Diagnostic testing and the challenges associated with vaccine development for porcine rotavirus

by

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B.S., Missouri State University, 2007

A Report

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Department of Diagnostic Medicine/Pathobiology
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2021

Approved by:

Major Professor
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Abstract

Rotavirus (RV) is one of the leading causes of acute gastroenteritis worldwide in children and young animals. In swine, this disease commonly infects neonatal and recently weaned piglets. A RV infection can be either asymptomatic or symptomatic depending on the RV strain, age of the pig, the immune status of the piglet, and environmental conditions. When symptoms are present, they include dehydration, loss of weight, mild to severe diarrhea, slowed growth, and possible death. Swine producers can face economic losses due to loss in production and the costs associated with treating the infection. This report discusses the ever evolving field of swine diagnostic testing, the complexity of RV infections within swine populations, the difficulties in developing a cross-protective vaccine for RV infections, and possible vaccine strategies used in other host species that can help further swine vaccine development.

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Acknowledgements

First, I would like to thank my major professor, Dr. Paige Adams. Thank you for all your support, guidance, and encouragement throughout this degree program. Her patience and dedication helped me grow as a graduate student. I would also like to thank my supervisory committee, Drs. Gary Anderson and Ellen Lowery, for their insight and support as well. I would also like to thank Dr. Erin Strait for all her guidance and encouragement throughout my entire master's program as well as my career. As a mentor, she challenged me to think deeper, step outside of my comfort zone, and helped me become a better scientist.

I would also like to thank a couple of people from my work family at Merck Animal Health. I would especially like to thank Michelle Allen and Craig Vester. Both managers have been supportive throughout this entire process and have helped me grow in my career at Merck. I will be forever grateful for the opportunities they have provided for me.

Another big thank you goes out to my family. My parents, Carla and Frank Lane, have been some of my biggest supporters throughout my life. Thank you for being the best cheerleaders and taking care of my little boy while I was finishing this report. Finally, I would like to thank my husband, Will. He has been supportive of my goal of finishing my master's degree, and I thank him for being the best father to our son.

Dedication

The report is dedicated to my husband, Will, and our son, Liam.

Chapter 1- Introduction to Rotavirus and Swine Rotavirus

Rotavirus (RV) is one of the major causes of acute gastroenteritis in humans and other animal species, including bovine, ovine, equine, swine, chickens, turkeys, canines, and bats. Globally, rotavirus causes 440,000 deaths in humans with many of those deaths occurring in developing countries (Parashar *et al.*, 2006). Approximately 1 billion dollars alone is spent annually on emergency room visits, hospitalizations, and physician associated costs in the United States (Shao *et al.*, 2016). This disease is not only a human concern, but it is also a livestock concern. RV is one of the leading causes of diarrhea in young piglets and calves. Bovine were the first livestock species to isolate and identify RV as a cause of diarrhea in 1969 (Mebus *et al.*, 1969). A few years later, in 1975, RV was isolated and detected in the diarrhea of a young piglet for the first time (Rodger *et al*., 1975; Woode *et al.*, 1976). RV infections can cause significant losses to livestock producers due to slow growth, anorexia, mild to severe diarrhea, and possible death due to dehydration of an animal (Chepngeno *et al.*, 2020).

In this report, the author will focus on swine RV diagnostic testing, the complexity of swine RV infections, the difficulties in creating cross-protective vaccines for swine, and potential vaccine applications from other species affected by RV for development of new swine RV vaccines.

Rotavirus Properties

The term "rota" comes from the Latin word for wheel. Under an electron microscope, RV appears as wheel-like structure (Chang *et al.*, 2012; Dhama *et al.*, 2008). Figure 1.1 a shows an electron micrograph of RV particles under an electron microscope. RV is described as a non-enveloped, icosahedral particle consisting of a triple-layered capsid containing 11 segments of double-stranded RNA. The 11 segments encode for six structural proteins including Viral

Protein 1 (VP1), Viral Protein 2 (VP2), Viral Protein 3 (VP3), Viral Protein 4 (VP4), Viral Protein 6 (VP6), and Viral Protein 7 (VP7) and five non-structural proteins (NSP), including NSP1, NSP2, NSP3, NSP4, and NSP5/6 (Crawford *et al.*, 2017; Molinari *et al.*, 2016). Figure 1.1 b shows a cross-sectional schematic of all the major structural proteins within the RV particle, and Figure 1.1 c shows the electrophoretic migration pattern of the 11 segmented RNA genome and the six structural proteins and five non-structural proteins associated with each RNA genome segment.

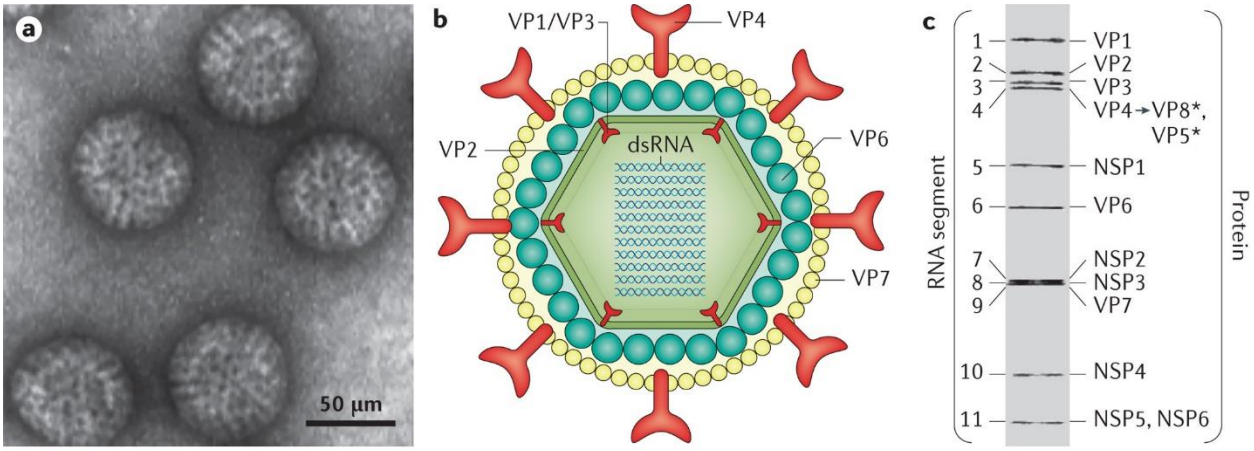

Figure 1.1 Rotavirus Particles and Structure

Sourced from Crawford S, Ramani S, Tate JE, Parashar UD, Svensson L, Hagbom M, Franco MA, Greenberg HB, O'Ryan M, Kang G, Desselberger U, Estes MK. (2017). Rotavirus infection. *Nat Rev Dis Primers*. 3:17083.

The outer layer of the triple-layer capsid particle is composed of two important viral proteins (VP), VP7 and VP4. The glycoprotein VP7 creates the smooth outer capsid layer of the particle, while sixty VP4 dimeric, spike proteins protrude from the outer capsid layer (Ludert *et al.*, 2002; Naseer *et al.*, 2017). The function of the spike protein is to facilitate virus attach to the mature enterocytes in the small intestine (Arias *et al.*, 2015). These two proteins induce virus neutralizing antibodies, which are important for RV classification, diagnostic tools, and vaccine development. The intermediate protein layer consists of VP6 (Desselberger, 2014). This viral

protein is used to separate RV into different species antigenically through serotypes or by nucleotide sequence. The inner or core capsid is composed of VP1, VP2, and VP3. The shell of the core is comprised of VP2, and the two other VPs, VP1 and VP3, are encased within the shell. VP1 is a viral RNA-dependent RNA polymerase, and VP3 is a viral capping enzyme (Desselberger, 2014).

The non-structural proteins are important in evading the host immune system, viral replication, and morphogenesis of the RV progeny. NSP1 is the most variable and is thought to be involved in host range restriction of RV. NSP2 and NSP5 are important in forming the viroplasms, where early morphogenesis and RNA replication occurs. NSP6 interacts with NSP5 and found within the viroplasms. The NSP4 is one of the most important non-structural proteins for several reasons. It acts as a receptor in the endoplasmic reticulum for newly formed double layer particles, it disrupts cellular electrolyte homeostasis by increasing Ca^{++} in the infected cell, it alters the plasma membranes permeability, and most importantly, it acts as a viral enterotoxin (Desselberger, 2014; Desselberger *et al.*, 2009).

Classification of Rotavirus

Rotavirus belongs to the genus *Rotavirus* in the virus family *Reoviridae* (Arias *et al.*, 2015; Chang *et al.*, 2012; Resende *et al.*, 2019). It is currently classified based on its antigenic characteristics and nucleotide sequences. The intermediate capsid, VP6, is used to differentiate the different RV species antigenically through serological techniques, genomic RNA electrophoretic patterns, and group-specific PCR (Sanekata *et al*., 2003). Currently, there are ten known groups or species of RV (Chepngeno *et al.*, 2019). These include Rotavirus A (RVA), Rotavirus B (RVB), Rotavirus C (RVC), Rotavirus D (RVD), Rotavirus E (RVE), Rotavirus F (RVF), Rotavirus G (RVG), Rotavirus H (RVH), Rotavirus I (RVI), and Rotavirus J (RVJ). RV

species known to infect both humans and animals are RVA, RVB, RVC, and RVH. The remaining RV species, RVD, RVE, RVF, RVG, RVI, and RVJ have been found to infect only animals (Chepngeno *et al.*, 2019; Molinari *et al*., 2016).

RV's two outer capsid proteins, VP7 and VP4, are the basis of the binary system used to classify RV by their serotype or genotype (Greenberg & Estes, 2009). Both proteins can induce neutralizing antibodies, and classification of each serotype is based off these characteristics. In neutralizing antibody tests, VP7 is classified as the G serotype, and VP4 is classified as the P serotype. The G stands for the glycosylated protein of VP7, and the P stands for the protease-sensitive protein of VP4. Similarly, in a sequencing test, VP7 is classified as a G genotype, and the VP4 is classified as the P genotype. The neutralizing antibody tests and sequencings tests for the G serotype and G genotype give similar results, so they are classified based on their G serotype (Gx). This is not the same for the P serotype and P genotype. The results do not agree between the neutralizing tests and sequencing tests. Therefore, P serotypes are labeled as Px and P genotypes have brackets P[x]. As more RV sequences were acquired, it became clear a better classification system was needed.

RVA is the most prevalent and documented species known to infect humans and animals in the world. To better characterize and classify the diversity of RVA, the Rotavirus Classification Working Group (RCWG) was established in April of 2008. Since its establishment, the group has developed, evaluated, and maintained a RV classification system based on nucleotide sequenced genotypes (Matthijnssens *et al.*, 2011).

In this classification system, a new strain of RVA is assigned a genotype based on the RVs 11 segmented genome. Different genotypes are based on a set of "established nucleotide percent cutoff values" (Matthijnssens *et al.*, 2008). The current percent nucleotide identity cutoff

values and number of genotypes per segment can be found in Table 1.1. The order of a new strain is described by its structural viral proteins and nonstructural viral proteins in the following order: VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6. Each of these structural and nonstructural proteins are abbreviated in the following order Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx. A proposed nomenclature for naming new RV strains has been suggested by the RCWG. This naming strategy is modeled similarly to the current naming strategy used by the "Influenza Virus Resource" for monitoring the current influenza strain (Matthijnssens *et al*., 2011). The nomenclature is as follows: RV group/species of origin/country for identification/common name/year of identification/G-and P-type. An example of a RVA strain using this nomenclature is RVA/Pig-tc/USA/Gottfried/1983/G4P[6].

Table 1.1 Diversity and Percent Identity Cutoff Value of RVA Genotypes Since 2015 Rotavirus Classification Working Group, 7th Meeting in Goa, India

Viral protein	Percent Identity Cutoff Value	Number of genotypes*	Genotype (acronym underlined)
VP7	80	28	Glycosylated (G)
VP4	80	39	Protease-sensitive (P)
VP6	85	21	Inner capsid (I)
VP1	83	14	RNA-Dependent RNA Polymerase (R)
VP2	84	14	Core protein (C)
VP3	81	13	Methyltransferase (M)
NSP1	79	24	Interferon Antagonist (A)
NSP2	85	14	NTPase (N)
NSP3	85	16	Translation enhancer (T)
NSP4	85	21	Enterotoxin (E)
NSP5	91	16	Phosphoprotein (H)

* The number of genotypes for each viral protein are updated from Matthijnssens & Theuns, 2015.

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

The last RCWG meeting was held on October 9, 2015, and from this meeting, 28 different G genotypes (VP7) and 39 P genotypes (VP4) found within the RVA species were approved by the group. All the updates for the different genotypes can be found in Table 1.1 **(**Matthijnssens & Theuns, 2015). Since the last RCWG meeting, more complete genomes have been collected for RVB and RVC. Researchers in these groups are trying to create proposed classifications systems similar to the RCWG.

A provisional whole genome classification system for Rotavirus B (RVB) has been updated and proposed by Shepherd *et al.* (2018), based on the RCWG classification guidelines for RVA. This system includes complete genomes consisting of porcine, human, caprine, bovine, and murine sources. The number of genotypes and percent nucleotide identity cutoff values can be found in Table 1.2.

Table 1.2 Diversity and Percent Identity Cutoff Value of RVB Genotypes by Shepherd *et al.*, 2018

Viral protein	Percent Identity Cutoff Value	Number of genotypes	Genotype (acronym underlined)
VP7	80	26	Glycosylated (G)
VP4	80	5	Protease-sensitive (P)
VP6	81	13	Inner capsid (I)
VP1	78	5	RNA-Dependent RNA Polymerase (R)
VP2	79	5	Core protein (C)
VP3	77	5	Methyltransferase (M)
NSP1	76	8	Interferon Antagonist (A)
NSP2	83	10	NTPase (N)
NSP3	78	6	Translation enhancer (T)
NSP4	76	4	Enterotoxin (E)
NSP5	79	7	Phosphoprotein (H)

Rotavirus C (RVC) also has a provisional whole genome classification system proposed by Suzuki & Hasebe (2017), based on the RCWG classification guidelines for RVA. Their system includes partial and complete genomes consisting of multiple porcine, human, ferret, bovine, and canine sources. The number of genotypes and percent nucleotide identity cutoffs values can be found in Table 1.3.

Table 1.3 Diversity and Percent Identity Cutoff Value of RVC Genotypes by Suzuki & Hasebe, 2017

Viral protein	Percent Identity Cutoff Value	Number of genotypes	Genotype (acronym underlined)
VP7	85	18	Glycosylated (G)
VP4	85	21	Protease-sensitive (P)
VP6	87	13	Inner capsid (I)
VP1	84	4	RNA-Dependent RNA Polymerase (R)
VP2	85	6	Core protein (C)
VP3	85	6	Methyltransferase (M)
NSP1	84	9	Interferon Antagonist (A)
NSP2	87	8	NTPase (N)
NSP3	85	6	Translation enhancer (T)
NSP4	81	5	Enterotoxin (E)
NSP5	80	4	Phosphoprotein (H)

Researchers are continuing to work on creating similar classification systems to RVA for all remaining RV species that do not yet have established systems.

Rotavirus Replication

Rotavirus replication is important to understand since many of the current vaccine development strategies are based on important structural proteins involved in the viral replication process. Much of our current understanding of RV replication is derived through *in vitro* work performed in permissive cell lines that allow RVs to replicate. An example of one of these cell lines is non-differentiated MA104 cells (African Green Monkey kidney cell line) typically used

to culture RVA species (Arias *et al.*, 2015). From this work, RV strains have been characterized by several features when it comes to preferences of cell surface molecules. Depending on the host species, some RV animal strains use the spike protein (VP4) bind to cellular glycans, such as sialic acids, while other animal strains and most human strains have been found to bind to histo-blood group antigens (Arias *et al.*, 2015; Kim *et al.*, 2017). The RV strains that bind to sialic acid are classified as either neuraminidase sensitive (binds to external sialic acid) or neuraminidase resistant (binds to internal sialic acid). The exact way these mechanisms work is still being investigated today through crystallographic studies in both human and animal RV strains.

Once attached, the spike protein found on the outer capsid of the triple-layered RV particle undergoes a proteolytic cleavage. In the presence of trypsin, the spike protein (VP4) is cleaved into two subunits, VP5 and VP8 (Yoder *et al.*, 2009). The VP8 subunit facilitates binding of RV to different cell surface receptors found on the host cell, and VP5 helps with viral penetration (Li *et al*., 2018). These two subunits are currently being targeted for use in subunit vaccines and will be discussed more in Chapter 4. The current working hypothesis is that RV penetrates the cell either through direct penetration or receptor-mediated endocytosis. The mechanism by which RV enters the cell is still poorly understood and is actively being investigated through crystallography studies of the VP8 structures (Liu *et al.*, 2017).

Once inside the host cell, the triple-layer particle sheds the outer capsid layer in the cytoplasm of the host cell mediated by low intracellular Ca^{++} and cellular enzymes. *In vitro* work has shown the glycoprotein, VP7, binds calcium to solubilize the outer protein layer of triple-layer RV particle (Ludert *et al.*, 2002). The remaining double-layered particle actively starts transcribing the 11 segments of RNA into capped viral mRNA in the cytoplasm. These

transcripts are either translated into the six viral structural proteins or five non-structural proteins (Desselberger *et al.*, 2009).

Once enough viral proteins are created, viral replication occurs within inclusion bodies found in the cytoplasm called viroplasms (so called "virus factories"). These specialty compartments consist of two non-structural proteins, NSP2 and NSP5. Within these viroplasms, VP1, VP2, VP3, VP6, and mRNA from each of the 11 segments create a double-layered particle. NSP4, found on the outside of the rough endoplasmic reticulum (ER) acts as a receptor and buds the newly formed double layer particle into the ER. Inside the ER, the double-layered particle becomes transiently enveloped but loses the envelope when the outer layer consisting of VP7 and VP4 is acquired. Currently, little is known regarding the transitory envelope that occurs in the ER. Once the particles have matured, the triple-layered particles are released from the host cell through cell lysis (Desselberger, 2014; Desselberger *et al.*, 2009).

Public Health Concerns and Zoonotic Potential

Rotaviruses are known to infect many host species, primarily targeting the very young. In humans, children under the age of five are the most affected by this disease. Prior to 2006, RV infections in the United States (US) caused over 3 million cases with over 60,000 hospitalizations, 500,000 doctor visits, and 20-40 deaths in humans (Desselberger, 2014). After the introduction of two live attenuated RV vaccines, RotaTek® (Merck) and RotaTrix® (GlaxoSmithKline), in 2006, RV cases, especially in developed countries, have significantly decreased, and as a result, mortality has also decreased. However, since multiple species can be infected with RV, there are public health concerns of zoonotic potential between human and animal.

The new RV classification system based on complete and incomplete genome analysis has helped researchers better understand RV evolution on a molecular level. Rotavirus has an 11 segmented genome and has been found to easily reassort both *in vivo* and *in vitro*. Martella *et al.* (2010) describes "the mechanisms driving rotavirus diversification include positive accumulation of single point mutations, inter-segmental recombination, rearrangement, and notably reassortment." These evolutionary events are leading to the emergence of new RVA G9 and G12 serotypes in both swine and humans (Shao *et al.*, 2016). A major concern with the emergence of the new RVA genotypes in humans and swine is that the currently licensed vaccines will not protect against these new strains.

Initially, RV strains were thought to only circulate and infect within a host species. However, it has been documented that interspecies transmission and reassortment between RV strains infecting pigs and horses, pigs and cattle, and pigs and humans has occurred (Chang *et al.*, 2012). *In vitro* and *in vivo* studies were completed by Shao *et al.* (2016) that investigated the pathogenesis of a human RVA (HRV) strain, Wa G1P[8], and a porcine RVA (PRV) strain, G9P[13], in gnotobiotic pigs. The investigators performed a complete genome sequence of the porcine RV strain G9P[13] and found it "possessed a human-like G9 VP7 genotype but shared higher overall nucleotide identity with historic PRV strains." Other findings from this study included: 1) the PRV G9P[13] strain had longer viral shedding and RVA detected in serum than the HRV G1P[8] strain in gnotobiotic pigs; 2) the PRV G9P[13] strain generated short-term complete cross-protection in pigs challenged with either 10^5 fluorescence-forming units of HRV G1P[8] strain or 10^5 fluorescence-forming units porcine RV G9P[13] challenge; and 3) the human strain G1P[8] strain had limited cross protection to 10^5 fluorescence-forming units porcine RV G9P[13] challenge. This is one of the first reports to describe possible cross-

protection between heterologous G and P genotypes. To safeguard humans, continual surveillance of porcine rotavirus genotypes is important for emerging genotypes with zoonotic potential.

Swine Rotavirus

Since 1975, RV has been found to be a leading cause of diarrhea in swine (Marthaler *et al*., 2014a; Rodger *et al*., 1975). All pigs are susceptible to RV infections, but nursing and weaned piglets are affected the most (Kahn & Line, 2005). By nature, the virus is hardy in the environment and has been documented in swine herds worldwide (Chang *et al.*, 2012; Dhama *et al.*, 2009). These infections tend to be endemic to a herd and can cause severe morbidity and mortality in young piglets. Outbreaks on commercial farms can be economically devastating for producers due to loss of production and the costs associated with treating the infection (Chandler-Bostock & Mellits, 2015). It has been difficult to associate the true cost of RV infections since not all RV species have been accurately identified and the pathogenesis of all RV species that infect swine is still unknown.

The United States Department of Agriculture conducts swine surveys every six to eight years. The last survey was conducted in 2012, and the final survey results can be found in the Swine 2012 Part III: Changes in the U.S. Swine Industry, 1995−2012. In this survey, swine producers were asked a variation of same question in 2000, 2006, and 2012 for pre-weaned piglets. An example of the survey question asked to swine producers included: “In the last 12 months, were any of the following disease problems known or suspected to have caused sickness or mortality in one or more preweaned (suckling) pigs?”. The percentage of sites that were found to answer RV as one of the diseases were as followed: 5.7 percent, 6.9 percent, and 19.4 percent, in 2000, 2006, and 2012, respectively. It is important to know the results are opinion-

based and not necessarily confirmed with diagnostic laboratory testing. However, it does show that swine producers are suspecting increases in RV cases within their herds over a span of years. The next National Animal Health Monitoring System's (NAHMS) swine study will be conducted in 2021, where it will investigate both small and large swine enterprises (United States Department of Agriculture, 2020).

RV Prevalence in Swine

Currently, five different species of RV known to infect pigs, include RVA, RVB, RVC, RVE, and RVH. RVA is the most common species found to infect and cause diarrhea in swine around the world (Molinari *et al.*, 2016). This was the first RV species to be detected and isolated in pigs in 1975 (Rodger *et al*., 1975; Woode *et al*., 1976). Currently, there are 12 porcine RVA G genotypes, including G1, G2, G3, G4, G5, G6, G8, G9, G10, G11, G12, and G26, and 13 porcine RVA P genotypes, including P[1], P[5], P[6], P[7], P[8], P[11], P[13], P[19],P[23], P[26], P[27], P[32], and P[34] (Naseer *et al.*, 2017). Of these, the most common G genotypes in swine include G3, G4, G5, G9, and G11, and the two most common P genotypes include: P[6] and P[7] (Lorenzetti *et al.*, 2011).

RVB was originally identified as an atypical rotavirus or rotavirus-like virus in the 1980's when the species was first detected in swine (Marthaler *et al.*, 2014b; Shepard *et al.*, 2017; Theil *et al.*, 1985). Since its discovery, RVB has been found circulating in herds in Japan, Germany, US, Canada, Mexico, and India. These infections tend to be sporadic in nature since low amounts of RVB are excreted in feces. Currently, 20 RVB G genotypes have been identified in swine. The two most dominant G genotypes in the USA include G16 and G20 (Shepard *et al.*, 2017).

In 1980, RVC was first detected in a 27-day old piglet in Ohio (Saif *et al.*, 1980). RVC is known to have the potential to induce subclinical infections or cause large outbreaks in swine (Marthaler *et al.*, 2014a). RVC has been detected in herds in the US, Brazil, Canada, and throughout Europe (Chepngeno *et al.*, 2019). It typically infects piglets between one and 20 days of age (Chepngeno *et al.*, 2020; Marthaler *et al.*, 2014a). Currently, there are seven RVC G genotypes, including G1, G3, G5, G6, G7, G8, and G9, and 6 P genotypes, including P[1], P[2], P[3], P[4], P[5], and P[6], known to infect pigs (Theuns *et al.*, 2016). Two of the most common RVC G genotypes are G3 and G6 (Vlasova *et al*., 2017).

To date, there has only been one porcine sample identified with RVE, and it was detected in field samples collected in the United Kingdom (Pedley *et al.*, 1986). At this time, the relevance and importance of this species is unknown.

Between the years of 1991-1995, a new species of RV was causing diarrhea in piglets less than 30 days of age in Japan (Suzuki & Inoue, 2018). It was not until 1999, when a new swine species RVH, strain SKA-1, was identified and isolated in MA104 cells (Wakuda *et al.*, 2011). Marthaler *et al*. (2014b) investigated the presence of RVH circulating in the US swine populations from 204 samples collected between 2006 and 2009. Thirty percent of the samples were found positive for RVH and in ten different states. In the US, RVH is commonly detected in piglets between 21-55 days of age and 55 days or older. It is hypothesized that piglets diagnosed with RVH at 21-55 days of age in the US is due to weaning practices. Piglets from different production sites are commonly mixed together at 21 days of age (Marthaler *et al.*, 2014b). Similarly, in 2012, Molinari *et al*. (2014) detected RVH in three fecal samples in 35-day old piglets in Brazil.

Transmission

Sows are often subclinical carriers of RV and shed RV infectious particles in their feces into the environment. Infections in piglets typically occur through direct contact with RV-infected feces from their dam prior to weaning or from other pigs post-weaning (fecal-oral transmission). These infections can also occur indirectly through contaminated food, water, and fomites (Chandler-Bostock & Mellits, 2015).

Pathogenesis

The pathogenesis for RV begins when the virus starts replicating in the cytoplasm of mature enterocytes (epithelial cells), which are located on the tips of the villi of the small intestines. As the RV infection progresses, "extensive cellular necrosis of the epithelium tissues of the small intestine develops, leading to villous atrophy, loss of digestive enzymes, reduction in absorption and increased osmotic pressure in the gut lumen and the onset of diarrhea" (Desselberger *et al.*, 2009).

Clinical Signs

In swine, the severity of the RV infection depends on host and viral factors. The virulence of a RV strain in a pig population can be affected by the virus strain, the dose of viral exposure, the age of the host, how many digestive cells are affected, the immune status of the host, the environment, nutrition of the host, and if concurrent infections are present in the host (Dewey *et al*., 2003; Kahn & Line, 2005; Will *et al.*, 1994). Through reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) researchers have found each RV species tends to infect different age groups. This will be discussed in more detail in Chapter 3. When clinical signs of a RV infection are present, they include dehydration, anorexia, depressed growth, and mild to severe diarrhea (Saif & Fernandez, 1996).

Rotavirus can infect all ages of pigs but typically infects neonates (<7 days of age) and weaned piglets (21 to 28 days of age). The clinical signs in neonates consists of watery diarrhea 12-48 hours after birth, which occurs when neonates do not receive enough protective maternal antibodies from the sow or after weaning when maternal antibodies tend to wane. In nursing piglets (5 to 21 days of age), diarrhea is often yellow or gray in color, and after two days, becomes gray and pasty. Diarrhea in nursing piglets can last for two to five days during an infection. Weaned piglets often have watery diarrhea with poorly digested feed found in it (Kahn & Line, 2005).

Lesions

Gross lesions of RV infections are often more severe in piglets one to 14 days of age. The stomach is typically full of food, and the small intestines appear to be thin-walled, flaccid, and dilated with a watery fluid (Chang *et al.*, 2012; Kahn & Line, 2005). Further distal in the digestive tract, the cecum and colon contain more liquid feces. The gross lesions become mild to absent at 21 days of age and older.

Experimental infections of RVA in gnotobiotic pigs have provided researchers a better understanding of what occurs at a microscopic level. Between 15 to 18 hours after oral inoculation with RV, the epithelial cells found on the apical tip of villi of the small intestines begin to degenerate. Within 16-24 hours, villus atrophy occurs by cells sloughing off and increases in severity by 24-72 hours post infection. At 48-72 hours, crypt epithelial hyperplasia is documented (Chang *et al.*, 2012). Figure 1.2 a-c shows histopathology results of edema, villous atrophy, and necrosis of villi in two- to three-day old piglets infected with RVA, RVB, and RVC, respectively. For comparison purposes, Figure 1.2 d shows histopathology of normal villi.

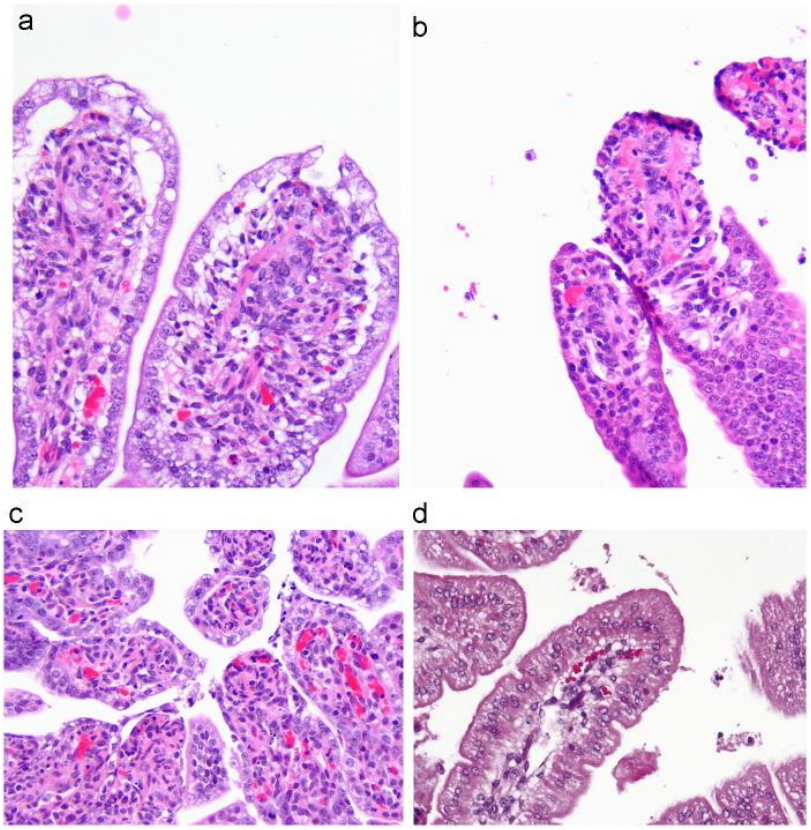

Figure 1.2 Histopathology of RVA, RVB, and RVC

Sourced from Marthaler D, Rossow K, Gramer M, Collins J, Goyal S, Tsunemitsu H, Kuga K, Suzuki T, Ciarlet M, and Matthijnssens J. (2012). Detection of substantial porcine group B rotavirus genetic diversity in the United States, resulting in a modified classification proposal for G genotypes. *Virology*. 433(1):85-96.

Diagnosis

A laboratory confirmation is needed to diagnosis a RV infection since diarrhea is a common sign in swine for other pathogens, including porcine epidemic diarrhea virus, transmissible gastroenteritis, *Escherichia coli*, *Clostridium perfringens*, and *Isospora suis*. Samples used to detect RV infections include fecal samples, intestinal tissues, and intestinal contents (Kahn & Line, 2005). The methods for identification include electron microscopy, polyacrylamide gel electrophoresis (PAGE), virus isolation, enzyme-linked immunosorbent assays (ELISAs), immunohistochemistry (IHC), reverse transcriptase-quantitative polymerase

chain reaction (RT-qPCR), and next generation sequencing. The advantages and limitations of each of these tests will be described in more detail in Chapter 2.

Control, Prevention, and Treatments

Eradication of RV in commercial swine herds is nearly impossible due to its ubiquitous nature. An infectious RV virus particle can survive in feces for over two years at 4ºC and in the environment for over nine months (Chandler-Bostock & Mellits, 2015). These virus particles are stable at a pH range of 3 to 9 (Chang *et al.*, 2012), and since RV is a non-enveloped virus, it tends to be more resistant to disinfection (Chandler-Bostock & Mellits, 2015).

In large, swine production systems, controlling and preventing RV can become more complex due to all the stakeholders involved in the process. Points of entry for RV infections can occur in these systems, including in farrowing barns, nursing barns, fattening or finishing barns, transportation vehicles, slaughterhouses as well as with contact with people (veterinarians and farm staff) at each phase of the production process. Lacapelle *et al.* (2017) used RV as an enteric molecular marker to evaluate the potential for spread between stakeholders in a large, swine production network. Their findings emphasized that transporters and slaughterhouses serve as major reservoirs and vectors for transmitting RV throughout a swine network.

Housing practices of swine must be considered when it comes to controlling and preventing RV. In Ontario, an epidemiological study found that "pigs raised in all-in, all-out nurseries were 3.4 times more likely to have a positive group A rotavirus diagnosis than pigs in continuous flow facilities" (Dewey *et al.*, 2003). In a continuous flow operation, sows are consistently breeding and farrowing. The rooms containing sows and piglets are constantly changing based on the individual animal. This means that the rooms are almost never completely empty, and so, extensive cleaning may not be performed on a regular basis. For those

producers who use continuous flow it is thought the piglets in these operations are exposed to RV in the environment and "able to develop an active immune response under the partial protection of maternal antibody" (Dewey *et al.*, 2003). However, in an all-in, all-out nursery the pigs move as a group based on age and weaning. Typically, there is down time between batches of pigs and cleaning can be done routinely. In this study, many of the all-in, all-out piglets were weaned early which could have contributed to the higher incidence of RV (Dewey *et al.*, 2003).

Animal husbandry and biosecurity measures are other ways to control and prevent RV infections within a swine network (Lachapelle *et al.*, 2016). Swine facilities should be constructed to reduce fecal build-up in farrowing and nursing areas, easy to clean, and the floors should be disinfected between different batches of swine (Chang *et al.*, 2012). It is also important to wash, disinfect, and dry all transportation vehicles and farm fomites, such as boots, between batches of animals to limit the spread of RV and other infectious pathogens (Lachapelle *et al.*, 2016). Other considerations made at the farm level are to limit transportation vehicles access and movement on the premises since these can serve as reservoirs.

Choosing the correct cleaning procedures and disinfectant is important in reducing RV loads in swine facilities. The amount of organic matter (feces) present in the environment can have a dramatic effect on how effective the disinfectant is against RV viral loads. In a controlled study in the United Kingdom by Chandler-Bostock & Mellits (2015), four commercially available disinfectants were investigated to see how efficacious the disinfectants were in the presence of low or high organic matter, which represents different farm environments. Bi-OO-cyst® manufactured by Biolink, a phenolytic (ether)-based disinfectant, was proven to reduce the RV loads on surfaces by more than 4 $\log_{10}$ in cell plaque assay in the presence of high organic matter. Two other common disinfectants manufactured by Evans Vanodine, Vanadox®

(peracetic acid) and GPC8™ (glutaraldehyde), were able to reduce RV viral loads by more than 4 $\log_{10}$ in a cell plaque assay with no organic matter or in the presence of low organic matter but not in the presence of high organic matter. Virkon S™, manufactured by DuPont (peroxygen compounds), had similar efficacy as Bi-OO-cyst® in the reduction of RV loads on surfaces by more than 4 $\log_{10}$ in cell plaque assay when no organic matter was present or in the presence of low organic matter, but it lost efficacy in the presence of high organic matter.

Two other ways to control RV infections in swine herds are by performing a planned exposure, a practice also known as "feedback" or through mass vaccination. Planned exposure occurs when RV-infected feces or intestinal contents from a farm field strain are fed to a sow, two-to-five weeks prior to farrowing (Robbins *et al.*, 2014). The goal is to prime the immune system of the sow to create maternally-derived, neutralizing antibodies in the colostrum and milk for their piglets (Tuanthap *et al.*, 2019). This practice is common in locations where a vaccine against a RV species is not available or in areas where RV infections are prevalent within a herd.

Currently, there are two United States Department of Agriculture (USDA) licensed vaccine products, ProSystem® Rota and ProSystem® RCE (Merck Animal Health, Madison, NJ), for use in swine in the US. These vaccines contain two, modified-live RVAs, one each from serotypes G4 (A1) and G5 (A2), either alone (ProSystem® Rota) or in combination with *Clostridium perfringens* type C and four *Escherichia coli* pilus antigens: K99, K88, F41, and 987P (ProSystem® RCE).

ProSystem® RCE is administered to pregnant sows in two, two mL doses given intramuscularly at five weeks and two weeks prior to farrowing (Merck Animal Health Product Bulletin: Prosystem® RCE). The purpose of the vaccine is to increase a sow's level of maternally-derived neutralizing antibodies against RVA, so the antibodies will be passed in the

colostrum and milk. This product is labeled to "aid in the prevention of rotaviral diarrhea, colibacillosis and enterotoxemia in their nursing piglets".

ProSystem® Rota contains only the two RVA serotypes and is given to piglets in two, one mL doses. The first dose is administered orally, and the second dose is administered intramuscularly, seven to ten days before weaning (Merck Animal Health Product Bulletin ProSystem® Rota). This vaccine is labeled "for use as an aid in the prevention of rotavirus diarrhea in young pigs". This vaccine is also commonly given to gilts to acclimate them prior to entering the sow farm as well. Even though there are vaccines available for RVA, RV outbreaks still occur in swine herds. Challenges for developing new RV vaccines in swine will be discussed in more detail in Chapter 3.

Currently, there are no antivirals treatments for RV infections in swine (Chang *et al*., 2012). Antibiotics are not effective in treating rotavirus; however, it can help piglets co-infected with secondary bacterial infections. Supportive treatments, including glucose/glycine electrolyte solutions and oral solutions with L-glutamine, help treat dehydration and weight loss caused by RV in young piglets (Chang *et al.*, 2012). A new supportive treatment, found that Resveratrol, "a stilbene and a naturally occurring phytoalexin produced by several plants in response to injury or protecting against microbial infections", when used as a feed additive in 30-day old piglets was able to reduce diarrhea in piglets experimentally infected with RV (Cui *et al.*, 2018). Other ways to reduce mortality is to keep the ambient temperature around 35ºC to minimize heat loss in young piglets. Also feeding weaned piglets' high-energy diets may also help reduce morbidity and mortality (Chang *et al.*, 2012).

Swine RV infections are prevalent in most herds; however, most infections are not fatal in swine unless the infection is complicated with concurrent infections. RV species and strains

need to be monitored in swine. It is well-documented that interspecies transmission and gene reassortment events occur between humans and pig RV. As a public health measure, a need for newer diagnostic tests to accurately detect all RV species in swine is needed. Surveillance of swine RV in commercial herds can help identify the emergence of new G and P genotypes of RV species.

Chapter 2- Diagnostic Testing for Swine Rotavirus

It is important for swine producers that there are diagnostics tests available to detect and accurately diagnose rotavirus (RV) infections within swine herds. Clinical signs alone are not enough to diagnose a RV infection since other enteric diseases have similar clinical signs, resulting in stunted growth, lethargy, and diarrhea. Diagnostic testing for swine RV has evolved since it was first detected in swine in 1975. Introduction of newer diagnostic tools, especially PCR, has helped expand our current understanding of how RV infections occur in the field. The prevalence of RV infections in swine herds has been difficult to estimate due to the lack of readily available diagnostic test for all the different RV species known to infect swine. There are several diagnostic tests available; however, each diagnostic test has its advantages and disadvantages.

Early Diagnostic Tools

The first method available to analyze feces, tissue, or intestinal contents for RV infections in veterinary diagnostic laboratories was with the use of electron microscopy (EM). Under EM, the virus appears as a wheel-like particle that measures 65-75 mm in diameter (Chang *et al.*, 2012). In swine RV infections, it is common to see both intact triple-layered infectious particles and double-layered particles with rough edges (Chang *et al.*, 2012). The major drawback of the method is that it lacks specificity and sensitivity. Under EM, the RV structures cannot be distinguished between different species of RV (Marthaler *et al.*, 2014a). Other disadvantages of this method are that these machines are very expensive, require specialized training, and not every veterinary diagnostic laboratory offers this service. This method is not often used to detect RV infections in samples today, but it is still used in scientific research.

Another tool used in early research for diagnosing RV infections was polyacrylamide gel electrophoresis (PAGE). In this method, the dsRNA is extracted from fecal or intestinal content samples, loaded into a polyacrylamide gel, and subjected to an electric current where the RNA migrates through the gel with the smaller RNA fragments moving faster than the larger RNA fragments. The gels are then stained with silver stain. The RNA fragments create electrophoretic migration patterns known as electro-pherotyping. For RV, the RNA segments cluster into four regions labeled I, II, III, and IV (Chang *et al.*, 2012). The number of bands found in each of the four regions corresponds to the four serogroups: A, B, C, and D. The respective RNA segment clusters for A, B, C, and D are described as 4:2:3:2, 4:2:2:3, 4:3:2:2, and 5:2:2:2, respectively (Chang *et al.*, 2012). In Figure 2.1, lane A represents the porcine RV serotype A (4:2:3:2), lane B represents porcine RV serotype B (4:2:2:3), and lane C represents porcine RV serotype C (4:3:2:2).

Figure 2.1 RV Electro-Pherotyping of Different Serotypes of Porcine Rotaviruses

Sourced from Janke BH, Nelson JK, Benfield DA, Nelson EA. (1990). Relative Prevalence of Typical and Atypical Strains among Rotaviruses from Diarrheic Pigs in Conventional Swine Herds. *J Vet Diagn Invest.* 2(4):308-311.

The PAGE method was originally used to distinguish between the RVA serogroup from the atypical RV strains consisting of RVB and RVC (Janke *et al.*, 1990). A disadvantage of PAGE is this test cannot distinguish between different RV strains, which need to be confirmed by other diagnostics tests (Chang *et al.*, 2012). Also, gels can be very time consuming. This method was popular in the early 1980s and 1990s, but they fell out of favor as new diagnostic tests for RV became available. Currently, the PAGE method is not performed regularly as a diagnostic test; however, it is still used in research to evaluate and characterize attenuated RV strains versus wild-type strains (Park *et al.*, 2019).

Diagnostic Methods Currently in Use

The gold standard for infectious RV diagnosis is isolation in cell culture. Although this method is available, it is not commonly used to diagnose RV for a couple of reasons. The first reason is not all RV species can be grown in cell culture. A second reason this detection method is not performed often is cell culture is very time consuming and labor-intensive. Over the past several years, faster and less labor-intensive methods have been developed.

RVA is the most well characterized, documented, and prevalent of all RV species. Most strains of RVA are easily isolated and serially passed in MA104 cells (African Green Monkey kidney cell line) with the addition of a proteolytic enzyme, such as pancreatin or trypsin. RVB, on the other hand, has had less success adapting to cell culture. Only one RVB strain has been described. It was successfully isolated in swine kidney cells (Sanekata *et al.*, 1996). Since this publication, no other successful propagation of RVB in cell culture has been published. The first RVC strain, Cowden, was adapted to serially propagate in both primary porcine kidney cells and

MA104 cells in the late 1980s (Saif *et al.*, 1988; Terrett & Saif, 1987). Since then, only a few other RVC strains have been isolated but not successfully propagated in cell culture. Rotavirus E (RVE) has only been identified in one sample in the United Kingdom (Pedley *et al.*, 1986), and not much work has been done since then. At least one strain of RVH has been adapted to grow in cell culture (Wakuda *et al*., 2011).

Another popular assay used in screening feces for suspected rotavirus is enzyme-linked immunosorbent assays (ELISAs). Several commercial kits are available for research purposes and are not intended for diagnostic use. Two of these kits include the Porcine Rotavirus Antigen ELISA Kit (Colorimetric) (Novus Biologics, catalog number NBP2-60133-1) and Pig Rotavirus Group Specific Antigen ELISA Kit (Sandwich) (LifeSpan Biosciences, catalog number LS-F10367-1). Both test kits use pre-coated microtiter plates with RV-specific antibody. A fecal sample is diluted in normal saline prior to addition to the pre-coated antibody plate. An equal amount of the diluted fecal sample and antibody with conjugated horseradish peroxidase (HRP) is added to the well. If the sample is positive for RV, it will bind to the coating antibody on the plate and will not be removed when the sample is washed with washing buffer. After the wash step, the substrate solution is added to the test well, and color development will increase in intensity if the sample is positive for RV. The reaction is stopped using a stopping solution and read on a microtiter plate reader. These assays are qualitative and tell the operator if RV is present in the sample or not. These tests tend to be specific to swine RV, but some cross-reactivity can occur between other species of RV. Since this test is qualitative, it often requires confirmation with another RV diagnostic test to confirm a RV infection. The advantage of an ELISA is that it is a fast and reliable screening tool for multiple fecal samples. However, a

downfall of this assay is that not all porcine RV species have commercially ELISA antibody kits available. Most commercially available kits tend to be only specific for the RVA species.

Identification of RVA antigen in the small intestine is commonly performed with immunohistochemistry (IHC). In this method, a piece of tissue is fixed in 10% formalin and placed on a slide. The slide is blocked, and an anti-RVA VP6 polyclonal antibody is added to the slide and incubated. A secondary, biotinylated antibody linked to streptavidin–peroxidase is then added. The slide is then incubated with a substrate and counterstained using Mayer hematoxylin (Almeida *et al.*, 2018). Figure 2.2 A shows sections of porcine IHC staining of porcine small intestines from a RVA outbreak that stained for both RVA and RVC, while Figure 2.2 B shows IHC staining from a RVC outbreak.

There are commercially available antibodies to RVA, RVB, and RVC. A limitation to this method is the lack of commercial monoclonal antibodies available for species RVE and RVH. This limits the study of the pathogenesis of these strains in tissues.

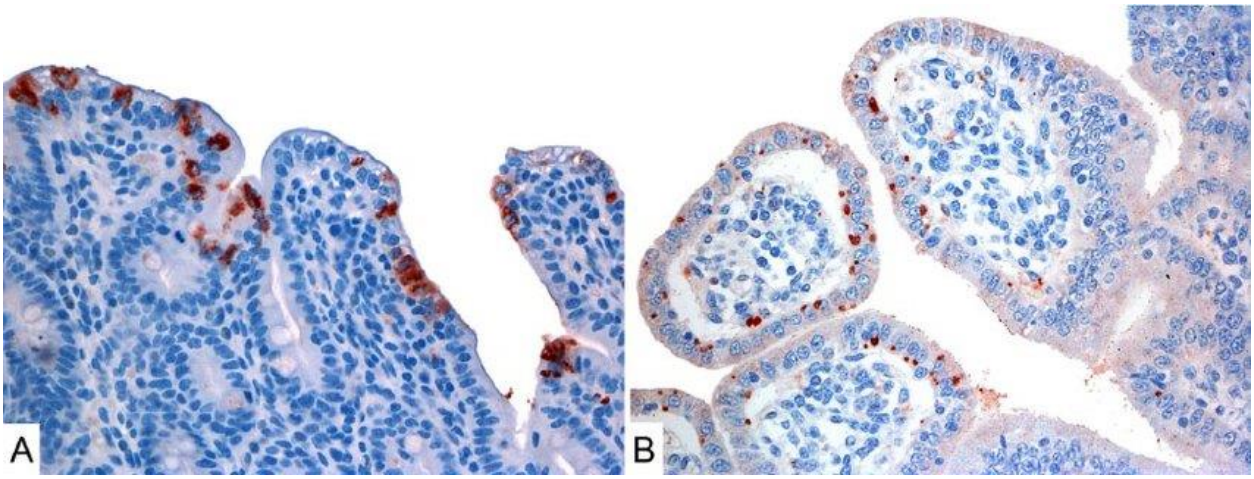

Figure 2.2 Immunohistochemistry of RVA Through Commercially Available Products and RVC Through a Research Group

Sourced from Almeida PR, Lorenzetti E, Cruz RS, Watanabe TT, Zlotowski P, Alfieri AA, Driemeier D. (2018). Diarrhea caused by rotavirus A, B, and C in suckling piglets from southern Brazil: molecular detection and histologic and immunohistochemical characterization. *J Vet Diagn Invest.* 30(3):370–376.

Molecular Based Diagnostic Assays

It is now commonplace in US veterinary diagnostic laboratories to use either reverse-transcriptase-polymerase chain reaction (RT-PCR) or reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR) to detect and distinguish between RVA, RVB, and RVC swine infections. These methods are fast and highly accurate. In the RT-PCR method, the dsRNA is extracted from a fecal sample or a piece of small intestine. The sample is then amplified using RV-specific primers and read on an agarose gel. This method works well; however, compared to RT-qPCR, it is slower and more labor-intensive. A downfall to this method is it only measures gene expression and may not correlate with live, infectious virus.

Marthaler *et al.* (2014a) developed and described the use of a multiplex, real-time (RT-qPCR) for RVA and RVC, which could be used with RT-qPCR for RVB. The RVA and RVC multiplex, method uses probes for each of the RV species to help identify different RV species. The test is rapid, accurate, and sensitive. However, one of the disadvantages is that it only detects nucleic acid. The test can tell an operator if the virus is present within the sample, not if the virus is viable. Another disadvantage to this method is the primers used in the method should be reviewed and updated as more porcine swine strains become sequenced (Amimo *et al.*, 2013). Emerging strains may not be identified if the current primers do not detect them.

Another newer method called *in situ* hybridization RNA-based chromogenic technique (ISH-RNA) was described in Resende *et al. (*2019) for use in formalin-fixed, paraffin-embedded tissues. In this method, the investigators used probes designed against the VP6 gene for RVA, RVB, and RVC to discriminate between the three species within a piece of intestinal tissues. The actual sequences of the probes used are proprietary to the company Advanced Cell Diagnostics, Newark, CA. This investigational technique was originally developed to investigate

the relationship between RT-qPCR-positive results for each species versus histological lesions found in the small intestines. Figure 2.3 shows images collected from this newly developed method. Figure 2.3 A shows a small intestine tissue sample with a mixed infection of RVA (green color) and RVC (red color) using (ISH-RNA) method , Figure 2.3 B shows small intestine tissue sample with an RVC (red color) infection using (ISH-RNA) method, and Figure 2.3 C shows small intestine tissue sample with an RVA (green color) infection using (ISH-RNA) method.

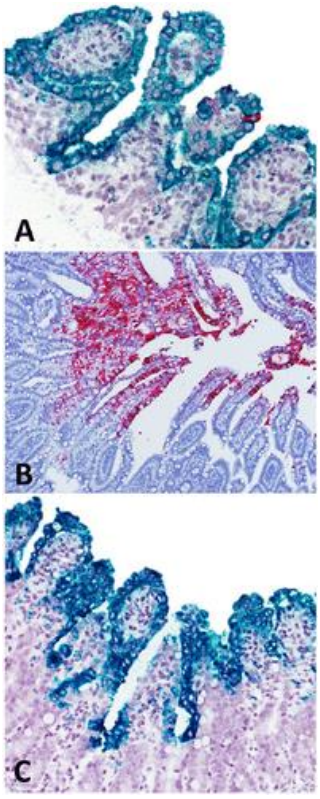

Figure 2.3 *In situ* Hybridization RNA-based Chromogenic Images

Sourced from Resende TP, Marthaler D, Vannucci FA. (2019). *In situ* hybridization detection and subtyping of rotaviruses in swine samples. *J Vet Diagn Invest.* 31(1):113-117.

One of the advantages of this ISH-RNA test is the probes target mRNA, which can only be detected in actively replicating virus. This newer technology could help researchers better

understand the pathogenesis of RVB and RVC. However, there are also disadvantages to this method. In the Resende *et al. (*2019) study, some the RT-qPCR-positive samples were not detected by this method. It was hypothesized that either the RV virus "infection may not be diffuse throughout the intestine, and sampling non-adjacent intestinal segments for PCR and ISH-RNA can result in two different outcomes", or one or more RV species are present but do not cause lesions in the intestines. Another reason this could have happened is ISH is less sensitive and not as specific as RT-qPCR.

Another tool being used by researchers to better understand RV strains in the field is next generation sequencing. This is a relatively new method in which complete genome analysis allows researchers to compare historic RV strain genome sequences to current RV strain genome sequences in the field (Chepngeno *et al.*, 2020). This is an important tool in better characterizing and understanding field RV strains. Some of the disadvantages of this method is it can be time consuming. The samples used in this testing method often originate from feces, serum, or tissue. The removal of all extraneous DNA and RNA from the host and other bacterial and viral infections within the sample is important. Another disadvantage of this method is the cost. This newer method requires special machines and people trained to do the analysis.

Since 1975, the diagnostic testing of RV infections in swine has evolved immensely. Faster, more accurate, and more specific diagnostic tests have led to a better understanding of RV infections in the field. The newer molecular methods have increased researchers understanding of RV species, like RVB and RVC, which are notoriously hard to isolate in cell culture. These tests are also crucial for developing better vaccines for swine RV.

Chapter 3- Challenges in Vaccine Development for Swine Rotavirus Vaccines

The current rotavirus vaccination strategy for swine is based on passive immunity. Sows are vaccinated while pregnant and their maternal neutralizing antibodies are passed in the colostrum and milk to their suckling young. The reason this is important is piglets are born agammaglobulinemic, and their adaptive immune systems are immature at birth (Saif & Fernadez, 1996; Chepngeno *et al*., 2019). This occurs because maternal antibodies do not cross the transplacental barrier in swine (Ward *et al*., 1996). To protect neonatal piglets, large numbers of maternal antibodies from a RV immune dam need to be acquired by ingesting colostrum within the first 36 hours of life (Chang *et al.*, 2012). The primary immunoglobulin found in swine colostrum is IgG, while the primary immunoglobin found in sow's milk is secretory IgA (Chang *et al*., 2012). As colostrum switches over to milk, the production of IgG declines, and IgA, which is "produced locally in the mammary gland by plasma cells originating in the intestine", becomes the predominant immunoglobulin (Saif and Fernadez, 1996). Vaccination or planned exposures are the current forms of prophylaxis against infections of rotavirus in swine.

Current RV Vaccines in the US

As described in Chapter 1, there is one USDA-approved live-attenuated RVA vaccine (Prosystem® RCE) licensed for pregnant sows, and there is one USDA-approved RVA vaccine licensed for use in piglets seven to ten days preweaning as well as acclimating gilts getting ready to enter a sow farm (ProSystem® Rota). Currently, it is difficult to estimate how many swine producers vaccinate their sows and piglets against RV with these vaccines since there is little to no published data available. However, when outbreaks occur in the field, producers often

assume a new RV genotype is circulating, leading to questions of whether the current vaccines are still effective (Naseer *et al.*, 2017). Another concern with these vaccines is the possibility of the live-attenuated strains causing immune pressure, driving the emergence of new RV genotypes in swine.

Lorenzetti *et al.* (2011) described a recent field case from Brazil, where an outbreak of diarrhea occurred in suckling and weaned piglets in a sow herd that was regularly vaccinated with a commercially available vaccine (Porcilis 2*4*3*, MSD Animal Health) containing two RVA strains, OSU strain (G5P[7]) and Gottfried (G4P[6]). In Brazil, both humans and piglets are affected with RVA P[6], and their close interaction could potentially cause interspecies transmission. Fecal samples from this swine herd outbreak were characterized, and it was discovered that "the immunological pressure induced by the commercial vaccine with a rotavirus containing a G4P[6] genotype of porcine origin (Gottfried strain) might have allowed the selection of PoRV-A strains with characteristics found in RV-A strains isolated in human hosts". It suggests "the emergence of RV-A with a new lineage of the G4 genotype". This example highlights the importance of monitoring both swine and human RV strains simultaneously for changes in circulating RV strains in the field.

RV strains with different G and P genotypes have little to no cross-protection, and RV strains with the same G and P genotypes have limited cross-protective activity (Saif and Fernandez, 1996). In different parts of the world, different strains of RVA are predominant. For example, in Korea, RVA genotype combinations including G5P[7], G8P[7], G9P[7], G9P[23], and G8P[1] have been detected (Park *et al.*, 2019). In this case, ProSystems® RCE and ProSystems® Rota would not be efficacious against three of these RVA genotypes (specifically, G8P[7], G9P[23], and G8P[1]). These mismatched field strains have led Park *et al.* (2019) to the

development of an updated tri-valent live attenuated vaccine based on three of Korea's predominant RVA strains, G8P[7] 174-1, G9P[23] PRG942, and G5P[7] K71, isolated from the field. This newly developed live-attenuated vaccine was found to be safe and efficacious in piglets challenged with virulent, RVA homologous strains. However, this vaccine is not currently licensed for use in Korea.

Swine producers have often used autogenous vaccines to fill in voids when commercial vaccines are not available or when new swine diseases have emerged on a farm. Production of autogenous vaccines can occur when sample tissues are obtained from an infected piglet or RV is isolated in a viral culture and sent to a company to develop an autogenous vaccine (Arsenakis *et al*., 2018). These autogenous vaccine-producing companies produce vaccines that are typically killed or inactivated and are herd-strain specific. The advantage of this type of vaccine is that it is tailored to the producers' herd. One of the challenges associated with this type of vaccine is not all RV strains, like RVB and RVC, are easily isolated in cell culture. Another problem is these vaccines a herd-strain specific, meaning this vaccine might not protect a swine herd at another location.

In January 2014, Harrisvaccines, Inc., recently acquired by Merck Animal Health, received licensure by the USDA for RVC vaccines (Merck Animal Health, 2014). These vaccines are herd-specific and are trademarked using the Sequivity™ RNA particle (RP) technology. These herd-specific vaccines are helpful for diseases that continually evolve and mutate like swine influenza as well as for viruses that cannot be produced easily using traditional vaccine production methods like RVB and RVC. There are four major steps followed to make these RP vaccines according to a Merck Animal Health Sequivity™ RNA white paper (2020). First, a veterinarian takes a sample from an infected animal within a herd and sends it to a

veterinary diagnostic laboratory. Second, the veterinary laboratory identifies the pathogen strain genome sequence and sends the sequence to Merck Animal Health electronically. In the third step, the gene of interest (GOI) for the pathogen is synthesized and inserted into the RNA production platform. In the final step, the newly developed RNA production platform is incubated, and the RPs are released from the production cells. The RP's are then harvested, purified, and formulated into a swine herd-specific RP vaccine. The advantages of these vaccines are they are pathogen specific to the farm's herd, they are designed to only deliver the GOI from the pathogen to professional antigen presenting cells (APCs), known as dendritic cells, and the RPs are safe and do not replicate themselves. A disadvantage to this method is it strain-specific to the herd.

Complexity of RV Infections Within Swine Herds

In the field, swine herds can encounter different RV species and genotypes. It has not been known until recently that the complexity of RV infections exists within swine populations. The implementation of RT-qPCR in veterinary diagnostic laboratories has found that conventional pigs are often co-infected with different species of RV at one time. Molinari *et al.* (2016) described a real-life example of a post-weaning outbreak of diarrhea in a swine herd in Brazil found to be infected with RVA, RVB, RVC, and RVH. The sow herd in this study was an all-in-all-out swine operation and was regularly vaccinated with a commercially available inactivated RVA vaccine containing RVA OSU strain (G5P[7]), *E. coli*, and *Clostridium perfringens* Type C and D. Forty-six percent of the diarrhea samples were found to contain a single RV species, while the remaining fifty-six percent were found to be infected with a mixture of RV species through RT-PCR testing for each species. RVC was the most prominent species

detected in the diarrhea samples. This study highlights that perceived vaccine failures might be occurring due to mixed infections and not because the vaccine is losing efficacy.

As described in Chapter 2, the pathogenesis of RVB and RVC has been hampered due to complications in successfully isolating RVB and RVC in cell culture. This has led to difficulties in preparing live-attenuated vaccines against the various RV species. However, it has also created a knowledge gap in the prevalence of different RV species within swine herds and what age group is vulnerable to infection by different RV species. Since the implementation of RT-qPCR in veterinary diagnostic laboratories, researchers have been able to detect RVA, RVB, RVC, and RVH in fecal samples sent to these laboratories. Marthaler *et al.* (2014a) performed an investigation on 7,508 swine samples sent to the University of Minnesota Veterinary Diagnostic Laboratory between December 2009 and October 2011. The samples were categorized into five age groups consisting of neonatal pigs (1-3 days old), suckling pigs (4-21 days old), growing pigs (22-55 days old), finishing pigs (55 days and older), and unknown age. From this study, 6,251 or 84% of the samples were positive for either single RV infections (RVA, RVB, or RVC) or mixed RV infections (RVAB, RVAC, RVBC, and RVABC). The results from this study found that RVA was commonly seen in all age groups but was more prevalent in the 21-51 days of age group. RVB and RVC were both seen in all age groups; however, RVB was more prevalent in 55 days or older group, and RVC was more prevalent in 1-20 days of age group. This study is important because it highlights that different RV species are more prevalent at different times during piglet development.

Implementation of RT-qPCR and next generation sequencing in the field of diagnostics has allowed researchers to conduct comparison studies between historic and current RV strains. A recent study performed by Chepngeno *et al.* (2020) compared the historic porcine RVC

Cowden (G1) strain and two new porcine RVC strains, RV0140 (G3) and RV0143(G6), in three-day old and three-week-old gnotobiotic piglets. Based on their findings, it was determined that the two new PCV strains demonstrated different levels of virulence from each other and the historic Cowden strain. The two newer strains had relatively higher clinical disease and higher RVC fecal shedding compared to the historic Cowden strain in both three-day-old and three-week-old piglets. At three weeks of age, the Cowden strain caused milder clinical disease and pigs at this age did not develop any diarrhea. The two newer strains appear to have, as yet undefined, pathobiological characteristics that contribute to their increased prevalence in three-day-old to three-week-old piglets.

Creating a single cross-protective vaccine that is effective for different RV species can be difficult. Current methods of developing live-attenuated vaccines for RVA will not work for RVB and RVC unless a permissive cell line is found. However, newer vaccine strategies, like RNA particles, have helped bridge the gap for making effective RVB and RVC vaccines for herd-specific strains. Therefore, looking into newer alternative strategies for vaccine development may prove to be more successful than conventional vaccine development strategies.

Chapter 4- Potential Vaccine Applications for Rotavirus Vaccines

Rotavirus (RV) was first discovered in humans in 1973, and research in new vaccines to prevent this disease started in the 1980's (Desselberger *et al.*, 2009). Currently, there are two live-attenuated vaccines—RotaTeq® (Merck) and Rotarix® (GlaxoSmithKline)—licensed for use in humans against RVA in many developed and developing countries. In children, these vaccines have been found to be safe and efficacious when tested in developed countries. However, in less developed countries, where rotavirus is more prevalent, these two vaccines have been shown to be less effective due to high maternal antibody titers and malnutrition. One of the complications seen with these live attenuated oral vaccinations is intussusception or bowel obstruction in children after vaccination (Li *et al.*, 2018; Xue *et al.*, 2015).

In human research, the gnotobiotic pig model is used to evaluate potential vaccines candidates. Pigs have similar physiology to humans and their immune responses are similar as well. When challenged with human strains of RVA, these pigs have been shown to exhibit clinical signs such as diarrhea and viral shedding similar to infants (Wang *et al.*, 2016). By extension, much of the discovery and development of human RV vaccines can be starting points for new swine RV vaccines.

Subunit Vaccines

Many researchers are focusing on subunit vaccines for human RV to meet the need of more safe and efficacious vaccines. Two advantages of a subunit vaccine are that they pose no risk for reversion to virulence and they do not require cell culture (Shepard *et al.*, 2017). This technique is intriguing for human RV researchers since RVB and RVC serogroups are notoriously harder to grow in cell culture, which limits the ability of producing live attenuated vaccines for these serogroups.

Some of the subunit work is still in its early stages of development and being performed in mice. As described in Chapter 1, VP4 is the spike protein that, in the presence of trypsin, cleaves into VP5 and VP8. VP8 is an important protein that helps RV attach to the host cell and can elicit neutralizing antibodies. Xue *et al.* (2014) started working on developing a recombinant subunit vaccine using the VP8 protein for RVA in *E. coli* plasmids. Four different recombinant VP8 proteins were developed in this study. These included the full length VP8 (1-231 amino acids), VP8-1 (26-231 amino acids), VP-2 (51-231 amino acids), and ΔVP8 (65-231 amino acids). Each recombinant VP8 protein was formulated using Freund's adjuvant at a final concentration of 10μg of protein. Each of the four recombinant vaccines was administered subcutaneously three times in five- or six-week-old mice. Serum was collected for each vaccination time point at which time mice were mated 14 days after the last injections. The mice pups were challenged with 5 x 10^6 $TCID_{50}$ with lamb rotavirus strain LLR (G10P[12]) at six-to-eight days of age. It was found that the novel, truncated version of VP8-1, which contained the lectin head (amino acids 26-231), gave higher neutralizing antibodies and higher protection against homologous challenge in mice pups than the other three recombinant VP8 proteins. The novel, truncated VP8-1 protein from this study was also found to be highly protective and a candidate for future development as a possible vaccine candidate for human RV.

Other researchers have looked at the outer capsid, VP4, as a possible vaccine candidate for RV. The full-length VP4 protein contains 776 amino acids. In a study by Li *et al.* (2018), the researchers described a recombinant subunit vaccine using a novel truncated VP4 of RVA. In this study, a truncated version of VP4 (which contained both the VP8 and VP5 body and stalk domains, 26-476 amino acids) was expressed in an *E. coli* plasmid. When the *E. coli* plasmid was injected into mice with aluminum adjuvant, high virus-neutralizing antibodies were obtained

and found to reduce the severity of diarrhea when challenged with a homologous rotavirus. This vaccine is thought to be a viable candidate for future development in human RV.

Virus-Like Particles

Another vaccination strategy being investigated for RV in humans is the use of virus-like particles (VLPs). This vaccination strategy has proven to be successful for human papillomavirus and human hepatitis viruses B and E. VLPs are attractive to many researchers since they are considered safe, do not require the use of additional adjuvants, and some VLP formulations have been proven to be stable outside of the cold chain (Lua *et al.*, 2015). This is especially important, since the current modified live RV vaccines, RotaTeq® and Rotarix®, require the use of electricity to power refrigerated units or freezers (cold chain) to keep the vaccines efficacious and stable. In developing countries, especially in remote villages, electricity is not always available which is one explanation for why these vaccines may not be successful in preventing RV infections.

Previous work performed by Azevedo *et al.* (2013) with VLPs using human VP2/VP6 as the outer domain displayed on the VLP proved to be immunogenic when administered intranasally in gnotobiotic pigs; however, once challenged, it did not correlate with protection. An alternate vaccination scheme was tried to see if the VLPs would induce protection when a piglet was primed with a live attenuated human RV and then vaccinated with VP2/VP6 VLP intranasally. The newer vaccination scheme was found to be immunogenic and partial protection was seen when challenged with a human RV strain. This work paved the way for formulating VLPs against RV's two outer capsid proteins, VP4 and VP7. Another group of researchers (Lua *et al.* 2015) were able to design a VLP that displayed RV's VP8 (18.1 kDa) domain on the VLP

of a murine polyomavirus. More research will need to be performed to see if these newly formed VLPs are worth pursuing as potential vaccine candidates for humans.

Future Research

There are many avenues of future research that can be looked at for improved understanding of RV infections and developing better vaccines. A few areas of investigation include better defining host cell and host species interactions, understanding the pathogenesis of all RV species that infect swine, and future research efforts in antivirals.

Currently, there are many questions on how RV replicates within a cell. Much of our current understanding about RV replication comes from *in vitro* tests that are based on MA104 cell cultures since these cells allow propagation of RVA. One area of research that should be completed is how different host species bind to different host cell surface receptors. Knowing this information would help improve our current understanding of the zoonotic potential of emerging RV strains in swine.

More research needs to be conducted on the pathogenesis in swine. As described in Chapter 3, swine infections with RV tend to be complex. Many of these infections tend to have mixed species of RV, often in combination with other concurrent viral and/or bacterial infections. Currently, it is not known in mixed RV species infections which species of RV is inducing disease or how much each species contributes to a given infection. Understanding the pathogenesis of all five species of RV that infect swine will help researchers have a better understanding of which species should be identified as candidates for future RV swine vaccines.

If vaccines cannot be made for every RV species, then the development of antivirals should be considered. La Frazia *et al*. (2013) studied the antiviral mechanisms of "nitazoxanide (NTZ), a thiazolide anti-infective licensed in the United States for treating diarrhea caused by

Cryptosporidium parvum and *Giardia lamblia* in children and adults". This antiviral has been found to be "effective in reducing clinical symptoms associated with rotavirus". In this study, two RVA strains, a simian RVA/SA11-G3P[2] strain and human RVA strain Wa-G1P[8] were investigated and shown to inhibit RV replication, but this antiviral did not inhibit RV infectivity, binding, or entry of the host cell. It does cause an alteration and size reduction of the viroplasms (NSP2 and NSP5), which appears to decrease dsRNA formulation. Further work would need to be performed to see if a safe and effective dose of nitazoxanide would be available for use in treating swine RV infections. These treatments could help producers limit viral replication and shedding of RV particles in the environment as well as reduce economic losses associated with RV infections.

Chapter 5- Discussion and Conclusions

Rotavirus (RV) is a leading cause of diarrhea in young animals and in children under the age of five. Currently, there are a limited number of vaccines available to prevent the spread of some RVA genotypes in both humans and swine. Outbreaks of RVB and RVC have been documented in both species. In swine, a newer vaccine option, known as Sequivity™, has helped to bridge the gap for vaccinating against RVA, RVB, and RVC on a herd-based level against homologous RV strains. However, equivalent RVB and RVC vaccines are not available for humans.

In this report, Chapter 2 discussed the evolving field of swine diagnostics. Many of the methods used early on, including EM and PAGE, are time consuming and often not sensitive enough to differentiate the different species of RV. As the RV diagnostic field advanced, other diagnostic tests, including virus isolation, immunohistochemistry, and ELISA, have become more mainstream. Each of these methods has their own challenges associated with them. Some RV species do not have commercially available antibodies, and some RV species, aside from RVA, are not able to be routinely isolated in cell culture. The real "game changer" occurred in the field of RV diagnostics when molecular tests, including RT-PCR and RT-qPCR, were developed for use in swine. This technology is rapid, sensitive, and specific to different species of RV. It is also being used to further our understanding of the epidemiology related to RV infections in the field.

The complexity of swine RV infections was discussed in Chapter 3. The use of molecular tests, such as RT-qPCR, has helped researchers investigate field samples collected over the years in veterinary diagnostic laboratories. From these samples, researchers have found RV infections can be caused by a single RV species, but more commonly, they occur with

multiple RV species. They have also allowed researchers to compare ages of pigs that are commonly infected with different RV species. Next generation sequencing allows researchers to compare historic RV strains with current RV strains in the field to help better understand the changes seen in the whole RV genome.

Chapter 3 of this report also described the challenges associated with developing swine RV vaccines. It is thought that the current RVA vaccines in use are causing immune pressure for newer genotypes emerging in the field. RVB and RVC have had limited success growing in cell culture; however, newer technologies, like Sequivity™, are helping swine producers fill in this absence of vaccine availability. Other problems occur when RV strains with different G and P genotypes provide little to no cross-protection, and even RV strains with the same G and P genotypes provide limited cross-protection.

Finally, in Chapter 4, different vaccines strategies used in other host species were discussed as possible vaccine strategies for use in swine RV vaccines. Two of the most promising avenues used in human RV vaccines are subunit vaccines and virus-like particles. Most of these vaccine strategies are using the outer capsid viral proteins, VP4 and its cleavage product, VP8. In the same chapter, future work, such as a better understanding of RV replication, RV pathogenesis, and the development of antivirals for use in swine treatment, were also discussed.

In conclusion, RV infections are a cause of diarrhea in young piglets as well as economic losses to livestock producers. It is well documented that interspecies transmission of RVs and gene reassortment events between human and pig RVs can occur. This report explored swine RV diagnostic testing, the complexity of swine RV infections, the difficulties of creating cross-

protective vaccines for swine, and the potential vaccine applications from other species affected by RV for development of new swine RV vaccines.

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