Ecology of Porcine Rotaviruses

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General Introduction

Rotaviruses (RVs) cause severe diarrhea in young animals including pigs and humans. Porcine RV infections cause severe economic losses for swine farmers. Since RV infections can be symptomatic or asymptomatic in pigs, economic losses are difficult to estimate. Recent studies are lacking to estimate the economic loss to farmers partly due to the historical lack of proper RV diagnostic assay, making any previous estimates inaccurate.

While the severity of disease can greatly differ due to many variables, RV morbidity and mortality are highest in piglets (<21 days of age) (Estes and Greenberg, 2013). The high mortality in piglets is due to many factors such as the greater size of the small intestine in proportion to overall body size, the destruction of the enterocytes during RV infection, and the inability to quickly restore intestinal function and structure. In addition, the piglet has an immature immune system and must rely on passive immunity from the sow to protect against a RV infection. As pigs reach 21 days and older, their ability to recover from a RV infection, and the immune response to a RV increases and mortality decreases.

Five RV species have been identified in swine: rotavirus A, B, C, E, and H (RVA, RVB, RVC, RVE, and RVH, respectively) (Estes and Greenberg, 2013). Rotavirus A (RVA) has commonly been identified as an important cause of diarrhea in pigs and numerous porcine RVA studies have been conducted, illustrating the importance of RVA in swineherds (Miyazaki et al. 2011; Amimo et al. 2013a; Miyazaki et al. 2013; Hong Anh et al. 2014; Li et al. 2014; Monini et al. 2014). However, research on RVB, RVC, RVE and RVH is only in its infancy.

RV epidemiology and molecular diversity depends on viral, host and environmental factors. Understanding the occurrence of RVA, RVB, RVC, and RVH within the pig population will allow us to ascertain predominant RV species in specific swine age groups. While several studies have investigated the occurrence and molecular diversity of RVA in swine, there is limited data available on the occurrence and molecular diversity

of RVB, RVC, and RVH. To prevent and control of RV infections in swineherds and estimate the economic losses associated with RV infections, more research is needed on RVB, RVC, RVE, and RVH, including the development and validation of new diagnostic tools to identify RV in an efficient and timely matter.

In conclusion, swine RVA has been known as the most important cause of diarrhea in swine, but the information on swine RVB, RVC, and RVH is severely lacking. Understanding the ecology of swine RV species is important to help prevent and control RV infections in swineherds. This thesis will 1) establish swine RVB as an important cause of diarrhea and assess the genetic diversity of the VP7 and VP6 gene segments, 2) develop real time RT-PCR assay to estimate the occurrence of RVA, RVB, and RVC in porcine samples, 3) assess the VP7 genetic diversity of porcine RVC strains, and 4) identify and estimate the emergence of RVH in the US swine population.

Chapter 1: Literature Review

Rotavirus Taxonomy (classification)

RVs belong to the *Reoviridae* family and are non-enveloped, icosahedral virion of approximately 100 nm in diameter including the spikes proteins. RVs from different serogroups or species share several similar features, such as i) a distinct morphologic appearance (wheel-like) by negative-stain electron microscopy, ii) biochemical properties, and iii) a genome consisting of 11 segments of double-stranded (ds)RNA, each coding a distinct viral structural protein (VP) or non-structural protein (NSP) (Estes and Greenberg, 2013). Traditionally, RV group classification was based on antigenic properties of the inner viral capsid protein 6 (VP6) and distinct dsRNA pattern on polyacrylamide gels (electropherotypes) (Both et al. 1994; Estes and Greenberg, 2013). Nowadays, RV group classification is based on amino acid sequence identity of VP6 (Matthijnssens et al. 2012). The International Committee on Taxonomy of Viruses (ICTV) currently recognizes 5 distinct groups or species (RVA, RVB, RVC, RVD, RVE), two tentative species (RVF, RVG), and an unassigned species (ADRV-N), now referred to RVH (Guglielmi et al. 2011; Matthijnssens et al. 2012).

History of discovery of porcine rotaviruses

In 1969, a diarrhea causing, reovirus-like particle in calves was first discovered (Mebus et al. 1969), which was able to establish an infection in piglets (Bridger and Woode, 1975; Woode and Bridger, 1975). This reovirus-like particle was later named as a duovirus or commonly known today as the Nebraska calf diarrhea virus (NCDV) rotavirus strain (Davidson et al. 1975; Woode et al. 1976). In addition, a swineherd was serologically positive to the NCDV RV strain, thus identifying natural RV infections and isolation of swine RVs from pigs (Woode and Bridger, 1975; Woode et al. 1976).

RVA

RVAs have been considered the most pathogenic and genetically diverse of all RV species infecting all animals and were first identified in pigs in 1975 (Martella et al. 2010; Matthijnssens et al. 2010b; Estes and Greenberg, 2013) While most RVA strains can be adapted to grow in cell culture, for some RVA strains such attempts have been

unsuccessful. Since adaption to cell culture may be difficult and time consuming, many porcine RVA genomes have been sequenced directly from the stool sample to identify genetic differences among porcine RVA strains.

Before the development of the genotyping methodology, serotyping was used to classify RVA strains (Estes and Greenberg, 2013). The serotyping involved the outer two outer capsid proteins, VP7 and VP4, defining the G and P serotypes, respectively. Serotyping utilized plaque reduction neutralization assays or fluorescent focus neutralization assays (Estes and Greenberg, 2013). Due to the ease of sequencing, serotyping is not routinely performed anymore.

RVB

Unlike porcine RVAs, porcine RVB strains were not identified until the 1980s (Bridger and Brown, 1985; Theil et al. 1985). While RVBs were identified 5 years after porcine RVAs, and have been described in cattle, lambs, rats, and humans, detailed RVB serological and molecular characterization is limited (Eiden et al. 1992; Chang et al. 1997; Tsunemitsu et al. 1999; Shen et al. 1999; Barman et al. 2004; Ghosh et al. 2007) The lack of RVB serological and molecular characterization may be due to the difficulty in propagating RVB in cell culture, which has also hindered RVB vaccine development(Bridger. 1994; Sanekata et al. 1996). Historically, swine RVB prevalence has been considered low.

RVC

Unlike the discovery of RVA, RVC (Cowden strain) was first identified in a piglet with diarrhea (Saif et al. 1980). Like RVB, RVC was identified in 1980, but limited RVC research continued throughout the subsequent years (Saif et al. 1980). Similarly to RVB, RVC is difficult to propagate in cell culture, lacks a proper serological typing and a RVC vaccine has yet to be developed (Saif et al. 1988; Tsunemitsu et al. 1991; Fujii et al. 2000). In addition to pigs, RVC causes gastroenteritis in cattle, ferrets, and humans (Rodger et al. 1982; Torres-Medina. 1987; Tsunemitsu et al. 1991; Chang et al. 1999;

Otto et al. 1999; Mawatari et al. 2004; Rahman et al. 2005; Banyai et al. 2006; Castello et al. 2009; Mitui et al. 2009; Araujo et al. 2011). Swine RVC can infect both the small and large intestine, infections can be subclinical or cause severe gastroenteritis, and cases can be either sporadic or in large outbreaks (Saif et al. 1980; Bohl et al. 1982). Many swine enteric pathogen studies fail to screen for RVC (Kongsted et al. 2013; Chan et al. 2013). Recently, multiple studies have identified RVC infections as important causes of diarrhea in swine (Moutelikova et al. 2013; Amimo et al. 2013b).

RVE

Limited information is available on porcine RVE and RVH (Bohl et al. 1982; Chasey et al. 1986; Janke et al. 1990; Wakuda et al. 2011). A single porcine RVE was described in 1985 based on viral double stranded RNA migration pattern in a polyacrylamide gel and failure to produce immunofluorescence antigen to convalescent antisera to RVA, RVB and RVC (Chasey et al. 1986). Subsequent RVE identifications have not been observed, which could be due to lack of a proper diagnostic assay specific for RVE. In addition, there is no dsRNA sequence information, which calls into question the classification and origin of the strain. Unfortunately, the original strain is no longer available for further analysis.

RVH

A single Japanese porcine RVH strain (SKA-1) from 1999 was identified based on sequencing data of the VP7, VP4, VP6, and NSP4 gene segments (Wakuda et al. 2011) The swine RVH VP6 nucleotide percent identity had a higher similarity to the VP6 of a novel human RVH strains J19 and B219 than to the swine RVB strains (Wakuda et al. 2011). In 2012, RVH VP6 sequences from 3 Brazilian porcine strains (BR63, BR60, and BR59) became available in GenBank (accession numbers KF021621, KF021620, and KF021619). Due to the recent discovery of RVH, detailed epidemiological, serological, and molecular characterization are warranted.

Genotypes identified in each RV species

In 2008, an RVA classification system was proposed based on nucleotide percent identity cut-off values distinguishing genotypes for each of the 11 gene segments (Matthijnssens et al. 2008a). In addition, the Rotavirus Classification Working Group (RCWG) was subsequently formed and is composed of worldwide RV researches to maintain the RVA classification system (Matthijnssens et al. 2008b). The classification systems nomenclature of Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx correlates to the RVA gene segments VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6, respectively. New proposed RVA genotypes are submitted to the RCWG for genotype assignment. The new classification allows RV researchers to identify novel genotypes within different host species. Commonly, a dual classification system (GxP[x]) is used to describe RVA strains. Worldwide, the predominant human RVA genotypes are G1P[8], G3P[8], G4P[8], G9P[8] (Wa-like) and G2P[4] (DS-1-like) found in combination with two human RVA constellations: I1-R1-C1-M1-A1-N1-T1-E1-H1 (Wa-like) and I2-R2-C2-M2-A2-N2-T2-E2-H2 (DS-1-like). Human Wa-like RVA strains and porcine RVA strains share a common origin, while the DS-1-like strains share a common origin with bovine RVA strains (Matthijnssens and Van Ranst, 2012). The third, minor human RVA genotype constellation is referred to as AU-1-like (I3-R3-C3-M3-A3-N3-T3-E3-H3), which share a common origin with cat or dog rotaviruses (Nakagomi and Nakagomi, 1989). Porcine RVA with the following G genotypes have been identified in pigs: G1, G2, G3, G4, G5, G6, G8, G9, G10, G11, and G26, while the P genotypes include P[1], P[3], P[5], P[6], P[7], P[8], P[13], P[19], P[23], P[26], P[27], P[32] and P[34] (Matthijnssens et al. 2011; Martel-Paradis et al. 2013; Monini et al. 2014; Hong Anh et al. 2014).

While the G and P genotypes from many porcine RVA strains have been sequenced, a limited number of complete genomes of porcine strains were initially available, with most of these strains having been isolated more than 30 years ago in the US (n=2), Mexico (n=1), or Venezuela (n=2). However, due to the ease of sequencing, many porcine complete RVA genomes are currently being sequenced (Martel-Paradis et al. 2013; Monini et al. 2014; Hong Anh et al. 2014). The porcine internal gene

constellation is I1-R1-C1-M1-A1-N1-T1-E1-H1 gene, which is the same as the human Wa-like genotype constellation. However, a number of porcine specific genotypes, such as I5, A8, and T7, are also readily found in swine (Matthijnssens and Van Ranst, 2012).

Since RVA is the most prevalent, more genomic data are available for porcine RVA than any of the non-RVA species. In 2009, the first swine RVB VP7 sequences were generated from Japan (Kuga et al. 2009). In 2012, 68 US RVB VP7 sequences were generated and in conjugation with previously published rat, human, bovine, and porcine RVB VP7 sequence (n=57), an 80% nucleotide cut-off value was proposed, generating 20 RVB G genotypes. Thus far, the Japanese swine RVB NSP1, NSP2, NSP5 gene segments have been sequenced while only American and Japanese VP6 sequences have been generated, suggesting a large undiscovered genetic diversity within porcine RVB strains (Suzuki et al. 2011; Suzuki et al. 2012a; Suzuki et al. 2012b). Further, complete genome sequencing of RVB strains is still needed to confirm potential reassortment events and the existence of a common porcine RVB gene constellation.

The porcine RVC Cowden strain has been completely sequenced yielding a G1-P[1]-I1-R1-C1-M1-A1-N1-T1-E1-H1 gene constellation (Soma et al. 2013). In 2007, with the generation of additional porcine RVC VP7 sequences, an amino acid percent cut-off classification was proposed, yielding porcine G genotypes G1, G3, G5, G6 and G1/4, while the human and bovine G genotypes were G4 and G2, respectively (Martella et al. 2007b).

Because the RVE sequence data are not available, no genotype information is known. While the complete genome sequence data are absent for porcine RVH strains, the glycosylated, protease-sensitive, inner capsid, and enterotoxin gene sequences (VP7, VP4, VP6, and NSP4, respectively) have been generated for the Japanese strain SKA-1 (Wakuda et al. 2011). Despite the additional availability of 3 Brazilian no classification system had been proposed, although a single porcine VP6 (I) genotype exists.

Rotavirus structure

RVs have a distinct "wheel like" structure by electron microscopy from which RV species received their name (*rota* means wheel in latin). The RV infectious particle contains a triple protein layered structure. The core consists of the viral protein 2 (VP2), which forms the innermost viral layer. The intermediate layer is composed of the VP6 while the outer layer consists of the glycoprotein VP7 and the cell attachment protein VP4 (Estes and Greenberg, 2013). Triple layered RV particles are required to retain infectivity, as the single (VP2) and double-layered (VP2 and VP6) particles are not infectious.

Rotavirus genes and proteins

The 11 dsRNA codes for six structural proteins (VP1-VP4, VP6, and VP7) and five or six non-structural proteins (NSP1-NSP6). The positive sense RNA contains 5' guanidine followed by a conserved set of nucleotides in the untranslated region (UTR), the open reading frame (ORF), and then another UTR ending with two cytidines at the 3' end. The largest gene segment, the VP1 with ~3.3 Kb encodes the RNA-dependent RNA polymerase (RdRp). VP2 is the innermost viral protein and is encoded by a gene segment slightly longer than the VP3 encoding gene segment, which acts as a Guanylytransferase and Methyltransferase. The VP6 encodes for the inner core protein. The VP4 and VP7 form the outer capsid of the RV virion and are used for viral attachment, which in turn stimulates neutralizing antibodies and are important in generating protective immunity. The nonstructural rotavirus genes have various functions in the replication cycle of the virus, viral morphogenesis and immune evasion. The NSP1 gene segment blocks the induction of interferon from infected cells. The NSP2 and NSP5 are the main building blocks for the viroplasm within an infected cell. The NSP3 binds to viral ssRNA to increase translation of viral RNA. The NSP4 is an enterotoxin, which modulates intracellular calcium levels. RVs are one of the few viruses, which produce an enterotoxin (Estes and Greenberg, 2013).

Epidemiology and Evolution

Investigating the RVA, RVB, and RVC occurrence within the pig population will allow us to ascertain predominant RV species in each age group. However, several studies have investigated only the porcine RVA prevalence and molecular diversity, with very limited RVB and RVC epidemiologic data being available (Miyazaki et al. 2011; Miyazaki et al. 2013; Martel-Paradis et al. 2013; Amimo et al. 2013a; Monini et al. 2014; Hong Anh et al. 2014). In addition, the porcine RVB and RVC genetic diversity is largely unknown. Studying the molecular diversity of RVB and RVC will increase our knowledge on strain diversity, which may impact the on success or failure of veterinary vaccines.

The porcine RV strain genetic diversity is evident, and point mutations or antigenic drift, in particular in the antigenic domains, contribute to the RV genetic diversity(Ghosh et al. 2007). All the contributing factors of antigenic drift are not known, but many of the point mutations can be contributed to the RNA-dependent RNA polymerase, which lacks proofreading mechanism, allowing for single polymerase nucleotide changes in the RNA(Estes and Kapikian, 2007). The average mutation rate for the RVA genome is estimated to be 5×10^{-5} *in vitro*, equating to 1 mutation per viral replication cycle (Blackhall et al. 1996). However, this *in vitro* mutation rate neglects to include host immune pressures, which can increase the mutation rate. Continued research suggested a RVA VP7 estimated nutation rate of $1.66\text{-}1.87 \times 10^{-3}$, which is higher than the *in vitro* mutation rate (Matthijnssens et al. 2010a)

In addition, RV reassortment events, RNA segments exchanged between different RV strains during dual infection within a host, may lead to the introduction of new RV gene segments into a naïve host population. While parental viral strains, host factors, and environmental factors all influence viral progeny, many RVA molecular epidemiologic studies have revealed reassortment events between RVA strains different animal species (Santos and Hoshino, 2005; Matthijnssens et al. 2008a; Matthijnssens et al. 2009; Martella et al. 2010; Yamamoto et al. 2011). Human Wa-like RVA strains and porcine RVA strains share a common origin. The interspecies transmission of RVA Wa-like gene constellation in human and swine is strengthened with the current partial genotyping data

of the common RVA G3, G4, G5, G9, and G11 genotypes circulating among swine (Miyazaki et al. 2011). While the frequency of interspecies transmission events is difficult to estimate for RVA strains, the limited complete genome genetic diversity makes identifying interspecies events difficult for RVB, RVC, and RVH.

RV rearrangement events occur when RNA fragments are concatenated, partially duplicated or partially deleted, yielding an increase or decrease in the gene segment length (Desselberger. 1996; Taniguchi et al. 1996). RV rearrangements have been documented *in vitro* and *in vivo* (Desselberger. 1996; Gault et al. 2001; Alam et al. 2008). While the precise rearrangement mechanisms are unknown, RNA template switching, which can occur between gene segments of the same virus, or between gene segments from distinct viruses in case of a during dual RV infections, and the loop model, results in partial head-to-tail duplications, have been proposed (Matthijnssens et al. 2006). RV intragenic recombination of the VP7 from different RV strains has been reported (Martella et al. 2007b). Portions of different RVA G genotypes or lineage have been found in a single RVA VP7 sequence while a porcine RVC VP7 strain contained both human and porcine VP7 fragments (Parra et al. 2004; Phan et al. 2007; Martella et al. 2007a; Martinez-Laso et al. 2009).

Pathogenesis and clinical signs

RV infects the mature enterocytes of the small intestine near the tip of the villi and causes clinical signs of diarrhea, fever, vomiting, decreased food intake, and weight loss. After RV infection, clinical signs of diarrhea occur before villus atrophy will occur. The diarrhea is believed to occur due to the enterotoxin produced by NSP4, which disrupts the tight junctions and secrets chloride from the enterocytes. Villus atrophy, blunting, inflammation, mononuclear infiltration of the lamina propria, and vacuolation of the enterocytes occurs between 24-72 hours post infection. Piglets are born with mature enterocytes, and the destruction of the villus decreases absorption of the nutrients and water, leading to dehydration and death in piglets. In older pigs, the villus regenerate and functions return to normal. In addition, RV infections are segmental, meaning villus

damage may not occur through the entire small intestine. It has been documented that RVA infections can lead to viremia and replication can occur in extra intestinal organs (Fragoso et al. 1986; Azevedo et al. 2005; Crawford et al. 2006; Kim et al. 2011).

Porcine RVA, RVB, and RVC all cause microscopic lesions and these have been well documented. It is important to note that the lesion severity can vary by RV species. Additional factors, such as RV strain, host immune status, and age of the host can contribute to disease severity. Since disease severity is difficult to access, diarrhea in a piglet is different from that of an older pig.

Transmission routes

The main RV transmission route in pigs is fecal-oral route, but aerosol transmission and contact with contaminated material are possible routes of infection (Estes and Greenberg, 2013). Large quantities of RV virions (10^{10-12}) are shed in one gram of feces and are resistance to physical inactivation. While RV infections in swine have an incubation period of approximately 5-7 days, longer incubation periods and viral shedding periods have been reported (Estes and Greenberg, 2013). Since RV infection can be asymptomatic, older pigs can serve as a source of RV infection. Introduction of asymptomatic infected animals, and pig movement within and between herds can increase the risk of infection in swine populations. Lack of complete long-term RV immunity in sows may play a key role in the transmission of RV strains to newborn piglets (Hoshino et al. 1988).

Porcine rotavirus vaccines

Swine RV prevention and control methods include vaccination and feedback. Feedback is the practice of taking feces or intestinal content from diarrheic pigs and feeding it back to sows. Prior to farrowing, sows are vaccinated or given feedback to enhance production of maternal antibodies. Focused and intensive RVA research continued throughout the years, resulting in the marketing of a porcine RVA vaccine (ProSystem RCE, Intervet/Merck Animal Health). The ProSystem RCE vaccine contains 3 porcine

RVA strains: OSU (G5P[7]), Gottfried (G4P[6]), and A2 (G9P[7]), which are used to stimulate maternal immunity when given to sows prior to farrrowing (Saif &Fernández, 1996). However, success rates of vaccine and feedback techniques vary for three main reasons: First, while the RVA vaccination rates are unknown in the United States, numerous studies have indicated vaccine failure, which could occur by a variety of factors including management of the pigs and antigenic differences between vaccine and field strains (Hoshino et al. 1988; Papp et al. 2013). Depending on the strain homology between circulating viruses and the vaccine, RV infection can still occur but viral shedding in reduced (Hoshino et al. 1988; Yuan et al. 2004). Second, pig producers cannot vaccinate against RVB and RVC because there are no vaccines for these RV species. While RVB and RVC subunit and autogenous vaccines are available, depending on strain homology, complete protection may not occur. Third, the feedback process is difficult to master and maintain. Without proper diagnostic assays to identify the RV concentration of the feedback, maternal antibodies may not be stimulated properly. Third, feedback material requires great care to insure RV viability to stimulate the maternal antibodies.

Detection methods

A variety of detection methods exist for the diagnoses RV infections. Since both bacterial and viral pathogens can cause clinical signs in pigs, diarrhea alone does not indicate a RV infection and RV infections in older pigs can be asymptomatic. RV isolation in cell culture is possible, but not used as a routine diagnostic assay. Stool or intestinal homogenates are added to African green monkey cell culture (MA104) and are passed a series of times (Estes and Greenberg, 2013). If the cells exhibit cytopathic effect (CPE), destruction of the cellular monolayer has occurred, indicating RV is growing in the cell culture. RV isolation is not a useful diagnostic assay for numerous reasons. While RVB, RVC, and RVH have been reported to be adapted to cell culture, only RVA can be routinely be isolated in cell culture. Furthermore, cell culture techniques are very time consuming and labor intensive. In addition, MA104 cell line can support the growth of a variety of swine pathogens, including Porcine Reproductive

and Respiratory Syndrome Virus, and a positive CPE does not indicate a positive RV result.

Electron microscopy (EM) was first used to diagnose a RV infection (Estes and Greenberg, 2013). The EM assay utilizes the RV morphology, the "wheel-like" structure, to identify an infection. A benefit of EM is the ability to detect all RV species. However, this strength is a limitation because it doesn't discriminate the different RV species to implement appropriate control and prevention strategies in pigs. With a detection limit of 10^5 to 10^6 RV particles per milliliter, EM lacks the sensitivity to detect a RV infection. In addition, EM is very time consuming and labor intensive.

Fluorescent Focus Assay (FFA) and Immune Electron Microscopy (IEM) utilize antibodies to detect RVA particles. However, antibodies to RVB and RVC are not routinely available. In addition, FFA requires the adaption of RV particles to cell culture, which is difficult with some RVA and non-RVA strains.

Enzyme-linked immunosorbent assay (ELISA) is routinely used to diagnose RVA infection in humans (Estes and Greenberg, 2013). The technique is relatively basic and simple. The plate is coated with antibodies against RVA, targeting the VP6 protein. The sample is added to the plate, and the antigen, if present in the sample, binds to the coating antibodies. The sample is washed away while the antigen stays bound to the coating antibodies. Then, the RV antigen is detected by adding an enzyme-conjugated anti-RV antibody, also known as a direct test. Alternatively, a secondary antibody to detect RV particle is added and as enzyme-conjugated detecting the secondary antibody is applied, known as an indirect test. Finally, a chromogenic substance is added, which reacts with the enzyme-conjugated antibody, resulting in a color formation, if the sample is positive while no reaction will occur if RV is absent. The reactions are read and recorded on a commercial ELISA plate reader. The ELISA assay is a fast and reliable method to detect RVA antigens infection. A commercial ELISA assay is available to detect anti-RVA antibodies in human serum. However, a commercial ELISA assays to detect anti-RVA

antibodies in swine is not available. Since the human and porcine RVA VP6 gene segments code for slightly different proteins, the commercial human ELISA kit cannot be used to diagnose porcine RVA infections and will not identify RVB, RVC, or RVH infections. While ELISA assays can indicate exposure to RV infection by the detection host RV specific antibodies, commercial porcine RVA-RVB and RVH ELISA are not available, but would be beneficial and are needed to access material immunity to RVs.

Multiple molecular diagnostic assays are available to detect RV infections. Polyacrylamide gel electrophoresis (PAGE) is used to analyze the 11 dsRNA genomic segment migration pattern (Estes and Greenberg, 2013). While a specific dsRNA pattern is recognized for each species, variation within each RV species is large and infections with multiple RV species can lead to inaccurate results. In addition, PAGE lacks sensitivity and is time consuming.

RNA hybridization assays bind the RV RNA to a nitrocellulose membrane, to be hybridized to a label probe of another RV, which can be beneficial to compare sequence similarity between strains. However, the process is time consuming, and sequencing of RV strains has replaced this technique in diagnostic laboratories. Restriction fragment length polymorphism (RFLP) is not routinely used in diagnostic laboratories. The RV dsRNA is converted to cDNA, which is digested by bacterial restriction enzymes to create a characteristic banding pattern. RFLP can be time consuming and has been replaced by gene segment sequencing due to the advances and ease of sequencing.

Reverse transcription-polymerase chain reaction (RT-PCR), is now widely used to detect RV infections. The RV dsRNA is extracted from the fecal or intestinal homogenate, amplified using RV specific primers, and then, the RT-PCR product is visualized on an agarose gel, which is referred to as conventional RT-PCR. Real time RT-PCR is faster than conventional RT-PCR due to the addition of a hydrolysis probe. The hydrolysis probe contains a fluorescent dye, which is released during the amplification of the RV RNA. A real time thermal cycler records the fluorescent signal

and hence the RV amplification, which generates a sigmoidal curve. Real time RT-PCR is extremely fast and accurate. In addition, by using specific primers and probes with different fluorescent dyes, multiple RV species can be detected within a single reaction. Since real time RT-PCR is extremely sensitive, precautionary steps must be taken to reduce cross contamination and false positives. Also, if single nucleotide polymorphisms (SNPs) occur in the dsRNA corresponding to the primer and probe sequences, the real time RT-PCR will not detect the RV dsRNA. The real time RT-PCR must be designed to recognize a conserved region of the RV genome to ensure accurate detection.

Conclusions

In conclusion, RVs are a significant cause of diarrhea in pigs, especially in young piglets. While porcine RVA is considered the most prevalent and pathogenic, porcine RVB and RVC are also important causes of diarrhea. The pathogenicity of RVH in pigs still needs to be confirmed. While RVA vaccination rates and efficacy are unknown in the United States. Currently, porcine RVB, RVC, and RVH vaccines are unavailable but are necessary to limit RV gastrointestinal disease in piglets. Due to the lack of proper RV serological detection assays, RT-PCR is the most robust, sensitive, and specific assay to detect porcine RV species. Understanding the pathogenicity, prevalence, and genetic diversity of porcine RVs is important for increasing pig health, sustaining profitable pork production, and advancing enteric virus research globally.

Chapter 2: Detection of Substantial Porcine Rotavirus B Genetic Diversity in the United States, resulting in a modified classification proposal for G genotypes

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Marthaler D, Rossow K, Gramer M, Collins J, Goyal S, Tsunemitsu H, Kuga K, Suzuki T, Ciarlet M, Matthijnssens J. Detection of substantial porcine group B rotavirus genetic diversity in the United States, resulting in a modified classification proposal for G genotypes. Virology. 2012 Nov 10;433(1):85-96. doi: 10.1016/j.virol.2012.07.006. Epub 2012 Aug 9.

Introduction

Rotaviruses (RVs) are a major etiological agent of acute viral gastroenteritis in young animals and children worldwide. RVs belong to the *Reoviridae* family, possess a genome composed of 11 segments of double-stranded RNA (dsRNA) and are currently classified into eight groups (A-H) based on antigenic properties and sequence based classification of the inner viral capsid protein 6 (VP6) (Both et al., 1994, Estes M, 2007, Matthijnssens et al., 2012, Trojnar et al., 2009). RVA is considered the most important RV group because of its high prevalence and pathogenicity in both humans and a variety of domestic and wild animals (Estes M, 2007, Martella et al., 2010, Matthijnssens et al., 2010). While human RVA and RVC have been described around the world, current reports indicate that human RVB strains have been described only in China (Chen et al., 1990, Dai et al., 1987, Fang et al., 1989, Hung et al., 1984), India (Kelkar and Zade, 2004, Lahon and Chitambar, 2011), and Bangladesh (Ahmed et al., 2004, Rahman et al., 2007, Saiada et al., 2011, Sanekata et al., 2003). While porcine RVB were first identified in the 1980s (Bridger and Brown, 1985, Theil et al., 1985) four other rotavirus groups, RVA, RVC, RVE, and RVH have also been described in pigs (Chasey et al., 1986, Janke et al., 1990, Wakuda et al., 2011). In addition to pigs, RVB strains have been also detected in cattle (Barman et al., 2004, Chang et al., 1997, Ghosh et al., 2007, Tsunemitsu et al., 1999), lambs (Shen et al., 1999), and rats (Eiden et al., 1992). No genetic information exists for porcine RVE strains, only one paper has reported swine RVH (Wakuda et al., 2011), and a limited number of RVC strains have been published (Collins et al., 2008, Lee et al., 2011, Martella et al., 2007a, Tsunemitsu et al., 1996).

RVA have been well characterized and hundreds of complete genomes have been sequenced. In 2008, a sequenced-based classification system was proposed for RVA strains, and for each of the 11 gene segments, a nucleotide percent identity cut-off value was determined to discriminate between genotypes based on the phylogenetic analyses and pairwise sequence identity profiles (Matthijnssens et al., 2008a). Subsequently, a Rotavirus Classification Working Group (RCWG) was formed to set the RVA classification guidelines and maintain the proposed classification system (Matthijnssens

et al., 2008b). To avoid misclassification of RVA strains not belonging to any of the established RVA genotypes, new sequences are submitted to the RCWG. The correct genotype is assigned, or a novel genotype is created. Currently, only RVA classification has been developed and maintained by the RCWG. Several other viruses such as astroviruses (Noel et al., 1995), sapoviruses (Schuffenecker et al., 2001), noroviruses (Zheng et al., 2006), hantavirus (Maes et al., 2009), and papillomaviruses (de Villiers et al., 2004) have been successfully classified using similar methodology.

Serological and molecular characterization of RVB strains is limited due to the difficulty of adapting RVB strains to cell culture (Bridger, 1994, Sanekata et al., 1996). Complete genome sequencing has been performed on several human RVB strains from Southeast Asia (Ahmed et al., 2004, Kobayashi et al., 2001, Yamamoto et al., 2010, Yang et al., 2004). A limited number of RVB gene segments from a rat and bovine have also been sequenced (Barman et al., 2004, Chang et al., 1997, Eiden et al., 1992, Ghosh et al., 2007, Kelkar and Zade, 2004, Petric et al., 1991, Rahman et al., 2007, Tsunemitsu et al., 1999). Due to the increased ease of sequencing, Kuga and colleagues (2009) sequenced the VP7 of 38 porcine RVB strains and constructed phylogenetic trees and pairwise identity frequency graphs for G genotype classification purposes (Kuga et al., 2009). Based on their analyses, they proposed 5 genotypes which were further divided into 12 clusters, using 67% and 76% nucleotide cut-off values, respectively (66% and 79%, respectively, on the amino acid level) (Kuga et al., 2009).

In order to better understand the role of RV in porcine gastroenteritis cases, stool samples and small intestines from pigs of different ages were tested for RVA, RVB, and RVC as well as common pathogens associated with porcine diarrhea. The VP7 open reading frame (ORF) for 68 swine RVB samples in the United States was determined, revealing a substantial genetic diversity of porcine RVB strains. The genotype classification cut-off values as proposed by Kuga and colleagues (Kuga et al., 2009) were modified after including the novel sequence data generated in this study. Based on phylogenetic and molecular evolutionary analyses, a modified classification system is

proposed currently describing 20 RVB VP7 genotypes based on the nucleotide cut-off value of 80%.

Materials and Methods

Origin of samples

Animal intestinal samples, with a history or current outbreak of diarrhea, are routinely submitted to the University of Minnesota Veterinary Diagnostic Laboratory for determination of etiological agents of gastrointestinal disease. Clinical signs of gastrointestinal disease are most commonly weight-loss and diarrhea. Between the months of August and September 2009, 173 porcine samples (119 different sites) from 14 US states were submitted for diagnostic investigation. Porcine samples from all age groups are routinely screened for *Salmonella enterica spp.*, TGEV, RVA, RVB, and RVC. Screening for other pathogens in addition to the former is dependent on the age of the pig: <10 days, *Escherichia coli*, *Clostridium difficile* and *C. perfringens*; 11 to 55 days, *E. coli*, coccidia; >55 days, *Lawsonia intracellularis* and *Brachyspira species*.

Electron microscopy

Ammonium molybdate (1%) prepared in water (pH = 5.5) was used to perform negative stain electron microscopy to visualize RV particles in samples.

Histopathology

Small intestine was fixed in 10% neutral buffered formalin, routinely processed, paraffin embedded, and stained using Harris's hematoxylin and eosin (HE).

Extraction of genomic material, reverse transcriptase-polymerase chain reaction (RT-PCR) amplification, and sequence analysis

Approximately, 3 grams of each sample was homogenized with 2 mL of gamma-irradiated Hyclone donor equine serum (Thermo Fisher Scientific, Waltham, MA) and centrifuged at 4,200 rpm for 1 hour. Subsequently, the viral RNA was extracted from the

homogenate supernatant using an Ambion MagMax extraction kit (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions.

Molecular detection by RT-PCR of RVA, RVC and TGEV were carried out according to controlled Standard Operating Procedures at the Veterinary Diagnostic Laboratory at the University of Minnesota (unpublished). The TGEV RT-PCR amplifies approximately a 120-nucleotide region of the spike protein while both the RVA and RVC RT-PCR amplify a region of approximately 100 nucleotides at the 3' end of VP6.

For the molecular detection of RVB strains, a modified RT-PCR protocol was used as described by Matthijnssens et al, 2008 (Matthijnssens et al., 2008a). Oligonucleotide primers: Forward RVB VP7 (5'-GGA AAT AAT CAG AGA TGG CGT-3', nucleotides 1-21 and Reverse RVB VP7 (5'-TCG CCT AGT CYT CTT TAT GC-3', nucleotides 759-778) were designed based on sequence alignments of one human and five bovine RVB VP7 sequences listed in GenBank (DQ869567, AB016818, U84141, U84472, U84473, and AF531910 respectively). Briefly, 5 µl of extracted RNA was denatured at 97°C for 3 min. RT-PCR was carried out using the Qiagen OneStep RT-PCR Kit (Qiagen/Westburg) in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems). Thermal cycling conditions were as follow: initial reverse transcription, 45°C for 30 min; PCR activation, 95°C for 15 min., followed by 40 cycles of 45 s at 94°C, 45 s at 48°C, and 2 min at 68°C, and a final extension of 7 min. at 72°C.

PCR amplicons, approximately 800 nucleotide in length, were purified using the QIAquick PCR purification kit (Qiagen/Westburg) and sequencing was performed using a fully automated ABI 3730xl DNA Analyzer (Perkin-Elmer) with ABI BigDye Terminator version 3.1 chemistry (Perkin-Elmer). Primer sequence walking was performed to provide double coverage of each sequence. Sequences were analyzed using the Seqman 8.0 program of the Lasergene software (DNASTAR, Madison, WI). Sequences were aligned using Clustal W (Thompson et al., 1994) and phylogenetic analysis was performed using MEGA 5 (Tamura et al., 2011). Kimura 2-parameter

correction at the nucleotide level and the Poisson correction parameter at the amino acid level were utilized to calculate the genetic distances (Matthijnssens et al., 2008b). The Neighbor-joining method was used to create the phylogenetic trees (Saitou and Nei, 1987). Amino acid profile and identity figures (sliding window of 1) were generated using Geneious Pro (Drummond et al., 2011)

To obtain suitable cut-off values for evolution based classification of the RVB VP7 genome segment, the percentage identities between the complete ORFs of the available RVB VP7 genome segments in GenBank (DB180, AF529214; DB101, AY158155; DB176, AF531910; WD653, U84141; Nemuro, AB016818; Mebus, U84473; ATI, U84472; CAL-1, AF184083; Bang373, AY238385; WH-2, AY215070; ADRV, M33872; MMR-B1, FJ81125; IDIR, D00911; WH-1, AY539856; Bang544, FJ851390; Bang117, Gu391306; IDH-084, GU377229; IC-008, GU377218; RVB/Cow-wt/USA/MN-10-1/2010/G3P[X], JQ288103; PB-F18, AB490417; PB-Taiheiyo, AB490418; PB-Kyushu, AB490419; PB-S3, AB490420; PB-S5- AB490421; PB-S13, AB490422; PB-S15, AB490423; PB-S19-1, AB490424; PB-S22-3, AB490425; PB-S24-AA, AB490426; PB-S26-1, AB490427; PB-S37-3, AB490428; PBS40-1, AB490429; PB-S40-11, AB490430; PB-S43-2, AB490431; PB-S43-11, AB490432; PB-S43-17, AB490433; PB-S48-1, AB490434; PB-S49-2, AB490435; PB-S49-13, AB490436; PB-S59-19, AB490437; PB-S61-4, AB490438; PB-7-2-4, AB490439; PB-7-5-5, AB490440; PB-14-29, AB490441; PB-14-31, AB490442; PB-23-5, AB490443; PB-23-10, AB490444; PB-23-44, AB490445; PB-68-C17, AB490446; PB-68-D5, AB490447; PB-68-E4, AB490448; PB-68-G4, AB490449; PB-70-H3, AB490450; PB-70-H5, AB490451; PB-71-H5, AB490452; PB-72-H3, AB490453; PB-72-I2, AB490454), as well as the sequences determined in this study (Table 2.1), were calculated using the pairwise distances program MEGA 5 (Tamura et al., 2011). Pairwise identity frequency graphs were constructed by plotting all the calculated pairwise identities in a graph with the percentage identities in the abscissa (X-axis) and the frequency of each of the calculated pairwise identities in the ordinate (Y-axis) (Ball, 2005).

Results

Small intestines or fecal samples from pigs with current or previous evidence of gastrointestinal disease (with or without diarrhea) are routinely examined by light microscopy for RV-like lesions and tested for RVA, RVC, and transmissible gastroenteritis coronavirus (TGEV) by RT-PCR at the University of Minnesota Veterinary Diagnostic Laboratory. Initially, one sample revealed RV-like lesions including villus atrophy, fusion, and necrosis by light microscopic but was negative for RVA and RVC by RT-PCR. Electron microscopy confirmed the presences of RV-like particles. The sample was found positive using an RT-PCR specially designed to detect the VP7 of RVB, and sequencing of the VP7 revealed a genetic identity of approximately 70% to bovine RVB strains, since porcine VP7 sequences were unavailable in GenBank at that time (August 2009).

Subsequently, 173 samples from pigs of all ages and residing in 119 different sites across the United States were retested for RVB by RT-PCR. Of the 173 samples, 156 samples (90.2%) were positive for RVA, RVB, and/or RVC while 17 samples (9.8%) were negative for RVs (Figure 2.2). RVA and RVC were found in 110 samples (63.6%) and 100 samples (57.8%), respectively. RVB was found in 81 samples (46.8%). The majority (72 of 81) of RVB positive intestines were simultaneously positive for either RVA or RVC. Among all of the pathogens tested, only RVB was identified in the intestinal samples from nine pigs. These 9 pig samples had typical RV lesions, such as those in Figure 2.1, and were confirmed to contain RV particles by electron microscopy, and were negative for RVA and RVC by RT-PCR. Three of the 173 samples (1.7%) were positive for TGEV. Of these three samples, one sample was also found to be positive for RVA.

In an attempt to estimate prevalence of RV detection by age of pig, the samples were divided by age groups as follows: <21 days, 21-55 days, and >55 days (Figure 2.2). The highest percentage of RVB positive samples was observed in the >55 days age group (72.7%), followed by the 21-55 days age group (64.2%), and the <21 days age group

(18.6%). The highest percentage of RVB-only positives were seen in the <21 days age group (12.9%), followed by the 21-55 days age group (2.5%). RVB only positive samples were absent in the >55 days age group. The highest percentage of RVB strains found in a mixed infection with either RVA or RVC was in the >55 days age group with RVA (27.3%). The highest RVB mixed infection with RVA and RVC was present in the 21-55 days age group (38.3%). Porcine enteric pathogens *Salmonella enteric spp.*, *E. coli*, *C. difficile* and *C. perfringens*, coccidia , *Lawsonia intracellularis* and weakly hemolytic *Brachyspira species* were detected in different age groups; however, the correlations between these pathogens and RV infections were outside the scope of this study.

We attempted to determine the complete RVB VP7 ORF from all 81 positive samples; however, 13 of the 81 samples contained more than one RVB strain, which resulted in the inability to generate an accurate full ORF sequence of the VP7 gene. Therefore, the VP7 of only 68 RVB strains identified in 50 different sites was successfully determined. Table 2.1 lists each of the 68 RVB strain names, clinical signs of diarrhea or lack thereof, RVA, RVC, and TGEV results, the age of the host, G genotype, state of origin, site and GenBank accession number. Of the 68 sequenced positive RVB samples, 36 had clinical signs of diarrhea, whereas in 6 samples, the presence of diarrhea was unknown. Single RVB G genotypes were detected in 46 sites while two G genotypes were found to co-circulate in 4 sites (G8/G12, G8/G16, G12/G16, and G12/G18,).

Using the 57 VP7 ORF nucleotide sequences of RVB available from GenBank, together with the 68 new VP7 sequences of RVB strains from this study, a phylogenetic tree (Figure 2.3) was constructed and a nucleotide sequence based pairwise identity frequency graph was generated (Figure 2.4). In the pairwise identity frequency graph, the typical pattern of clearly resolved peaks is apparent. The use of a 68% cut-off value resulted in 5 distinct clades in the phylogenetic tree (Figure 2.3, left dashed line), with a limited overlap between the inter- (red) and intra-cluster (orange and green) identities. Four of these clades contained RVB strains from only one host species (murine, human

and two clusters of porcine RVB), while clade 3 contained both bovine and porcine RVB strains (Figure 2.3). A second suitable cut-off value of 80% resulted in the identification of 20 G genotypes (Figure 2.3, the right dashed line) in the phylogenetic tree, with a very limited overlap between inter- (red and orange) and intra-genotype (green) identities. These 20 different genotypes contained RVB isolated from a single host species (G1: murine, G2: human, G3 and G5: bovine, and G4, G6-G20: porcine) (Figure 3). Table 2.2 shows the nucleotide and amino acid identity ranges among RVB strains belonging to the same G genotype. As could be expected the nucleotide identity ranges were above the determined 80% cut off value for the majority of the genotypes. Only the range among RVB strains belonging to the G12 genotype extended slightly below 80%.

Alignment of the deduced amino acid VP7 sequences from the 20 genotypes is shown in Figure 2.5. Six conserved cysteine residues (29, 84, 108, 137, 148, and 189) were present in the selected representative RVB strains, suggesting that these may be important to the folding, stability, and function of the VP7 protein. An additional cysteine residue was present at residue 13 of genotypes G3 to G5 and G11 to G20. RVB VP7 sequences contained 1 to 4 potential N-linked glycosylation sites: a conserved site at residues 47-49, and additional sites including residues 64-66, 107-109, and 242-245 for genotypes G11 to G20. Putative signal cleavage sites were located at residues 15-16 (Ala and Gln, respectively) and were conserved except for G1 (IDIR) and G8. The VP7 amino acid sequences for the 125 RVB strains (grouped by genotype) used in the phylogenetic analysis are presented in Figure 2.6. The data show that the genetic diversity (both conserved and highly variable amino acid positions) across the VP7 amino acid sequence was evenly distributed with an apparent slightly higher level of conservation in the region at the C-terminal.

Discussion

Kuga and colleagues (Kuga et al., 2009) and Collins and colleagues (Collins et al., 2008) reported higher prevalence of RVA and RVC than RVB in pigs. However, our results also indicate that nearly half (46.8%; 81/173) of the tested samples contained RVB,

which is higher than reported previously. Interestingly, the data reported in this study suggest that co-infection of RVB with RVA, and/or RVC can appear in pigs without concurrent clinical diarrhea. While porcine RVA or RVC infections can appear as both symptomatic and asymptomatic, it is important to remember that asymptomatic animals can still have remarkable gastrointestinal lesions of varying severity. Diarrhea is only one clinical sign associated with a RV infection. Less obvious clinical signs are associated with malabsorption of nutrients by villi with lesions, and may be manifested by loss of body weight, insufficient weight gain, lethargy, and/or mild dehydration. Our findings suggest that RVB can be a primary enteric pathogen in pigs. To that end, we found nine samples (eight of which yielded accurate gene sequences) positive by the RVB VP7 RT-PCR that were not only associated with diarrhea and had the presence of intestinal lesions typical of a RV infection, but also had RV particles identified by electron microscopy. These same samples were negative for other enteric viral or bacterial pathogens tested (*Escherichia coli*, *Clostridium difficile* and *C. perfringens*, coccidian, *Lawsonia intracellularis* and *Brachyspira species, details of testing not shown*), supporting the possibility that RVB can be an important cause of porcine enteritis. In addition, since all the samples were positive by the RVB VP7 RT-PCR designed specifically to detect only RVB, it is highly unlikely that the intestinal lesions and RV particles by electron microscopy were associated with other RV groups. However, we cannot absolutely rule out the possible presence of other RV groups such as RVE or RVH. These data suggest that along with RVA and RVC, screening for RVB is important and should be actively incorporated into testing schemes for etiological agents of gastrointestinal disease in pigs.

In piglets <21 days of age, 65.7% of the positive samples appeared as single infections either with RVA (15.7%), RVB (12.9%), or RVC (37.1%). A few studies have investigated the prevalence of RVA and RVC in young piglets and have indicated that RVA infection in piglets is more common (Martella et al., 2007a). However, in the <21 days old age group, we found a higher percentage of RVC (50.0%) than RVA (27.1%). Although this observation could be a sampling artifact, the difference may be due to differences in control measures between RVA and RVC. A RVA vaccine (ProSystems

RCE, Merck, Whitehouse Station, NJ) is commercially available for swine in the United States, but there is no RVC vaccine available. While the vaccination rates are unknown, the use of vaccination may impact the detection and prevalence of RVA in pigs. Because of the lack of routine detection and commercial RVC vaccine, other control measures such as feedback have been used to prevent RVC infections. Feedback material contains feces or intestinal content from sows and piglets, which is fed to sows generally 4-8 weeks before farrowing to stimulate maternal antibodies to virus therein, providing passive immunity to piglets against RV (Hoshino et al., 1988).

Multiple infections with genetically variant RVA strains have been described before, but the high detection rate (24.3%) of RVA/RVB/RVC co-infections in pigs described in our study is novel. A Japanese study suggested that comingling weaned piglets with different immunological histories and fecal shedding profiles exposes the piglets to other RVA strains, possibly leading to the multiple RVA infections within a group of nursery pigs (Miyazaki et al., 2011). We identified four sites from which samples positive for RVB contained more than one RVB genotype. In addition, the RT-PCR assay identified 13 RVB positive samples co-infected with more than one RVB genotypes, suggesting that like RVA, multiple RVB genotypes can co-circulate within one site. In addition to multiple RVB infections, our data shows that mixed RVA/RVB/RVC infections occur rather frequently as well. In samples from the 21-55 days old age group, there was a higher percentage of mixed RVA, RVB, and/or RVC infections than single RV infections. RVA was most prevalent; however, the prevalence of mixed RVA/RVB/RVC infections was higher than expected. In the >55 days old age group, all samples tested were positive for RVA. The continuous infection with RVA even in more mature pigs may be due to the limited cross protection between RVA genotypes (Hoshino et al., 1988). Nearly half (45.5%) of the samples from the >55 day old age group were positive for all 3 RV groups tested, indicating that pigs continue to be infected throughout their early life, as suggested by Miyazaki and colleagues (2011). Interestingly, the presence of RVB only, RVC only, or the combination of these two were not found in the >55 days old age group, and this leads to several intriguing hypotheses,

all of which require further investigation. The presence of multiple RV group infections in older pigs might suggest that the ability of RVB or RVC to cause disease in older animals as the only infecting pathogen is more limited than the ability of RVA strains. Furthermore, this observation might suggest that pigs >55 days of age do not get gastrointestinal disease upon infection with a single RV infection, and that a co-infection of multiple RV pathogens might be needed to cause clinical disease in these older animals. A primary confounding factor in interpreting any of these hypotheses is that there is no current method to measure existing RVB or RVC immunity. Until immunity measures are established, the significance, pathogenesis, and ecology of multiple RV infections in older pigs will remain unclear.

An 89% amino acid cut-off value was found to give a good correlation between G serotypes and G genotypes of RVA strain (Matthijnssens et al., 2008a). The standardized classification system for RVA developed by the RCWG, also demonstrated a good correlation between the 89% amino acid cut-off value and the 80% nucleotide cut-off value which is now routinely used to classify VP7 gene segments of RVA strains into 35 G genotypes (Matthijnssens et al., 2008b). In 2007, Martella and colleagues proposed a classification system for the RVC VP7 gene segment based on phylogenetic data, and an 89% amino acid cut-off value was used to distinguish at least 6 G genotypes (Martella et al., 2007b). Kuga and colleagues used phylogenetic analyses and pairwise identity frequency graphs of 50 VP7 sequences from RVB strains available at that time for classification purposes (Kuga et al., 2009). Their pairwise identity frequency graphs revealed the presence of two suitable nucleotide cut-off values: 67% and 76% (66% and 79% on the amino acid level). Our new analyses with a total of 125 available RVB VP7 sequences, including the 68 porcine RVB VP7 sequences determined in this study, revealed a similar picture as was observed by Kuga and colleagues, with slightly higher nucleotide cut-off values of 68% and 80%. Kuga and colleagues used their calculated 67% and 76% nucleotide cut-off values to define 5 G genotypes and 12 clusters inside these genotypes, respectively. Based on our most recent analyses, we propose to use the higher cut-off value (80%) to define 20 G genotypes for RVB VP7 sequences instead of

the lower cut-off value (68%). The rationale for this choice is that recent data revealed that the genetic diversity observed among RVA and RVB strains is in the same order of magnitude (Matthijnssens et al., 2012, Trojnar et al., 2009), opposed to what was believed in the past, which suggested that the genetic diversity of RVB strains was significantly larger than that of RVA strains (Eiden et al., 1992, Kuga et al., 2009, Tsunemitsu et al., 2005). Our calculations showed that an 80% nucleotide cut-off value for VP7 of RVB was suitable, which is the same cut-off used to distinguish G genotype for RVA strains. In addition, a correlation between RVB G genotypes based on a 68% nucleotide-cut-off value and G serotypes is unlikely. Although serotyping analyses cannot be performed currently due to the lack of methods to easily adapt RVB strains to cell culture, it is a reasonable assumption that a roughly similar number of amino acid changes (and corresponding nucleotide changes) have to be present into VP7 of RVA and RVB strains before two strains can be considered to belong to the distinct serotype (Green et al., 1988). Since the amino acid residues, which form the epitopes responsible for the serotypes specificity are currently unknown for VP7 of RVB, it is difficult to speculate on the correlation between RVB VP7 serotypes and genotypes, and future serological assays will have to be performed to study these relationships. However, for the majority of the RVB genotypes, the amino acid identity ranges among RVB strains belonging to the same G genotype is also above the 89% cut off value (Table 2.2) as previously determined for RVA strains to discriminate serotypes (Nishikawa et al., 1989). For a few RVB genotypes (G7, G12 and G16) the diversity extends below the 89% amino acid cut off value, which was also observed for a few RVA G genotypes such as G1-G4 and G6 (Matthijnssens et al., 2008a).

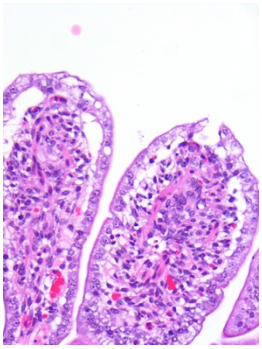
To update and maintain this classification system for RVB VP7 sequences, as has been done for RVA strains; we propose that assignment of VP7 sequences of RVB strains to potentially new genotypes would be done by the RCWG.

In conclusion, the genetic diversity observed in VP7 sequences of porcine RVB strains suggests that RVB strains have been circulating in the porcine population for a

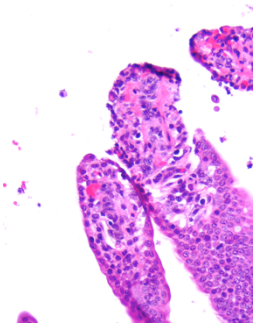
prolonged time (at least since the 1980s) and may be more prevalent than originally thought. Failure to detect the true prevalence of RVB strains in the swine population may have been overshadowed by the high prevalence, pathogenicity, and zoonotic properties of RVA. Most likely, the lack of proper diagnostic assays has concealed the prevalence of RVB in the swine population. The newly developed RT-PCR can be used to further increase our diagnostic capabilities and future studies on the molecular evolution and epidemiology of RVB strains. Moreover, the increased number of VP7 sequences of RVB strains has permitted its classification into 20 G genotypes based on an 80% nucleotide cut-off value by using the approach and guidelines used by the RCWG to classify RVA strains, leading to further comprehensive standardization of RV genotyping.

Figure 2.1. Sections of jejunum from 2-3 day old pigs with typical lesions of acute RV enteritis by 200X magnification. Villi are characterized by edema, early epithelial necrosis, and sloughing to overt epithelial necrosis with denuded villus tips.

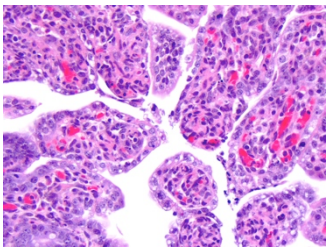
a) RVA infection



b) RVB infection



c) RVC infection



d) Normal villi

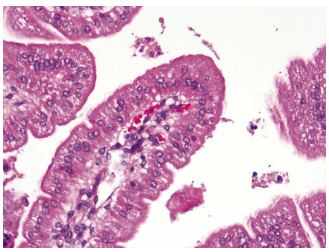
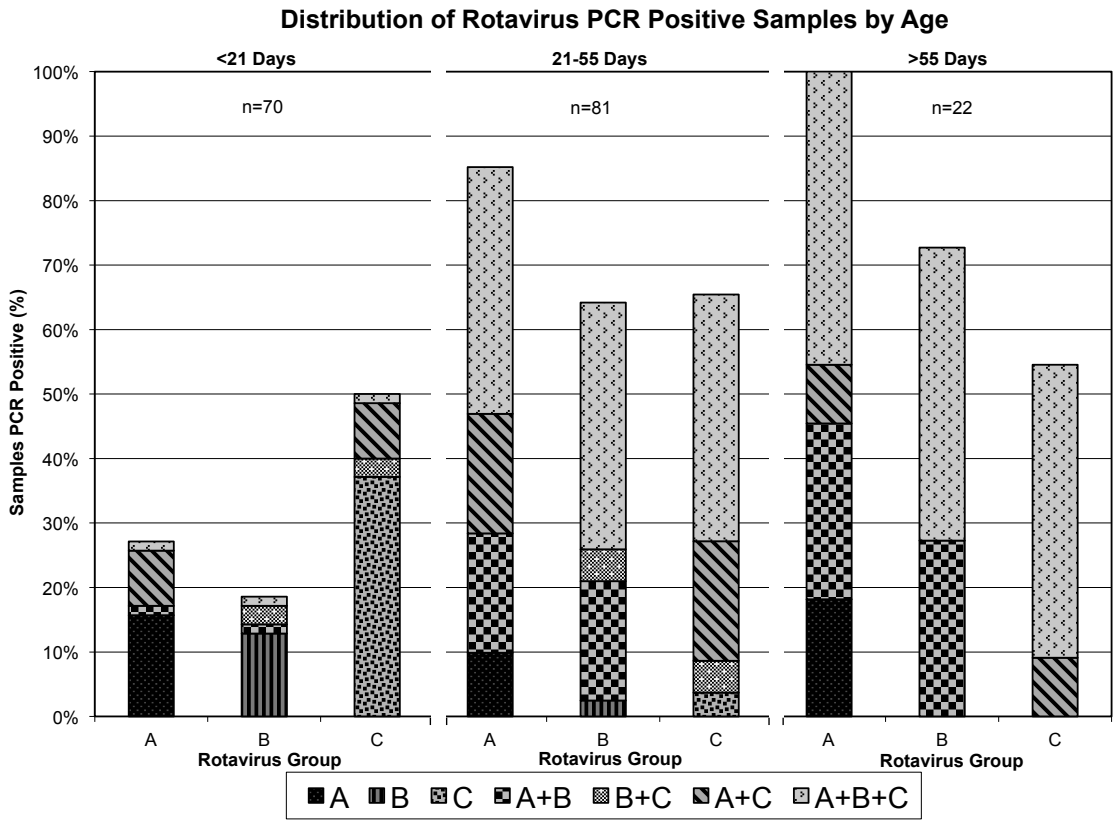


Figure 2.2

a) Histogram of distribution of RVA, RVB and RVC RT-PCR positive porcine samples by 3 age groups: <21 days, 21-55 days, and >55 days.

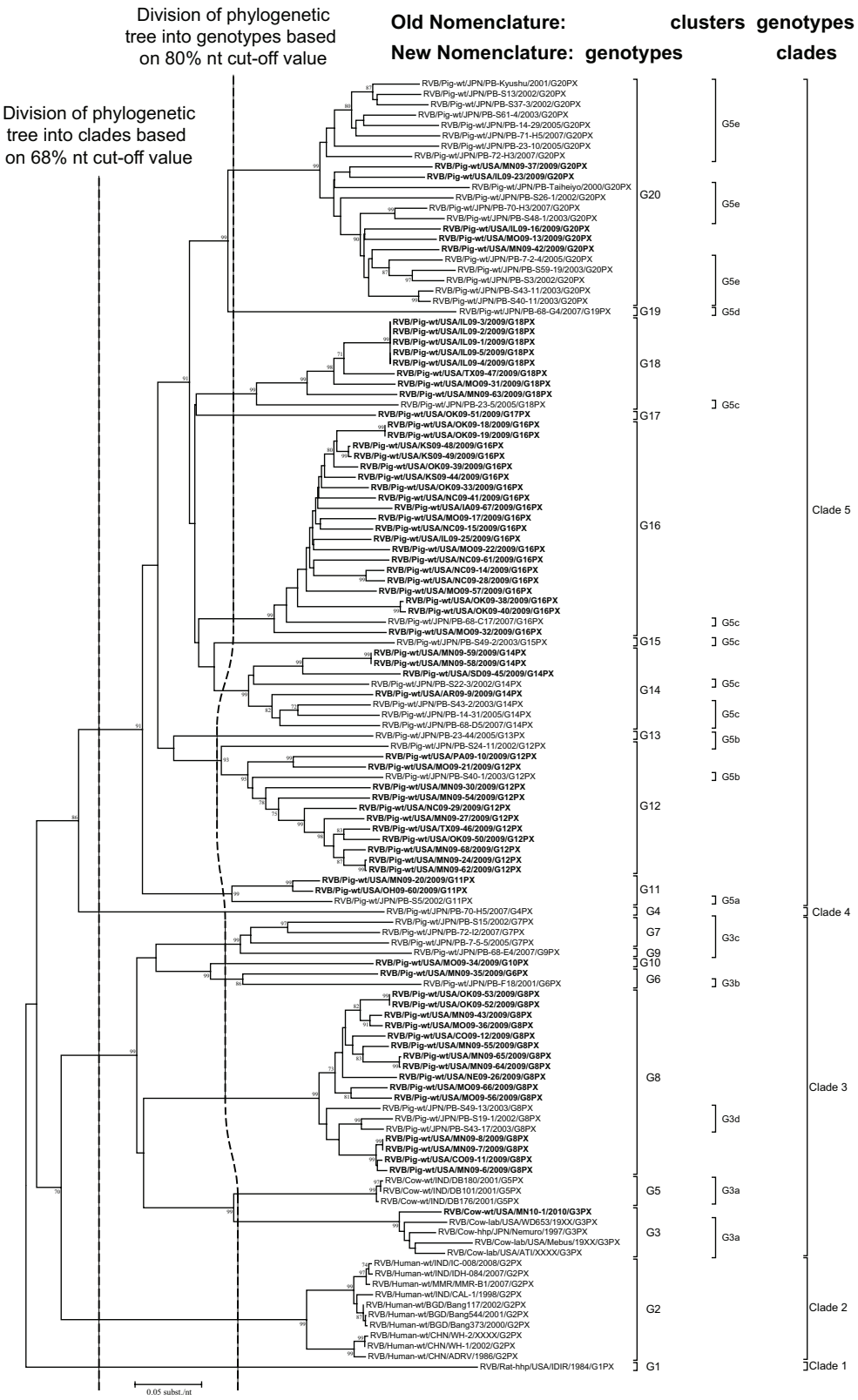


b) Numberic values of positive samples by age group.

Distribution of Samples by Group and Age

Category	<21 Days (n=70)		21-55 Days (n=81)		>55 Days (n=22)		Total (n=173)	
Total Positive	56	80.0%	78	96.3%	22	100.0%	156	90.2%
Negative	14	20.0%	3	3.7%	0	0.0%	17	9.8%
Total RVA positives	19	27.1%	69	85.2%	22	100.0%	110	63.6%
Total RVB positives	13	18.6%	52	64.2%	16	72.7%	81	46.8%
Total RVC positives	35	50.0%	53	65.4%	12	54.5%	100	57.8%
RVA only	11	15.7%	2	2.5%	4	18.2%	17	9.8%
RVB only	9	12.9%	2	2.5%	0	0.0%	11	6.4%
RVC only	26	37.1%	3	3.7%	0	0.0%	29	16.8%
RVA and RVB	1	1.4%	15	18.5%	6	27.3%	22	12.7%
RVB and RVC	2	2.9%	4	4.9%	0	0.0%	6	3.5%
RVA and RVC	6	8.6%	15	18.5%	2	9.1%	23	13.3%
RVA, RVB, and RVC	1	1.4%	31	38.3%	10	45.5%	42	24.3%

Figure 2.3. Phylogenetic tree of RVB VP7 nucleotide sequences. Numbers left of the node characterize the bootstrap (1,000 replicates) for each cluster. Bootstrap values less than 50% are not shown. The left dashed line represent the 68% cut-off value, resulting in 5 clades (or genotypes in the old classification of Kuga and colleagues), and the right dashed line represents the 80% nucleotide cut-off value, resulting in the 20 new G genotypes. The new and old clasification system are shown on the right side of the figures in brackets.


Division of phylogenetic tree into genotypes based on 80% nt cut-off value
Division of phylogenetic tree into clades based on 68% nt cut-off value
Old Nomenclature: clusters genotypes
New Nomenclature: genotypes clades
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RVB/Pig-wt/JPN/PB-71-H5/2007/G20PX
RVB/Pig-wt/JPN/PB-23-10/2005/G20PX
RVB/Pig-wt/JPN/PB-72-H3/2007/G20PX
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RVB/Pig-wt/USA/CO09-11/2009/G8PX
RVB/Pig-wt/USA/MN09-6/2009/G8PX
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RVB/Cow-wt/IND/DB176/2001/G5PX
RVB/Cow-wt/USA/MN10-1/2010/G3PX
RVB/Cow-lab/USA/WD653/19XX/G3PX
RVB/Cow-hhp/JPN/Nemuro/1997/G3PX
RVB/Cow-lab/USA/Mebus/19XX/G3PX
RVB/Cow-lab/USA/ATI/XXXX/G3PX
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RVB/Human-wt/CHN/WH-2/XXXX/G2PX
RVB/Human-wt/CHN/WH-1/2002/G2PX
RVB/Human-wt/CHN/ADRV/1986/G2PX
RVB/Rat-hhp/USA/IDIR/1984/G1PX
G20
G19
G18
G17
G16
G15
G14
G13
G12
G11
G4
G7
G9
G10
G6
G8
G5
G3
G2
G1
G5e
G5d
G5c
G5b
G5a
G3c
G3b
G3d
G3a
Clade 5
Clade 4
Clade 3
Clade 2
Clade 1
0.05 subst./nt

Figure 2.4. Pairwise identity frequency graph using 125 available RVB VP7 ORF nucleotide sequences. Two suitable nucleotide cut-off values (68% and 80%) are depicted by vertical solid lines.

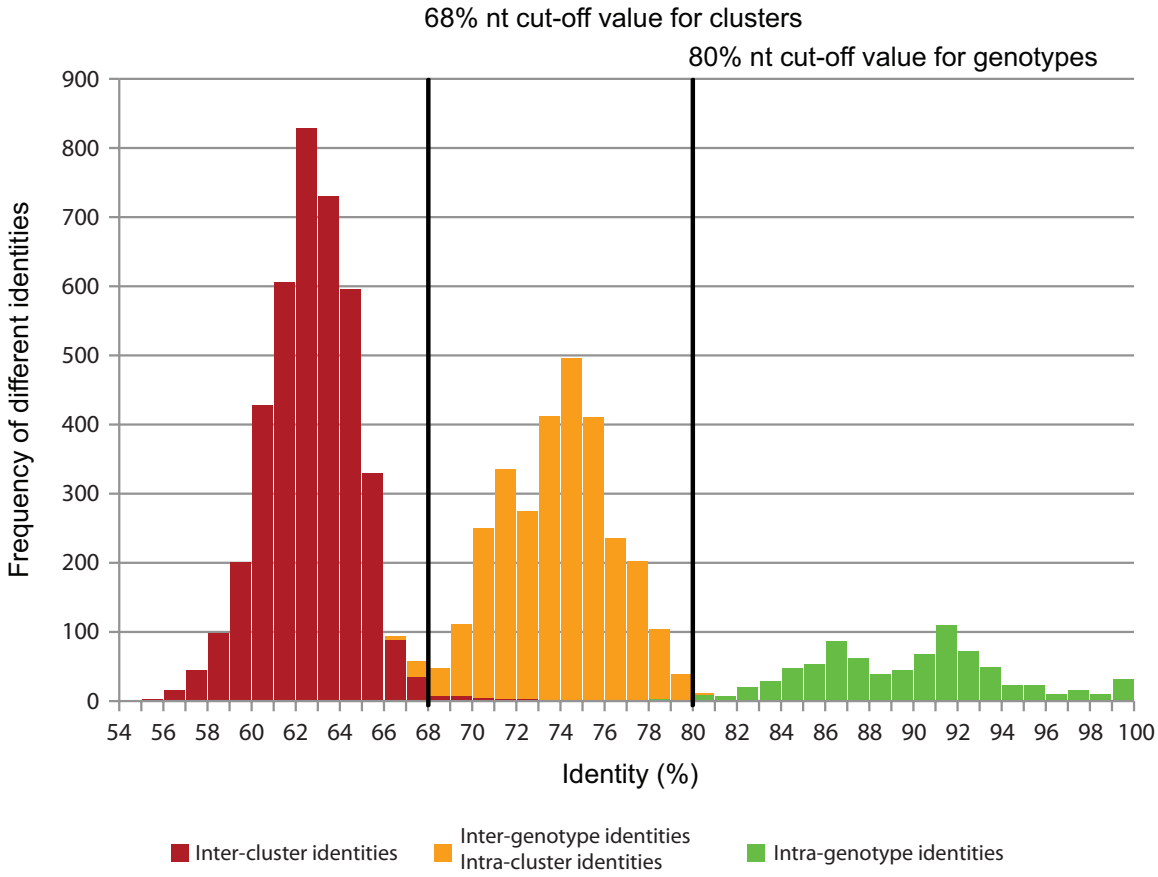


Figure 2.5. Multiple amino acid sequence alignment of selected RVB strains including the new and previously described VP7 genotypes. Matching amino acids are indicated with dots; gaps are shown by dashes; potential N-linked glycosylation sites are underlined; cysteine residues are boxed; insertion sites are indicated by addition symbols; ambiguous amino acids are represented by question mark, and putative signal cleavage sites are indicated by carets. Conserved residues within the 125 strains are shaded, while asterisks indicate highly divergent positions.

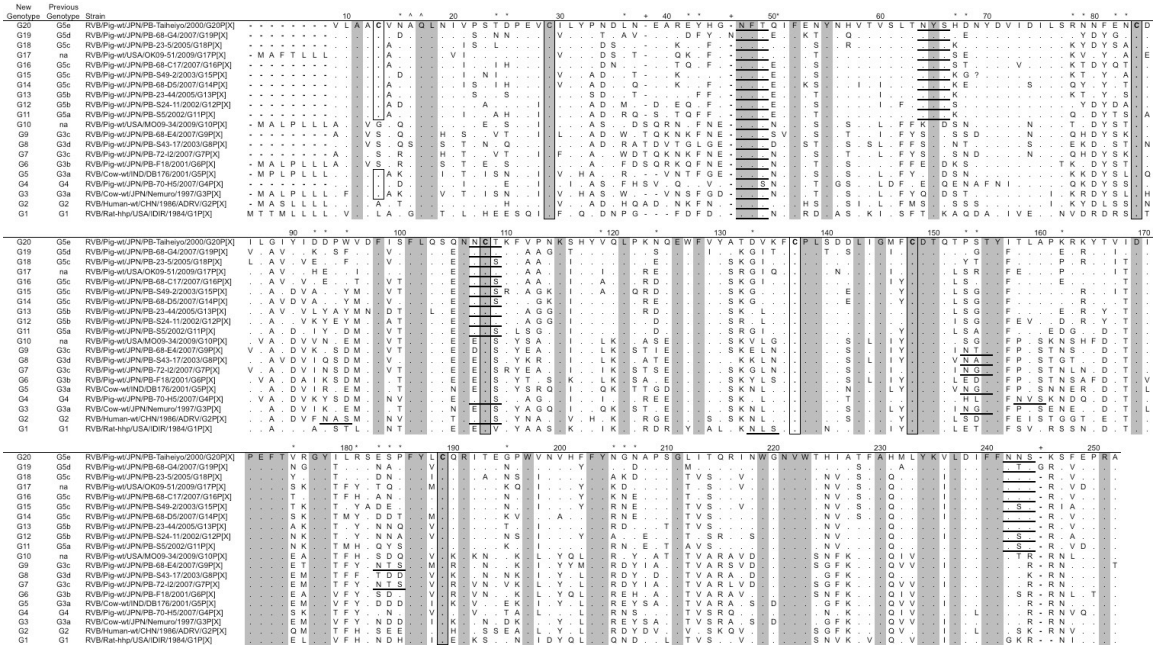
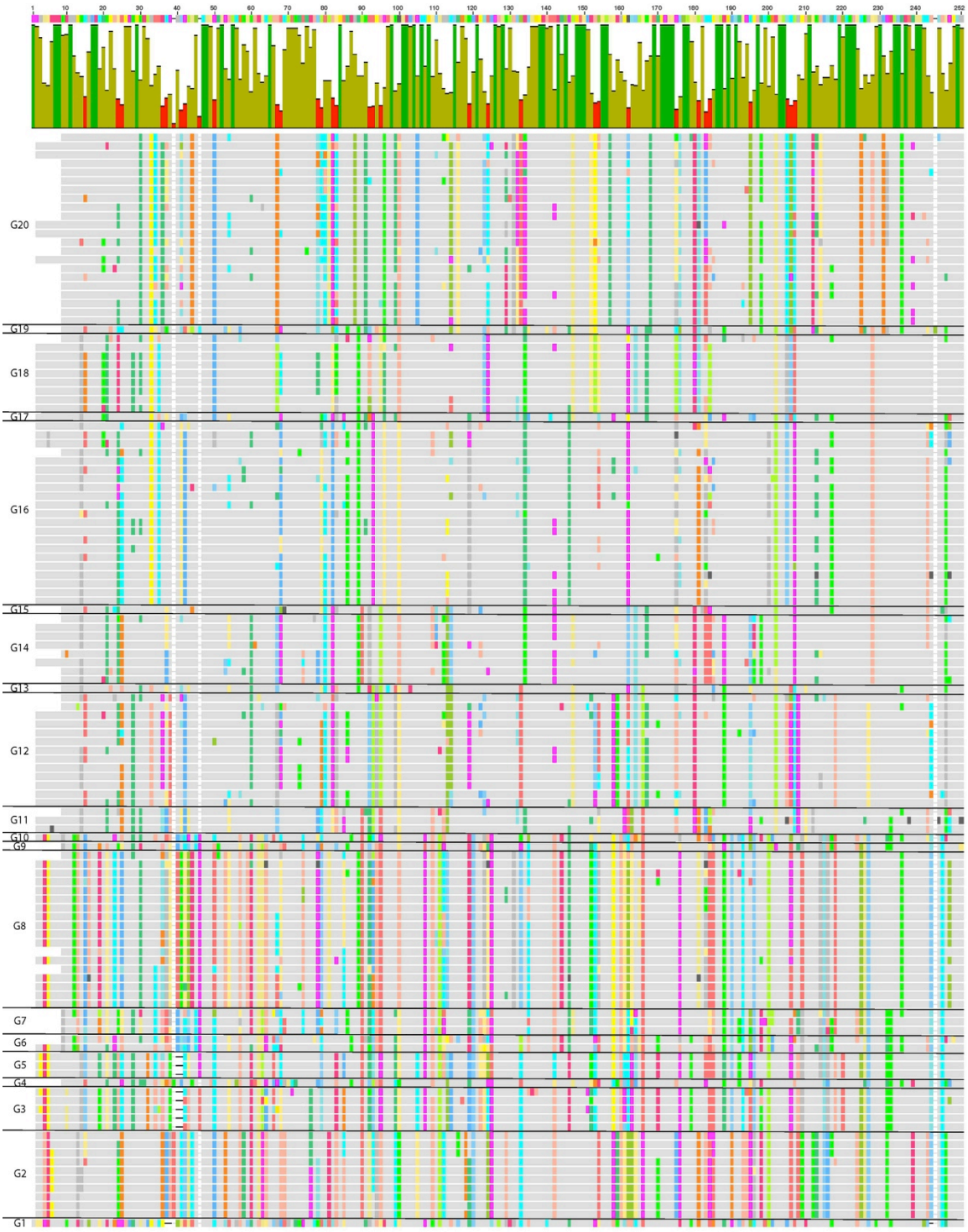


Figure 2.6. Visual representation of the alignment for the 125 RVB amino acid sequence divided into the 20 proposed genotypes. The designated G genotype is listed on the left side. The consensus amino acid sequence is listed at the top of the chart where each color represents a specific amino acid. The histogram represents diversity within each amino acid position where peaks (dark green) represent conserved residues while valleys (red) represent divergent amino acid residues.


1
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200
210
220
230
240
252
G20
G19
G18
G17
G16
G15
G14
G13
G12
G11
G10
G9
G8
G7
G6
G5
G4
G3
G2
G1

Table 2.1. Distribution of RVB sequences by diarrhea status, PCR results (RVA, RVC, and TGEV), age, G genotype, state, site, and accession number (n=68).

NCBI Name	Diarrhea Status	RVA Result	RVC Result	TGE Result	Age	G Genotype	State	Site	Acession Number
RVB/Pig-wt/USA/IL09-1/2009/G18P[X]	+	-	-	-	1-10 days	G18	Illinois	1	JQ043748
RVB/Pig-wt/USA/IL09-2/2009/G18P[X]	+	-	-	-	1-10 days	G18	Illinois	1	JQ043749
RVB/Pig-wt/USA/IL09-3/2009/G18P[X]	+	-	-	-	1-10 days	G18	Illinois	1	JQ043750
RVB/Pig-wt/USA/IL09-4/2009/G18P[X]	+	-	-	-	1-10 days	G18	Illinois	1	JQ043751
RVB/Pig-wt/USA/IL09-5/2009/G18P[X]	+	-	-	-	1-10 days	G18	Illinois	1	JQ043752
RVB/Pig-wt/USA/MO09-32/2009/G16P[X]	+	-	-	-	4 days	G16	Missouri	23	JQ043779
RVB/Pig-wt/USA/MN09-42/2009/G20P[X]	+	-	-	-	3 days	G20	Minnesota	31	JQ043789
RVB/Pig-wt/USA/MN09-59/2009/G14P[X]	+	-	-	-	3-10 days	G14	Minnesota	42	JQ043806
RVB/Pig-wt/USA/IL09-25/2009/G16P[X]	+	-	+	-	4 weeks	G16	Illinois	16	JQ043772
RVB/Pig-wt/USA/MN09-30/2009/G12P[X]	+	-	+	-	4 weeks	G12	Minnesota	21	JQ043777
RVB/Pig-wt/USA/MN09-58/2009/G14P[X]	+	-	+	-	3-10 days	G14	Minnesota	42	JQ043805
RVB/Pig-wt/USA/MN09-6/2009/G8P[X]	+	+	-	-	6 weeks	G8	Minnesota	2	JQ043753
RVB/Pig-wt/USA/NC09-14/2009/G16P[X]	+	+	-	-	5 weeks	G16	North Carolina	8	JQ043761
RVB/Pig-wt/USA/MO09-17/2009/G16P[X]	+	+	-	-	3 weeks	G16	Missouri	10	JQ043764
RVB/Pig-wt/USA/MN09-35/2009/G6P[X]	+	+	-	-	4 weeks	G6	Minnesota	26	JQ043782
RVB/Pig-wt/USA/NC09-41/2009/G16P[X]	+	+	-	-	4 weeks	G16	North Carolina	30	JQ043788
RVB/Pig-wt/USA/MN09-43/2009/G8P[X]	+	+	-	-	15 weeks	G8	Minnesota	32	JQ043790
RVB/Pig-wt/USA/MO09-56/2009/G8P[X]	+	+	-	-	5.5 weeks	G8	Missouri	41	JQ043803
RVB/Pig-wt/USA/OH09-60/2009/G11P[X]	+	+	-	-	4 weeks	G11	Ohio	43	JQ043807
RVB/Pig-wt/USA/MN09-64/2009/G8P[X]	+	+	-	-	18 days	G8	Minnesota	47	JQ043811
RVB/Pig-wt/USA/IA09-67/2009/G16P[X]	+	+	-	-	4 weeks	G16	Iowa	49	JQ043814
RVB/Pig-wt/USA/MN09-68/2009/G12P[X]	+	+	-	-	5 weeks	G12	Minnesota	50	JQ043815
RVB/Pig-wt/USA/MN09-20/2009/G11P[X]	+	+	+	-	24 days	G11	Minnesota	12	JQ043767
RVB/Pig-wt/USA/MO09-21/2009/G12P[X]	+	+	+	-	4-5 weeks	G12	Missouri	13	JQ043768
RVB/Pig-wt/USA/MO09-22/2009/G16P[X]	+	+	+	-	4-5 weeks	G16	Missouri	13	JQ043769
RVB/Pig-wt/USA/IL09-23/2009/G20P[X]	+	+	+	-	4 weeks	G20	Illinois	14	JQ043770
RVB/Pig-wt/USA/MN09-24/2009/G12P[X]	+	+	+	-	4 weeks	G12	Minnesota	15	JQ043771
RVB/Pig-wt/USA/NE09-26/2009/G8P[X]	+	+	+	-	14-16 weeks	G8	Nebraska	17	JQ043773
RVB/Pig-wt/USA/MN09-27/2009/G12P[X]	+	+	+	-	6 weeks	G12	Minnesota	18	JQ043774
RVB/Pig-wt/USA/NC09-28/2009/G16P[X]	+	+	+	-	4 weeks	G16	North Carolina	19	JQ043775
RVB/Pig-wt/USA/TX09-46/2009/G12P[X]	+	+	+	-	10 weeks	G12	Texas	35	JQ043793
RVB/Pig-wt/USA/TX09-47/2009/G18P[X]	+	+	+	-	10 weeks	G18	Texas	35	JQ043794
RVB/Pig-wt/USA/MO09-57/2009/G16P[X]	+	+	+	-	5.5 weeks	G16	Missouri	41	JQ043804
RVB/Pig-wt/USA/NC09-61/2009/G16P[X]	+	+	+	-	4 weeks	G16	North Carolina	44	JQ043808
RVB/Pig-wt/USA/MN09-62/2009/G12P[X]	+	+	+	-	3 weeks	G12	Minnesota	45	JQ043809
RVB/Pig-wt/USA/MN09-65/2009/G8P[X]	+	+	+	-	18 days	G8	Minnesota	47	JQ043812
RVB/Pig-wt/USA/MN09-7/2009/G8P[X]	-	+	-	-	20 weeks	G8	Minnesota	3	JQ043754
RVB/Pig-wt/USA/CO09-12/2009/G8P[X]	-	+	-	-	14 weeks	G8	Colorado	6	JQ043759
RVB/Pig-wt/USA/MO09-34/2009/G10P[X]	-	+	-	-	3 weeks	G10	Missouri	25	JQ043781
RVB/Pig-wt/USA/OK09-40/2009/G16P[X]	-	+	-	-	4 weeks	G16	Oklahoma	29	JQ043787
RVB/Pig-wt/USA/MN09-55/2009/G8P[X]	-	+	-	-	5 weeks	G8	Minnesota	40	JQ043802
RVB/Pig-wt/USA/MN09-63/2009/G18P[X]	-	+	-	-	5 weeks	G18	Minnesota	46	JQ043810
RVB/Pig-wt/USA/MN09-8/2009/G8P[X]	-	+	+	-	20 weeks	G8	Minnesota	3	JQ043755
RVB/Pig-wt/USA/AR09-9/2009/G14P[X]	-	+	+	-	3 weeks	G14	Arkansas	4	JQ043756
RVB/Pig-wt/USA/PA09-10/2009/G12P[X]	-	+	+	-	6 weeks	G12	Pennsylvania	5	JQ043757
RVB/Pig-wt/USA/CO09-11/2009/G8P[X]	-	+	+	-	14 weeks	G8	Colorado	6	JQ043758
RVB/Pig-wt/USA/MO09-13/2009/G20P[X]	-	+	+	-	3 weeks	G20	Missouri	7	JQ043760
RVB/Pig-wt/USA/NC09-15/2009/G16P[X]	-	+	+	-	4 weeks	G16	North Carolina	8	JQ043762
RVB/Pig-wt/USA/IL09-16/2009/G20P[X]	-	+	+	-	4 weeks	G20	Illinois	9	JQ043763
RVB/Pig-wt/USA/OK09-18/2009/G16P[X]	-	+	+	-	5 weeks	G16	Oklahoma	11	JQ043765
RVB/Pig-wt/USA/OK09-19/2009/G16P[X]	-	+	+	-	5 weeks	G16	Oklahoma	11	JQ043766
RVB/Pig-wt/USA/NC09-29/2009/G12P[X]	-	+	+	-	35 days	G12	North Carolina	20	JQ043776
RVB/Pig-wt/USA/OK09-38/2009/G16P[X]	-	+	+	-	4 weeks	G16	Oklahoma	29	JQ043785
RVB/Pig-wt/USA/OK09-39/2009/G16P[X]	-	+	+	-	4 weeks	G16	Oklahoma	29	JQ043786
RVB/Pig-wt/USA/KS09-44/2009/G16P[X]	-	+	+	-	28 days	G16	Kansas	33	JQ043791
RVB/Pig-wt/USA/KS09-48/2009/G16P[X]	-	+	+	-	3 weeks	G16	Kansas	36	JQ043795
RVB/Pig-wt/USA/KS09-49/2009/G16P[X]	-	+	+	-	3 weeks	G16	Kansas	36	JQ043796
RVB/Pig-wt/USA/OK09-50/2009/G12P[X]	-	+	+	-	3 weeks	G12	Oklahoma	37	JQ043797
RVB/Pig-wt/USA/OK09-51/2009/G17P[X]	-	+	+	-	3 weeks	G17	Oklahoma	37	JQ043798
RVB/Pig-wt/USA/OK09-52/2009/G8P[X]	-	+	+	-	5 weeks	G8	Oklahoma	38	JQ043799
RVB/Pig-wt/USA/OK09-53/2009/G8P[X]	-	+	+	-	8 weeks	G8	Oklahoma	39	JQ043800
RVB/Pig-wt/USA/MN09-54/2009/G12P[X]	-	+	+	-	5 weeks	G12	Minnesota	39	JQ043801
RVB/Pig-wt/USA/OK09-33/2009/G16P[X]	unknown	-	+	-	10 days	G16	Oklahoma	24	JQ043780
RVB/Pig-wt/USA/MN09-37/2009/G20P[X]	unknown	+	-	-	3 weeks	G20	Minnesota	28	JQ043784
RVB/Pig-wt/USA/SD09-45/2009/G14P[X]	unknown	+	-	-	14 days	G14	South Dakota	34	JQ043792
RVB/Pig-wt/USA/MN09-66/2009/G8P[X]	unknown	+	-	-	18 days	G8	Missouri	48	JQ043813
RVB/Pig-wt/USA/MO09-31/2009/G18P[X]	unknown	+	+	-	21 days	G18	Missouri	22	JQ043778
RVB/Pig-wt/USA/MO09-36/2009/G8P[X]	unknown	+	+	-	3 weeks	G8	Missouri	27	JQ043783

Table 2.2 RVB VP7 nucleotide and amino acid pairwise frequencies comparison within genotypes.

G genotype	nucleotide	amino acid
G1	na	na
G2	91.0-99.9	95.5-100
G3	92.5-95.3	92.0-99.0
G4	na	na
G5	99.4-99.9	99.5-100
G6	80.5	85.6
G7	82.1-87.0	87.6-94.0
G8	89.4-100	93.0-100
G9	na	na
G10	na	na
G11	87.3-96.6	93.0-97.0
G12	78.3-99.9	85.1-100
G13	na	na
G14	81.2-100	92.0-100
G15	na	na
G16	83.6-100	87.1-100
G17	na	na
G18	82.3-100	89.6-100
G19	na	na
G20	80.0-98.1	90.0-98.0

Chapter 3: VP6 Genetic Diversity, Reassortment, Intragenic Recombination and Classification of Rotavirus B in American and Japanese Pigs

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Introduction

Rotaviruses (RVs) are a significant cause of diarrhea in animals. RVs belong to the family *Reoviridae* and contain an eleven segmented, double strand RNA genome. RVs are classified into eight groups or species (A-H) based on antigenic properties and sequencing of the viral protein 6 (VP6) (Bohl et al. 1982; Estes and Kapikian, 2007; Trojnar et al. 2009; Matthijnssens et al. 2012). The RV triple-layered icosahedral capsid is composed of an outer layer (VP7 and VP4), an intermediate layer (VP6), and the inner core (VP1, VP2 and VP3) (Estes and Kapikian, 2007). Due to the increased use of sequencing, sequence based classification systems have complemented serotyping and are now widely used (Matthijnssens et al. 2008a; Marthaler et al. 2012; Marthaler et al. 2013). In 2008, a RVA nucleotide sequenced based classification system was developed for all eleven gene segments, which is maintained by the Rotavirus Classification Working Group (RCWG) (Matthijnssens et al. 2008a; Matthijnssens et al. 2011). The 11 RVA genome segments VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6 are referred to as the Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx genotypes, respectively (Matthijnssens et al. 2008b). Following the identification of multiple genetically divergent swine RVB VP7 sequences in 2012 in the United States, a RVB VP7 genotyping classification system was developed based on an 80% nucleotide cut-off value, yielding 20 RVB G genotypes (Marthaler et al. 2012).

RVB strains were initially associated with human adult diarrhea in southeast Asia, and RVB infections have also been documented sporadically in rats, cattle, and lambs (Hung et al. 1984; Dai et al. 1987; Fang et al. 1989; Eiden et al. 1992; Chang et al. 1997; Shen et al. 1999; Kelkar and Zade, 2004; Rahman et al. 2007; Ghosh et al. 2007; Chitambar et al. 2011). Furthermore, RVBs have been identified as a significant cause of porcine diarrhea (Bridger and Brown, 1985; Kuga et al. 2009; Marthaler et al. 2012). The difficulty in adapting RVB to cell culture has hindered serological analyses, and RVB strains have only been characterized at the genetic level (Bridger. 1994; Ghosh et al. 2007; Kuga et al. 2009; Suzuki et al. 2011; Suzuki et al. 2012a; Suzuki et al. 2012b).

Because the swine RVB VP6 genetic diversity has been studied to a limited extent, we sequenced the RVB VP6 gene segment from 80 American and Japanese pigs to understand the genetic diversity and reassortment dynamics within and between host species, with future plans to develop a RVB VP6 enzyme-linked immunosorbent assay (ELISA) to assess immunity against RVB in the swine population.

Material and Methods

Generation of RVB VP6 sequences

A VP6 specific RT-PCR was performed on the RNA extracts from 64 RVB USA strains, from which the VP7 gene segment was analyzed previously, using the Qiagen OneStep RT-PCR Kit (Qiagen/Westburg) with previously described thermal cycling conditions and published primers GB6-1 and GB6-3 (Ahmed et al. 2004; Marthaler et al. 2012). RT-PCR products were run in a 2% ethidium bromide gel, bands of ~1,200 nucleotides were visualized under UV light, and the RT-PCR products were purified using the Qiagen QIAquick PCR kit (Qiagen/Westburg). The published primers GB6-1 to GB6-3 were submitted to the University of Minnesota Genomics Center (UMGC) for Sanger sequencing on a ABI 3730xl DNA Analyzer (Perkin-Elmer) using the ABI 3.1 BigDye Terminator v3.1 kit (Perkin-Elmer) (Ahmed et al. 2004). Additional primers were designed to obtain a minimal coverage of 2X across the VP6 gene segment. The chromatograms were analyzed using Lasergene Seqman 10.0 software (DNASTAR, Madison, WI). The 16 Japanese RVB strains were identified, extracted, and sequenced using a single primer amplification method as described previously (Wakuda et al. 2005; Suzuki et al. 2012a; Suzuki et al. 2012b).

Sequence alignment and phylogenetic tree creation

The novel porcine (n=80) and the available GenBank (n=30, RUBV282, GQ358715; DB176, GQ358713; RUBV226, GQ358714; Nemuro, AB106542; NIV-005626, JQ904201; Bang544, FJ851392; 10913, JQ904209; NIV-005623, JQ904200; NIV-0948756, JQ904207; NIV-04623, JQ904202; IDH-084, GU377228; 9222, JQ904208; 11037, JQ904210; NIV-957971; 1995; JQ904199; NIV-04624, JQ904203; NIV-

0632252; 2006; JQ904204; NIV-1048101, JQ904211; NIV-005625, JN009779; NIV-094456, JN009777; NIV-04622, JN009778; Bang373, AY238389; Bang117, GU391305; WH-1, AY539858; NIV-076222, JQ904205; CAL-1, AB037931; MMR-B1, FJ811827; IC-008, GU377217; NIV-0924341, JQ904206; ADRV, M55982; IDIR, M84456; 9222, JQ904221.) RVB VP6 sequences were aligned using Clustal W in Geneious Pro (Thompson et al. 1994; Drummond et al. 2011). The JModelTest program determined the General Time-Reverse Substitution model (GTR) with a proportion of invariable sites (+I) and rate variation among sites (+G) as appropriate nucleotide substitution model using Maximum Likelihood (PHYML) (Guindon and Gascuel, 2003; Guindon et al. 2010; Darriba et al. 2012). The Prottest program determined the LG (+G) substitution model as the appropriate amino acid substitution model (Drummond and Strimmer, 2001; Guindon and Gascuel, 2003; Abascal et al. 2005). The porcine RVB VP7 (G) sequences available from GenBank were used to construct phylogenetic trees (IDIR, D00911; RUBV282, DQ531557; RUBV226, DQ531551; DB176, AF531910; Nemuro, AB016818; WH-1, AY539856; NIV-005625, JN009782; NIV-04622, JN009781; NIV-094456, JN009780; MMR-B1, FJ811825; Bang373, AY238385; Bang544, FJ851390; Bang117, GU391306; IDH-084, GU377229; IC-008, GU377218; NIV-1048101, JQ904224; 11037, JQ904223; 10913, JQ904222; NIV-0948756, JQ904220; NIV-0924341, JQ904219; NIV-076222, JQ904218; NIV-0632252, JQ904217; NIV-04624, JQ904216; NIV-04623, JQ904215; NIV-005626, JQ904214; NIV-005623, JQ904213; NIV-957971, JQ904212; CAL-1, AF184083; ADRV, M33872; PB-F18, AB490417; PB-Taiheiyo, AB490418; PB-Kyushu, AB490419; PB-S3, AB490420; PB-S5- AB490421; PB-S13, AB490422; PB-S15, AB490423; PB-S19-1, AB490424; PB-S22-3, AB490425; PB-S24-AA, AB490426; PB-S26-1, AB490427; PB-S37-3, AB490428; PBS40-1, AB490429; PB-S40-11, AB490430; PB-S43-2, AB490431; PB-S43-11, AB490432; PB-S43-17, AB490433; PB-S48-1, AB490434; PB-S49-2, AB490435; PB-S49-13, AB490436; PB-S59-19, AB490437; PB-S61-4, AB490438; PB-7-2-4, AB490439; PB-7-5-5, AB490440; PB-14-29, AB490441; PB-14-31, AB490442; PB-23-5, AB490443; PB-23-10, AB490444; PB-23-44, AB490445; PB-68-C17, AB490446; PB-68-D5, AB490447; PB-68-E4, AB490448; PB-68-G4, AB490449; PB-70-H3, AB490450; PB-

70-H5, AB490451; PB-71-H5, AB490452; PB-72-H3, AB490453; PB-72-I2, AB490454). All phylogenetic trees were built in Geneious Pro.

Pairwise identity frequency graphs.

Nucleotide and amino acid pairwise identity frequency graphs were constructed with the identity on the x-axis and the frequency on the y-axis to determine the appropriate nucleotide and amino acid cut-off values (Ball. 2005).

Intragenic recombination

In addition, the porcine RVB VP6 sequence alignment was tested with the Recombination Detection Program (RDP) under default setting to search for intragenic recombinant RVB VP6 sequences (Martin et al. 2010). To confirm the RDP results, the sequences were split at the suggested intragenic recombinant position, and separate nucleotide phylogenetic tree were constructed.

Results

Sequence alignment

The genome segment 6 (encoding VP6) of 80 novel porcine RVB VP6 strains were sequenced. The gene 6 segments were between 1271 and 1273 nucleotides in length, excluding the primer binding sites. The 5' UTR contained no INDELs (INnsertions/DELetions), while the 3' untranslated region (UTR) contained multiple INDELs (Figure 3.1). The single rat, 4 bovine, 25 human, and 80 porcine strains RVB VP6 open reading frames (ORF) were aligned (n=110), yielding 23 extremely diverse nucleotide positions (containing A, C, G, and T) and 434 (36.9%) conserved positions within the RVB VP6 ORF. Porcine samples CO09-12, IL09-16, NE09-26, MO09-36, KS09-44, NC09-61, MN09-65, MO09-66, and IA09-67, isolated in the USA from 2009, contained more than one RVB strain as indicated by degenerate bases within the nucleotide sequence.

The RVB VP6 protein (391 amino acids) contained 150 conserved amino acids positions and 24 hypervariable positions containing 5 or more amino acids variants (Table 3.1). Fourteen hypervariable amino acid positions (29, 87, 116, 130, 172, 205, 226, 240, 241, 293, 306, 344, 371, and 375) contained 5 different amino acids, while 7 hypervariable positions contained 6 different amino acids (113, 145, 221, 262, 303, 359, and 372). Hypervariable positions 12, 19, and 348 contained 7 different amino acids while position 290 was the most hypervariable site with 8 different amino acids (M=69, N=12, S=11, E=6, Q=6,V=3, T=2, and I=1).

Phylogenetic analysis

Nucleotide and amino acid pairwise identity frequency charts of the 110 RVB sequences were constructed (Fig 3.2). Based on the pairwise identity charts and the phylogenetic trees, an 81% nucleotide percent identity cut-off value and an 89% amino acid cut-off value were appropriate and yielded 13 RVB VP6 (I) genotypes (Fig 3.3). A single I genotype each was detected for the rat (I1), human (I2), and bovine (I3) RVB strains, while the 10 additional I genotypes were detected among porcine RVB strains. The large RVB I13 genotype contained half (n=40) of the porcine strains. With nucleotide and amino acid percent values slightly below the proposed cut-off value, the RVB I13 genotypes had the highest nucleotide and amino acid intra-genotype diversity of 80.7% and 88.5%, respectively (Table 3.2). In addition, the RVB genotypes I10 and I13 had the highest nucleotide and amino acid inter-genotype similarities (83.4% and 92.3%, respectively), which is slightly higher than the proposed nucleotide and amino acid percent cut-off value.

Intragenic recombination

The porcine RVB VP6 sequence alignment was tested with the RDP. At approximately nucleotide position 555, the Japanese strain PB-85-I3 from 2008 was indicated as an intragenic recombinant of Japanese RVB strains closely related to PB-70-H5 and PB-Taiheiyo strains isolated in 2007 and 2000, respectively. The PB-85-I3 sequence

clustered in different branches when different regions of the ORF (nucleotides 1-554 and 555-1176) were used, indicating recombination (Fig 3.4).

Reassortment events among VP7 and VP6 of RVB strains

To investigate the frequency of reassortment events between VP7 and VP6 gene segments of porcine RVB strains, we constructed a phylogenetic tree for the VP6 gene segment and color-coded the strains according to their VP7 G genotype (if known) (Fig 3.3a). In addition, a RVB VP7 phylogenetic tree was color-coded according to the VP6 I genotype (Fig 3.3b) to investigate a potential linkage between these two gene segments.

The RVB I genotypes from the rat (I1) and human (I2) strains were associated with a single G genotype, G1 and G2, respectively. The bovine RVB I3 genotype contained the G genotypes G3 and G5. The porcine RVB I6 genotype, which contained a single sequence, has an unknown RVB G genotype. Representing a limited number of strains, the porcine RVB I4, I7, I8, I9, and I10 genotypes were only found in combination with a solitary G genotype (G7, G8, G12, G8, and G4, respectively). The porcine RVB I5 genotype encompassed two G genotypes (G6 and G7). In addition, porcine RVB I12 encompassed two genotypes (G18 and G20). The porcine RVB I11 genotype was naturally associated with 6 RVB G genotypes (G8, G11, G12, G14, G17, and G18). Moreover, the widely circulating RVB I13 genotype (n=40) was also associated with 6 RVB G genotypes (G8, G12, G14, G16, G18 and G20).

Inversely, the RVB G4, G6, G11, G16 and G17 genotypes were associated with a solitary RVB I genotype (I10, I5, I11, I13, and I11, respectively). The RVB G7, G14, and G20 genotypes were associated with two I genotypes (I4 and I5, I11 and I13, and I12 and I13, respectively). The RVB G12 and G18 genotypes encompassed 3 I genotypes (I8, I11, and I13; and I11, I12, and I13, respectively). The RVB G8 genotype was found in combination with the greatest number of I genotypes (I7, I9, I11, and I13) while the RVB I genotypes were unavailable for the G9, G10, G13, G15, and G19 genotypes.

Discussion

To our knowledge, this is the first report of porcine RVB VP6 sequences, and the first RVB VP6 sequences to be released from the United States. The porcine RVB VP6 sequences contained several INDELs in the 3' UTR. Unfortunately, human and bovine RVB VP6 3'UTR sequences were not available for comparison. While the particular importance of the VP6 3' UTR had not been determined for the RVB, some interesting difference may exists between host species. Future RVB sequencing efforts should include the ORF, 5' and 3' UTR.

We identified nine RVB VP6 strains that contained ambiguous bases, indicating multiple variants within these samples. Additionally, we indicated that the RVB VP6 gene segment of Japanese strain PB-85-I3 was an intragenic recombinant between closely related Japanese strains PB-70-H5 and PB-Taiheiyo. Both the PB-85-I3 and PB-70-H5 were from the same farm in the Chiba Prefecture, but the PB-Taiheiyo strain was from the Fukushima Prefecture. While a single RVC and several RVA intragenic recombinant strains have been identified, this is the first identification of a RVB intragenic recombinant strain (Parra et al. 2004; Phan et al. 2007; Martella et al. 2007a; Martinez-Laso et al. 2009). Since RVA, RVB, and RVC intragenic recombinant strains have been identified, future RV studies should utilize intragenic recombinant software before starting in-depth phylogenetic analysis because these strains can generate inaccurate phylogenetic trees and hence incorrect conclusions.

The RVA and RVB G genotypes share the same nucleotide (80%) and amino acid (89%) cut-off value. However, an 85% nucleotide cut-off value for the RVA VP6 gene segment has identified 16 I genotypes from 11 host species, while an 81% nucleotide cut-off value for the RVB VP6 gene segment identified 13 I genotypes in only 4 host species (Matthijnssens et al. 2011). Thus far, only three porcine RVA VP6 genotypes (I1, I2, I5) have been recognized while ten porcine RVB VP6 genotypes (I4-I13) have been identified (Shi et al. 2012; Okitsu et al. 2013). Moreover, the RVB I genotypes from the RVB G9, G10, G13, G15 and G19 genotypes have not determined, and more RVB I

genotypes may exist within the porcine population. Overall, these observations suggest more genetic diversity within porcine RVB VP6 gene segment than in the porcine RVA VP6 gene segment. Furthermore, while the G3 and G5 bovine RVB strains from Japan and India, respectively, share the same I genotype (I3); an American bovine VP6 sequence has yet to be determined, potentially resulting in additional RVB I genotypes.

At first glance, the RVB I genotype diversity appears to be region and host specific. However, the RVB I13 genotype contains both Japanese and American strains, suggesting the spread or prevalence of certain RVB genotypes worldwide. Also, our analyses also revealed a surprisingly high frequency of reassortment events involving the porcine RVB VP7 and VP6 gene segments, which further confirms that RVB co-infections must occur frequently. On the other hand, the RVB G4 and G16 genotypes were only associated with genotypes I10 and I13, respectively. While this may be a sampling artifact within the G4 genotype (only one strain has been identified), the RVB G16 genotype contained 18 strains, all belonging to the I10 genotype, suggesting that certain genotype combinations (i.e. G16/I10) avoid reassortment compared to other genotype combinations. Further complete genome analyses of porcine RVB strains will enhance our understanding on the reassortment dynamics of RVB strains.

In conclusion, the sequencing of 80 American and Japanese porcine RVB VP6 sequences increases our understanding of the genetic diversity and phylogenetic relationships of RVB strains. Based on nucleotide pairwise identity chart and the phylogenetic tree, we propose an 81% nucleotide percent cut-off, generating 13 RVB I genotypes. Moreover, rat, human, and bovine RVB strains consist of a single I genotype each, suggesting that the RVB I genotype may be species specific (although it may need to be confirmed by testing additional RVB strains from these species). In contrast, we identified 10 porcine RVB I genotypes, implying a large genetic diversity among RVB strains circulating in the swine population in the United States, Japan, and most likely in other pig populations worldwide.

Figure 3.1 RVB VP6 alignment illustrating insertions and deletions (INDELs) in the 3' UTR.

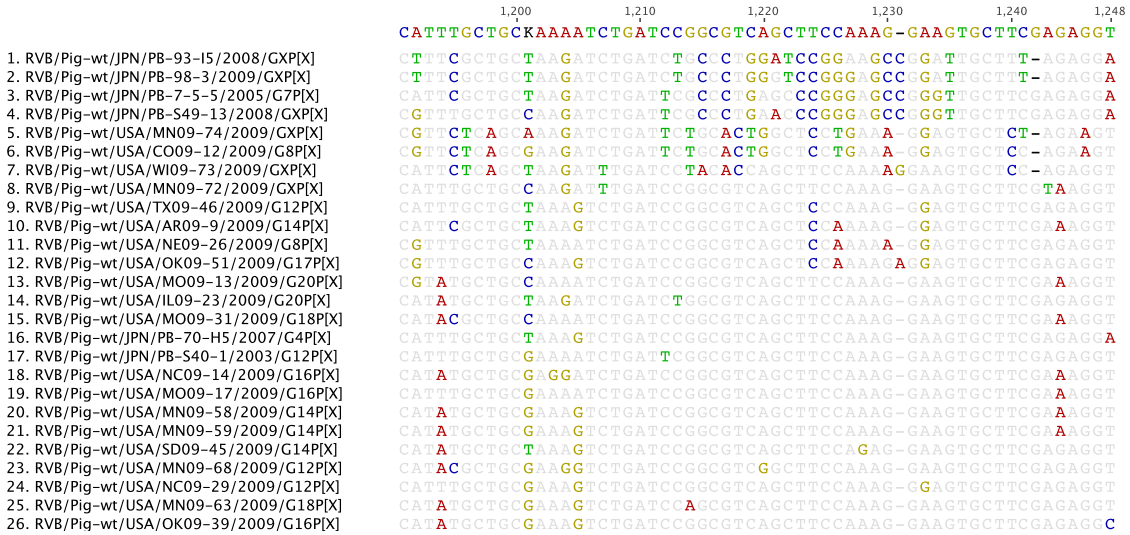
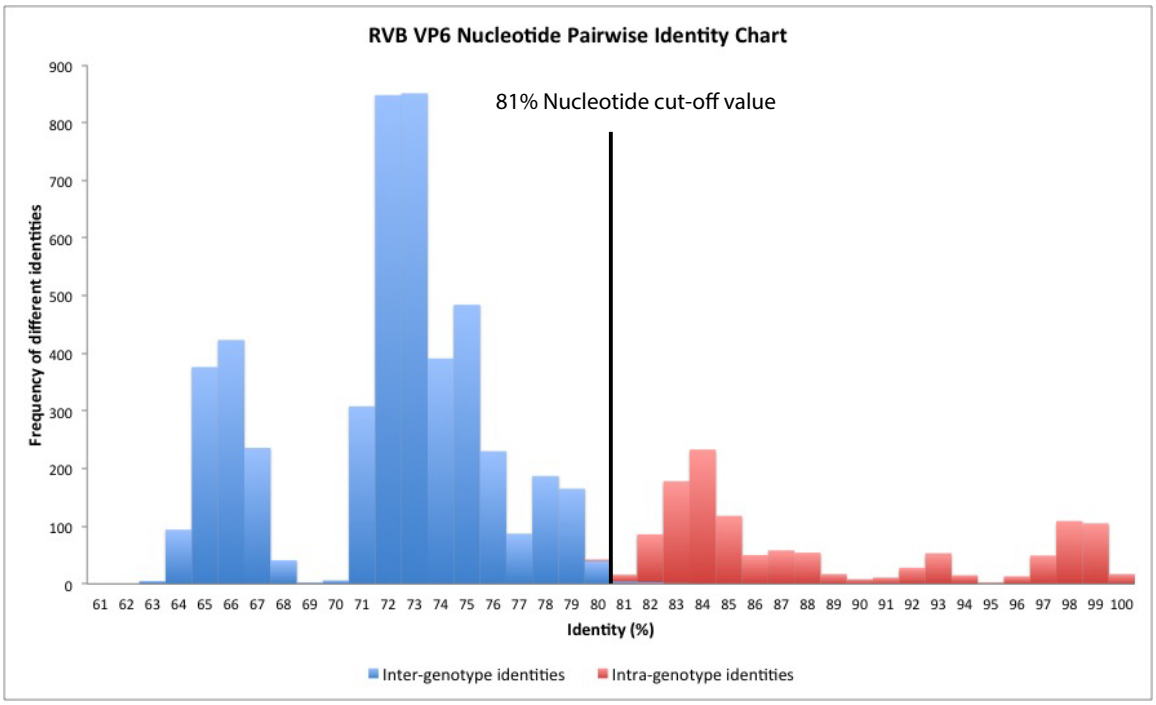


Figure 3.2. The RVB nucleotide (a) and amino acid (b) pairwise identity histograms illustrating the proposed 81% and 89% cut-off values, respectively.



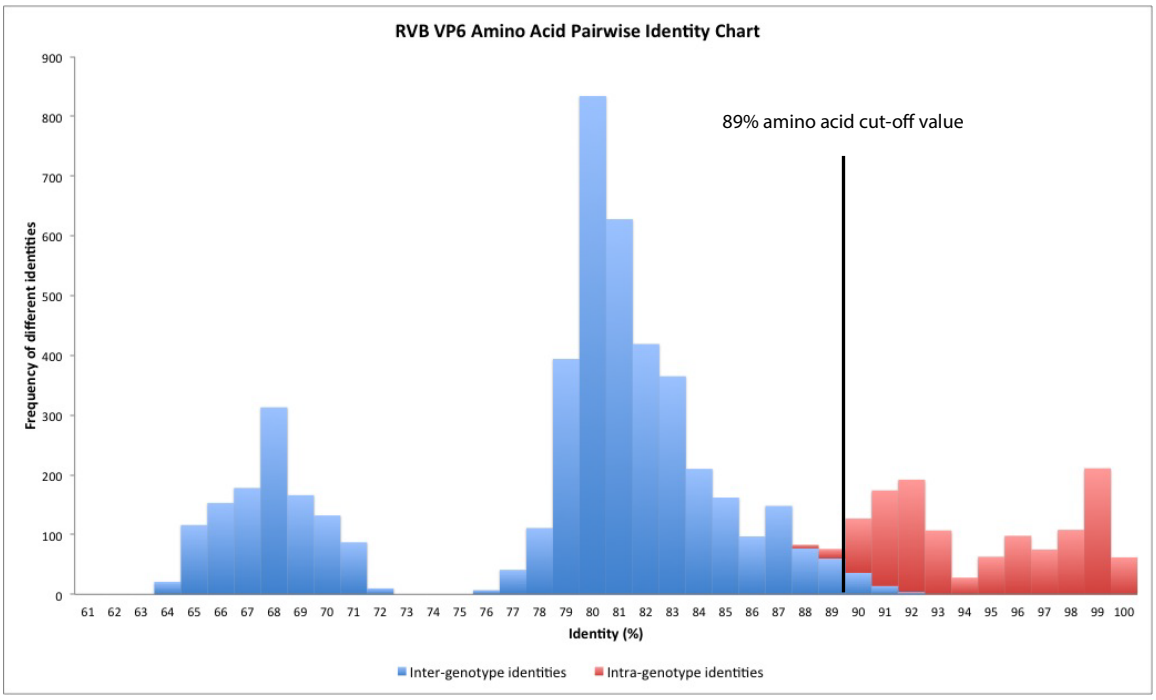
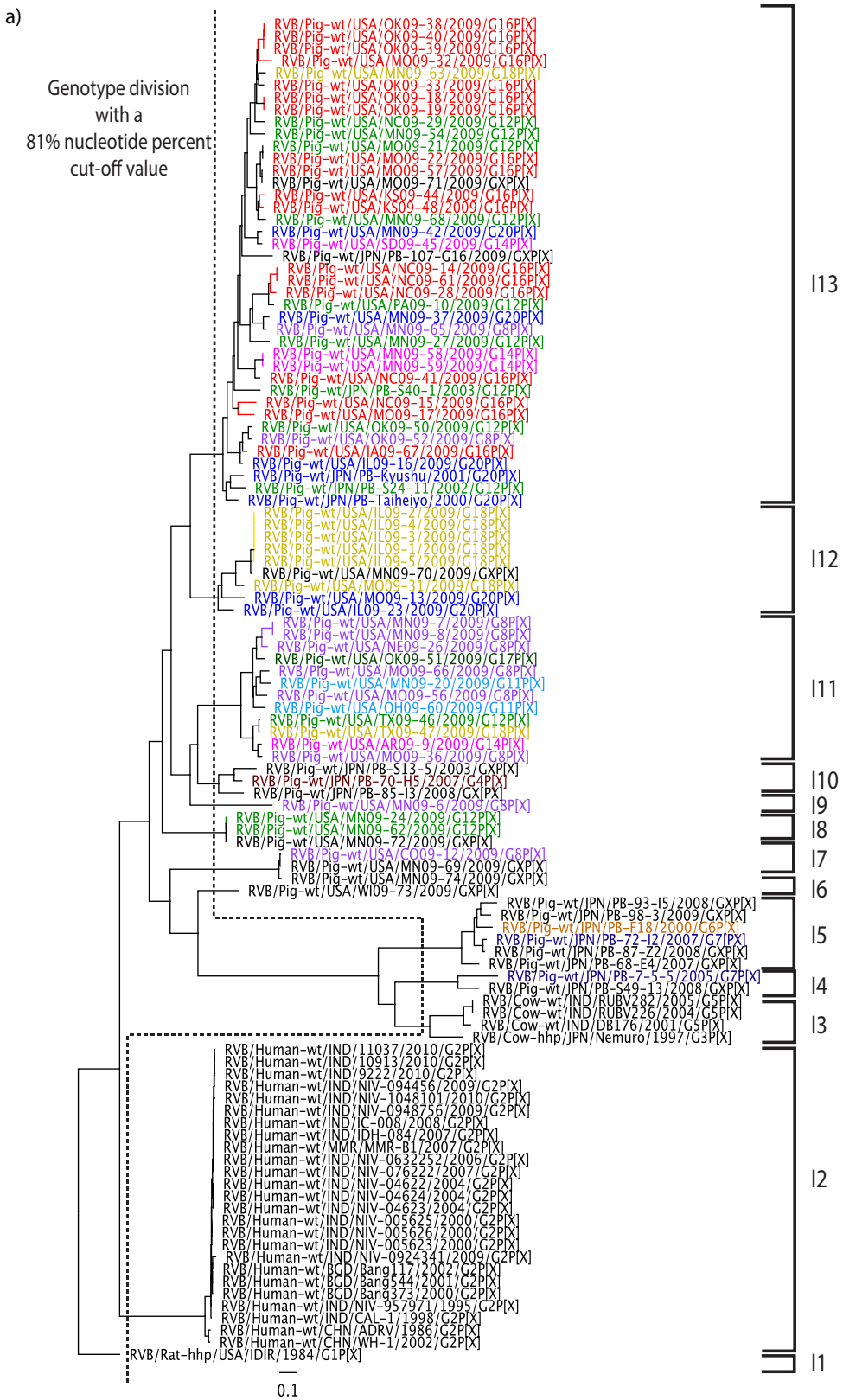


Figure 3.3. RVB VP6 phylogenetic tree with the proposed 81% nucleotide cut-off value, yielding 13 I-genotypes (a). The porcine strains with an available VP7 sequence have been colored according their VP7 genotype. The RVB G genotypes were unavailable for 13 porcine RVB strains due to the lack of original RV infected fecal or intestinal material for VP7 sequencing. The RVB VP7 phylogenetic tree was colored according to the available VP6 genotypes (b).


a)
Genotype division
with a
81% nucleotide percent
cut-off value
RVB/Pig-wt/USA/OK09-38/2009/G16P[X]
RVB/Pig-wt/USA/OK09-40/2009/G16P[X]
RVB/Pig-wt/USA/OK09-39/2009/G16P[X]
RVB/Pig-wt/USA/MO09-32/2009/G16P[X]
RVB/Pig-wt/USA/MN09-63/2009/G18P[X]
RVB/Pig-wt/USA/OK09-33/2009/G16P[X]
RVB/Pig-wt/USA/OK09-18/2009/G16P[X]
RVB/Pig-wt/USA/OK09-19/2009/G16P[X]
RVB/Pig-wt/USA/NC09-29/2009/G12P[X]
RVB/Pig-wt/USA/MN09-54/2009/G12P[X]
RVB/Pig-wt/USA/MO09-21/2009/G12P[X]
RVB/Pig-wt/USA/MO09-22/2009/G16P[X]
RVB/Pig-wt/USA/MO09-57/2009/G16P[X]
RVB/Pig-wt/USA/MO09-71/2009/GXP[X]
RVB/Pig-wt/USA/KS09-44/2009/G16P[X]
RVB/Pig-wt/USA/KS09-48/2009/G16P[X]
RVB/Pig-wt/USA/MN09-68/2009/G12P[X]
RVB/Pig-wt/USA/MN09-42/2009/G20P[X]
RVB/Pig-wt/USA/SD09-45/2009/G14P[X]
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RVB/Pig-wt/USA/NC09-14/2009/G16P[X]
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RVB/Pig-wt/USA/PA09-10/2009/G12P[X]
RVB/Pig-wt/USA/MN09-37/2009/G20P[X]
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RVB/Pig-wt/USA/MN09-27/2009/G12P[X]
RVB/Pig-wt/USA/MN09-58/2009/G14P[X]
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RVB/Pig-wt/USA/OK09-50/2009/G12P[X]
RVB/Pig-wt/USA/OK09-52/2009/G8P[X]
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RVB/Pig-wt/USA/IL09-3/2009/G18P[X]
RVB/Pig-wt/USA/IL09-1/2009/G18P[X]
RVB/Pig-wt/USA/IL09-5/2009/G18P[X]
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RVB/Pig-wt/USA/MO09-66/2009/G8P[X]
RVB/Pig-wt/USA/MN09-20/2009/G11P[X]
RVB/Pig-wt/USA/MO09-56/2009/G8P[X]
RVB/Pig-wt/USA/OH09-60/2009/G11P[X]
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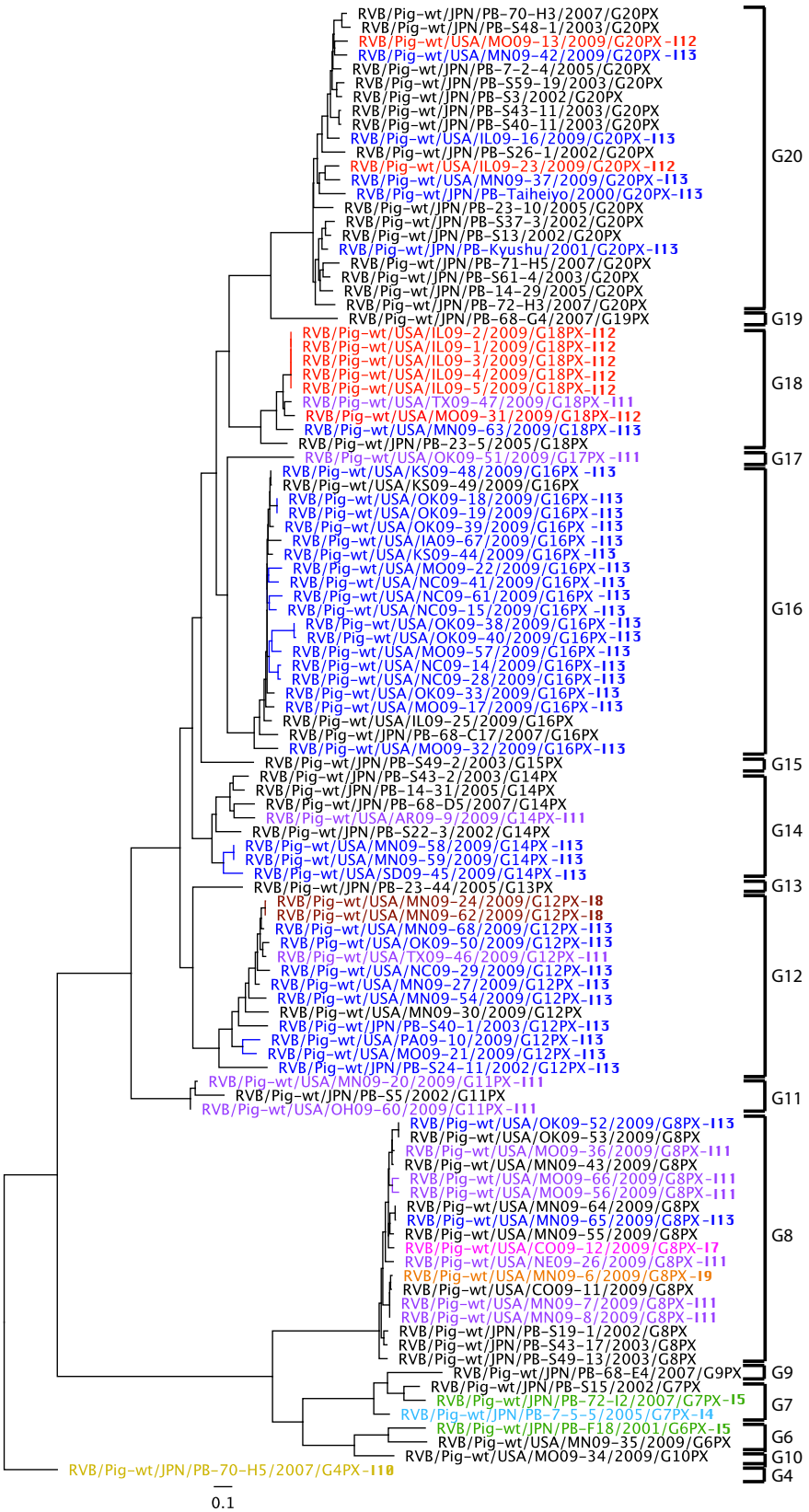
RVB/Human-wt/BGD/Bang544/2001/G2P[X]
RVB/Human-wt/BGD/Bang373/2000/G2P[X]
RVB/Human-wt/IND/NIV-957971/1995/G2P[X]
RVB/Human-wt/IND/CAL-1/1998/G2P[X]
RVB/Human-wt/CHN/ADRV/1986/G2P[X]
RVB/Human-wt/CHN/WH-1/2002/G2P[X]
RVB/Rat-hhp/USA/IDIR/1984/G1P[X]
0.1
I13
I12
I11
I10
I9
I8
I7
I6
I5
I4
I3
I2
I1

b)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RVB/Pig-wt/USA/MN09-42/2009/G20PX-I13
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RVB/Pig-wt/USA/MN09-37/2009/G20PX-I13
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RVB/Pig-wt/USA/OK09-38/2009/G16PX-I13
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RVB/Pig-wt/USA/MO09-17/2009/G16PX-I13
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RVB/Pig-wt/USA/OH09-60/2009/G11PX-I11
RVB/Pig-wt/USA/OK09-52/2009/G8PX-I13
RVB/Pig-wt/USA/OK09-53/2009/G8PX
RVB/Pig-wt/USA/MO09-36/2009/G8PX-I11
RVB/Pig-wt/USA/MN09-43/2009/G8PX
RVB/Pig-wt/USA/MO09-66/2009/G8PX-I11
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RVB/Pig-wt/USA/MN09-6/2009/G8PX-I9
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RVB/Pig-wt/JPN/PB-S15/2002/G7PX
RVB/Pig-wt/JPN/PB-72-I2/2007/G7PX-I5
RVB/Pig-wt/JPN/PB-7-5-5/2005/G7PX-I4
RVB/Pig-wt/JPN/PB-F18/2001/G6PX-I5
RVB/Pig-wt/USA/MN09-35/2009/G6PX
RVB/Pig-wt/USA/MO09-34/2009/G10PX
RVB/Pig-wt/JPN/PB-70-H5/2007/G4PX-I10
0.1
G20
G19
G18
G17
G16
G15
G14
G13
G12
G11
G8
G9
G7
G6
G10
G4

Figure 3.4. Nucleotide phylogenetic trees illustrating the recombinant RVB strain PB-85-I3 (green) and the parental RVB strains PB-70-H5 (blue) and PB-Taiheiyo strain (red).

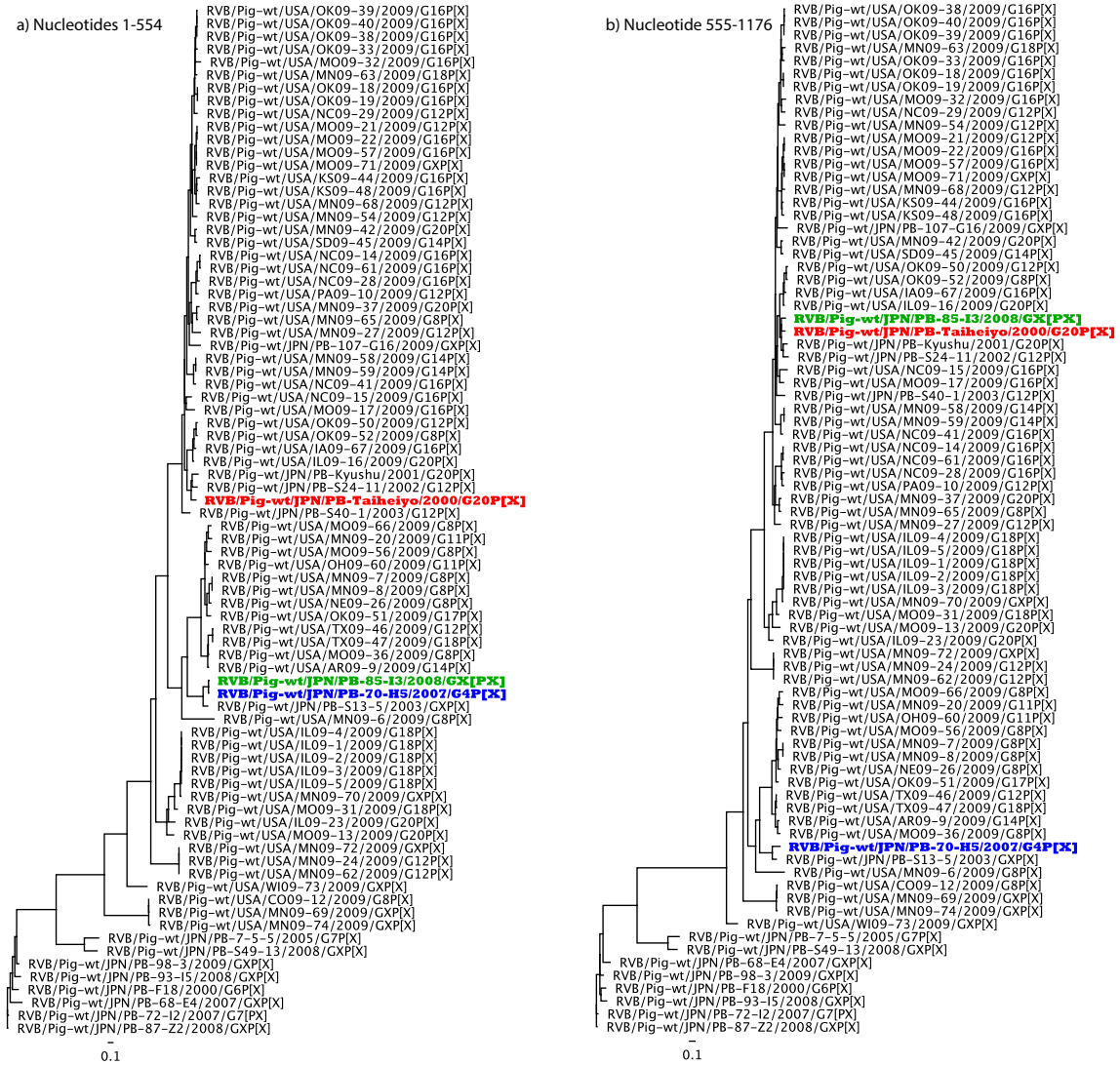

Table 3.1. Amino acid alignment of 13 representative RVB I genotypes. Dots represent the same amino acid as the reference strain, IDIR. Colors represent number of amino acid per position from the 110 RVB VP6 aligment: white =1 (n=150), green = 2 (n=110), yellow = 3 (n=76), orange = 4 (n=30), purple = 5 (n=14), blue = 6 (n=7), brown = 7 (n=3), and red = 8 (n=1).

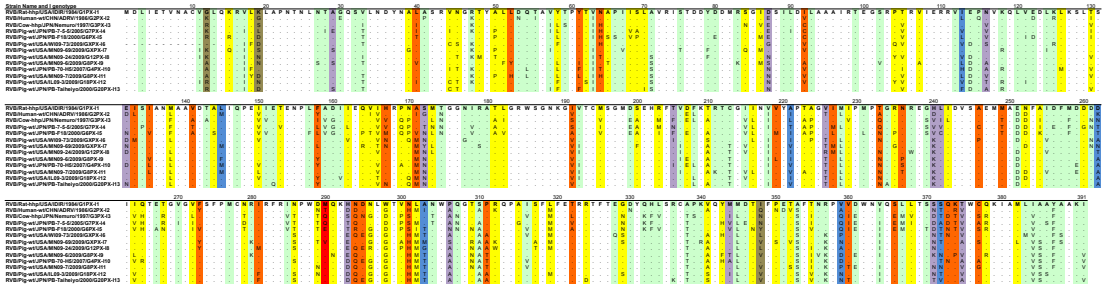

Table 3.2. RVB VP6 nucleotide (top) and amino acid (bottom) percent identities.

	I1	I2	I3	I4	I5	I6	I7	I8	I9	I10	I11	I12	I13
I1	NA NA	71.9-73.6	67-68.4	65.3-66.4	64.7-66.4	72.4	71-71.3	73.9-74	72.1	72.1-73.2	70.5-72.8	70.6-73.6	69.9-73.7
I2	83.4-84.4	92.4-100 96.7-100	65.6-66.8	66.5-67.9	63.9-66.2	72.2-73.2	71.5-72.2	72.3-73.7	71.3-72.4	71-72.9	71.1-74.2	71.2-73.8	71.3-74.7
I3	72.1-72.6	69.6-71.6	81.5-100 96.7-100	74.3-75.9	73.6-75.3	68.3-68.9	66.3-67.3	67.2-68.2	65.5-66.2	66.7-68.5	65.4-68	66-68.4	64.8-68.3
I4	69.3-69.6	68.5-70.6	83.9-85.4	83.2 95.9	72.5-74.3	68.6-69.1	66.6-66.8	65.7-66.9	64.1-64.6	63.8-65.7	63.9-66.5	66.2-68.3	63.4-67.1
I5	67.1-68.5	67.1-69.3	81.3-82.4	78.2-81.3	84.1-97.2 96.9-99.2	67.1-68.7	65.7-66.8	65.7-66.8	64.9-65.5	64.7-67	65.4-67.8	64.5-66.4	64.2-68.5
I6	79.6	80.2-81.7	69.6-70.9	69.6-69.8	66.9-67.5	NA NA	73.8-74.5	75.5	72	73.9-74	74.9-76.4	76-76.6	73.4-76.2
I7	78-78.3	78.3-79.8	66.8-67.5	65.2-66.8	64-65	77.5-77.8	97.6-99.1 98.2-98.7	73.1-73.7	72.1-72.5	71.6-73.1	71-73.3	73.2-75.6	72-74.9
I8	81.1	80.6-81.8	68.8-69.1	68.8-69.8	66.5-67.1	84.4	81.9-82.4	99.9-100 100	75.7	76-77.1	74.6-76.4	77-78	73.7-76.7
I9	79.8	78.5-79.8	66.2-67	66.8-67.5	66.5-67.3	79.4	76.7-77.2	82.1	NA NA	75.5-77.2	75-76.5	74.6-76.3	73.6-76.1
I10	79.3-79.5	79.5-81.6	68.5-71.1	67-68.8	65-68.5	82.3-83.9	76.7-77.7	84.1-84.9	82.6-85.2	82.7-87.8 91.6-95.9	76.6-79.8	75.2-79.8	74.2-83.4
I11	77.7-79.8	77.8-81.3	67.9-70.8	68.1-70.8	66.8-69.9	81.5-83.9	76.2-79.3	81.9-85.4	82.4-85.7	83.1-88	83.6-100 90.9-100	73.5-76.6	73.4-77.5
I12	80.6-82.4	81.1-83.6	70.2-72.9	70.3-71.9	67.5-68.9	85.2-86.4	79.5-81.1	85.4-87.2	81.9-83.4	84.7-89	82.6-86.2	83.3-100 95.2-100	76.7-80.8
I13	78.8-81.6	78.3-82.9	66.5-71.4	65.2-69.1	64.5-68.5	81.7-85.6	77.2-80.8	81.5-85.9	82.4-86.7	83.4-92.3	79.6-85.7	84.7-92.1	80.7-100 88.5-100

Chapter 4: Rapid Detection and Occurrence of Porcine Rotavirus A, B, and C by RT-qPCR in Diagnostic Samples

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Introduction

Rotaviruses (RVs) belong to the *Reoviridae* family and are a leading cause of severe diarrhea in many animals. The non-enveloped, 11-segmented double stranded (ds) RNA virus has a triple layered protein capsid (Estes and Greenberg, 2013). RVs are classified into eight groups or species (A-H) based on serological assays and sequencing of the viral protein 6 (VP6) (Matthijnssens et al. 2012; Estes and Greenberg, 2013).

Currently, five of the eight RV species (RVA, RVB, RVC, RVE, and RVH) have been detected in pigs (Saif et al. 1980; Wakuda et al. 2011; Estes and Greenberg, 2013) Considered the most common and pathogenic RV infection, swine RVA was first identified in 1975 and had been well documented as an important cause of swine diarrhea (Ciarlet et al. 1995; Ciarlet et al. 2002; Miyazaki et al. 2011; Miyazaki et al. 2013; Amimo et al. 2013a). While RVB was identified only 5 years after porcine RVA, the inability of RVB to grow in cell culture has hindered its further serological and molecular characterization (Bridger and Brown, 1985; Theil et al. 1985; Bridger. 1994; Sanekata et al. 1996). While porcine RVB reports have been sporadically published, until recently, RVB was not considered an important cause of pig diarrhea (Kuga et al. 2009; Marthaler et al. 2012). RVC was first discovered in a piglet with diarrhea in 1979 (Saif et al. 1980) Although a few swine RVC strains have been adapted to grow in cell culture, routine RVC propagation in cell culture is difficult(Saif et al. 1988; Tsunemitsu et al. 1991; Saif et al. 1996; Marthaler et al. 2013; Amimo et al. 2013b). Swine RVC can cause subclinical or severe gastroenteritis infections and can be associated with sporadic cases or large outbreaks (Saif et al. 1980; Bohl et al. 1982; Collins et al. 2008). RVE has only been identified from a single porcine sample, rendering its epidemiological prevalence or importance in question (Chasey et al. 1986). The first detection of swine RVH was in 1999 in Japan (Wakuda et al. 2011), followed by 3 additional RVH strains isolated in 2012 in Brazil. Recently, the widespread distribution of swine RVH strains in the US was reported in multiple age groups (Marthaler et al., submitted), although its role in pathogenesis is still unknown.

The prevalence of RVA, RVB, and RVC has been difficult to estimate because of the lack of proper diagnostic assays. Electron microcopy lacks the sensitivity, specificity, and the ability to discriminate RV species. While several commercial serological assays are available for RVA, commercial RVB and RVC antibody detection assays are unavailable. Conventional gel-based RT-PCR assays are an excellent tool to diagnose RV infections, but are time consuming and not very sensitive. A multiplex RVA and RVC RT-qPCR and a RVB RT-qPCR were developed to rapidly detect and distinguish RVA, RVB, and RVC infections to understand the causative agent of diarrhea.

Material and Methods

Samples

Swine samples (n=7508), collected from the US, Mexico, and Canada, were submitted to the University of Minnesota Veterinary Diagnostic Laboratory for enteric pathogen screening between December 2009 and October 2011. Samples were categorized by age: 1-3 days (neonatal piglets), 4-21 days (suckling piglets), 22-55 days (nursey pigs), >55 days (finishing pigs), and unknown age. Submitted samples may contain demographic information including: animal age, state of origin, and clinical signs of weight-loss and diarrhea. The sample set and extraction were described earlier (Marthaler et al. 2013). In addition, 38 swine RVA and 40 RVC positive samples were identified by conventional RT-PCR method as described previously (Gouvea et al. 1991; Matthijnssens et al. 2008a). The RVB RT-qPCR assay was tested with the previously identified positive 81 RVB samples (Marthaler et al. 2012).

Qualitative RT-qPCR

Qualitative RT-qPCR (RT-qPCR) primer and hydrolysis probes were designed based on RVA, RVB, and RVC VP6 sequences available from GenBank (Table 4.1). The RVA and RVC primers and hydrolysis probes were multiplexed into a single RT-qPCR (RVAC RT-qPCR assay). A few months after using the RVAC RT-qPCR assay, RVB positive samples were detected that were negative for RVA and RVC (Marthaler et al. 2012). Although attempts were made to multiplex the RVB-specific RT-qPCR with the

RVAC RT-qPCR, the RVB RT-qPCR assay required lower annealing temperature than the RVAC RT-qPCR, which prevented the multiplexing for all three rotavirus species into one RT-qPCR assay. The RVAC and RVB RT-qPCR assays utilized the Ambion Path-ID™ Multiplex One-Step RT-PCR Kit® (Life Technologies, Austin, TX) described in Table 4.2. The RVAC RT-qPCR assay used the following thermal cycling conditions on the 7500 Fast Real-Time PCR System (Life Technologies, Austin, TX): reverse transcription, 10 min at 45°C; Taq activation, 10 min at 95°C; followed by 45 cycles of 15 sec at 95°C, and 45 sec at 60°C. The RVB RT-qPCR followed the previous thermal cycling conditions, with a modification of the annealing temperate to 50°C. Although the RT-qPCRs were run for 45 cycles for sigmoidal curve visualization, samples with Cq values >40 were considered RV negative. The RVAC and RVB RT-qPCR assays were tested against 60 porcine pathogens (Porcine Cytomegalovirus, North American Porcine Reproductive and Respiratory Syndrome, European Porcine Reproductive and Respiratory Syndrome, Pseudorabies virus, Swine Influenza virus H1, Swine Influenza virus H2, Swine Influenza virus H3, Transmissible Gastroenteritis virus, Porcine Respiratory Corona virus, Porcine Hemagglutinating Encephalomyelitis virus, Encephalomyocarditis virus, Porcine Enterovirus, Classical Swine Fever, Hepatitis E virus, Porcine Parvovirus Type I, Porcine Adenovirus, Porcine Circovirus Type I, Porcine Circovirus Type II, Porcine Picornavirus, Porcine Lymphotropic Gamma Herpes Virus 1, Porcine Lymphotropic Gamma Herpes Virus 2, Porcine Hokovirus, Beta-hemolytic *Escherichia coli,* Non-beta-hemolytic *Escherichia coli, Pasteurella multocida, Salmonella cholerasuis, Actinobacillus pleuropneumoniae, Bordetella bronchiseptica, Clostridium perfringens type A, Clostridium perfringens type C, Salmonella typhimurium, Brachyspira hyodysenteriae, Brachyspira pilosicoli, Brachyspira murdochii, Brachyspira intermedia, Brachyspira innocens, Actinobacillus suis, Actinobacillus rossi, Actinobacillus minor,,Actinobacillus indolicus,Actinobacillus equuli, Actinobacillus pyogenes, Streptococcus suis, Enterococcus durans, Yersinia enterocolitica, Campylobacter coli, Campylobacter jejuni, Staphylococcus aureus, Mycoplasma hyorhinis, Mycoplasma hyopneumoniae, Mycoplasma hyosynoviae, Haemophilus parasuis, Leptospira species, Erysipelothrix rhusiopathiae,*). gBlock Gene Fragments

(IDT, Coralville, IA) containing the RVA, RVB, and RVC RT-qPCR targets were purchased to estimate the detection limit of the RT-qPCR assays.

Analysis of Samples

The RV infectious status of the samples was defined as RVA, RVB, RVC, RVAB, RVAC, RVBC, and RVABC. Counts of positive and negative samples were performed to calculate RVA, RVB, and RVC, RVAB, RVBC, RVAC, and RVABC infection percentage.

Results

Development of the RT-qPCR Assays

Individual RVA and RVC RT-qPCR assays were designed, but were combined and optimized into the RVAC RT-qPCR. During optimization of the RVAC RT-qPCR assay, 6 samples had sigmoidal curves below the threshold. Sequencing of the 6 samples indicated that in the A-1 hydrolysis probe, nucleotide positions 13 and 15 were an A and T, instead of G and Y, respectively (Table 4.1). Hydrolysis probe A-2 was designed and incorporated in the RVAC RT-qPCR assay. The swine RVB RT-qPCR assay was problematic to design due to the genetic diversity of the VP6 gene segment. The RVB RT-qPCR assay contained forward primers A and B, reverse A and the hydrolysis probe and detected the previously describe 81 RVB positive samples.

For the RVAC RT-qPCR assays, the RVA standard curve had a slope of -3.345, which corresponded to 99.03% efficiency while the RVC standard curve had a slope of -3.382, corresponding to 97.45% efficiency (Figure 4.1). The RVB standard curve generated a slope of -3.417, corresponding to 96.17% efficiency. The RT-qPCR assays had an estimated detection limit of approximately 40 DNA copies per reaction. The RT-qPCR assays did not detect 60 other known porcine pathogens tested, and did not cross-react with RVA, RVB, or RVC.

Percentage of RVA, RVB, and RVC infections in Pigs

Between December 2009 and October 2011, 7508 porcine intestinal samples, fecal

samples or rectal swabs were tested for RVA, RVB, and RVC by RT-qPCRs. Table 4.3 illustrates the distribution of the 7508 samples across the 4 age groups: 1-3 days (n=954), 4-20 days (n=2144), 21-55 days (n=2538), and >55 days (n=1207) while the ages of the pigs were unknown for the remaining 665 samples. Of the 7508 samples, 4465 samples (62%) were positive for RVA, 2388 samples (33%) were positive for RVB, and 3833 samples (53%) were positive for RVC, while 1350 samples (18%) were negative for RVA, RVB, and RVC. As expected, RVA infections were common across all age groups but the highest percentage (84%) of RVA infections was detected in the 21-55 day age group. RVB and RVC infections were also common, but RVB was most prevalent (50%) in the >55 day age group, while the highest percentage (62%) of RVC infections was detected in the 1-3 day age group.

The distribution of RV infections across all age groups, including exclusive infection by RVA, RVB or RVC and any specific combination of RV co-infection is shown in Figure 4.1. The highest percentages of RVA only infections were detected in the 4-20 and 21-55 day age groups (20% in each age group). The highest percentage of RVB only infection was detected in the >55 day age group (7%), indicating that the majority of the samples in this age group represented co-infection of RVB with either RVA or RVC. In turn, the highest percentage of only RVC infection was detected in the 1-3 day age group (46%). In the 1-3, 4-20 and 21-55 age groups, co-infections of RVA and RVC were the most common dual co-infection, 11%, 13%, and 22%, respectively. The highest percentage of RVA and RVB co-infections were detected in the 21-55 and >55 day age groups (12% in each age group). The percentage of RVB and RVC co-infections was rather low (2-3%) across all age groups, except in the >55 day age group (7%). Co-infections with RVA, RVB, and RVC were predominant (30%) in the 21-55 day age group.

Discussion

A RVAC and RVB RT-qPCR assays was described to detect porcine rotaviruses. Since RV species are classified by the VP6 (Matthijnssens et al. 2008b; Estes and Greenberg,

2013), RT-qPCR assays targeting the VP6 segment generated a species-specific assay. While a gBlock Gene Fragment was used to estimate the detection limit of the RT-qPCR assay, the gBlock Gene Fragment does not account for any variation in the reverse transcription reaction, and the can only serve as a reference. The RT-qPCR assays did not cross-react with the 60 common swine pathogens and specifically detected RVA, RVB, and RVC only.

Due to the genetic diversity of swine RV species, designing RT-qPCR can be difficult. During RT-qPCR re-optimization with the hydrolysis probe A-2 RVA, an increased concentration of 800 nm reduced the sigmoid curve of 5 RVA samples below the threshold while decreasing the hydrolysis probe A-2 concentration to 200 nm increased sigmoidal curves of the 5 RVA samples. In the multiplex RVAC RT-qPCR, increasing the RVA and RVC primer concentrations to 600 nm slight improved the RVA and RVC Cq value. The initial RVB RT-qPCR only detected 76 samples positive samples. Introduction of the reverse primer B at 800 nm and reducing the thermal cycler's annealing temperature to 50°C resulted in the detection of all 81 positive RVB samples. Optimization and rigorously validation of RT-qPCR is necessary and must be completed before implementation the RT-qPCR for diagnostic testing.

Accurate pathogen detection associated with clinical disease is important in preventing and controlling disease, especially for pig farmers. Commercial swine RVB and RVC antibody detection assays are lacking, and electron microcopy is not able to differentiate different RV species. RT-qPCR assays are a fast and accurate method to detect and distinguish RV species. Although many manuscripts have reported detection of porcine RVA, RVB, and RVC by conventional RT-PCR assays, RT-qPCR assays are more sensitive to detect porcine RVs(Gouvea et al. 1990; Gouvea et al. 1991; Marthaler et al. 2012). Loop-mediated isothermal amplification (LAMP) has gained popularity to detect pathogens due to the minimum requirements necessary to run the assay. However, LAMP lacks sensitivity compared to real time PCR (Harper et al. 2010). Currently, the US swine veterinary diagnostic laboratories are utilizing RT-qPCR assays, not LAMP, to detect swine pathogens.

Since RVB and RVC have been identified as important swine enteric pathogens, all future prevalence swine RV studies should include RVB and RVC. Current porcine RVA epidemiological studies cannot be applied to porcine RVB and RVC since each RV species had a high detection rate in different age groups, and therefore, most likely different epidemiology. While RVA, RVB and RVC were all detected at high percentages in the 21-55 day age group (84%, 47%, and 59%, respectively), RVB was associated more with older pig infection while RVC was associated with baby piglet infections. These epidemiological findings are important and need to be addressed in future RV studies and to ultimately prevent RVB and RVC infections.

Conclusion

In conclusion, a swine RVAC multiplex RT-qPCR assay and RVB RT-qPCR assay was designed, which is a rapid and sensitive diagnostic tool to detect swine RVA, RVB, and RVC. The assays are currently in use in our laboratory, which serves as a major diagnostic laboratory for veterinary samples in the US. Swine RVA, RVB, and RVC are important enteritis pathogens of swine, each RV species have is own etiology. Future swine RV research should include RVA, RVB, and RVC to understand complex interactions of swine RVs.

Figure 4.1. Standard curve of the RVAC and RVB RT-qPCR assays.

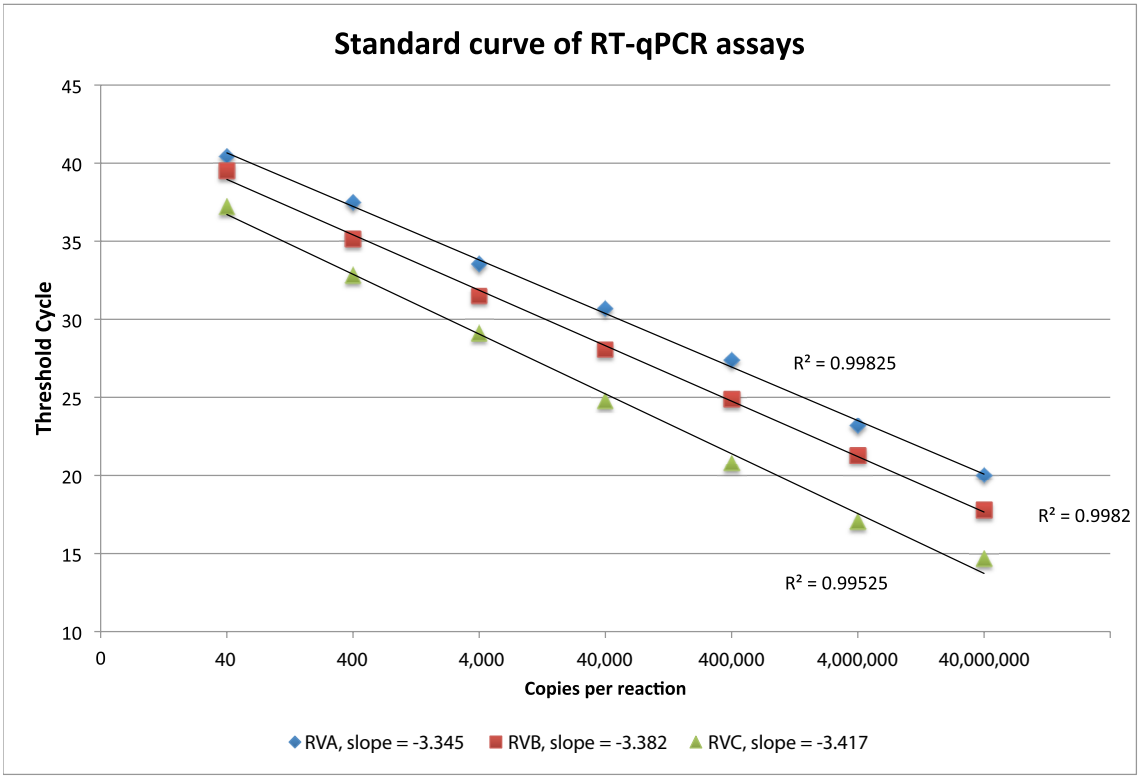

Figure 4.2. Distribution of RVA, RVB, and RVC by age.

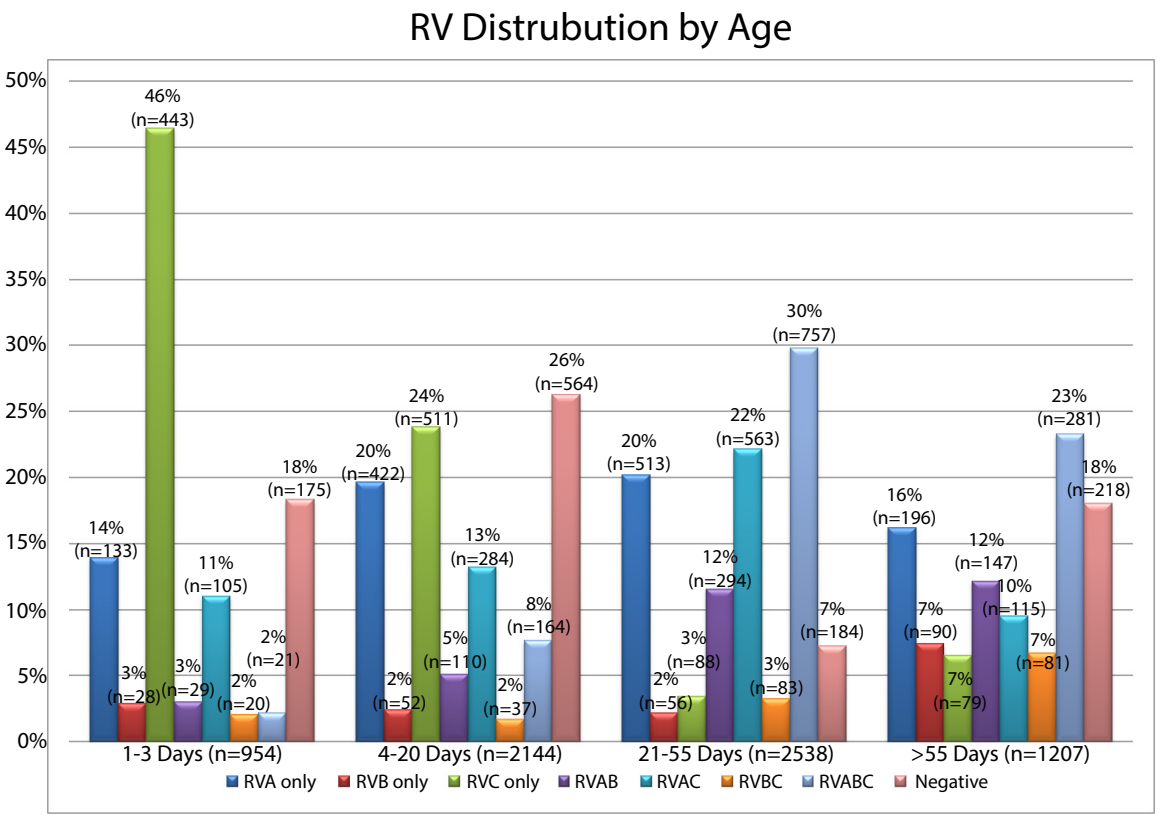

Table 4.1. List of RVA, RVB, and RVC primer and hydrolysis probes.

RV Species	Primer sequence
Porcine RVA	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 **A**A**T** TTT CAA AC-MGB-3'
Porcine RVC	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 CGC ATG A-TAMRA-3'
Porcine RVB	Forward A Primer 5'-GGT TTA AAT AGC CCA ACC GGT GC-3'
	Forward B Primer 5'-GGT TTA AAT AGC CCA ACC GAC GC-3'
	Reverse A Primer 5'-TGC AAT TTR ATG CAT GCG TT-3'
	Reverse B Primer 5'-GTR TTY AAA TTS GTR TTT GGC GCT A-3"
	Probe 5'-FAM-AGC ATG GAT CTG ATY GAA ACR GT-MGB-3'

Table 4.2. RVAC and RVB RT-qPCR components

Porcine Rotavirus A and C Multiplex RT-qPCR

Component	Stock Concentration	Volume (µl)	Final Concentration
Porcine A-1 Probe	5 µM	2	400nM
Porcine A-2 Probe	5 µM	1	200nM
Porcine A Forward Primer	40 µM	0.375	600nM
Porcine A Reverse Primer	40 µM	0.375	600nM
Porcine C Probe	5 µM	2	400nM
Porcine C Forward Primer	40 µM	0.375	600nM
Porcine C Reverse Primer	40µM	0.375	600nM
2X Multiplex RT-PCR Buffer	2X	12.5	
Multiplex RT-PCR Enzyme Mix		1	
Final Master Mix Volume		**20**	
RNA template		**5**	
Total RT-PCR reaction		**25**	

Porcine Rotavirus B real time RT-qPCR

Component	Stock Concentration	Volume (µl)	Final Concentration
Forward A	40 µM	0.07	100 nM
Forward B	40 µM	0.07	100 nM
Reverse A	40 µM	0.13	200 nM
Reverse B	40 µM	0.5	800 nM
Probe	10 µM	0.5	200 nM
2X Multiplex RT-PCR Buffer	2X	12.5	
Multiplex RT-PCR Enzyme Mix		2.5	
Rnase, Dnase free water		3.73	
Final Master Mix Volume		**20**	
RNA template		**5**	
Total RT-PCR reaction		**25**	

Table 4.3. Distribution of Positive and Negative samples by Age.

	1-3 Days (n=954)			**4-20 Days (n=2144)**			**21-55 Days (n=2538)**			**>55 Days (n=1207)**			**Total (n=7508)**	
	N+	N-	% +	N+	N-	%	N+	N-	%	N+	N-	%	N+	%
Total RVA	288	666	30%	980	1164	46%	2127	411	84%	739	468	61%	4465	62%
Total RVB	98	856	10%	363	1781	17%	1190	1348	47%	599	608	50%	2388	33%
Total RVC	589	365	62%	996	1148	46%	1491	1047	59%	556	651	46%	3833	53%

Chapter 5: Phylogenetic Analysis and Classification of Porcine Rotavirus VP7 C Sequences from the United States and Canada

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Introduction

Rotaviruses (RVs) belong to the *Reoviridae* family, possess a genome composed of 11 dsRNA segments, and are classified into eight groups or species (RVA-RVH) based on their antigenic properties and sequence diversity of the inner viral capsid protein 6 (VP6) (Both et al. 1994; Estes and Kapikian, 2007; Matthijnssens et al. 2012). RV species share features such as the same morphology (a wheel-like appearance by negative contrast electron microscopy), a similar genomic organization, and a genome encapsulated in a triple layered capsid (Estes and Kapikian, 2007). The outer capsid components VP7 and VP4 of RVA elicit serotype-specific neutralizing antibodies and have been used to develop a dual classification system (G and P-types, respectively) (Estes and Kapikian, 2007). RVA strains are well known for their high prevalence and pathogenicity in humans and many other animal species. RVB, RVC, and RVH are also known to infect humans and a limited number of animal species. RVD, RVE, RVF and RVG have only been detected in birds or pigs while no human infections have been reported to date (Estes and Kapikian, 2007; Martella et al. 2010; Matthijnssens et al. 2010b; Wakuda et al. 2011). RVA, RVB, and RVC are known to frequently infect pigs, whereas only single reports of RVE and RVH infecting pigs have been published (Bohl et al. 1982; Chasey et al. 1986; Janke et al. 1990; Wakuda et al. 2011).

Initially named pararotavirus, RVC was first detected in a 27-day old piglet with diarrhea from a herd in Ohio in 1980 (Saif et al. 1980). Additionally, RVC has been shown to cause diarrhea and lesions of both the small and large intestine in experimentally infected gnotobiotic piglets (Saif et al. 1980; Bohl et al. 1982). RVC infections can be subclinical or cause severe gastroenteritis in pigs of all ages and can be associated with sporadic episodes or large outbreaks (Collins et al. 2008). In addition to pigs, RVC is a known cause of viral gastroenteritis in a variety of animals, including cattle (Tsunemitsu et al. 1991; Chang et al. 1999; Mawatari et al. 2004), dogs (Otto et al. 1999), ferrets (Torres-Medina. 1987), and also humans (Rodger et al. 1982; Rahman et al. 2005; Banyai et al. 2006; Castello et al. 2009; Mitui et al. 2009; Araujo et al. 2011).

Due to difficulties in propagating RVCs in cell culture, a proper serological typing assay has not been developed to analyze RVCs (Saif et al. 1988; Tsunemitsu et al. 1991; Fujii et al. 2000). In 2007, a proposed RVC VP7 sequence-based classification system, using an 89% amino acid percent identity cut-off value was developed to distinguish at least 6 RVC G genotypes (Martella et al. 2007b). RVC genotypes G1 and G2 were initially assigned to porcine strain Cowden and bovine strain Shintoku, respectively, whereas the prototype porcine RVC strain HF was assigned to the G3 RVC genotype (Tsunemitsu et al. 1992; Tsunemitsu et al. 1996). The VP7 genes of human RVC strains show a high degree of similarity and cluster together generating a single G4 RVC genotype. The porcine strain 134/04-18 was assigned to the G5 genotype, and porcine strains 344-04-7, 43/06-22, 134/04-2, and 43/06-16 constituted the G6 genotype, while the porcine strain 42/05-21 remained unassigned (Jiang et al. 1995; Jiang et al. 1996; Adah et al. 2002; Schnagl et al. 2004; Rahman et al. 2005; Banyai et al. 2006; Abid et al. 2007; Kuzuya et al. 2007; Martella et al. 2007b; Khamrin et al. 2008; Castello et al. 2009; Medici et al. 2009; Mitui et al. 2009; Araujo et al. 2011; Moon et al. 2011). Sequencing analyses of the human Ehime (G4PX), bovine Shintoku (G2PX) and porcine Cowden (G1PX), WH (G1PX), and HF (G3PX) RVC strains revealed a minimum of 8 variable regions (VR-1 to VR-8) in the RVC VP7 protein, much like it has been demonstrated for the RVA VP7 protein (Nishikawa et al. 1989; Tsunemitsu et al. 1992; Tsunemitsu et al. 1996; Ciarlet et al. 1997).

To date, several hundred RVA genomes have been completely sequenced (Matthijnssens and Van Ranst, 2012). In 2008, a RVA classification system for all 11 genome segments was proposed, using a nucleotide cut-off values to distinguish genotypes for each gene segment based on phylogenetic analyses and pairwise sequence identity profiles (Matthijnssens et al. 2008b). RVAs belonging to the same G genotype share at least 80% nucleotide similarity while an 89% amino acid correlation was found between G serotypes and G genotypes (Matthijnssens, et al. 2008). Subsequently, the Rotavirus Classification Working Group (RCWG) was established to maintain the guidelines and formally recognize new RVA genotypes. More recently, in conjugation

with the RCWG, we used the same nucleotide sequence based approach to establish an 80% nucleotide cut-off value to distinguish 20 VP7 G-genotypes for RVB strain (Marthaler et al. 2012).

At the University of Minnesota Veterinary Diagnostic Laboratory, it became apparent that non-RVA strains were causing enteritis and clinical disease in very young pigs (≤3 days) in multiple locations across the United States. A RVC RT-PCR was developed to study the ecology of porcine RVC infections (Marthaler et al. 2012). In the present study, we investigated the genetic diversity of RVC strains associated with clinical signs of diarrhea and weight loss in pigs by sequencing the VP7 encoding gene segment of porcine RVC-positive samples from eight American states and a Canadian province in 2009, 2010, and 2011, generating 70 RVC VP7 sequences. Using the novel sequence data generated in this study, the proposed 89% amino acid classification cut-off value for RVC VP7 genotypes as described by Martella and colleagues (Martella et al. 2007b) was complemented with an 85% nucleotide cut-off value based on pairwise identity frequency profiles and phylogenetic analyses, resulting in 9 VP7 RVC genotypes, G1 to G9.

Material and Methods

Origin of samples

The University of Minnesota Veterinary Diagnostic Laboratory routinely receives feces, fecal swabs, intestinal, and lung tissue samples for the diagnosis of swine disease. Clinical signs may or may not be reported on the submission forms. In general, the samples are rarely complemented with serum and may be a composite of more than one animal. Respiratory and digestive tissue samples from the same pigs arrive in separate bags. If the respiratory and digestive tissues are received in one bag, the samples are not tested due to the potential cross contamination of pathogens between tissues. Between December 2009 and October 2011, 7,520 porcine samples from herds in the US and Canada were submitted to our laboratory for analysis, and 3,447 were confirmed to contain RVC by the RVC VP6 RT-PCR (Marthaler et al., 2012). We determined the VP7

gene segment of 70 porcine RVC strains from 8 American states (Illinois, Colorado, Ohio, North Carolina, Minnesota, Nebraska, Tennessee, and Arkansas) and one Canadian province (Manitoba), as a validation of the new RVC VP6 RT-PCR, or upon client request, to better understand RVC ecology within each swineherd. Lung tissues with histological lesions, but negative for routinely tested respiratory pathogens (influenza A virus, porcine reproductive and respiratory syndrome virus, porcine circovirus type 2, *Mycoplasma hyopneumoniae, M. hyorhinis, M. hyosynovaie, and H. parasuis*), are also screened for RVA, RVB, and RVC by RT-PCR independently from the RV result in fecal or intestinal samples (Marthaler et al. 2012). When RVC was detected for the first time in lung tissue (IL10-31, G6PX), the available serum sample was also screened for RVC by RT-PCR in search of a RVC viremia. However, because serum is rarely submitted along with feces, fecal swabs, intestinal, and lung tissues, extensive screening of RVC in serum by RT-PCR cannot routinely be performed.

Histopathology

Small intestine and lung tissue samples were fixed in 10% neutral buffered formalin, embedded in paraffin, and stained using Harris's hematoxylin and eosin (HE) (Marthaler et al. 2012).

Extraction of genomic material, reverse transcriptase-polymerase chain reaction amplification, and sequence analysis

A 2 mL solution of gamma-irradiated Hyclone donor equine serum (Thermo Fisher Scientific, Waltham, MA) and approximately 3 grams of each sample were emulsified, centrifuged at 4,200 rpm for 1 hour, and the supernatant was transferred into a new vial. Viral RNA was extracted using an Ambion MagMax extraction kit (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. The VP7-specific RT-PCR was carried out using previously published protocol by Marthaler and colleagues and previously published VP7 primers by Rahman and colleagues (Rahman et al. 2005; Marthaler et al. 2012). Four µL of the PCR product was mixed with 2 µL of loading dye (Sigma, St. Louis, MO) and 4 µL of TAE buffer. Then, the mixture was loaded into a 2%

ethidium bromide stained agarose gel and run for 30 minutes at 140V. The PCR bands were visualized under UV light, and the remaining PCR product was purified using the QIAquick PCR purification kit (Qiagen/Westburg), under recommended conditions, and sequenced using Sanger technology at the University of Minnesota Genomic Center (a fully automated ABI 3730xl DNA Analyzer (Perkin-Elmer) with ABI BigDye Terminator version 3.1 chemistry (Perkin-Elmer). The Seqman 8.0 program of the Lasergene software (DNASTAR, Madison, WI) was used to analyze sequences during primer walking to ensure double coverage of each sequence. Sequence alignments were performed using Clustal W (Thompson et al. 1994) while MEGA 5 (Tamura et al. 2011) was used to conduct phylogenetic analysis. Genetic distances were calculated using the Kimura 2-parameter correction at the nucleotide level (Matthijnssens et al. 2008b). Construction of phylogenetic trees utilized the Neighbor-joining method (Saitou and Nei, 1987). Geneious Pro produced the amino acid identity profile (sliding window of 1) (Supplemental data 3) (Drummond et al. 2011). The percentage identities of the RVC VP7 complete ORFs from this study (n=70) (Table 2) and published sequences in GenBank (n=82; Solano, AF120478.1; T97/126, AF225552; WH, U31749; JA398231; Y11-1, AB648916; CUK-5, HQ833830; CAU10-312, HQ896715; Moduganari, AF323979; Shintoku, U31750; HF, U31748; CUK-6, HQ323754; YNR001, HQ185657; BK0830, HQ185678; BS347, HQ185637; Icheon, GU199223; KA4/1017, AF225562; Uppsala/1004, AF225560; T97/330, AF225558; T97/258, AF225556; T97/190, AF225554; KA4/1018, AF225563; KA4/949, AF225561; T97/451, AF225559; T97/277, AF225557; T97/194, AF225555; T97/167, AF225553; 88-220, M61100; Cowden, M61101; Arg1563, FJ477249; Arg165, FJ477247; Arg1731, FJ477250; Arg996, FJ477248; Arg47, FJ477246; 43/06-16, EF464656; 344-04-7, EF464654; 118/05-4, EF464652; 42/05-21, EF464650; 118/05-18, EF464648; 43/06-22, EF464657; 134/04-2, EF464655; 134/04-18, EF464653; 118/05-1, EF464651; 118-05-27, EF464649; DhakaC13, AY754824; V966, AY803726; CHRV/A90L, AY392446; CHRV/A93M, AY392447; Jajeri, AF323982; GUP188, AB499615; Y09-1, AB533519; Y08-3, AB533517; Y08-1, AB533515; Y09-2, AB533520; Y08-4, AB533518; Y08-2, AB533516; Y06-1, AB533514; CMH004/03, EF641111; 1GA/05/Cork, EU624403;

OH567, AB281654; OK595, AB281652; AE53, AB281655; T360, AB281653; OK239, AB281651; 208, FJ807866; OT-99, AB086969; KW290, AB086967; OK459, AB086965; KC48, AB086963; KW408, AB086968; KU166, AB086966; I57, AB086964; G7, AB086962; BCN9, AM118022; BCN21, AM118023; BCN6, AM118021; V508, AY803725; V460, AY803724; Yamagata, AB108681; K9304, AB004250; OK450, D87544; OK118, D87543; Sequence 33 from Patent EP2297186, JA398219; Sequence 45 from Patent, EP2297186) were used to achieve the cut-off values for evolution based classification via the pairwise distances program MEGA 5 (Tamura et al. 2011). To construct the pairwise identity frequency graphs, all the calculated pairwise identities were plotted on the X-axis while the frequencies of the calculated pairwise identities were plotted on the Y-axis (Ball. 2005).

Results

Intestinal samples, fecal samples or rectal swabs from pigs of all ages are routinely tested for RVA, RVB, RVC, and Transmissible Gastroenteritis Virus (TGEV) by RT-PCR; other porcine enteric pathogens, such as *Salmonella enterica spp.*, *E. coli*, *C. difficile* and *C. perfringens*, *L. intracellularis* and *Brachyspira spp* are also tested by a variety of methods. Among 7,520 samples submitted from December 2009 to October 2011, RVC was successfully detected in 3,447 samples (46%). From these 3,447 samples, 16% was detected in very young pigs (≤3 day old), 21% in young pigs (4-20 day old), 42% in post-weaning pigs (21-55 day old), 13% in older pigs (>55 day old), and 8% in pigs of unknown age (Figure 5.1). Affected porcine intestinal tissue samples had lesions characterized by villus tip epithelial cell (enterocyte) swelling with cytoplasmic vacuolar change progressing to enterocyte necrosis and sloughing, resulting in denuded villus tips (Figure 5.2). In our experience, these acute intestinal lesions can be lost due to post-mortem autolysis if intestinal samples that are not fixed in formalin within 15 minutes of euthanasia or death. Thirty-four percent (1156/3447) of the RVC positive samples were negative for RVA and RVB, indicating that RVC is an important cause of enteritis in pigs of all ages (Figure 5.3). However, the highest percentage of single RVC infections were found in very young (≤3 days), and young pigs (4-22 days) in 78% and 65% of the RVC

positive samples, respectively, whereas this percentage was much lower (between 6% and 39%) in pigs in the older age groups (Figure 5.3).

Because RVA has been shown to infect extra intestinal organs (Blutt et al. 2003; Azevedo et al. 2005; Zhao et al. 2005; Crawford et al. 2006; Kim et al. 2011), a subset of 635 porcine lung samples with histological lesions of bronchiolitis or alveolitis, but negative for common swine respiratory pathogens (influenza A virus, porcine reproductive and respiratory syndrome virus, porcine circovirus type 2, *Mycoplasma hyopneumoniae, M. hyorhinis, M. hyosynovaie,* and *H. parasuis*), were screened for RVA, RVB and RVC using RT-PCR assays (Marthaler et al. 2012). Our analyses revealed a higher occurrence of RVA (50%) in these lung tissue samples, followed by RVC (10%) and RVB (5%) (Table 5.1). Mixed RV species were also identified in 12% of the samples (Table 1). The RVC VP7 strain (IL10-31, G6PX) was sequenced from a porcine lung tissue sample with histopathological lesions of bronchiolitis, clinical signs of pneumonia (coughing), but negative for common swine respiratory pathogens. Furthermore, a RVC strain (IL10-32, G6PX) was also sequenced from the intestinal sample of the same animal. The corresponding serum sample was also available for testing, but RVC was not detected in this pig's serum sample. The RVC VP7 sequences of IL10-31 and IL10-32 were identical (data not shown), belonging to the G6 genotype.

To better understand the genetic diversity of porcine RVC strains circulating in 30 herds from eight American states and a Canadian province between January 2009 and October 2011, 65 RVC samples were selected for sequencing of the VP7 gene segment, which yielded 70 sequences. However, the samples were obtained from both sub-clinically infected pigs and clinically infected pigs with RV-like histopathological lesions and signs of diarrhea and weight loss. Table 5.2 lists the names of the 70 RVC strains, together with the clinical signs (diarrhea and weight loss), whether or not co-infections with RVA and/or RVB strains were detected by RT-PCR, the age of the host, sample type, G-genotype assigned to RVC strains, state or province of origin, herd number, and GenBank accession number. Of the 70 sequenced RVC strains, 61 were obtained from

pigs with diarrhea, 2 from pigs without diarrhea, and the presence or absence of diarrhea was unknown for the remaining 7 strains. Clinical data on whether or not the pigs lost weight was available for 26 RVC positive samples, and 14 of these samples also contained RVA (n=11) or RVB (n=3) strains. Weight loss was observed in 19 pigs, either infected with the G1 (n=2) or G6 (n=17) RVC strains while 7 pigs did not report weight loss as a clinical sign and were infected with either G1 (n=4) and G6 (n=3) RVC strains (Table 2). Only a single sample contained a RVC strain (MN09-7, G6PX) that was not associated with clinical signs of diarrhea or weight-loss, whereas another single G6 RVC strain (MN09-6, G6PX) was associated with weight-loss in the absence of diarrhea. Since samples submitted to the laboratory are mostly from pigs with gastrointestinal disease it is difficult to establish an association between genotype and disease as normal, uninfected control samples are not routinely available.

Using the 70 complete ORF sequences of the VP7 gene generated from the RVC strains identified in this study and 82 RVC VP7 ORF sequences available from GenBank (2 bovine, 64 human, and 16 porcine; Solano, AF120478.1; T97/126, AF225552; WH, U31749; JA398231; Y11-1, AB648916; CUK-5, HQ833830; CAU10-312, HQ896715; Moduganari, AF323979; Shintoku, U31750; HF, U31748; CUK-6, HQ323754; YNR001, HQ185657; BK0830, HQ185678; BS347, HQ185637; Icheon, GU199223; KA4/1017, AF225562; Uppsala/1004, AF225560; T97/330, AF225558; T97/258, AF225556; T97/190, AF225554; KA4/1018, AF225563; KA4/949, AF225561; T97/451, AF225559; T97/277, AF225557; T97/194, AF225555; T97/167, AF225553; 88-220, M61100; Cowden, M61101; Arg1563, FJ477249; Arg165, FJ477247; Arg1731, FJ477250; Arg996, FJ477248; Arg47, FJ477246; 43/06-16, EF464656; 344-04-7, EF464654; 118/05-4, EF464652; 42/05-21, EF464650; 118/05-18, EF464648; 43/06-22, EF464657; 134/04-2, EF464655; 134/04-18, EF464653; 118/05-1, EF464651; 118-05-27, EF464649; DhakaC13, AY754824; V966, AY803726; CHRV/A90L, AY392446; CHRV/A93M, AY392447; Jajeri, AF323982; GUP188, AB499615; Y09-1, AB533519; Y08-3, AB533517; Y08-1, AB533515; Y09-2, AB533520; Y08-4, AB533518; Y08-2, AB533516; Y06-1, AB533514; CMH004/03, EF641111; 1GA/05/Cork, EU624403;

OH567, AB281654; OK595, AB281652; AE53, AB281655; T360, AB281653; OK239, AB281651; 208, FJ807866; OT-99, AB086969; KW290, AB086967; OK459, AB086965; KC48, AB086963; KW408, AB086968; KU166, AB086966; I57, AB086964; G7, AB086962; BCN9, AM118022; BCN21, AM118023; BCN6, AM118021; V508, AY803725; V460, AY803724; Yamagata, AB108681; K9304, AB004250; OK450, D87544; OK118, D87543; Sequence 33 from Patent EP2297186, JA398219; Sequence 45 from Patent, EP2297186), pairwise identity frequency graphs were constructed on the nucleotide and amino acid level (Figure 4), and a phylogenetic tree was created on the nucleotide level (Figure 5). Based on the pairwise identity frequency graphs and phylogenetic analyses, an 85% percent identity cut-off value on the nucleotide level, and an 89% percent identity cut-off value on the amino acid level were found suitable to divide the phylogenic tree into 9 G genotypes G1 to G9, each of which contain RVC strains isolated from a single host species (G1, G3, G5-G9: porcine, G2: bovine, and G4: human) (Figure 5). In addition to the 9 G-genotypes, 4 larger clades could be observed in the phylogenetic tree composed of: i) G4, G7, G1, G9 and, G5; ii) G8 and G6; iii) G3; and iv) G2. The secondary peaks in the pairwise identity frequency graphs at 84-87% in the upper panel and 82-84% in the lower panel, represent pairwise identities between strains belonging to genotypes from the same larger clades, whereas the large peak at 76-82% in the upper panel and 76-80% in the lower panel, represent pairwise identities between strains belonging to genotypes from different larger clades. Table 5.3 displays the inter- and intra-genotype diversity on the nucleotide and amino acid level. The lowest intra-genotype similarities, on the nucleotide and amino acid level were observed in genotypes G5 (88.6% and 87.5%, respectively) and G6 (88.4% and 85.3%, respectively) with the amino acid percentages slightly below the current 89% amino acid cut-off value. The other seven genotypes showed nucleotide and amino acid values above the proposed percent identity and current amino acid identity cut-off values (Table 5.3). The highest inter-genotype nucleotide similarities were observed between G5 and G9, and G6 and G8, 84.6-85.9% and 83.1-86.1% respectively, with values slightly above the 85% nucleotide cut-off value. The most common VP7 genotypes detected were G6 (70%), followed by G5 (17%), G1 (12%), and G9 (1%) (Table 5.2). In the

majority of the herds, only a single RVC genotype was detected, but RVC strains belonging to two distinct G genotypes were found to co-circulate in herds 10 and 13 (G5/G6), 22 (G5/G9), and 23 (G1/G6).

RVC strain representatives for each genotype and geographical location were selected (both from this study and published in GenBank), and their VP7 amino acid sequences were aligned (Figure 5.6). The 8 variable regions (VR-1 to VR-8) as described by Tsunemitsu and colleagues are boxed in Figure 5.6 (Tsunemitsu et al. 1992). Conserved cysteine residues were located at positions 3, 140, 170, 196, 201, 212, 253, and 258 while additional cysteine residues were located at positions 7 (G2 strains), 10 (G1-G5, some G6, and G7-G9 strains), 14 (G1, G2, and G5-G9 strains), and 44 (G1-G7, and G9 strains). Conserved potential N-linked glycosylation sites were present at residues 67-69 and 225-227, while additional potential N-linked glycosylation sites were located at residues 152-154 (for G4 and some G6 and G9 strains), 274-276 (G2 strains), and 318-320 (some G1 strains). The majority of the conserved amino acid positions possessed a Leucine or Isoleucine (positions 21, 117, 144, 232, 255, 265 and 296), followed by Aspartic acid or Asparagine (positions 95, 174, 226, 233 and 236, while only a single conserved Glutamic acid or Glutamine was observed at position 228 (Figure 5.6). The 152 RVC VP7 sequences used in the phylogenetic analysis also displayed specific amino acid profiles for the 9 proposed G genotypes (Figure 5.7). A histogram showing the amino acid genetic diversity across different G genotypes is located at the top of the figure with valleys (red) indicating regions with a great diversity, whereas peaks (dark green) represent conserved residues, with the C-terminal (260-336) apparently containing more conserved regions.

Discussion

Historically, RVA has been considered the primary causative agent of viral diarrhea; however, RVB and RVC were found to be also important causes of gastroenteritis in swine populations (Marthaler et al. 2012). Previous studies have confirmed that RVC infections can cause both clinical and subclinical infections in pigs, and in this study, we

identified multiple RVC strains genetically distinct from previously known strains (Saif et al. 1980; Bohl et al. 1982; Collins et al. 2008). Interestingly, while the G3 RVC genotype was first described in 1985 in the United States, our study failed to identify any additional G3 RVC strains, which may be a result of sampling bias. While co-infections of RVA, RVB and RVC have been well documented (Collins et al. 2008; Kuga et al. 2009; Marthaler et al. 2012), we cannot rule out the rare, but possible additional infection of RVE or RVH strains and their contribution to virulence. Further research on swine RVA, RVB, RVC, RVE and RVH is still needed to better understand their prevalence, pathogenesis, and relationship among each other.

In pigs, RVC infections are not limited to a single age group. We previously identified RVC in all three age groups; <21 days (suckling piglets), 21-55 days (post-weaning), and >55 days (finishing) (Marthaler et al. 2012), and the current analyses revealed that RVC genotypes G1, G5, G6, and G9 infected all three age groups, implying that RVC G genotypes are also not restricted to single age group. Additionally, 78% of the RVC positive samples in the ≤3 day ago group were negative for RVA and RVB, implying that RVC is an important cause of enteritis in very young pigs. Moreover, continued RVC screening and sequencing in all porcine age groups will be important to enhance our understanding of the genetic diversity of RVC strains, their transmission dynamics and occurrence over time.

The ability of RVA to cause viremia has been well documented in some animals, and the respiratory secretions after infection of the upper and/or lower epithelial cell has been attributed to the viremia (Fragoso et al. 1986; Azevedo et al. 2005; Crawford et al. 2006; Kim et al. 2011). Crawford and colleagues noted an increased level of lymphocytes and macrophages, RVA replication and aerosolization in lung tissue from 4-day old rat pups (Crawford et al. 2006). In our current study, RVA was detected in 50% of the lung tissues screened while RVC was detected in only 10% of the lung samples, suggesting that RVA is the predominant RV capable of causing histopathological lung lesions. RVC was detected in lung tissue with histopathological lesions of bronchiolitis from one pig

with diarrhea and no other pathogens were detected in this pig. Sequence analysis of the VP7 of the RVC strain revealed that the strain belonged to the G6 genotype. The identical VP7 sequence was detected in the intestinal samples from the same pig. To our knowledge, this is the first report of RVC detection in lung tissue. However, the detection of RVC RNA in lung tissue does not provide solid evidence of RVC replication in lung tissue. Further studies are needed to determine the replication, aerosolization, transmission, and viremia of RVC in porcine lung tissue.

With only 2 sequences available, bovine RVCs strains share 93.6% nucleotide and 96.1% amino acid percent identity between each other. Human RVC strains represent nearly half of the sequences in the phylogenetic analysis (49%) and form a genetically conserved genotype (at least 93.2% and 94.3% identical on the nucleotide and amino acid level, respectively), whereas porcine RVC strain demonstrated a genetic identity as low as 71.0% and 68.1% on the nucleotide and amino acid level, respectively. This large genetic diversity observed for porcine RVC strains compared to human and bovine RVC strains, suggest that swine are possibly the main host for RVC strains. The wide spread genetic diversity of porcine RVC strain in the United States may be related to the movement of pigs from nurseries to finishing farms as described for influenza A virus (a segmented RNA virus) by Nelson and colleagues where genetic diversity was associated with the movement of pigs from the Southern region to the Midwest region of the United States (Nelson et al. 2011). To further explore this hypothesis, future research on RVC strains diversity should incorporates both spatial and temporal information to determine if epidemiological factors contribute to RVC pathogenesis and diversity.

A previous phylogenetic study on the VP7 of RVC proposed an 89% amino acid cut-off value resulting in 6 G genotypes while one porcine strain 42/05-21 remained unassigned (Martella et al. 2007b). Here, we propose an 85% nucleotide cut-off value, yielding 9 G genotypes, which is largely in agreement with the previous amino acid based classification. The proposed RVC VP7 nucleotide percent identity cut-off value of 85% is higher than the RCWG's established 80% nucleotide percent identity cut-off

values for VP7 of RVA and RVB (Matthijnssens et al. 2008b; Marthaler et al. 2012). However, the RVA, RVB, and RVC coincidently have an amino acid percent identity cut-off value of 89%. Moreover, the correlation between RVC serotype and genotype has been poorly studied, and fluorescent-focus neutralization assays demonstrated a minimal cross reactivity between the Cowden (pig) and Shintoku (bovine) RVC strains, suggesting that these RVC strains belonged to two different serotypes (Tsunemitsu et al. 1992). Because adapting RVC to cell culture is difficult, the relationship between G genotypes (cut-off value 85% on the nucleotide level and 89% on the amino acid level) with G serotypes will remain unknown until further serological assays are performed. We propose that the RCWG further maintains and updates the newly proposed RVC VP7 G genotype classification system using the 85% nucleotide cut-off value in conjugation with established RVA and RVB genotypes.

In summary, while RVA infections have overshadowed the importance of RVC in swine, RVC infections continue to be a cause of diarrhea in a variety of swine age groups, especially in very young (≤3 days old) and young piglets (4-20 days old). While the correlation between clinical signs and genotype could not be established firmly, genotypes G1, G5, and G6 were most predominant in the porcine population. The identification of RVC in lung tissue highlights the possibly viremic aspect of RVC strains, as is known for RVA strains. Additionally, an 85% nucleotide percent cut-off value was proposed resulting in 9 VP7 G-genotypes.

Figure 5.1. Age distribution of RVC positive samples, n=3,447.

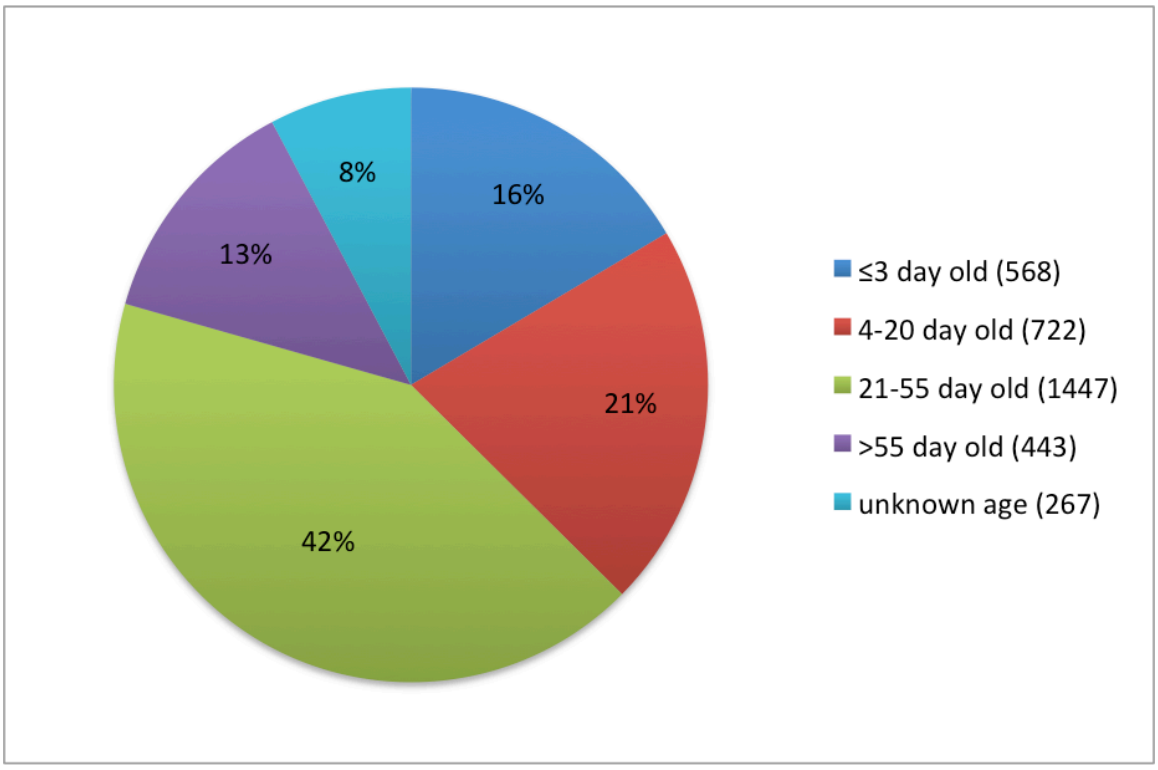

Figure 5.2. Hematoxylin and eosin stained jejunum from a 3-day piglet (10X magnification). Tissue architecture was revealed by eosin staining (pinking) while nuclei were stained with hematoxylin staining (blue). Normal villi (a): The arrows indicate colostrum, which is being absorbed by the villi. Acute enteritis (b): The arrows indicate enterocyte necrosis in the three center villi.

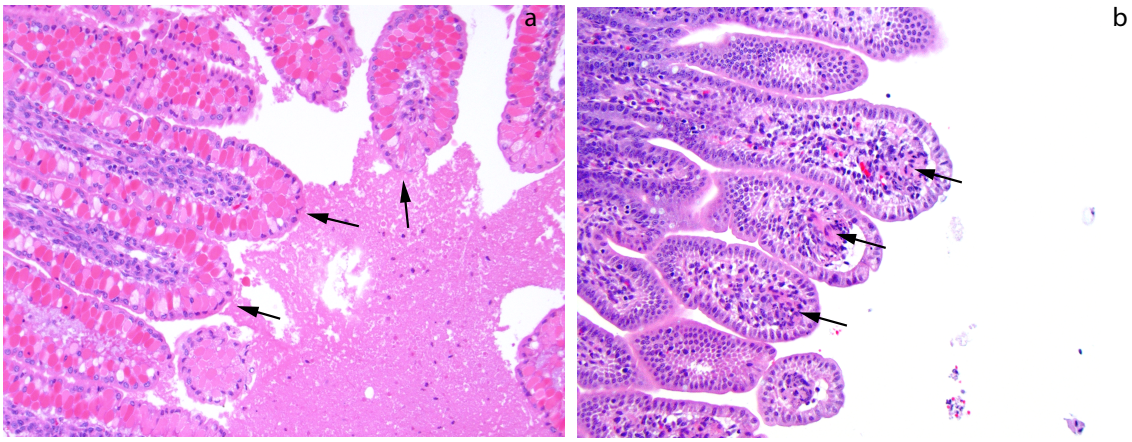

Figure 5.3. Histogram illustrating the co-infection rates of RVC with RVA and/or RVB per age group.

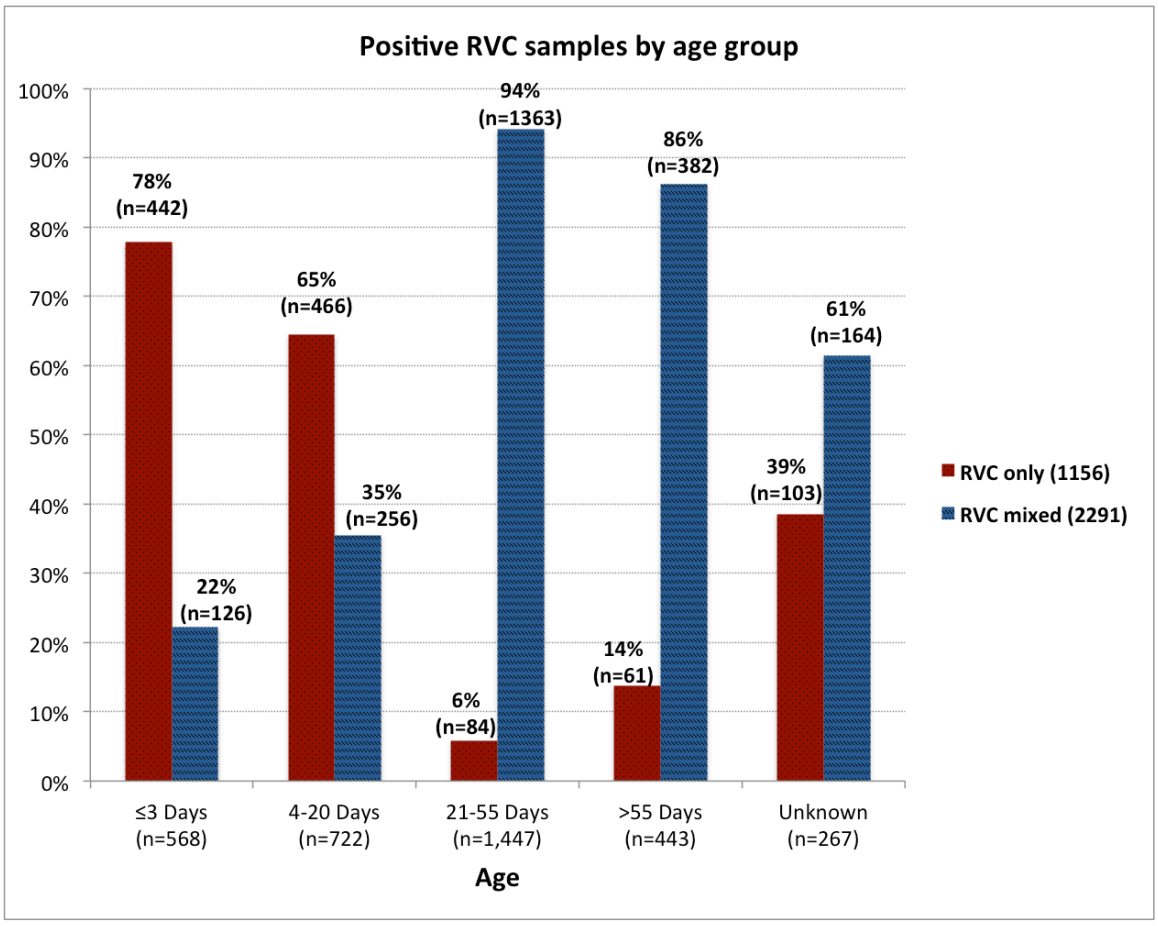

Figure 5.4. Nucleotide and amino acid pairwise identity frequency graphs of the VP7 ORF of 152 RVC strains. The proposed 85% nucleotide (bottom) and current 89% amino acid (top) cut-off value are represented by the vertical lines.

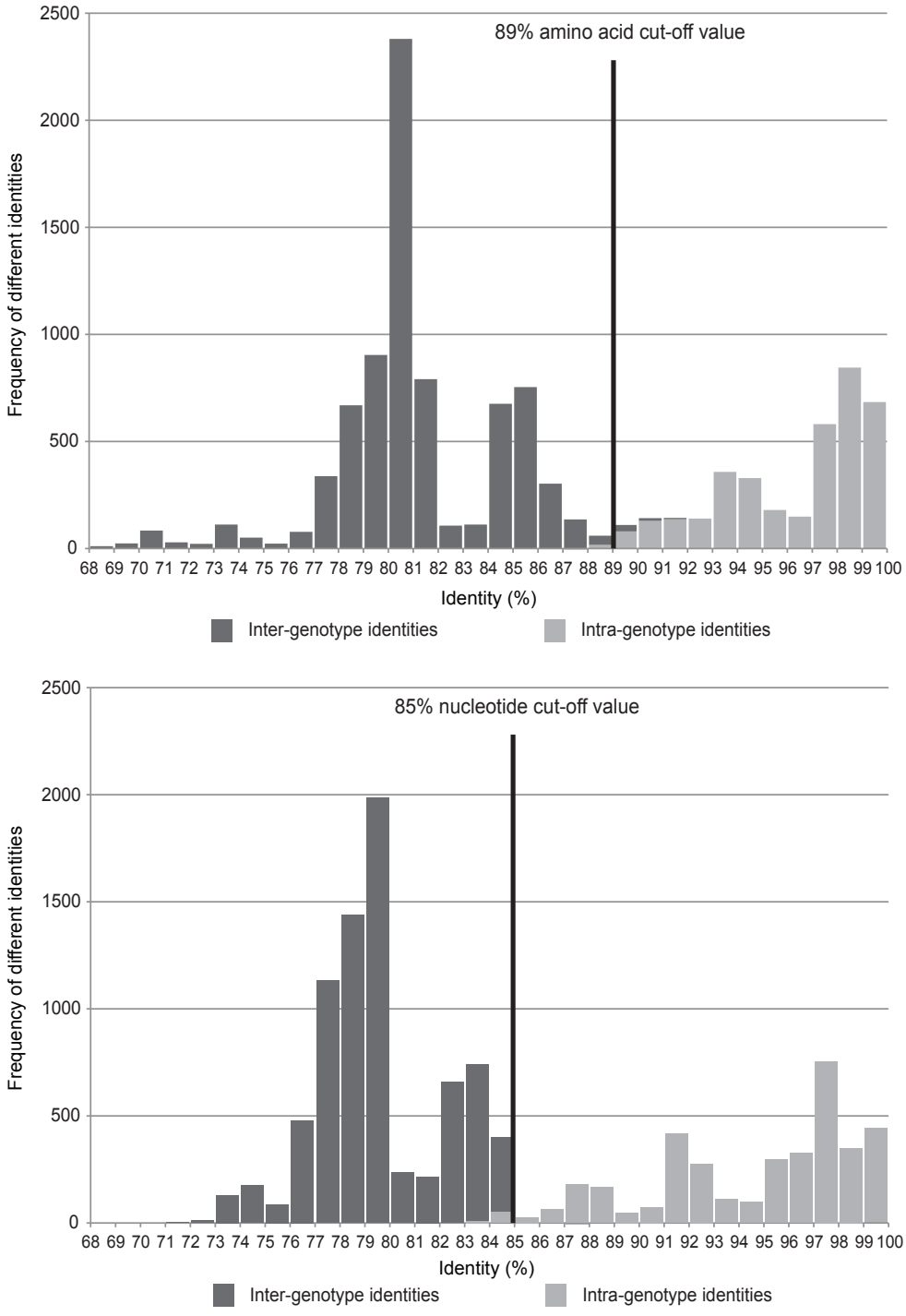

Figure 5.5. Phylogenetic trees of nucleotide VP7 sequences from 152 RVC strains. The sequences from this study are bolded. The G4 cluster was replaced by a triangle. The dashed line represents the 85% nucleotide identity cut-off value, establishing 9 G genotypes. Bootstrap values less than 70% are not shown.

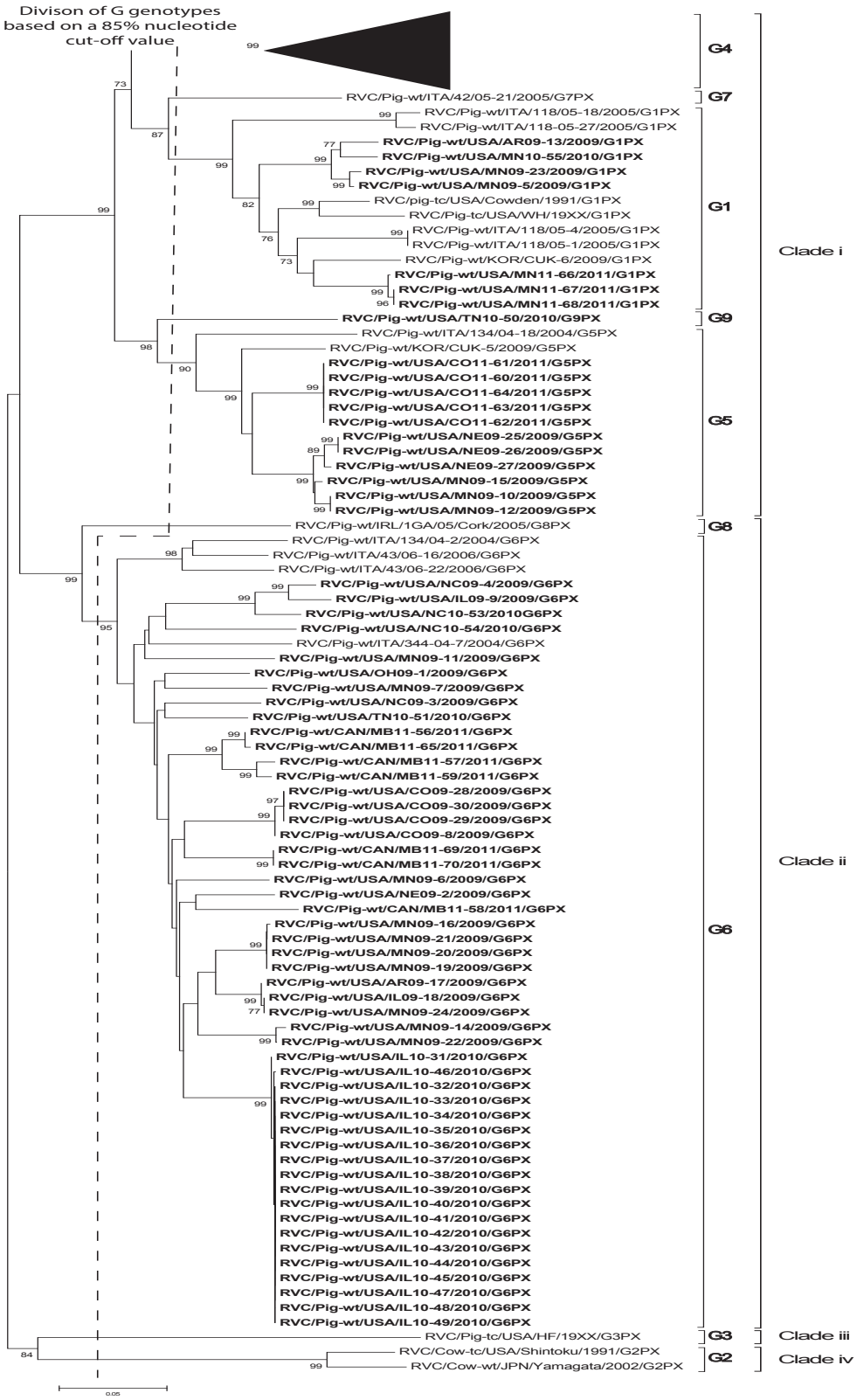
Divison of G genotypes based on a 85% nucleotide cut-off value
G4
G7
RVC/Pig-wt/ITA/42/05-21/2005/G7PX
RVC/Pig-wt/ITA/118/05-18/2005/G1PX
RVC/Pig-wt/ITA/118-05-27/2005/G1PX
RVC/Pig-wt/USA/AR09-13/2009/G1PX
RVC/Pig-wt/USA/MN10-55/2010/G1PX
RVC/Pig-wt/USA/MN09-23/2009/G1PX
RVC/Pig-wt/USA/MN09-5/2009/G1PX
RVC/pig-tc/USA/Cowden/1991/G1PX
RVC/Pig-tc/USA/WH/19XX/G1PX
RVC/Pig-wt/ITA/118/05-4/2005/G1PX
RVC/Pig-wt/ITA/118/05-1/2005/G1PX
RVC/Pig-wt/KOR/CUK-6/2009/G1PX
RVC/Pig-wt/USA/MN11-66/2011/G1PX
RVC/Pig-wt/USA/MN11-67/2011/G1PX
RVC/Pig-wt/USA/MN11-68/2011/G1PX
G1
RVC/Pig-wt/USA/TN10-50/2010/G9PX
G9
RVC/Pig-wt/ITA/134/04-18/2004/G5PX
RVC/Pig-wt/KOR/CUK-5/2009/G5PX
RVC/Pig-wt/USA/CO11-61/2011/G5PX
RVC/Pig-wt/USA/CO11-60/2011/G5PX
RVC/Pig-wt/USA/CO11-64/2011/G5PX
RVC/Pig-wt/USA/CO11-63/2011/G5PX
RVC/Pig-wt/USA/CO11-62/2011/G5PX
RVC/Pig-wt/USA/NE09-25/2009/G5PX
RVC/Pig-wt/USA/NE09-26/2009/G5PX
RVC/Pig-wt/USA/NE09-27/2009/G5PX
RVC/Pig-wt/USA/MN09-15/2009/G5PX
RVC/Pig-wt/USA/MN09-10/2009/G5PX
RVC/Pig-wt/USA/MN09-12/2009/G5PX
G5
Clade i
RVC/Pig-wt/IRL/1GA/05/Cork/2005/G8PX
G8
RVC/Pig-wt/ITA/134/04-2/2004/G6PX
RVC/Pig-wt/ITA/43/06-16/2006/G6PX
RVC/Pig-wt/ITA/43/06-22/2006/G6PX
RVC/Pig-wt/USA/NC09-4/2009/G6PX
RVC/Pig-wt/USA/IL09-9/2009/G6PX
RVC/Pig-wt/USA/NC10-53/2010G6PX
RVC/Pig-wt/USA/NC10-54/2010/G6PX
RVC/Pig-wt/ITA/344-04-7/2004/G6PX
RVC/Pig-wt/USA/MN09-11/2009/G6PX
RVC/Pig-wt/USA/OH09-1/2009/G6PX
RVC/Pig-wt/USA/MN09-7/2009/G6PX
RVC/Pig-wt/USA/NC09-3/2009/G6PX
RVC/Pig-wt/USA/TN10-51/2010/G6PX
RVC/Pig-wt/CAN/MB11-56/2011/G6PX
RVC/Pig-wt/CAN/MB11-65/2011/G6PX
RVC/Pig-wt/CAN/MB11-57/2011/G6PX
RVC/Pig-wt/CAN/MB11-59/2011/G6PX
RVC/Pig-wt/USA/CO09-28/2009/G6PX
RVC/Pig-wt/USA/CO09-30/2009/G6PX
RVC/Pig-wt/USA/CO09-29/2009/G6PX
RVC/Pig-wt/USA/CO09-8/2009/G6PX
RVC/Pig-wt/CAN/MB11-69/2011/G6PX
RVC/Pig-wt/CAN/MB11-70/2011/G6PX
RVC/Pig-wt/USA/MN09-6/2009/G6PX
RVC/Pig-wt/USA/NE09-2/2009/G6PX
RVC/Pig-wt/CAN/MB11-58/2011/G6PX
RVC/Pig-wt/USA/MN09-16/2009/G6PX
RVC/Pig-wt/USA/MN09-21/2009/G6PX
RVC/Pig-wt/USA/MN09-20/2009/G6PX
RVC/Pig-wt/USA/MN09-19/2009/G6PX
RVC/Pig-wt/USA/AR09-17/2009/G6PX
RVC/Pig-wt/USA/IL09-18/2009/G6PX
RVC/Pig-wt/USA/MN09-24/2009/G6PX
RVC/Pig-wt/USA/MN09-14/2009/G6PX
RVC/Pig-wt/USA/MN09-22/2009/G6PX
RVC/Pig-wt/USA/IL10-31/2010/G6PX
RVC/Pig-wt/USA/IL10-46/2010/G6PX
RVC/Pig-wt/USA/IL10-32/2010/G6PX
RVC/Pig-wt/USA/IL10-33/2010/G6PX
RVC/Pig-wt/USA/IL10-34/2010/G6PX
RVC/Pig-wt/USA/IL10-35/2010/G6PX
RVC/Pig-wt/USA/IL10-36/2010/G6PX
RVC/Pig-wt/USA/IL10-37/2010/G6PX
RVC/Pig-wt/USA/IL10-38/2010/G6PX
RVC/Pig-wt/USA/IL10-39/2010/G6PX
RVC/Pig-wt/USA/IL10-40/2010/G6PX
RVC/Pig-wt/USA/IL10-41/2010/G6PX
RVC/Pig-wt/USA/IL10-42/2010/G6PX
RVC/Pig-wt/USA/IL10-43/2010/G6PX
RVC/Pig-wt/USA/IL10-44/2010/G6PX
RVC/Pig-wt/USA/IL10-45/2010/G6PX
RVC/Pig-wt/USA/IL10-47/2010/G6PX
RVC/Pig-wt/USA/IL10-48/2010/G6PX
RVC/Pig-wt/USA/IL10-49/2010/G6PX
G6
Clade ii
RVC/Pig-tc/USA/HF/19XX/G3PX
G3
Clade iii
RVC/Cow-tc/USA/Shintoku/1991/G2PX
RVC/Cow-wt/JPN/Yamagata/2002/G2PX
G2
Clade iv
0.05

Figure 5.6. Amino acid alignment including new and previous described RVC VP7 genotypes. Dots represent identical amino acid; dashes indicate gaps; shaded areas represent cysteine residues; underlined residues indicate potential N-linked glycosylation sites; question marks indicate ambiguous amino acids; addition symbols represent insertions. Gray areas represent conserved amino acids within the 152 strains. Leucine or Isoleucine are indicated by a J. Aspartic acid or Asparagine indicated with a B while Z represents Glutamic acid or Glutamine.

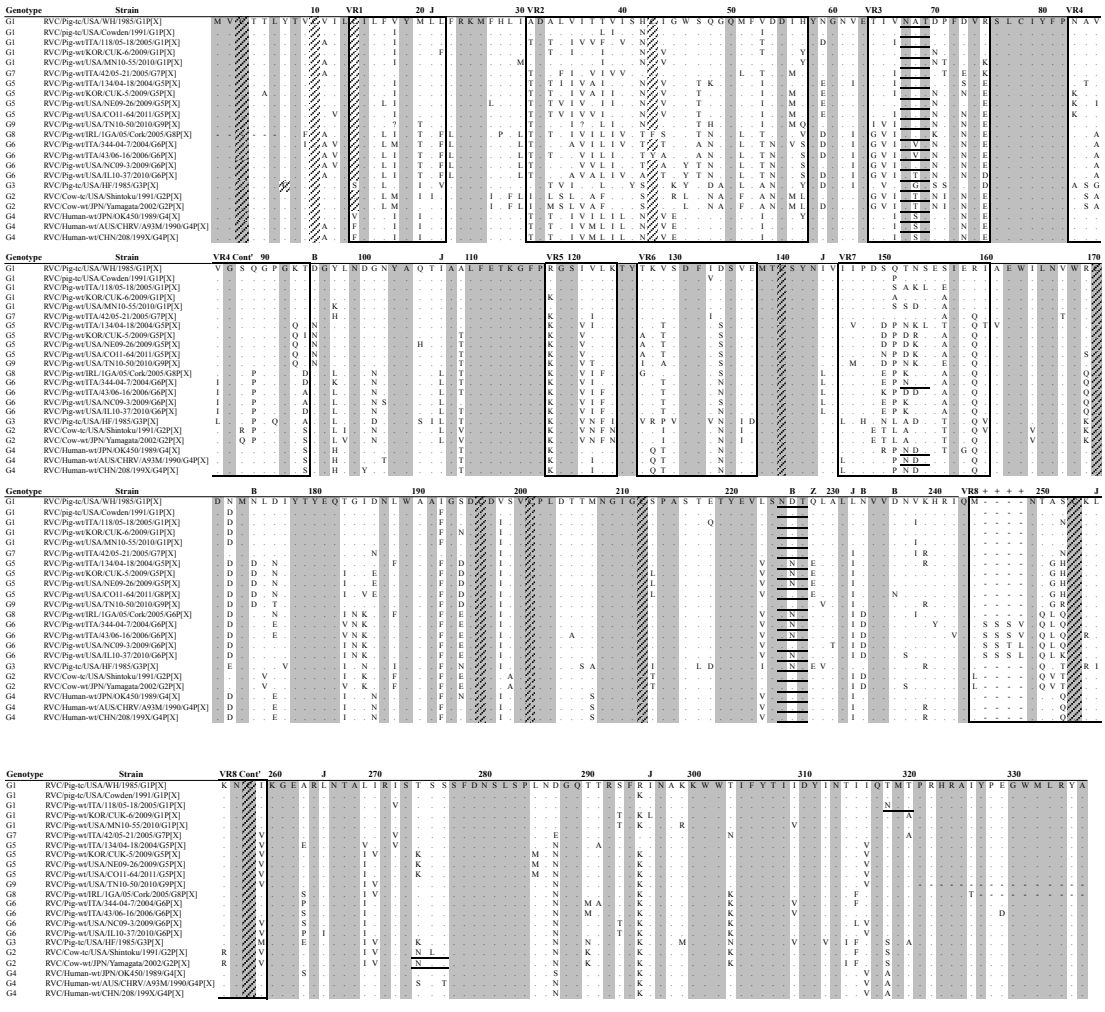

Figure 5.7. Amino acid alignment of the 150 RVC VP7 strains of the 9 proposed G genotypes, indicated on the left side of the chart. Each color represents a specific residue. The histogram at the top of the chart represents the consensus amino acid sequence where the valleys (red) represent diverse residues while the peaks (dark green) illustrate conserved residues.

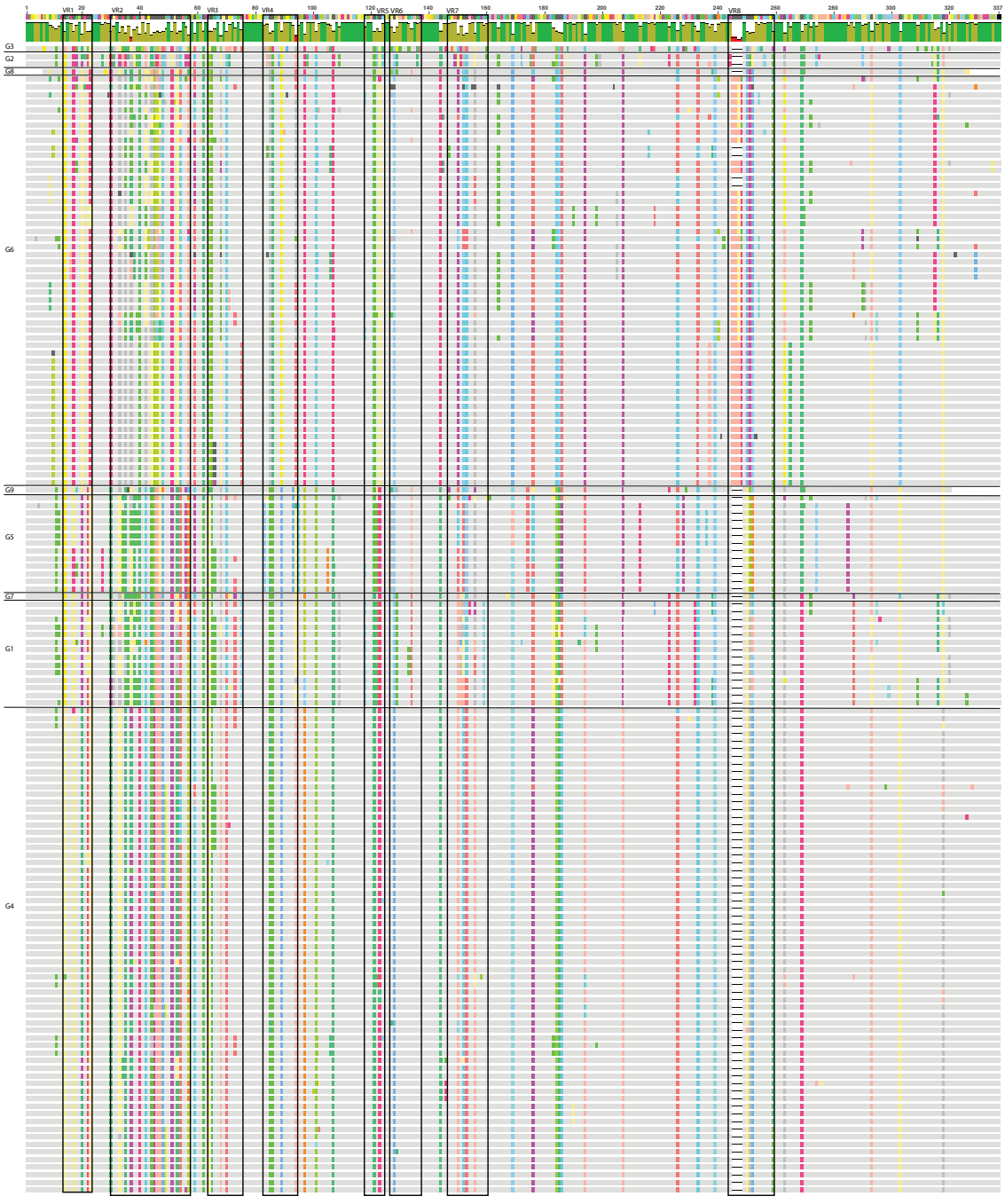

Table 5.1. RV distribution in lung samples (n=635).

Total RVA positives	317	50%
Total RVB positives	33	5%
Total RVC positives	64	10%
RVA only	246	39%
RVB only	11	2%
RVC only	15	2%
RVA and RVB	18	3%
RVA and RVC	49	8%
RVB and RVC	0	0%
RVA, RVB, and RVC	4	1%
Total Positives	343	54%
Negative	292	46%

Table 5.2. Distribution of RVC sequences by diarrhea status, weight loss status, PCR result (RVA and RVB), age, sample type, G genotype, state, site, and accession number (n=70).

Strain Name	Diarrhea Status	Weight loss Status	RVA Result	RVB Result	Age	Sample Type	G Genotype	State or Providence	Herd	Accession Number
RVC/Pig-wt/USA/IL10-49/2010/G6P[X]	+	+	-	-	2-3 days	Intestine	G6	Illinois	21	JX273346
RVC/Pig-wt/USA/IL10-48/2010/G6P[X]	+	+	-	-	2-3 days	Intestine	G6	Illinois	21	JX273345
RVC/Pig-wt/USA/IL10-47/2010/G6P[X]	+	+	-	-	2-3 days	Intestine	G6	Illinois	21	JX273344
RVC/Pig-wt/USA/IL10-46/2010/G6P[X]	+	+	-	-	2-3 days	Intestine	G6	Illinois	21	JX273343
RVC/Pig-wt/USA/IL10-45/2010/G6P[X]	+	+	-	-	2-3 days	Intestine	G6	Illinois	21	JX273342
RVC/Pig-wt/USA/IL10-44/2010/G6P[X]	+	+	-	-	2-3 days	Intestine	G6	Illinois	21	JX273341
RVC/Pig-wt/USA/IL10-43/2010/G6P[X]	+	+	-	-	2-3 days	Intestine	G6	Illinois	21	JX273340
RVC/Pig-wt/USA/IL10-42/2010/G6P[X]	+	+	-	-	2-3 days	Intestine	G6	Illinois	21	JX273339
RVC/Pig-wt/USA/IL10-41/2010/G6P[X]	+	na	-	-	3-8 weeks	Intestine	G6	Illinois	21	JX273338
RVC/Pig-wt/USA/IL10-40/2010/G6P[X]	+	na	-	-	3-8 weeks	Intestine	G6	Illinois	21	JX273337
RVC/Pig-wt/USA/IL10-39/2010/G6P[X]	+	na	-	-	3-8 weeks	Intestine	G6	Illinois	21	JX273336
RVC/Pig-wt/USA/IL10-38/2010/G6P[X]	+	na	-	-	3-8 weeks	Intestine	G6	Illinois	21	JX273335
RVC/Pig-wt/USA/IL10-37/2010/G6P[X]	+	na	-	-	3-8 weeks	Intestine	G6	Illinois	21	JX273334
RVC/Pig-wt/USA/IL10-36/2010/G6P[X]	+	na	-	-	3-8 weeks	Intestine	G6	Illinois	21	JX273333
RVC/Pig-wt/USA/IL10-35/2010/G6P[X]	+	na	-	-	3-8 weeks	Intestine	G6	Illinois	21	JX273332
RVC/Pig-wt/USA/IL10-34/2010/G6P[X][c]	+	na	-	-	3-8 weeks	Intestine	G6	Illinois	21	JX273331
RVC/Pig-wt/USA/IL10-33/2010/G6P[X][c]	+	na	-	-	3-8 weeks	Lung	G6	Illinois	21	JX273330
RVC/Pig-wt/USA/IL10-32/2010/G6P[X]	+	na	-	-	3-8 weeks	Intestine	G6	Illinois	21	JX273329
RVC/Pig-wt/USA/IL10-31/2010/G6P[X]	+	na	-	-	3-8 weeks	Intestine	G6	Illinois	20	JX273328
RVC/Pig-wt/USA/CO11-64/2011/G5P[X]	+	na	-	-	4 days	Intestine	G5	Colorado	30	JX273361
RVC/Pig-wt/USA/CO11-63/2011/G5P[X]	+	na	-	-	4 days	Intestine	G5	Colorado	30	JX273360
RVC/Pig-wt/USA/CO11-62/2011/G5P[X]	+	na	-	-	4 days	Intestine	G5	Colorado	30	JX273359
RVC/Pig-wt/USA/CO11-61/2011/G5P[X]	+	na	-	-	4 days	Intestine	G5	Colorado	30	JX273358
RVC/Pig-wt/USA/CO11-60/2011/G5P[X]	+	na	-	-	4 days	Intestine	G5	Colorado	30	JX273357
RVC/Pig-wt/USA/CO09-30/2009/G6P[X]	+	na	-	-	4 days	Intestine	G6	Colorado	20	JX273327
RVC/Pig-wt/USA/CO09-29/2009/G6P[X]	+	na	-	-	4 days	Intestine	G6	Colorado	20	JX273326
RVC/Pig-wt/USA/CO09-28/2009/G6P[X]	+	na	-	-	4 days	Intestine	G6	Colorado	20	JX273325
RVC/Pig-wt/CAN/MB11-70/2011/G6P[X]	+	-	-	-	7 days	Intestine	G6	Manitoba, Canada	29	JX273367
RVC/Pig-wt/CAN/MB11-69/2011/G6P[X]	+	-	-	-	7 days	Intestine	G6	Manitoba, Canada	29	JX273366
RVC/Pig-wt/USA/OH09-1/2009/G6P[X]	+	+	-	na	3-8 weeks	Intestine	G6	Ohio	1	JX273298
RVC/Pig-wt/USA/NC09-4/2009/G6P[X]	+	+	-	na	2 days	Intestine	G6	North Carolina	4	JX273301
RVC/Pig-wt/USA/MN09-5/2009/G1P[X]	+	+	-	na	2-3 days	Intestine	G1	Minnesota	5	JX273302
RVC/Pig-wt/USA/NE09-26/2009/G5P[X]	+	na	-	na	17-21 days	Fecal	G5	Nebraska	19	JX273323
RVC/Pig-wt/USA/NE09-2/2009/G6P[X]	+	na	-	na	3 days	Intestine	G6	Nebraska	2	JX273299
RVC/Pig-wt/USA/NC09-3/2009/G6P[X]	+	na	-	na	2-4 weeks	Intestine	G6	North Carolina	3	JX273300
RVC/Pig-wt/USA/NC10-54/2010/G6P[X]	+	+	+	-	6 weeks	Intestine	G6	North Carolina	24	JX273351
RVC/Pig-wt/USA/TN10-51/2010/G9P[X][d]	+	na	+	-	6 weeks	Intestine	G9	Tennessee	22	JX273348
RVC/Pig-wt/USA/TN10-50/2010/G5P[X][d]	+	na	+	-	6 weeks	Intestine	G5	Tennessee	22	JX273347
RVC/Pig-wt/USA/NC10-53/2010/G6P[X][e]	+	na	+	-	6 weeks	Intestine	G6	North Carolina	23	JX273350
RVC/Pig-wt/USA/NC10-52/2010/G1P[X][e]	+	na	+	-	6 weeks	Intestine	G1	North Carolina	23	JX273349
RVC/Pig-wt/CAN/MB11-65/2011/G6P[X]	+	na	+	-	7 days	Intestine	G6	Manitoba, Canada	27	JX273362
RVC/Pig-wt/USA/MN09-22/2009/G6P[X]	+	+	+	na	3 days	Intestine	G6	Minnesota	16	JX273319
RVC/Pig-wt/USA/MN09-21/2009/G6P[X]	+	+	+	na	3 days	Intestine	G6	Minnesota	16	JX273318
RVC/Pig-wt/USA/MN09-20/2009/G6P[X]	+	+	+	na	3 days	Intestine	G6	Minnesota	16	JX273317
RVC/Pig-wt/USA/MN09-19/2009/G6P[X]	+	+	+	na	3 days	Intestine	G6	Minnesota	16	JX273316
RVC/Pig-wt/USA/AR09-13/2009/G1P[X]	+	+	+	na	5 week old	Intestine	G1	Arkansas	11	JX273310
RVC/Pig-wt/USA/NE09-27/2009/G5P[X]	+	na	+	na	17-21 days	Fecal	G5	Nebraska	19	JX273324
RVC/Pig-wt/USA/NE09-25/2009/G5P[X]	+	na	+	na	17-21 days	Fecal	G5	Nebraska	19	JX273322
RVC/Pig-wt/USA/MN09-24/2009/G6P[X]	+	na	+	na	3-8 weeks	Intestine	G6	Minnesota	18	JX273321
RVC/Pig-wt/USA/MN09-16/2009/G6P[X][b]	+	na	+	na	22 days	Intestine	G6	Minnesota	13	JX273313
RVC/Pig-wt/USA/MN09-15/2009/G5P[X][b]	+	na	+	na	22 days	Intestine	G5	Minnesota	13	JX273312
RVC/Pig-wt/USA/MN09-12/2009/G5P[X]	+	na	+	na	20-33 days	Intestine	G5	Minnesota	10	JX273309
RVC/Pig-wt/USA/MN09-11/2009/G6P[X][a]	+	na	+	na	20-33 days	Intestine	G6	Minnesota	10	JX273308
RVC/Pig-wt/USA/MN09-10/2009/G5P[X][a]	+	na	+	na	20-33 days	Intestine	G5	Minnesota	10	JX273307
RVC/Pig-wt/USA/IL09-9/2009/G6P[X]	+	na	+	na	4 weeks	Intestine	G6	Illinois	9	JX273306
RVC/Pig-wt/USA/IL09-18/2009/G6P[X]	+	na	+	na	4 weeks	Intestine	G6	Illinois	15	JX273315
RVC/Pig-wt/USA/AR09-17/2009/G6P[X]	+	na	+	na	4 weeks	Intestine	G6	Arkansas	14	JX273314
RVC/Pig-wt/USA/MN09-23/2009/G1P[X]	+	-	+	na	3-8 weeks	Intestine	G1	Minnesota	17	JX273320
RVC/Pig-wt/USA/MN11-68/2011/G1P[X]	+	-	+	+	3-8 weeks	Fecal	G1	Minnesota	27	JX273365
RVC/Pig-wt/USA/MN11-67/2011/G1P[X]	+	-	+	+	3-8 weeks	Fecal	G1	Minnesota	27	JX273364
RVC/Pig-wt/USA/MN11-66/2011/G1P[X]	+	-	+	+	3-8 weeks	Intestine	G1	Minnesota	27	JX273363
RVC/Pig-wt/USA/MN10-55/2010/G1P[X]	na	na	-	-	13 weeks	Stomach	G1	Minnesota	25	JX273352
RVC/Pig-wt/CAN/MB11-56/2011/G6P[X]	na	na	-	-	28 days	Intestine	G6	Manitoba, Canada	26	JX273353
RVC/Pig-wt/USA/CO09-8/2009/G6P[X]	na	na	-	na	2-3 days	Intestine	G6	Colorado	8	JX273305
RVC/Pig-wt/CAN/MB11-59/2011/G6P[X]	na	na	+	-	28 days	Intestine	G6	Manitoba, Canada	29	JX273356
RVC/Pig-wt/CAN/MB11-58/2011/G6P[X]	na	na	+	-	28 days	Intestine	G6	Manitoba, Canada	28	JX273355
RVC/Pig-wt/CAN/MB11-57/2011/G6P[X]	na	na	+	-	28 days	Intestine	G6	Manitoba, Canada	28	JX273354
RVC/Pig-wt/USA/MN09-14/2009/G6P[X]	na	+	+	na	3 weeks	Intestine	G6	Minnesota	12	JX273311
RVC/Pig-wt/USA/MN09-6/2009/G6P[X]	-	+	-	na	28 days	Intestine	G6	Minnesota	6	JX273303
RVC/Pig-wt/USA/MN09-7/2009/G6P[X]	-	-	-	na	3-10 days	Intestine	G6	Minnesota	7	JX273304

*Strain names sharing the same superscript were derived from the same sample or animal

*na = data was not available

Table 5.3. RVC VP7 genotypes nucleotide (bottom) and amino acid (top) percent identities.

	G1	G2	G3	G4	G5	G6	G7	G8	G9
G1	90.1-100 91-100	72.6-75	68.7-71.4	83.4-88.6	81.3-85.5	75.6-82.8	88.3-91.0	75.3-78.2	82.5-86.3
G2	73.2-75.6	96.0 93.6	72.6-72.9	72.6-74.7	71.4-74.4	75.9-80.9	74.1-74.4	75.6-76.6	74.2
G3	71.0-74.6	73.2-73.3	na na	69.9-71.7	68.1-69.9	69.3-72.2	69.0	68.7	68.2
G4	80.7-84.9	73.3-75.4	72.4-74.4	94.3-100 93.2-100	83.1-87	78.6-82.8	85.5-88.3	78.2-80.1	84.1-86.3
G5	78.4-82.2	73.1-74.8	71.7-73.7	81.9-84.4	88.6-1 87.5-1	76.8-81	83.1-84.3	77.5-80.1	87.6-89.8
G6	74.7-78.9	75.1-78.9	72.7-75.6	76.1-80.8	75.5-79.6	88.4-1 85.3-1	77.1-81.9	87.7-91.5	78.3-81.8
G7	82.7-84.9	75.4-75.6	73.0	83.0-84.9	79.9-81.2	75.9-78.1	na na	78.5	84.7
G8	74.6-76.9	75.6-75.8	73.3	77.5-79.3	76.6-78.3	83.1-86.1	76.1	na na	79.9
G9	81.3-83.1	74.7-75.1	74.1	82.1-84.1	84.6-85.9	77.0-79.1	81.5	77.6	na na

Chapter 6: Widespread rotavirus H in Domesticated pigs, United States

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Introduction

Rotaviruses (RVs) are one of fifteen distinct genera contained in the *Reoviridae* family, and are a major cause of severe diarrhea in humans and animals worldwide(Estes and Greenberg, 2013). According to the International Committee on Taxonomy of Viruses, the rotavirus genus is divided into five antigenically distinct groups or species (RVA, RVB, RVC, RVD, RVE), two tentative species (RVF, RVG), and an unassigned species (ADRV-N), recently confirmed to be distinct from the other RV species, and now referred to as RVH(Guglielmi et al. 2011; Matthijnssens et al. 2012).

In total, 3 human Asian RVH strains (ADRV-N, J19, B219) (Yang et al. 1998; Yang et al. 2004; Alam et al. 2007; Jiang et al. 2008; Nagashima et al. 2008) and a porcine RVH strain (SKA-1) (Wakuda et al. 2011) have been identified between 1997 and 2002 and in 2012, 3 Brazilian porcine RVH strains BR63, BR60, and BR59 (GenBank accession numbers KF021621, KF021620, and KF021619) have been identified, bringing the total of known RVH strains to only 7. To investigate the presence of RVH in US swine, we screened 204 porcine samples collected between 2006 and 2009.

Material and methods

Sample selection

The University of Minnesota Veterinary Diagnostic Laboratory (UMNVDL) routinely receives porcine intestinal or fecal samples from pig farms across the US to determine the causative agents of gastrointestinal disease, which are screened by RT-PCR for transmissible gastroenteritis coronavirus (TGEV), RVA, RVB, and RVC, and a variety of bacterial pathogens by bacterial cultures(Marthaler et al. 2012; Marthaler et al. 2013). In June 2012, a porcine intestinal sample (MN123) from a pig in a farm in Minnesota showed histological RV-like lesions (blunt or nude villus tips) by light microscopy but the sample was negative for RVA, RVB, and RVC by RT-PCR. Another porcine intestinal sample (AR7.10-1) from a pig in a farm in Arkansas was positive for RVA, RVB, and RVC. Both samples (MN123 and AR7.10-1) were screened for RVH with RVH VP7-, VP6-, and NSP4-specific primers. The porcine intestinal sample MN123 was

negative for RVH with all primer sets; however, the AR7.10-1 sample was positive for RVH with the RVH VP6-specific primers. The RVH VP6-specific primers amplified a band of approximately 1200 nucleotides and sequencing of the band revealed a nucleotide percent identity of 90% to the swine Japanese RVH SKA-1 strain, identifying an RVH strain for the first time in the US. From a previous RV study, the extracted RNA from 204 RVA-, RVB- and/or RVC-positive samples collected between 2006 and 2009 was readily available and utilized to determine if RVH was also present in those samples. For the screening of RVH, only the RVH VP6-specific primers were employed because the RVH VP7- and NSP4-specific primers had failed to detect RVH in the sample AR7.10-1.

Histology, extraction of genomic material, and reverse transcriptase-polymerase chain reaction (RT-PCR) amplification

Small intestine samples were fixed in 10% neutral buffered formalin, embedded in paraffin, and stained using Harris' hematoxylin and eosin (HE), as described previously (Marthaler et al. 2012). Porcine intestinal or fecal samples were homogenized with Hyclone donor equine serum (Thermo Fisher Scientific, Waltham, MA) at a 2:3 ratio for 15 minutes, then undergo 3000 g centrifugation for 1 hour, as described previously (Marthaler et al. 2013). The total nucleic acid was extracted using the Ambion MagMax 1836 extraction kit following the manufacturer's instructions (Life Technologies, Carlsbad, CA).

For the molecular detection of RVH strains, VP7, VP6, and NSP4-specific primers were designed (Forward RVH_VP7_1-20: 5'- GGCAATTTGAAGCCATGTTG-3' and Reverse RVH_VP7_804-781: 5'- CATAACGGATTTCTCAACGTTATG-3', Forward RVH_VP6_1-23: 5'-GGCAATTTCTTGCTACAAGTGAC-3' and Reverse RVH_VP6_1203-1181: 5'-GGGTATATTTTATTTGCTATACTACTACGG-3', and Forward RVH_NSP4_1-24: 5'-GGCATTTTGTTCATCACAAATCACG-3' and Reverse RVH_NSP4_721-698: 5'-CTACCAAGCTATGTTTCCATCCAT-3') based on sequence alignment with Clustal W of the VP7, VP6, and NSP4 sequences of the single porcine

(SKA-1) and the three human (ADRV-N, J19, B219) RVH strains (Thompson et al. 1994; Yang et al. 2004; Alam et al. 2007; Jiang et al. 2008; Nagashima et al. 2008; Wakuda et al. 2011). The RT-PCR reaction utilized the Qiagen OneStep RT-PCR kit (Qiagen/Westburg) under recommended manufacturer's instructions and following the previously described thermal cycling conditions (Marthaler et al. 2012; Marthaler et al. 2013). RT-PCR products were visualized, purified, and Sanger sequenced using previously described methods (Marthaler et al. 2012; Marthaler et al. 2013). The nucleotides peaks from the traces files were visually evaluated and had a minimum of 2X consensus coverage in Lasergene Seqman 10.0 program (DNASTAR, Madison, WI). In Geneious Pro, the novel, and Genbank RVH (SKA-1, AB576626; B219, DQ168033; ADRV-N, AY632080; J19, DQ113902; BR63, KF021621; BR60, KF021620; BR59, KF021619) RV VP6 nucleotide sequences available from GenBank were aligned with Clustal W (Thompson et al. 1994). The nucleotide and amino acid phylogenetic trees were constructed with the Neighbor-Joining method (Saitou and Nei, 1987). A time-scaled phylogeny was inferred for the swine RVH VP6 sequences using a Bayesian MCMC approach, available in the BEAST package (BEASTv1.8.0) (Drummond et al. 2002; Drummond et al. 2005; Drummond et al. 2006; Drummond and Rambaut, 2007; Minin et al. 2008; Drummond and Suchard, 2010; Drummond et al. 2012). A general-time reversible model of nucleotide substitution was implemented, with a gamma distributed among-site rate variation. Using a relaxed molecular clock and a Bayesian skyline population (BSP) prior, the Markov Chain Monte Carlo (MCMC) chain was run for 300 million generations, with sub-sampling every 30,000 iterations. The initial 10% of the chain was discarded as burn-in, and a Maximum clade credibility (MCC) tree was summarized using TreeAnnotator (v.1.8.0). Times to the most recent common ancestor (TMRCAs) were identified on key nodes using FigTree (v1.4.0).

Statistical analysis

The Fisher's exact test was used to determine if the number of positives and negatives samples were significantly different between age groups. Using the age groups that contained RVH positive samples (4-20, 21-55, and >55 day age groups), a logistic

regression model was used to estimate the odds of RVH infection by age group, and the trend of RVH infection by age was tested using the Wald chi-square.

Sequence analysis

The ORF alignment (1191 nucleotides) of the 3 human (ADRV-N, J19, B219), 4 porcine (SKA-1, BR63, BR60, BR59), and the 30 novel porcine American RVH VP6 sequences revealed 682 identical sites (57%). When compared to the human RVH sequences, the US porcine RVH VP6 sequences had two nucleotide deletions at 1223 and 1224 in the 3'UTR. The RVH VP6 396 amino acid alignment of the American (n=30), Chinese (n=2), Bangladeshi (n=1), Japanese (n=1), and Brazilian strains (n=3) revealed 291 identical sites (74%). While most of the identified polymorphisms involved two different amino acids, polymorphisms involving 3 amino acids were identified at positions 44, 48, 150, 201, 220, 258, 353, and 378 (data not shown). Sequencing of the 30 novel RVH strains revealed 6 samples (IA5.5-3, MN4.53-6, MN4.87-5, MN32.15-1, AR7.32-4, and AR7.10-1) that contained mixed RVH infections or quasi-species, as indicated by ambiguous nucleotides within each the sequences (data not shown). Each ambiguous nucleotide translated only one amino acid.

Results

We identified RVH in a porcine intestinal sample (RVH/Pig-wt/USA/AR7.10-1/2012/GXP[X]) submitted from a farm in Arkansas in 2012. Subsequently, we rescreened 204 available RVA-, RVB-, and/or RVC-positive porcine samples, collected between 2006 and 2009 from 16 US states, for RVH. The samples were from 5 different age groups: 1-3 days (n=21), 4-7 days (n=23), 8-20 days (n=19), 21-55 days (n=110), and > 55 days (n=9), while 22 samples were of unknown age.

RVH was identified in 30 of the 204 porcine (15%, including sample AR7.10-1). RVH strains were identified in samples from 10 US states (Fig 6.1). The first US sample was identified on November 7, 2006. The majority of the RVH positive samples (n=20/111, 18%) were detected in 21 to 55 day-old pigs, while RVH was not detected in

1 to 3 day-old piglets (Fig 6.2). A limited number of samples also were positive for RVH in the 4 to 20 day-old group (n=5/42, 12%) and the >55 day-old age group (n=5/9, 56%) (Fig 6.2). A significant difference between the number of positive and negative samples was observed between age groups (p=0.036, Fisher's exact test). While the odds of being RVH positive in the 21-55 day age group was not significant (OR=1.63, p=0.36), the odds of being RVH positive in the >55 day age group was significant (OR=5.92, p=0.031) compared to 4-20 day age group. However, the trend of increased RVH positivity by age group was not significant (p=0.94, Wald chi-square test). While only 5 samples with RVH were identified in pigs co-infected with RVA and RVB, co-infections of RVH with RVA (n=1), RVB (n=1), RVA and RVC (n=1), or RVB and RVC (n=1) were also identified but not significantly different (p>0.05, Fisher's exact test) (Fig 6.3). RVH and RVC co-infections were not identified. The majority of RVH samples (n=21, 70%) were identified from pigs co-infected with RVA, RVB, and RVC, which was significantly different to any other RVH co-infections (p<0.001, Fisher's exact test). Of these 21 RVH RVA, RVB, RVC, and RVH co-infected samples, 15 were from the 21-55 day age group (Fig 6.3).

The American porcine RVH VP6 sequences (GenBank accession numbers KF757260-KF757289) exhibited a nucleotide percent identity of 91-100% and shared a nucleotide percent identity of 89-92% and 85-87% to Japanese porcine strain SKA-1 and Brazilian porcine strains BR63, BR60, and BR59, respectively (Table 6.1). The American porcine and human RVH VP6 sequences shared a nucleotide percent identity of 70-73%. The American porcine RVH VP6 sequences had an amino acid identity of 97-100%, while having an amino acid identity of 97-98% and 96-98% with the Japanese and the Brazilian porcine strains, respectively. The US porcine and human RVH VP6 sequences had an amino acid identity of 75.3-76.8% (Table 6.1). The nucleotide and amino acid pairwise identity charts (Fig 6.4) and phylogenetic trees (Fig 6.5) suggest the existence of at least two distinct RVH VP6 (I) clusters/genotypes, containing human and porcine strains respectively.

Compared to other rotaviruses species, the American RVH VP6 sequences shared the highest nucleotide and amino acid identities with RVG (51-53% and 39-41%, respectively) and RVB (47-52% and 34-39%, respectively) (Table 6.2). The RVH, RVG, and RVB VP6 nucleotide phylogenetic tree clustered in one large branch while those of RVA, RVC, RVF, and RVD clustered separately in another large branch (Fig 6.5). The RVH evolutionary rate (substitution/site/year) from BEAST was estimated at 2.6×10^{-3} [CI: 5.83×10^{-4} - 4.46×10^{-3}]. Based on the estimate of the tMRCA for the VP6 gene segment, American RVH strains have been circulating in the US swine for at least a decade and possibly much longer (tMRCA=1963-2002, 95% HPD) (Fig 6.6). The American and Japanese RVH VP6 sequences diverged between 1955-1993, 95% HPD, whereas the divergence of the Brazilian RVH VP6 sequences from the American and Japanese RVH VP6 sequences was estimated to be 1832-1991, 95% HPD.

Conclusion

Our data indicate that RVH is widespread in US swineherds. Although the samples analyzed were already known to be positive for RV species A, B, and/or C, it is remarkable that RVH was still identified in 15% of the samples. In the US, piglets are weaned at 21 days of age and then mixed with other piglets from different production sites, which may explain the higher rate of RV co-infections observed in the 21-55 day age group(Marthaler et al. 2012; Marthaler et al. 2013). These findings suggest that RVH is under-diagnosed in US swineherds and requires further surveillance efforts.

Our phylogenetic analysis indicates that the RVH population circulating in US swine is evolutionarily distinct from that found in humans and as well as from swine in Brazil and Japan. Although our low sample number and sequencing of a single gene (VP6) makes it difficult to fully assess the genetic diversity of RVH in US swine herds, the lack of spatial structure observed in the tree indicates extensive gene flow of RVH between swineherds located in different US regions. Inferring the circulation of RVH in US swineherds is difficult due to the low sample size, although our time-structured phylogenetic analysis indicates at least one decade of circulation. Although American

swine are routinely transported to South America, the phylogeny indicates that the VP6 gene of American swine RVH viruses is more closely related to the Japanese strain SKA-1 than to the three Brazilian strains included in this analysis.

In conclusion, we have identified RVH in 30 samples from pigs co-infected with RVA, RVB, and/or RVC in the United States, indicating that RVH has been circulating in American swine for at least one decade, and perhaps for longer. The human and porcine RVH VP6 sequences clustered into separate branches in the phylogenetic tree, but the presence of RVH in swine clearly raises the possibility of interspecies transmission. As the swine samples were co-infected with RVA, RVB, and/or RVC, the role of RVH is pathogenesis remains unknown, but represents a target of future molecular epidemiological studies.

Figure 6.1. Geographical distribution of RVH-positive porcine samples in the US. States containing RVH positive samples are colored in red while negative RVH sampled states are colored green.

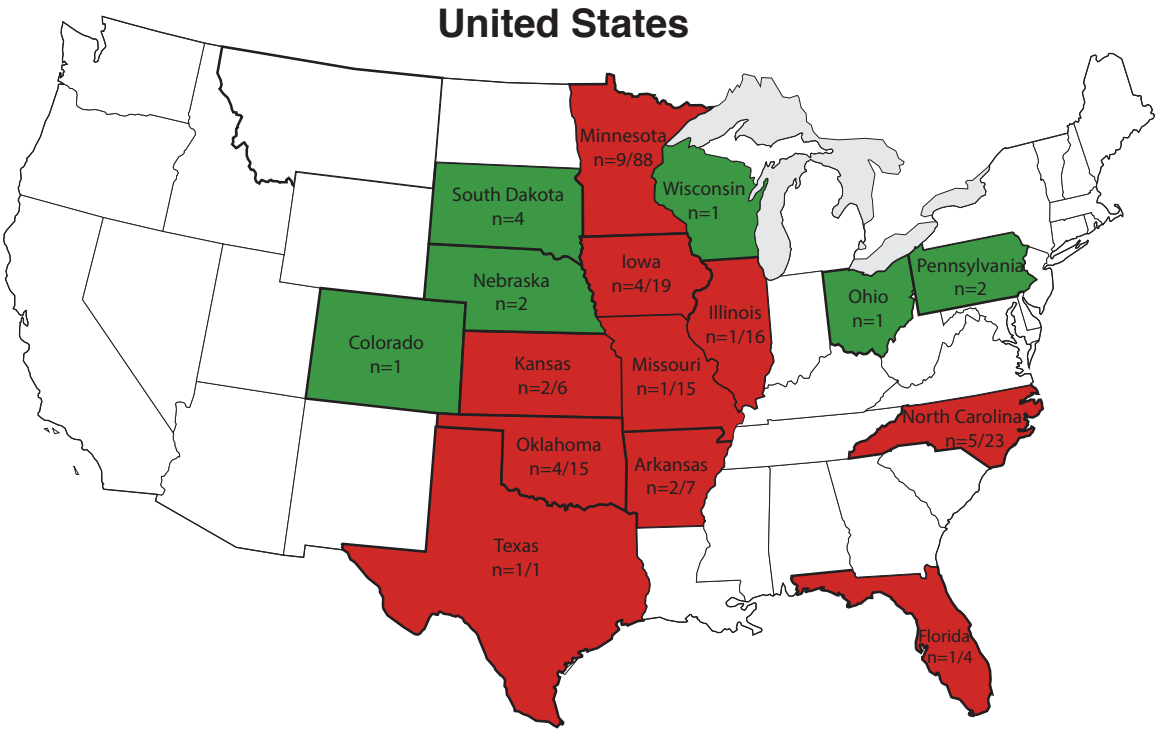

Figure 6.2. Distribution of RVH-positive samples by pig age group.

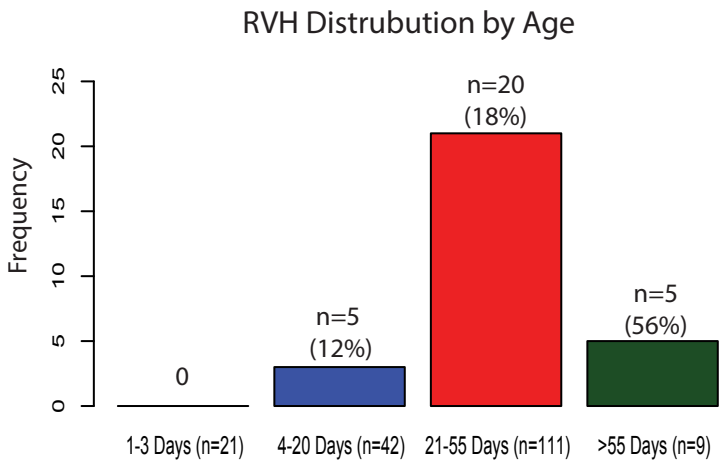

Figure 6.3. Distribution of RVH-positive samples and age group in pigs co-infected with RVA, RVB, and/or RVC. The color blue represent samples from the 4-20 day age group, and the color red represents samples from the 21-55 day age group while the color green represents samples from >55 day age group.

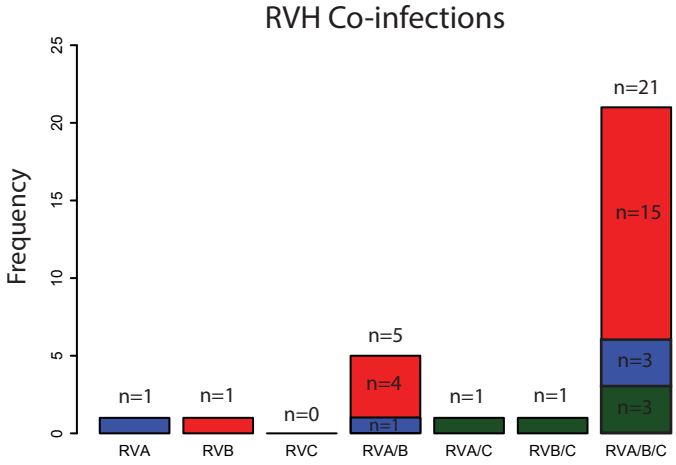

Figure 6.4. RVH VP6 nucleotide and amino acid pairwise identity charts.

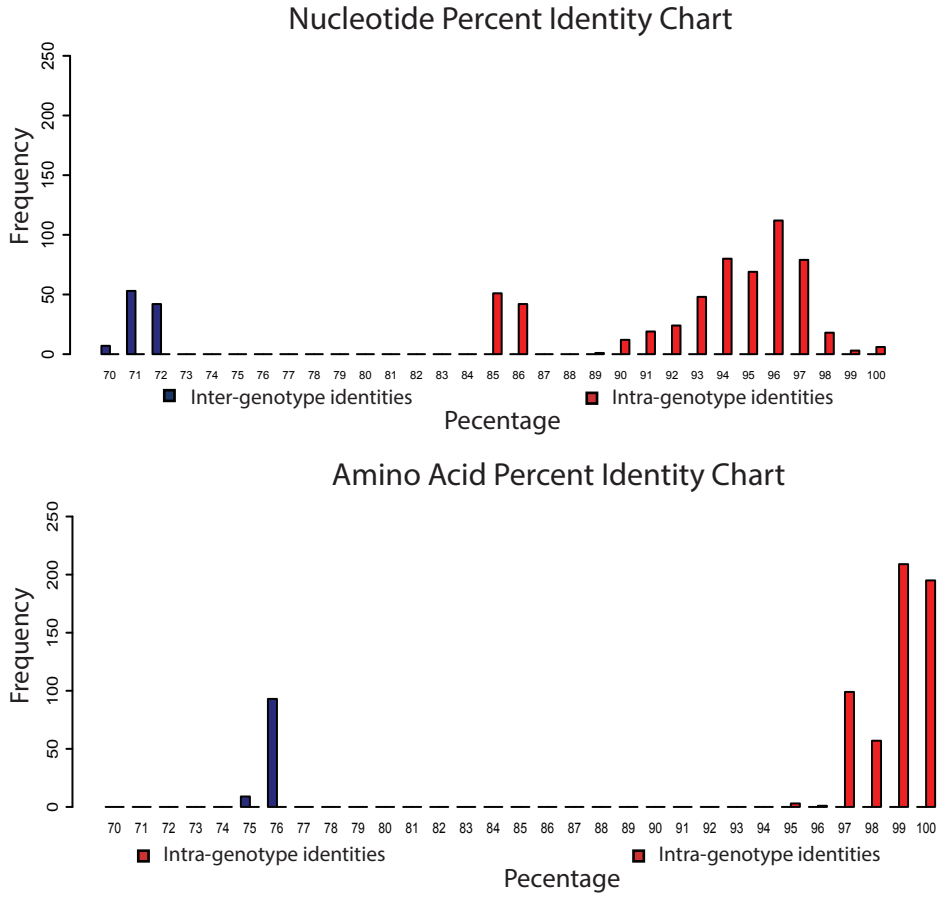

Figure 6.5. Nucleotide Neighbor-Joining phylogenetic tree of RVA-RVD and RVF-RVH VP6 sequences. Blue strains are from the US, green strains are from Brazil, and the red strain is from Japan while the purple strains are from humans.

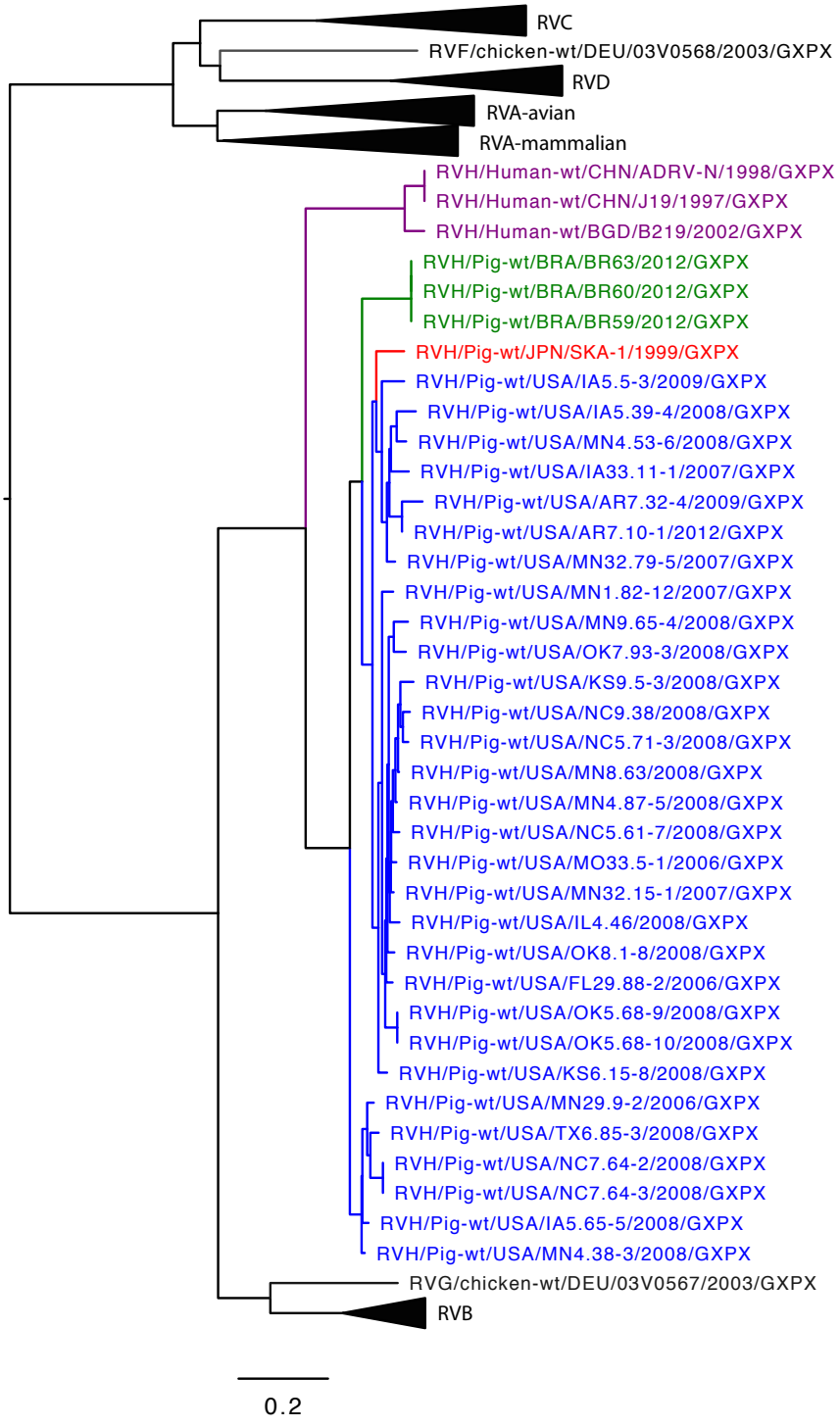

Figure 6.6. A time-scaled phylogeny of swine RVH VP6 sequences using a Bayesian MCMC approach. The blue shaded region represents the American TMCRA range, the red shaded region represents the American and Japanese RVH TMCRA range, and the green shaded region represents the TMRCA range for all swine RVH VP6 sequences.

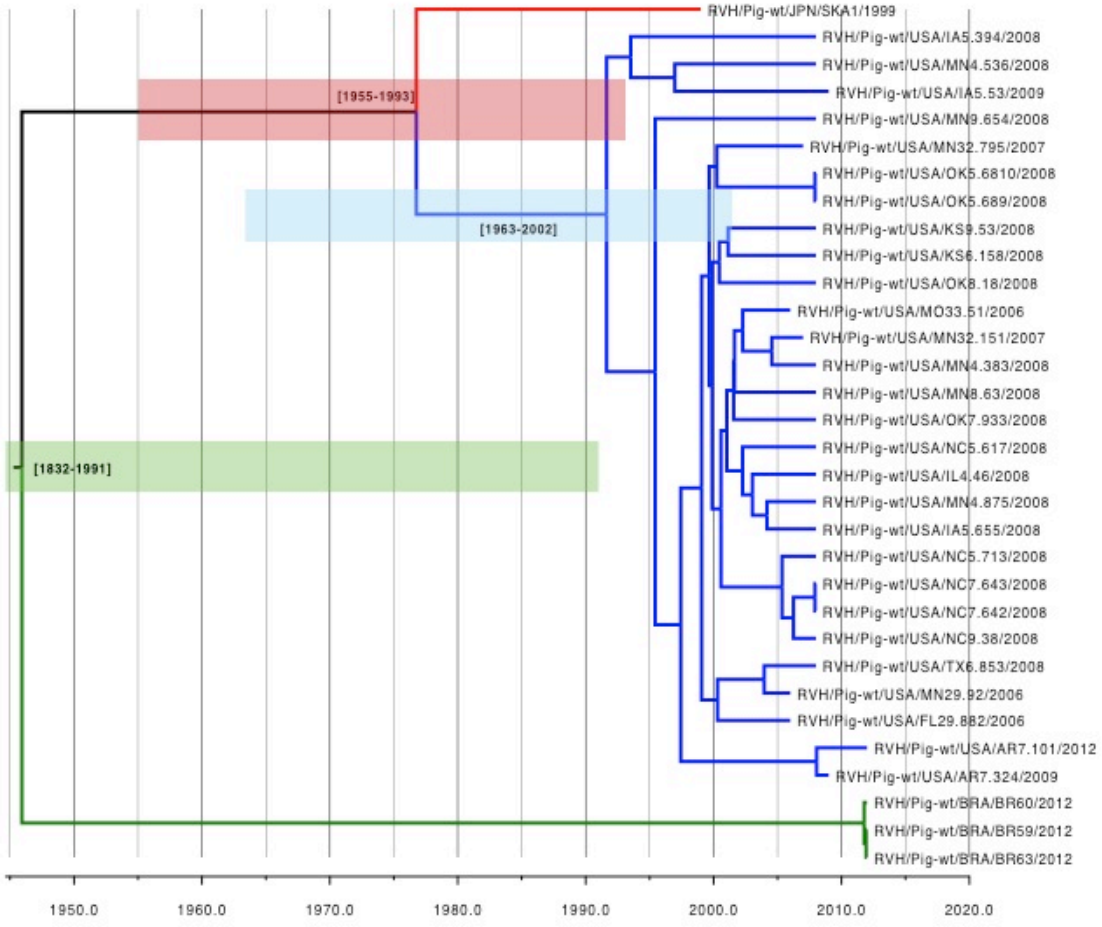

Table 6.1. RVH nucleotide (top) and amino acid (bottom) percent identities.

	American porcine RVH	Japanese porcine RVH	Brazilian porcine RVH	Human RVH
American porcine RVH	91-100 97-100	89.2-91.9	85.2-86.8	70.4-72.8
Japanese porcine RVH	96.5-98.2	na na	85.5	71.7-72.3
Brazilian porcine RVH	95.7-97.7	97	100 100	71.1-71.2
Human RVH	75.3-76.8	76.5-76.8	75.8-76	94-100 98.7-100

Table 6.2. RV nucleotide (top) and amino acid (bottom) percent identities.

	RVA	RVB	RVC	RVD	RVF	RVG	RVH
RVA	65.2-100 65-100	29.7-36.2	48.47-55.7	46.4-52.1	46.3-50.8	32.9-36.7	31.7-36.2
RVB	7.5-11.3	64.8-100 66.2-100	30.5-34.4	29.2-32.9	30.1-32.9	50.7-57.1	47.4-51.7
RVC	36.3-42.9	10.6-13.9	81.4-100 87.1-100	47.2-49.8	47.4-48.3	33.8-34.2	31.5-34.6
RVD	33.3-39.9	10.4-12.7	34.7-35.4	90.1-99.6 98.2-99.7	49.8-50.7	33-34	31.9-34.4
RVF	31.8-37.2	11.3-13.4	32.7-33.9	36.6-37.6	100 100	32.3	31-32.2
RVG	11.1-13.5	46.1-49.4	14.4-14.6	12-12.5	11.1	na NA	50.7-52.2
RVH	9.9-13.1	34.4-39.4	13.4-14.7	14.5-16.8	12.6-14	39.1-41.4	70.4-100 75.3-100

Chapter 7: General Discussion and Conclusions

General discussion and conclusion

Rotaviruses are an important cause of diarrhea in pigs and every commercial pig will experience one or multiple RV infections in its lifetime. The lack of proper diagnostic assays to identify RV infections has limited our ability of swine veterinarians to prevent and control RV infections in pig farms. While RVA has been considered the most prevalent and pathogenic, RVB, RVC, and RVH are also important enteric pathogens of swine. While studies on RVA disease ecology is well described in the literature, little information is available for swine RVB, RVC, and RVH, implying the lack of knowledge regarding RV disease ecology in swine. Therefore, the aim of this thesis was to understand the ecology and genetic diversity of RVB, RVC, and RVH in commercial swineherds.

The first chapter provides background information on porcine RV species, which clearly indicated a wealth of knowledge on porcine RVA strains and a lack of information on porcine RVB, RVC and RVH strains. Understanding the ecology and genetic diversity of RVB, RVC, and RVH infections will help swine veterinarians diagnose RV infections. The second chapter established RVB as an important cause of diarrhea in swine by identifying RVB in swine samples in the absence of other known enteric pathogens. Porcine RVB infections were identified in 46.8% of the samples with the majority of the samples containing RVA or RVC infections. The highest percentage of RVB strains (72.7%) was identified in older pigs, which seems to have similar disease ecology as RVB in human. Using the available RVB VP7 sequences from GenBank and the 68 RVB VP7 sequences generated from this study, an 80% nucleotide percent identity cut-off value was generated, which resulted in the establishment of 16 RVB G genotypes in swine populations. This study uncovered considerable genetic diversity within and between porcine RVB strains.

To further investigate the genetic diversity of porcine RVB strains and to develop a RT-qPCR to detect porcine RVB strains, the VP6 gene segment was sequenced as part of the work described in chapter 3. In collaboration with my colleagues in Japan, 80 porcine RVB VP6 sequences were generated. Using these 80 novel porcine RVB VP6 sequences and the RVB VP6 sequences available in GenBank, an 81% nucleotide percent

identity cut-off value was proposed, generating 13 RVB I genotypes. Of the 13 RVB I genotypes, 10 I genotypes were identified in swine. The genetic diversity of the RVB VP6 gene segment in swine is greater than the RVA VP6 gene segment, in which 2 RVA I genotypes have been identified in swine. Since the VP7 and VP6 RVB gene segments were sequenced, RVB reassortment events could be investigated. While some porcine RVB I genotypes (I4, I7, I8, I9, and I10) were only identified with a single G genotype (G7, G8, G12, G8, and G4, respectively), the RVB I11 genotype was identified with 6 RVB G genotypes. In contrast, some RVB G genotypes (G4, G6, G11, G16 and G17) were identified with a single RVB I genotype (I10, I5, I11, I13, and I11, respectively) while the RVB G8 genotype was identified with 4 I genotypes (I7, I9, I11, and I13). The results indicate that swine RVB reassortment events are common between the VP7 and VP6 gene segments.

The additional aim of chapter 3 was to investigate the gene diversity of the RVB VP6 gene segment, and thus the work described in chapter 4 utilized the RVB VP6 genetic information to develop a RT-qPCR to detect RVB in swine. In addition, a multiplex RT-qPCR (RVAC RT-qPCR) assay was developed to simultaneously detect RVA and RVC in swine samples. The RVA RT-qPCR component of the RVAC RT-qPCR assay had an efficiency of 99.03% while the RVC component had an efficiency of 97.45%. The RVB RT-qPCR had an efficiency of 96.17%. The RV RT-qPCR assays were sensitive, specific, discriminative and allowed for the investigation of possible RV co-infections in a fast and accurate manner. Between December 2009 and October 2011, the RV RT-qPCR assays identified porcine RVA, RVB, and RVC in 62%, 33%, and 53%, respectively, of the porcine samples tested. While the predominant RV species identified was RVA, our findings also indicate RVC is also as an important enteric pathogen with RVC detection in over 50% of the samples.

Chapter 5 further investigates RVC as an important enteric pathogen in swine. RVC was detected in the majority of the samples were from pigs in the 21-55 day age group. Interestingly, RVC was only detected in 78% of the 1-3 day age group and 65% of of the 4-20 day age group. These results imply that RVC alone, not just RVA as previously reports suggest, is a primarily cause of piglet diarrhea in the United States.

While the complete pathogenesis and disease ecology are still unclear, swine veterinarians can use this information to implement RVC controls measures on swine farms.

To further understand RV ecology, chapter 6 investigated the presence of RVH in the United States. From an archival sample set of 204 RV positive samples, RVH was identified in 15% of the samples from 10 different states. A majority of the positive RVH samples (67%) were detected in the 21-55 day age group while RVH was not identified in the 1-3 day age group. Co-infections of RVH, RVA, RVB, and RVC were identified in a majority of the samples. The American RVH VP6 sequence data indicated the Japanese RVH strain is a more closely related ancestor than the Brazilian RVH strains. In addition, the data indicates the American RVH strains have been circulating for at least a decade and possibly as early as 1963. While the study does not address RVH prevalence or pathogenesis of porcine RVH, this initial work can be used as a foundation for additional RV epidemiological studies.

Future studies should include the five RV described here in, namely RVA, RVB, RVC, and RVH, to more fully understand the interaction between RV species, and their effects on swine health. While this thesis established porcine RVB and RVC as important enteric pathogens in the United States, the prevalence of RVB and RVC in other countries is unknown. The identification of porcine RVH in the United States also underscores the need for global RV studies in animal and human populations. In addition, this thesis highlights the genetic diversity of porcine RVB, RVC, and RVH strains. Increasing our knowledge regarding the genetic diversity of RV species will allow for the development of future prevention and control measures, which can be used by swine veterinarians and producers to minimize the impact of RV on the well being of pigs.

While the studies described here in further our knowledge on porcine RVs, the studies have limitations, such as bias, since the samples were collected from a diagnostic laboratory. The data from these studies may not reflect the RV occurrence in swine farms. Nevertheless, these studies provide valuable insight into the complex ecology of RVs in swine.

In conclusion, this thesis has advanced the knowledge of the epidemiology and genetic diversity of RV in swine. In addition, this thesis has described the complex interactions of porcine rotaviruses, which can be used by researchers, veterinarians, and pork producers to investigate gastrointestinal disease outbreaks more completely, and eventually develop vaccines to prevent and limit the spread of RV in swine.

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