.1 Animals

Pregnant cross-bred sows were purchased from a commercial breeding herd and delivered to Iowa State University (ISU) Livestock Infectious Disease Isolation Facility (LIDIF) approximately one week prior to the expected farrowing date. On day 113 of gestation, caesarian
section surgeries were performed on the sows for piglet derivation. Neonatal piglets were separated from dams immediately upon birth and did not receive colostrum. Therefore, caesarian-derived and colostrum-deprived (CDCD) neonatal piglets were used in this study. All CDCD piglets included in this study were triaged at birth with navels clamped, cut, and sprayed with gentile iodine. Piglets received an iron and antibiotic injection (Excede®, Pfizer) per labeled directions.

2.2 Study design

Piglets were randomly divided into eight treatment groups with six piglets each. Piglets were inoculated with virus-free media (negative control) or with PoRV A, B, or C singularly and all possible combinations via orogastric gavage. Table 1 summarizes the study design. Study termination points were 24 and 72 hours post inoculation (hpi); half of the pigs in each group were euthanized at 24 hpi and the remaining piglets at 72 hpi.

2.3 Housing and care

All animals were housed in the ISU LIDIF. Piglets were individually housed in new plastic totes which were commercially purchased and each treatment group was kept in a separate temperature controlled room with HEPA filtration. Piglets were fed milk replacer (Esbilac; Pet-Ag, Hampshire, IL) three times a day (7 am, 12 pm, and 7 pm). All feedings were done by oral-gastric lavage using an 8-gauge French catheter. Different group of caretakers were assigned to each group. Strict shower-in/shower-out was practiced to prevent cross contamination between treatments.

2.4 Inoculum and inoculation

Rotaviruses used in this experiment were obtained from clinical cases during diagnostic investigation. The PoRV A isolate (ISU2010035768) were made and propagated in MA-104
cells using 1µg/ml trypsin in infection media as previously described (13). The titer of PoRV A was $10^6$ plaque-forming units (PFU)/ml. PoRV B (ISU2011025309A) and C (ISU2010025060P) were prepared from feces through a series of purification by laboratory techniques and amplification in CDDC piglets in a similar manner previously described (14, 15).

Each rotavirus inoculum was then adjusted to contain approximately 1,000 PFU/ml equivalent. For that purpose, all rotavirus materials were serially diluted and tested by gel-based PCRs as previously described (16, 17). The rotavirus B and C were PCR-negative after 1:1000 dilutions while the rotavirus A were PCR-negative at 1:1000000 dilution. The rotavirus A was then diluted 1:1000 in cell culture media to match its virus amount up with the virus amount in the rotavirus B and C inocula. The gel-based PCR was also used to confirm specificity of each inoculum. The amount of rotavirus RNA in the rotavirus A and C inocula was assessed by a quantitative real-time RT-PCR (ISUVDL-9.3284) and was confirmed to be similar.

Piglets were orogastrically inoculated with virus(es) as shown in Table 1 approximately 4 hrs post-surgery using an 8-gauge French catheter. Singularly infected groups (groups 2, 3, & 4) received 3 mL of inoculum orally. Dual infected groups (groups 5, 6, & 7) received 1.5 mL of each group and group 8 (triple infection) received 1 mL of each group. In this way, all pigs were given a similar total number of rotavirus genomic copies per pig.

2.5 Sample collection

Rectal swabs were taken from all piglets prior to inoculation and every 12 hours thereafter. At necropsy, rectal swabs, serum, colonic contents, and tissues of five segments of small intestine (i.e., duodenum, proximal jejunum, mid jejunum, distal jejunum, and ileum) were collected from all pigs. For consistency in sampling, the entire small intestine was extracted from
each pig, the mesentery cut away, and then folded back on itself several times (3 times). The same locations were cut from each pig as previously described (18).

2.6 PCR

Fecal swabs were tested by a quantitative real-time RT-PCR targeting VP6 gene of each rotavirus group with primers and probes listed in Table 2. The PCR for PoRV A and C was described previously (8). The PCR for PoRV B was developed specifically for the virus used in this study. The primers and probe were designed by Primer Express 3.0.1 (ThermoFisher Scientific, Waltham, MA) based on VP6 gene sequence of PoRV B which was obtained in a previously study (19).

2.7 Histopathology

After 48 h fixation in neutral buffered formalin, tissue sections were trimmed, processed, and embedded in paraffin. Four micron thick sections were cut and stained with hematoxylin and eosin by following the standard protocol (SOP #9.191) at Iowa State University Veterinary Diagnostic Laboratory (ISUVDL). Five perceived full length villi and crypts in each microscopic section of intestine were measured using a computerized image system (Olympus DP72 camera, cellSens Standard, Waltham, MA) (18). All assessment was done by one pathologist at a time and repeated by the second pathologist for reproducibility and confirmation.

2.8 Immunohistochemistry (IHC)

The IHC was done by following the standard protocol at ISUVDL (SOP #9.369). Formalin-fixed, paraffin-embedded intestinal tissues were cut and mounted on positively charged glass slides and dried at 60°C for 30 minutes, then loaded to the Leica Bond III autostainer (Leica Biosystems, Buffalo Grove, IL). The slides then were deparaffinized with xylene, three times, 5 minutes each and rehydrated with 100% alcohol, 95% alcohol, 70% alcohol, tap water,
and distilled water. Immunohistochemical staining was done using 10A11 and 11H3 murine monoclonal antibodies (MAbs) which were produced against PoRV A and C, respectively, in our laboratory previously (19). The antigen detection was semi-quantitatively estimated by a score system based on the following criteria: 0 = no signal, 1 = 1–20% of villous enterocytes within the section showing a positive signal, 2 = 21–50% of villous enterocytes showing a positive signal, and 3 = greater than 50% of villous enterocytes showing a positive signal. All slides were read by two designated pathologists, one at a time for consistency and reproducibility.

2.9 In situ hybridization (ISH)

An ISH-based test was used to detect RNA of PoRV B in intestinal tissues using an RNAscope® probe because a MAb-based IHC did not work for PoRV B. The probe specific for VP6 gene of the PoRV B strain used in the study was designed by Advanced Cell Diagnostics (Newark, CA, USA). The test was done by following manufacturer’s instructions. Briefly, the intestinal tissue embedded paraffin blocks were cut into 5µm sections. The ribbons were put in 45°C water bath and mounted on Superfrost™ Plus slides (ThermoFisher Scientific, Waltham, MA). The slides were air-dried overnight and then were baked at 60°C for 1 hour. The deparaffinization was done by incubating the slides two times in xylene for 5 minutes each at room temperature (RT) and then washed two times in 100% ethanol for 1 minutes each at RT. The dried slides were incubated with RNAscope® Hydrogen Peroxide for 10 minutes at RT and washed three to five times with water. The slides were boiled in 1X retrieval buffer provided in the kit for 30 minutes and then washed immediately with water and finally with 100% ethanol. Next, the protease provided in the kit was applied to the slides and incubated for 30 minutes at 40°C. Then the following steps were performed with two times washing for 2 minutes each in between. First, the probe was hybridized to RNA in the samples for 2 hours at 40°C. Next, Amp
1 to 6 was incubated with the sample in a step-by-step fashion for 30 minutes with Amp 1, 3, and 5 and for 15 minutes with Amp 2, 4, and 6. The Amp 1 to 4 were incubated at 40°C while Amp 5 and 6 were incubated at RT. The hybridization was visualized by incubating with DAB for 10 minutes at RT then washed with distilled water. The slides were stained with 50% hematoxylin for 2 minutes and washed with distilled water. Subsequently, the slides were dehydrated with 70% ethanol for 2 minutes, two times with 100% ethanol for 2 minutes each, and with xylenes for 5 minutes, at RT. The slides were dried at RT for 10 minutes and mounted with Cytoseal™ XYL (ThermoFisher Scientific) for 5 minutes. Finally, all slides were observed under an optical microscope for scoring. The amount of PoRV B RNA was semi-quantitatively scored based on the following criteria: 0 = no signal, 1 = 1–20% of villous enterocytes within the section showing a positive signal, 2 = 21–50% of villous enterocytes showing a positive signal, and 3 = greater than 50% of villous enterocytes showing a positive signal. All slides were read at the same time by the same person for consistency.

2.10 Statistical analyses

Mean villous length by intestine sections and group were subjected to an analysis of variance (ANOVA) with mean comparison using an all pairs Tukey-Kramer adjacent. The same was done for crypt length. The viral shedding titers were compared using restricted maximum likelihood method. IHC and ISH scores were compared using a generalized linear mixed (GLIMMIX) by Statistical Analysis System (SAS) software version 9.3 (SAS Institute, Cary, NC). Significant differences were determined when p value was equal to or less than 0.05.
III. Results

3.1 Clinical signs

Diarrhea was not observed in any of the negative control pigs during the 74-hour study period after inoculation. In contrast, 50% and 100% of pigs in all of the virus-inoculated groups became diarrheic at 24 hpi and 48 hpi, respectively. Diarrhea, characterized by watery or creamy and yellow feces, continued until termination of the study. Clinically, there was no discernable difference in the severity of diarrhea between singular or co-infected groups. At necropsy, virus-inoculated pigs were variably dehydrated and thin and had fecal staining on the peritoneum, whereas control piglets were normal with no fecal staining and had formed feces within the colon.

3.2 Pathology

Grossly, small intestines of virus-inoculated pigs were segmentally thin-walled and spiral colons were distended by a large amount of watery contents. There was no apparent difference in the severity of gross lesions among the inoculated groups.

Microscopically, virus-inoculated pigs had varying degrees of villous atrophy in small intestines. The degree of villous atrophy, as measured by villous length, for sham- and virus-inoculated groups is summarized in Figure 1 (pigs necropsied at 24 hpi) and Figure 2 (pigs necropsies at 72 hpi). Pigs inoculated with PoRV A had significant villous atrophy only in mid jejunum at 24 hpi (p=0.035) and in all segments except duodenum at 72 dpi (p<0.05). In comparison, pigs inoculated with PoRV B or C had significant villous atrophy at all segments from 24 hpi (p<0.05). All co-infected pigs had significant (p<0.05) villous atrophy in all segments at all times except duodenum of group A/B and A/B/C at 24 hpi.
There was no significant statistical difference in crypt depth across all treatment groups necropsied at 24 hpi. Group A, B, A/C and A/B/C had crypts in duodenum or/and proximal jejunum significantly longer than those of sham negative control and group C at 72 hpi.

### 3.4 Immunohistochemistry (IHC) and in situ hybridization (ISH)

PoRV A and C antigens in intestinal tissues were specifically detected by immunohistochemical staining with the MAb 10A11 and 11H3, respectively (Figure 4A and 4C), while the MAb 10B1 failed to detect the PoRV B antigen in any of the examined intestinal tissues for unknown reasons. As an alternative, an RNAScope® ISH technique was used and could detect PoRV B RNA in the tissues (Figure 4B). Using IHC and ISH techniques, distribution of PoRV A, B and C in intestinal tissues after experimental inoculation were assessed.

In singularly infected groups, the PoRV B RNA and PoRV A and C antigens were detected in villous enterocytes at both 24 hpi and 72 hpi. Distribution-wise, PoRV A, B and C were detected in all small intestinal segments at 24 hpi. However, at 72 hpi, PoRV A was detected only in proximal jejunum and duodenum while PoRV B and C were found in all segments. There was no significant difference in the IHC/ISH scores of PoRV A, B or C among all intestine segments. Overall, PoRV A antigen was detected predominantly in jejunum (proximal, mid, distal) and ileum at 24 hpi compared to that at 72 hpi. In contrast, PoRV B and C distributions in all of the intestinal segments between the two time points were not significantly different (Figure 5).

In co-infected groups, differences in distribution of PoRV A, B, and C between 24 hpi and 72 hpi depended on combinations of virus inocula. In group A/B and A/C, no significant difference in the distribution of the individual virus in all intestinal segments was observed
between the two time points. In group B/C and A/B/C, however, PoRV C antigen was predominant in all the segments of small intestine at 24 hpi compared to that at 72 hpi.

Comparisons between IHC or ISH scores of the PoRV in singular groups and those of co-infected groups were illustrated in Figure 6. The significant difference in PoRV A IHC scores were found between group A and groups A/B, A/C, and A/B/C in the jejunum at 24 hpi (p<0.05) and between group A and groups A/C and A/B/C in the ileum at 24 hpi (p<0.05). There was, however, no difference in IHC scores between group A and any of co-infected groups at 72 hpi. For PoRV B, there was no significant difference in PoRV B ISH scores between group B and groups A/B, B/C, and A/B/C in any of the intestinal segments at both time points. Regarding PoRV C, the significant differences in IHC score were observed between PoRV C group and co-infected groups (A/C, B/C, and A/B/C) in duodenum, mid jejunum and proximal jejunum at 72 hpi although there was no difference between singularly infected and co-infected groups at 24 hpi.

3.5 Fecal shedding of virus

Fecal shedding of PoRV A, B and C from singularly and co-infected pigs is summarized in Table 5 (proportion). Fecal shedding titers of individual PoRV group over time in singularly and co-infected groups are illustrated in Figure 7 and Figure 8, respectively.

First detection of fecal shedding from PoRV A infected pigs was at 24 hpi. Fecal shedding continued in 1 of 3 pigs until necropsy at 72 hpi (Table 5), but the viral titer was continuously decreased from 24 hpi to 72 hpi (Figure 7). While PoRV A titers in feces at 24, 36, 48, and 60 hpi were not statistically different, viral titer at 24 hpi was significantly higher (p<0.0001) than that at 72hpi. PoRV B fecal shedding was detected in the majority of inoculated piglets at 12 hpi and continued in all pigs until their designated necropsy days (Table 5). The titer
of PoRV B in feces at 12 hpi was significantly lower (p<0.0001) than that at any other sampling times (Figure 7). It is interesting to note that the mean PoRV B titer in feces was gradually decreased from 24 to 48 hpi and then increased back from 48 to 72 hpi although any of these differences were not statistically significant. The fecal shedding of PoRV C at a high titer was detected as early as 12 hpi and remained statistically unchanged until the end of the study (Table 5 and Figure 7). Overall, the peak shedding of PoRV A, B, and C in feces ranged from 24 to 36 hpi. The statistical difference in fecal viral titers between virus groups were found only at 12 hpi between group A and groups B and C but not between B and C.

When fecal shedding of individual PoRV group was compared between singularly infected and co-infected groups (Figure 8), there were no statistically significant differences between groups except PoRV A shedding in group A/C versus that in group A, A/B, and A/B/C at 24 hpi (p<0.0001) and PoRV C shedding in group C and A/C versus that in group A/B/C at 12 hpi (p<0.005). However, when fecal shedding titers of individual rotaviruses within each co-infected group were compared (Figure 9), the difference in virus shedding was observed depending on the combinations. In group A/B, the significant difference in shedding of PoRV A and PoRV B was found only at 12 hpi (P <0.05); however, in group A/C, the significant difference was observed at 24 hpi (P <0.0001). In group B/C, there was no statistical difference between PoRV B and C shedding over time. In group A/B/C, there was a significant difference between the shedding of PoRV A or C and that of PoRV B at 12 hpi (P <0.05) and between the shedding of PoRV A or B and that of PoRV C at 72hpi.

IV. Discussion

Since diagnostic laboratory data suggested that PoRV A, B, and C have a different prevalence by age group (8), the current study was conducted to assess if PoRV A, B and C
differ in their pathogenicity and pathogenesis in CDCD neonatal piglets by exposing the pigs to individual group or combinations. All inoculated neonatal CDCD piglets in this study developed diarrhea irrespective of group of the virus to which the pigs were exposed. In singularly infected piglets, diarrhea was developed between 24 and 48 hpi, which is similar to previous observations (14,20,21,22). The severity of diarrhea caused by each of group A, B, and C was not different either, which is contradictory to the previous observation that PoRV A and C caused more severe diarrhea and dehydration than PoRV B (23). Such a difference may have been attributed to variation of virulence among rotavirus strains used or of experiment conditions between studies cited in the review article. Furthermore, the onset and severity of diarrhea in piglets was comparable between singularly infected group and co-infected group, suggesting no synergistic or additive consequence of multiple infections. The lack of synergistic or additive effect of co-infection was also apparent with pathology in small intestine. It could be speculated that such lacking might have been attributed to that the total amounts of viruses in co-infected groups were adjusted to be the same as those in singular infected pigs in order to have individual pigs the same viral burden. However, the total amount of PoRV shed in co-infected pigs were higher than that in singularly infected pigs as shown in Table 5, suggesting that each serogroup of PoRV replicated to its full capacity. It is then uncertain that a lower challenge dose of each serogroup in co-infected group (i.e., $1 \times 10^3$ PFU for co-infected as compared to $3 \times 10^3$ PFU for singularly infected) had a negative impact leading to no synergistic or additive effect, as the difference in viral dose seemed to be negligible in the current study. Thus, it may be necessary to assess a minimum infectious dose of each serogroup and study dose response of outcome in pigs exposed to mixed infection.
All rotavirus challenged piglets developed microscopic lesions of infection, i.e., atrophic enteritis, suggesting that the viruses used in this study are virulent in the CDCD piglets. Except PoRV A infected piglets, microscopic lesions were present in all examined segments of small intestine. In addition, virus antigens (PoRV A and C) or virus RNA (PoRV B) were found in all small intestinal segments of piglets singularly infected with the corresponding virus at 24 hpi, suggesting that these viruses can equally infect the epithelial cells throughout the small intestine. The ability of all 3 PoRV groups to infect all intestinal segments is in agreement with previous studies (21, 22, 24). At 72 hpi, PoRV B RNA and PoRV C antigen were still present in all intestinal segments of the singularly infected groups. However, PoRV A antigen was only found in duodenum and proximal jejunum but not in ileum and mid and distal jejunum that had significant villous atrophy. A reason could be that there were not many cells harboring PoRV A in ileum and mid and distal jejunum due to villous atrophy at 72 hpi. This agrees with shedding data that PoRV A shedding titer at 72 hpi was around $10^{3.1}$ copies/10µl, reduced more than $10^3$ copies from that at 24 hpi while PoRV B and C shedding titers were $10^{5.9}$ and $10^{4.7}$ copy/10µl, respectively. It would mean that PoRV A might replicate faster than PoRV B or C so that it kills cells rapidly. An interesting finding was that PoRV A group had significant longer villi in duodenal segment at both 24 hpi and 72 dpi compared to other segments in the same group as well as those of PoRV B, C or B/C group although the virus antigen were found by IHC in these regions at the same time. While some investigators reported that PoRV A infected duodenum have longer villi than those in other regions of the small intestine (11, 15, 25), others reported that group A rotavirus caused villous atrophy throughout the entire small intestine (14, 24, 26). Such a discrepancy may be due to differences of virus strains used in those studies.
There was no significant difference in crypt lengths between PoRV C only group and negative control until 72hpi, suggesting that the intestinal mucosal recovery in these pigs was slower than that of the other groups, such as A, B, A/C, and A/B/C in which the crypt depth of duodenum and/or proximal jejunum was increased at 72hpi. Because the replacement time for epithelia of small intestinal mucosa in neonatal pigs has been reported to be 7 to 10 days (27), the significance of the observed difference in the intestinal mucosal recovery from the disease between treatments could not be assessed as the study was terminated at 3 dpi. It is unknown why the crypt depth of duodenum was increased in PoRV A infected pigs when the virus seemed not to negatively affected the duodenum.

Singularly infected pigs did not show a significant difference in the virus shedding titer from 24 to 72hpi. Fecal shedding pattern among PoRV groups was, however, different. The fecal shedding of PoRV B and C was detected by 12 hpi and remained in a high titer until the termination of the study (72 hpi). In contrast, PoRV A shedding was detected at 24 hpi, not at 12 dpi, and gradually decreased from 60 hpi to 72 hpi. This observation suggests that PoRV B and C may have longer shedding than PoRV A and was in accordance with the result of IHC and ISH showing that PoRV A was detectable only in duodenum and proximal jejunum at 72 hpi. This may be related to the difference in replication behavior of the viruses. It can be speculated that by 72 hpi, PoRV A may have destroyed villous epithelium so extensively that there were not many enterocytes left for virus replication. Interestingly, the shedding of individual PoRV in co-infected pigs was lower than those in singular groups, which may reflect the fact that pigs were exposed to a lower dose of individual PoRV when received multiple groups since all inocula were adjusted to contain the same amount of rotaviruses in total. However, such a difference was not statistically significant after 24 hpi. In fact, the total amount of all PoRV groups shed in co-
infected groups were higher than that in singular infected group. Any negative impact, if any, of lower challenge dose of individual PoRV group on clinical presentation or pathology in co-infection groups would be negligible as compared to singular infection groups.

There was a difference in the virus amount in the intestinal tissues over time between PoRV A- and PoRV B and C-infected pigs, indicating that the replication pattern of PoRV A is different from those of PoRV B and C used in this study. The amount of PoRV A antigens in the small intestine at 72 hpi was significantly lower than that at 24 hpi, which is different from a previous report (24) in which the immunofluorescence scores of PoRV A in duodenum, jejunum and ileum at 3 dpi were higher than those at 1 dpi. In comparison, the amount of PoRV B and C at 24 hpi was not significantly different from that at 72 hpi. The PoRV C data of our study is also different from a previous study (22) reporting that the number of cells positive for PoRV C by immunofluorescence at 48 hpi was lower than that at 36hpi. It appears that rotaviruses seem have different replication pattern among not only groups but also the strains within the same group.

In conclusion, PoRV A, B, and C were equally pathogenic to CDCD neonatal piglets under conditions presented in the study regardless singular infection or co-infection with multiple groups. However, it appears that the viruses of different groups may have different replication behavior/capability in the gut, resulting in difference in the distribution of the microscopic lesions at 24 hpi as well as the starting of virus shedding time point between the groups. Therefore, while this neonatal piglet study could not support the difference in the pathogenicity among PoRV A, B as we postulated, and C, it may be worthwhile to study the replication and pathogenicity of PoRV A, B and C in sows concerning lactogenic immunity to piglets as such a study could explain the reported high prevalence of PoRV C in rotavirus-associated pre-weaning diarrhea.
References


Table 1. Experimental design: One day-old CDCD piglets were randomized into eight treatment groups and inoculated with sham (i.e., negative control) or different rotaviral groups singularly or in combination.

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. of pigs</th>
<th>Age</th>
<th>Inoculum</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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</tr>
<tr>
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<tr>
<td>8</td>
<td>6</td>
<td>1 days</td>
<td>Rotavirus group A, B, &amp; C</td>
</tr>
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**Table 2.** Sequences of oligonucleotide primers and probes used in quantitative reverse transcriptase-polymerase chain reaction assays to detect porcine rotavirus (PoRV) A, B, and C in specimens.

<table>
<thead>
<tr>
<th>Target (reference)</th>
<th>Primer sequence</th>
</tr>
</thead>
</table>
| PoRV A (8)         | Forward Primer 5′-GCT AGG GAY AAA ATT GTT GAA GGT A-3′  
                     Reverse Primer 5′-ATT GGC AAA TTT CCT ATT CCT CC-3′  
                     Probe A-1 5′-FAM-ATG AAT GGA AAT GAY TTT CAA AC-MGB-3′  
                     Probe A-2 5′-FAM-ATG AAT GGA AAT AAT TTT CAA AC-MGB-3′ |
| PoRV B*            | Forward Primer: 5′-ATG GAT CTG ATC GAA ACA GTG AAC-3′  
                     Reverse Primer 5′- TTG GAG CAA GTA NAA GAA TAC GTT TCT-3′  
                     Probe 5′-FAM-CATGCGTCAAATTTG-MGB-3′ |
| PoRV C (8)         | Forward Primer 5′-ATG TAG CAT GAT TCA CGA ATG GG-3′  
                     Reverse Primer 5′-ACA TTT CAT CCT CCT GGG GAT C-3′  
                     Probe 5′-VIC-GCG TAG GGG CAA ATG GC ATG A-TAMRA-3′ |

*The primers and probe were designed in this study.*
Table 3. Distribution of porcine rotavirus (PoRV) A, B or C in different regions of the small intestine of pig at 24 and 72 hours post inoculation (hpi) with one of the 3 groups as measured by immunohistochemistry (A and C) or in-situ hybridization (B)

<table>
<thead>
<tr>
<th>Region</th>
<th>PoRV A</th>
<th>PoRV B</th>
<th>PoRV C</th>
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<td></td>
<td>24 hpi</td>
<td>72 hpi</td>
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<tr>
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<td>+++</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
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<td>– –</td>
<td>+++</td>
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<tr>
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<tr>
<td>Mid jejunum</td>
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<td>– –</td>
<td>+++</td>
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<td>1.7</td>
</tr>
</tbody>
</table>

*+ or – is equal to one pig

§Average immunohistochemistry or in-situ hybridization score (0 = no signal, 1 = 1–20% of villous enterocytes within the section showing a positive signal, 2 = 21–50% of villous enterocytes showing a positive signal, and 3 = greater than 50% of villous enterocytes showing a positive signal)
Table 4. Distribution of porcine rotavirus (PoRV) A, B or C in different regions of the small intestine of pig at 24 and 72 hours post inoculation (hpi) with combination of the groups as measured by immunohistochemistry (A and C) or in-situ hybridization (B)

<table>
<thead>
<tr>
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<th>A/C A</th>
<th>A/C C</th>
<th>B/C A</th>
<th>B/C C</th>
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<td>+</td>
<td>1.3</td>
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<td>+--</td>
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<td>1.0</td>
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</tr>
<tr>
<td>Proximal jejunum</td>
<td>+--</td>
<td>+++</td>
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<td>+++</td>
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<tr>
<td>Duodenum</td>
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<td>---</td>
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<td>+--</td>
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</tr>
</tbody>
</table>

*++ or – is equal to one pig

†average immunohistochemistry or in-situ hybridization score (0 = no signal; 1 = 1–20% of villous enterocytes within the section showing a positive signal, 2 = 21–50% of villous enterocytes showing a positive signal; and 3 = greater than 50% of villous enterocytes showing a positive signal)
Table 5. Proportion of pigs shedding porcine rotavirus A, B and/or C in feces and PCR-estimated titers (in the parenthesis) of individual rotavirus in fecal swabs from piglets inoculated singularly (A, B, C) or with different combinations (A/B, A/C, B/C, A/B/C) of rotaviral groups at given hours post infection (hpi)

<table>
<thead>
<tr>
<th>Virus inoculum</th>
<th>Fecal Shedding pattern at a given time after experimental inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12 hpi</td>
</tr>
<tr>
<td>A</td>
<td>0* (N/A)</td>
</tr>
<tr>
<td>B</td>
<td>50 (3.8)</td>
</tr>
<tr>
<td>C</td>
<td>83 (3.98)</td>
</tr>
<tr>
<td>A/B</td>
<td>0.0 / 33 (0.0 / 2.7)</td>
</tr>
<tr>
<td>A/C</td>
<td>50 / 83 (2.5 / 4.5)</td>
</tr>
<tr>
<td>B/C</td>
<td>67 / 50 (3.3 / 2.3)</td>
</tr>
<tr>
<td>A/B/C</td>
<td>0 / 17 / 0 (0.0 / 2.4 / 0.0)</td>
</tr>
</tbody>
</table>

*% positives for each target group  
*Not Applicable  
§geometric mean genomic copy number/10µl of respective porcine rotavirus group (log_{10})
Figure 1. Average villous height per small intestinal location (duodenum, proximal jejunum, mid jejunum, distal jejunum, ileum) of piglets at 24 hours post inoculation with sham (i.e., negative) or porcine rotavirus A, B and/or C singularly or in combination.
Figure 2. Average villous height per small intestinal location (duodenum, proximal jejunum, mid jejunum, distal jejunum, ileum) of piglets at 72 hours post inoculation with sham or porcine rotavirus A, B and/or C singularly or in combination.
Figure 3. Average crypt depth per small intestinal location of (duodenum, proximal jejunum, mid jejunum, distal jejunum, ileum) of piglets at 72 hours post inoculation with sham or porcine rotavirus A, B and/or C singularly or in combination.
Figure 4. Microphotography of immunohistochemical staining of porcine rotavirus (PoRV) A (A) and PoRV C (B) infected enterocytes in small intestine by monoclonal antibodies specific for VP6 protein of respective group and *in situ* hybridization of PoRV B (C) using RNAcope® probe specific for VP6 gene of the virus.
**Figure 5.** The distribution and level of porcine rotavirus (PoRV) A, B, and C in various small intestine regions (duodenum, proximal jejunum, mid jejunum, distal jejunum and ileum) at 24 hours post inoculation (hpi) and 72 hpi as determined by either monoclonal antibody-based immunohistochemistry (IHC; for PoRV A and C) and RNAScope® in-situ hybridization (ISH; for PoRV B). Average immunohistochemistry or in-situ hybridization score (0 = no signal, 1 = 1–20% of villous enterocytes within the section showing a positive signal, 2 = 21–50% of villous enterocytes showing a positive signal, and 3 = greater than 50% of villous enterocytes showing a positive signal)
Figure 6. Comparison of the distribution and level of porcine rotavirus (PoRV) A, B and C in various small intestine regions (duodenum, proximal jejunum, mid jejunum, distal jejunum, ileum) of piglets inoculated with the virus singularly (A, B, C) or in combination (A/B, A/C, B/C, A/B/C) at 24 hours post inoculation (hpi) and 72 hpi as determined with monoclonal antibody-based immunohistochemistry (IHC; for PoRV A and C) and RNAScope® in-situ hybridization (ISH; for PoRV B). Average immunohistochemistry or in-situ hybridization score (0 = no signal, 1 = 1–20% of villous enterocytes within the section showing a positive signal, 2 = 21–50% of villous enterocytes showing a positive signal, and 3 = greater than 50% of villous enterocytes showing a positive signal)
Figure 7. Titer of porcine rotavirus (PoRV) A, B or C in fecal swabs collected from CDCD neonatal piglets inoculated with one of the rotaviruses at given hours post inoculation (HPI). Virus titers were estimated using quantitative RT-PCR specific for VP6 gene of each PoRV group.
Figure 8. Titer of porcine rotavirus (PoRV) A, B or C in fecal swabs collected from CDCD neonatal piglets inoculated with one or in combinations of the rotaviruses at given hours post inoculation (HPI). Virus titers were estimated using quantitative RT-PCR specific for VP6 gene of each PoRV group.
Figure 9. Titer of porcine rotavirus (PoRV) A, B or C in fecal swabs collected from CDCD neonatal piglets inoculated with combinations of the rotaviruses at given hours post inoculation (HPI). Virus titers were estimated using quantitative RT-PCR specific for VP6 gene of each PoRV group.
CHAPTER 4. ISOLATION AND MOLECULAR CHARACTERIZATION OF A PORCINE ROTAVIRUS C

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Abstract

Group C porcine rotaviruses have been found predominantly in piglets of pre-weaning ages; however, there is no commercial vaccine available to mitigate the problem. One of reasons is that virus isolation and sustainable propagation has been difficult and not reproducible. Since MA-104 cell based virus isolation protocol failed to support isolation of non-group A rotaviruses, attempts were made to establish a reliable virus isolation method using different cell lines for porcine rotavirus C. As a result, a porcine rotavirus C strain (IA/2015) was successfully isolated and maintained using IPEC-1 cell based roller culture system with incorporation of a relatively high concentration of trypsin. By sequencing for VP4, V6, and VP7, the genotypes of the virus was identified as G6P[5]I5. The isolate and the newly established virus isolation/cultivation method may be useful for pathobiology studies, diagnostics and vaccine development.

I. Introduction

The rotaviral enteric disease is common in pigs, which causes considerable economic loss to the swine industry (1). The causative agent, porcine rotavirus (PoRV), belongs to genus Rotavirus in the family Reoviridae. Rotaviruses are divided into ten groups (A-J) base on the
cross-antigenicity and sequence of VP6 protein/gene (2-5). Of them, serogroup A, B, C, E, and H have been reported to cause or implicated in enteric disease in pigs (6, 7).

Generally rotavirus is fastidious to be isolated. Porcine rotavirus A has been isolated using an African green monkey kidney derived cell line MA-104 with the presence of a proteolytic enzyme, such as trypsin, in cell culture media (8, 9, 10). The successful rate of PoRV A isolation using MA-104 cells was reported to be approximately 40% in one study (11). Isolation of non-group A porcine rotaviruses, however, has been problematic and sparse because MA-104 cells are not permissive to them. Although there were reports of successful isolation of PoRV B using swine kidney (SKL) cells (12) and PoRV C using primary pig kidney cells (13), none of those protocols has been reproducible as no other PoRV B or C isolates are not available.

There is no commercial non-group A porcine rotavirus isolates or vaccines available either. As a result, researches of pathobiology, immunology, diagnostics, and prophylaxis for non-group A porcine rotaviruses have been hindered even though data from the veterinary diagnostic community recently demonstrated identification of non-group A rotaviruses in diarrheic pigs as frequently as rotavirus A, raising the need for an effective laboratory method to isolate and propagate non-group A rotaviruses.

IPEC-1 cells are derived from a mixture of jejunum and ileum tissues from a neonatal piglet while IPEC-J2 cells are isolated from porcine ileum tissue. Both cell lines were originated from 1-day old piglets (14). Because these two cell lines are made of non-transformed cells and can form polarized columnar cell monolayers, they have been considered good in vitro tools for studying the interaction of host cell-virus, bacteria, or toxin and metabolism of some substances (15,16,17). IPEC-J2 was reported to support the growth of porcine enteric coronaviruses, such as transmissible gastroenteritis virus (TGEV) and porcine epidemic diarrhea virus (PEDV) (18),
and especially, porcine rotavirus A (19). Thus, IPEC-1 and IPEC-J2 cells could also support isolation and propagation of non-group A rotaviruses. The present study was conducted to determine if an in vitro virus isolation system for non-group A rotaviruses especially PoRV C can be established using any of these IPEC cell lines.

II. Materials and Methods

2.1 Cells

The IPEC-1 and IPEC-J2 were kindly provided by Dr. Dennis Black (University of Tennessee Health Science Center, Memphis, Tennessee) and Dr. Bruce D. Schultz (Kansas State University, Manhattan, Kansas), respectively. IPEC-1 was also purchased from a commercial source (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). The cells were cultured in DMEM/F12 media (ThermoFisher Scientific, Walham, Massachusetts) supplemented with 5% fetal bovine serum (FBS) (Atlas Biological, Fort Collins, Colorado), 5ng/ml epidermal growth factor (Sigma-Aldrich, St. Louis, Missouri), 1% insulin-transferrin-selenium (ThermoFisher Scientific), and antibiotics containing 100 units/ml penicillin and100µg/ml streptomycin (Sigma-Aldrich), hereafter growth media. The cells were maintained in 37°C with 5% CO₂. The subculture was mostly done at the splitting ratio of 1:3 and the confluent monolayer formed in 2 days.

2.2 Inocula

Inocula were prepared from intestinal contents which were determined to be positive for PoRV B or C by PCR testing. The intestinal contents were obtained from experimental neonatal pigs orally inoculated with PoRV B (2011025309A) or PoRV C (2010025060H) purified from a positive intestinal content of CDCD pig as described elsewhere (20). Briefly, the fecal samples were diluted 1:10 in phosphate-buffered saline (PBS), pH 7.2, and then centrifuged for 15
minutes at 5000 x g. The resulting supernatants were filtered through 0.2µm Costar membrane filters (Sigma-Aldrich) before use. The PoRV A isolate (ISU2010035768) was used as positive control.

2.3 Virus isolation

The virus isolation was attempted as previously described (13) with modifications. Cells were seeded to Corning roller culture tubes, 16x125mm in size (Sigma-Aldrich) at the rate of 3 x 10^5 cells/tube in 5ml of growth media. All tubes were inserted into a roller drum placed in a water-jacketed CO₂ incubator. When cell monolayers were formed (generally 2 days after seeding), the cells were washed 3 times with DMEM/F12 supplemented with the penicillin-streptomycin approximately 3 hours before inoculation. One hour before inoculation, 95µl of each processed fecal sample were mixed with 5µl trypsin (200µg/ml) and incubated for one hour at 37°C. Afterward, cell monolayers were incubated with 100µl of the treated inoculum for 2 hours at 37°C. Each inoculum was run in duplicate. Finally, 2ml of the maintenance media were added to each tube. The maintenance media was the same as growth media without FBS which was supplemented with 5µg/ml trypsin (ThermoFisher Scientific) and 10% tryptose phosphate broth (Sigma-Aldrich). The cells were checked every day for cytopathic effect (CPE) in 3 days. Subsequently, the cells were frozen-and-thawed 3 times and used as inoculum for the next cell culture passage. Subculture was done in the identical manner as described above. After each passage, the cell supernatant was tested by a real time RT-PCR for PoRV C as described below. The inoculum was considered as negative when the cycle threshold (Ct) value was higher than 30.
2.4 Confirmation of virus growth

2.4.1 Real-time RT-PCR

One hundred and forty microliters of cell culture media from inoculated cells were taken to extract RNA using QIAamp® Viral RNA Mini kit (QIAGEN, Valencia, California) as per procedure recommended by the manufacturer. Subsequently, the RNA extract was tested by real-time RT-PCRs specific for PoRV A, B and C as previously reported (21). The 10-fold serial dilutions of plasmids containing known copy numbers of PoRV C VP6 gene were used as standards to estimate viral titers.

2.4.2 Immunofluorescence assay

The IPEC-1 cells were seeded at the concentration of 3 x 10^5 in a Lab-Tek™ SlideFlask chamber (ThermoFisher Scientific) which, after that, was attached to a 15ml plastic centrifuge tube and placed in a roller drum. Two days later, resulting cell monolayers were inoculated with 100µl of the trypsin-treated virus inoculum and incubated for 2 hours at 37°C, then replenished with 2ml of the maintenance media. After 24 hours post inoculation, the cells were fixed with 2ml of cold 80% acetone aqueous solution. The cells were first incubated with a monoclonal antibody specific for VP6 of PoRV C (2010025060H) (22) for 1 hour at 37°C. After washing 3 times with PBS, the presence of virus-antibody mixture were visualized by staining with goal anti-mouse IgA+IgG+IgM (H+L) polyclonal antibody conjugated with fluorescein isothiocyanate (Seracare, Milford, MA). After washing, the stained cells were observed under a fluorescent microscope for the rotavirus-specific signal.

2.4.3 Immunoelectron microscopy (IEM)

Thirty milliliters of cell-cultured virus material were clarified by ultracentrifugation at 100000 x g on a 35% sucrose cushion as previously described (23). The resulting pellet was
resuspended in TNC buffer (0.4mM Tris/HCl, 2mM NaCl, 10µM CaCl₂) and ultracentrifuged again in the identical manner as described above. Then 200 µl of deionized water was added to the pellet and left overnight at 4°C. The virus was resuspended by dispersing the pellet using a syringe with 18G needle. The virus was incubated with anti-PoRV C VP6 monoclonal antibody produced in our laboratory (22) for 1 hour at 37°C and centrifuged for 15000 x g for 30 minutes. Then, the pellet containing virus was stained with 2% uranyl acetate and examined under a transmission electron microscope at Roy J. Carver High Resolution Microscopy Facility, Iowa State University.

2.5 Sequencing and sequence analysis

The V4, VP6, and VP7 genes were amplified by using the primers listed in Table 1. The amplified products were purified by QIAquick gel extraction kit (QIAGEN) by following manufacturer’s recommended procedures and sequenced by the Sanger method at the Iowa State University DNA Facility. Sequences were assembled by Lasergene’s SeqMan Pro (DNASTAR®, Madison, Wisconsin). The sequences of VP4, VP7, and VP6 genes were then comparatively analyzed with other corresponding sequences of rotaviruses available in GenBank® (National Center for Biotechnology Information, Bethesda, MD) using Lasergene’s MegAlign (DNASTAR®). In addition, the phylogenetic analyses were completed by using maximum likelihood method in MEGA software, version 7 (24).

III. Results

3.1 Virus isolation

PoRV A-inoculated IPEC-1 cell lines in the first passage showed CPE manifested by cells death and detaching from growing surface of cell culture tubes from 48 hours post inoculation. However, the cytolytic changes by PoRV B or C in IPEC-1 or IPEC-J2 were not
clearly discernable between inoculated cells and uninoculated cells (i.e., negative control) by the
time cell culture fluids were ready to be harvested for testing.

When cell culture supernatants collected after each passage of virus material were tested
by PCR to monitor virus growing, the Ct value of PoRV C-inoculated IPEC-J2 cell culture
supernatant after the 5th passage was 33.0 which was higher than that of the original inoculum
(Ct=28.0), whereas the Ct value of PoRV C-inoculated IPEC-1 cell culture supernatant after the
5th passage was 22.9 which was lower than that of the original inoculum. Since IPEC-J2 seemed
not to support PoRV C growth, the cell was not used in further blind passages of PoRV C
material. The Ct value of PoRV B-inoculated IPEC-1 and IPEC-J2 cell culture supernatant after
the 5th passage are 35.9, which were higher than that of the original inoculum (Ct=27.5),
suggesting that both IPEC cells did not support PoRV B growth. No further blind passage of
PoRV B was made after passage 5.

As shown in Table 2, the titer of PoRV C appeared to be gradually increased from
passage 1 (10^{4.8} genomic copy number/10\mu l) to passage 7 (10^{7.6} genomic copy number/10\mu l) in
IPEC-1 cells as estimated by quantitative real-time RT-PCR and remained stable during further
cell culture passages, suggesting that the virus was growing and adapted to IPEC-1 cells. All cell
culture supernatants collected from PoRV C inoculated IPEC-1 cells during serial passages were
negative by the real-time RT-PCR for PoRV A and PoRV B.

Under electron microscope, wheel-shaped non-enveloped virus particles of 60 nm in
diameter, which was similar to the morphology of members of Reoviridae, were observed from
cell culture supernatant harvested after 20 passages (Figure 1). Since virions were aggregated by
adding PoRV C-specific antibody for IEM, the virus in the cell culture supernatants was
determined to be a rotavirus. Immunofluorescence microscopy using PoRV C VP6-specific
monoclonal antibody (11H3) confirmed the presence of PoRV C in IPEC-1 cells after 24 hours post inoculation, suggesting viral growth in the cell. The infected cells with fluorescence were clearly visible in the cytoplasm whereas there was no fluorescence from sham-inoculated negative control cells (Figure 2). The PoRV C isolate was then designated IA/2015.

### 3.2 Molecular characterization of porcine rotavirus C IA/2015 isolate

VP4, VP7, and VP6 sequences of the IA/2015 isolate were successfully obtained. For VP4 gene, the nucleotide similarity between the IA/2015 and 23 reference strains representing 8 P genotypes (P1 to P8) ranged from 69.9% to 88.9% (Table 3). Of them, VP4 sequence of the isolate showed the highest similarity to those belonging to the P[5] genotype, ranging from 81.2% to 88.9%. As such, the IA/2015 isolate was classified as P[5] when the 83% nucleotide identity was applied as cut-off as previously described (25). The phylogenetic analysis based on VP4 sequences showed that the IA/2015 strain was closely related to and clustered with PoRV C KOR/07-60-4, USA/RV0143, JPN/CJ13-6, and JPN/134-9, which were all P[5] genotype, as illustrated in Figure 3.

The nucleotide similarity of VP7 gene between the IA/2015 isolate and 32 PoRV C reference strains representing 13 G types ranged from 48.6% to 94.7% as summarized in Table 4. The isolate was classified as G6 when the 85% nucleotide identity was applied as cut-off as previously proposed (26). Phylogenetically, the new isolate was clustered with G6 PoRV C CAN/MB11-58, USA/NE09-2, CAN/MB-11-57, and USA/IL10-49 (Figure 4).

Based on the 90% nucleotide identity of VP6 as cut-off (25), the IA/2015 isolate of PoRV C was determined to be I5 genotype. The VP6 nucleotide identity between the new isolate and other PoRV C representing 7 I types ranged from 80.0% to 93.5% as shown in Table 5. Figure 5
illustrates that the isolate is phylogenetically close to and clustered with KOR/06-92-1, KOR/06-146-2, USA/RV0143, and USA/RV0104 based on VP6 sequence.

IV. Discussion

Porcine rotavirus C is an important enteric viral pathogen in pigs and has been most commonly identified in pre-weaning piglets with diarrhea (1-21 days of age) as compared to PoRV A and B being common in older piglets (21, 26). Frequent identification of PoRV C in neonatal and nursing piglets raised the need for better prophylaxis leading to good lactogenic immunity in young piglets. However, it has been historically a great challenge, if not possible, to isolate PoRV C, resulting in lack of virus isolates which can be used to study pathobiology or to develop diagnostics and prophylactic methods including vaccines. The current study shed a light on a possible in vitro method for isolating PoRV C reliably as the study showed successful isolation of PoRV C from intestinal contents using IPEC-1 cells as well as sustainable maintenance of the virus in the cells over 20 serial passages. The same method was also applied to a limited number of clinical specimens submitted to Iowa State University Veterinary Diagnostic Laboratory which were positive for PoRV C by PCR and resulted in successful isolation of PoRV C (data not shown), suggesting that the newly established method can be an effective in vitro virus isolation technique for group C porcine rotaviruses with a reasonable recovery rate.

The IPEC-1 cells are immortalized enterocytes originated from a mixture of ileum and jejunum tissue from 1-day old piglets. As mature enterocytes of small intestine is the main natural host cell for rotaviruses in pigs, IPEC-1 cells were expected to support porcine rotavirus infection and replication and to maintain the natural characteristics of the virus, which was demonstrated in the current study for PoRV A and C but not for PoRV B. Reasons for the failure of cultivating PoRV B in IPEC-1 cells are unknown and remain to be further investigated.
Nonetheless, IPEC-1 cells may serve as an *in vitro* model for studying the pathobiology of porcine rotaviruses.

Proteases, such as pancreatin and trypsin, are known to be required for *in vitro* rotavirus cultivation (27). For isolation of PoRV C, use of a high concentration of such a proteolytic enzyme in media was recommended (28). However, a high concentration of trypsin (5µg/ml) in the maintenance media was somewhat detrimental to viability and integrity of IPEC-1 cells in this study. As a consequence, monitoring for the development of virus-associated CPE was not as clear and easy as should be since approximately 20 to 30% of cells died and were detached from the growing surface of cell culture plasticware unrelated to virus infection or growth after 2 or 3 days post inoculation. Therefore, it was necessary to confirm the virus replication in IPEC-1 cell culture using other laboratory methods such as PCR or immunofluorescence test besides CPE observation. Furthermore, because several blind passages were necessary to confirm the isolation and propagation of PoRV C, use of a PCR-based assay in a quantitative manner was found to be beneficial for rapid screening of cell culture media for virus growth.

Unlike rotavirus A, there is no thorough classification system for group C rotaviruses. Nevertheless, it is important to determine G and P types of an isolate as VP7 and VP4 are known to contain neutralizing epitopes related to cross-protective immunity (29, 30). To determine the G genotype of isolate, the 85% (26) or 86% (31) VP7 nucleotide identity to rotaviruses with known G types has been proposed as cut-off, which result in 9 or 13 G genotypes, respectively. Of them, G1, 3, 5, 6, 7, 8, 9, 10, 12, and 13 have been detected in clinical specimens from diarrheic pigs (32). The PoRV C IA/2015 isolate was determined to be G6 and closely related to the North America PoRV C strains CAN/MB11-58, USA/NE09-2, CAN/MB11-57, and USA/IL 10-49 based on VP7 sequence. Noteworthy, G6 has been the most common G type detected in
pigs associated with rotavirus infection in the U.S., Canada (26), Brazil (33), and Italy (34). It has also been reported in Ireland (35), Korea (36), and Czech Republic (32), indicating that G6 may be widely distributed in pig-producing regions worldwide. For P type classification, Suzuki et al (37) initially proposed 80% VP4 nucleotide identity as cut-off, which classified rotaviruses into 6 P types (1-6). The 83% identity as cut-off has also been suggested by other investigators (25), (32) which divides rotaviruses into 7 or 8 genotypes. Based on VP4 sequence, the isolate was typed to be P[5] and was phylogenetically closer to Korean strain KOR/07-60-4 of PoRV C than USA/RV0143 strain of PoRV C. It should be noted though that such a phylogenetic relationship may not reflect the real situation because there are a limited number of PoRV C VP4 sequences from US swine available in the public domain. Thus, more VP4 gene sequencing is necessary to have better understanding of PoRV C epidemiology and genotype frequency in US swine. Interestingly, the G6P[5] has been reported to be dominant in Japan (37) and Brazil (33). Although G6P[5] has been identified in US swine (38), its prevalence is not known.

In conclusion, IPEC-1 cell based roller culture system with incorporation of a high concentration of trypsin in the maintenance media may be an effective in vitro virus isolation and cultivation technique for group C porcine rotaviruses. As the newly established method provides sustainable replication of PoRV C at a relatively high titer, the method may also be useful for development of whole virus-based vaccines and diagnostics for PoRV C.
References


genes and will contribute to a comprehensive, generally accepted classification system. Infect Genet Evol 44:106-113.


Table 1. Primers used for PCR amplification and sequencing of VP7, VP4, Vp6 gene.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Reference</th>
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<td>GCTGTCTGACAAAACTGGTC</td>
<td>GCCACATGATCTTTGTTTACGC</td>
<td>(38)</td>
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<tr>
<td>VP6</td>
<td>GGCATTTAAAATTCATTTCAC</td>
<td>AGCCACATAGTTCACATTTC</td>
<td>(39)</td>
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Table 2. Real-time RT-PCR assessment of porcine rotavirus C growth in IPEC-1 cells over 20 continuous passages

<table>
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<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
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<tr>
<td>Observed Ct value in cell culture supernatant</td>
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<td>24.9</td>
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<td>20.5</td>
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<td>Estimated genomic copy numbers/10µl</td>
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<td>$10^{6.9}$</td>
<td>$10^{7.0}$</td>
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Table 3. Nucleotide sequence identity for VP4 between porcine rotavirus C IA/2015 and reference strains whose sequences are available in GenBank

<table>
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<tr>
<th>GenBank accession number</th>
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Figure 1. Electron microscopic photography of porcine rotavirus (PoRV) C particles from IPEC-1 cell culture supernatant which were aggregated by PoRV C-specific monoclonal antibody
Figure 2. Immunofluorescence staining of porcine rotavirus C IA/2015 in IPEC-1 cells at 24 hours post inoculation (left) by porcine rotavirus C VP6-specific monoclonal antibody as compared to negative staining of sham-inoculated IPEC-1 (right)
**Figure 3.** Phylogenetic relationship of porcine rotavirus C IA/2015 isolate (indicated by the red square) with other group C porcine rotaviruses based on VP4 sequence
Figure 4. Phylogenetic relationship of porcine rotavirus C IA/2015 isolate (indicated by the red square) with other group C porcine rotaviruses based on VP7 sequence.
Figure 5. Phylogenetic relationship of porcine rotavirus C IA/2015 isolate (indicated by the red square) with other group C porcine rotaviruses based on VP6 sequence
CHAPTER 5. GENERAL CONCLUSIONS

Historically, rotavirus A has been believed to be the major cause of rotavirus-associated diarrhea in young pigs even though the presence of non-group A rotaviruses in swine was reported, probably due to difficulty of isolating non-group A rotaviruses and lacking of specific reagents. With advance in molecular diagnostic techniques, non-group A rotaviruses have also been frequently identified in diarrheic pigs with or without rotavirus A. Notably, rotavirus C was most commonly detected in diarrheic neonatal and nursing piglets while detection of rotavirus A was more common in association with post-weaning piglet diarrhea according to diagnostic data. Furthermore, it was not uncommon to detect more than one group or genotype of rotavirus in feces from diarrheic pigs. Knowing that rotavirus infection is ubiquitous in pig populations, it was necessary to better understand the pathogenesis and pathogenicity of different genotypes of rotaviruses to explain the observed age disparity in detection frequency of various genotypes and devise a better intervention strategy. It was also necessary to develop reagents and laboratory tools specific for non-group A rotaviruses in order to support pathobiology studies and aid diagnostic investigation and prevention.

The current study demonstrated that all group A, B, and C rotaviruses were equally pathogenic to immunologically naïve neonatal piglets when given oro-gastrocally under the conditions presented during the study. The viruses caused comparable clinical signs including diarrhea although fecal shedding of rotavirus B and C started earlier than rotavirus A. Interestingly, concurrent infection of more than one serogroup did not exacerbate the severity of diarrhea. All serogroups were able to cause atrophic enteritis although distribution of villous atrophy within small intestine varied between the serogroups. Nonetheless, there was no difference in the distribution of viral antigens or nucleic acid among all fragments of small
intestine examined between serogroups. While the current study did not elucidate why rotavirus C infection would be more common in neonatal and nursing piglets pathogenicity-wise, a similar study should be conducted in older pigs, particularly adult swine to assess each rotavirus serogroup’s capability of replication and inducing effective mucosal and lactogenic immunity in those pigs.

In addition to gain of understanding on the pathogenicity of three rotavirus serogroups in newborn pigs, the current study produced reagents and tools valuable for studying non-group A rotaviruses in vitro and in vivo. First, murine monoclonal antibodies (mabs) specific for VP6 of each of porcine rotavirus A, B and C were successfully produced, leading to the development of immunohistochemistry (IHC) for porcine rotavirus C in intestinal tissues which was not available. The mabs specific for porcine rotavirus B and C that were produced in this study are ‘the first’ of its kind to our knowledge and can be useful for development of various diagnostic tests (e.g., antigen-capturing ELISA, competitive or blocking ELISA for serology). The availability of mab-based IHC for porcine rotavirus C may aid accurate diagnosis of rotavirus-associated diarrhea leading to a better intervention when concurrent detection of more than one rotavirus serogroup in feces from diarrheic pigs is not uncommon. In this sense, an in situ hybridization using RNAscope® probe developed in this study for porcine rotavirus B should provide the same utility.

Second, a group C porcine rotavirus was successfully isolated along with an IPEC-1 cell based roller culture system for isolating and maintaining rotavirus C in vitro. There has been only one porcine rotavirus C isolate (Cowden strain) which is not available anymore. Then porcine rotavirus C isolate IA/2015 (G6P[5]I5) made in this study would be the second of its kind and might be the only porcine rotavirus C isolate currently available. The isolate can serve as a reference strain and would be useful for future pathobiology studies and vaccine
development. The newly established virus isolation method may provide an opportunity to get more rotavirus C isolates although the protocol should be evaluated on a large number of diagnostic specimens for its effectiveness.
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