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Detection of substantial porcine group B rotavirus genetic diversity in the United States, resulting in a modified classification proposal for G genotypes

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ABSTRACT

Rotavirus (RV) is an important cause of gastrointestinal disease in animals and humans. In this study, we developed an RT-PCR to detect RV group B (RVB) and characterized the VP7 (G) gene segment detected in porcine samples. One hundred seventy three samples were tested for RV group A (RVA), RVB, and C (RVC) by RT-PCR and examined for RV-like lesion using histopathology. A majority (86.4%) of the samples had mixed RV infections and co-infections of RVA/RVB/RVC were detected at a higher rate (24.3%) than previously reported. RVB was identified in 46.8% of the 173 samples. An adapted VP7 classification was developed using previously published ($n=57$) and newly sequenced ($n=68$) RVB strains, resulting in 20 G genotypes based on an 80% nucleotide identity cutoff value. Our results revealed a broad genetic diversity of porcine RVB strains, suggesting RVB has been the cause of common/pre-existing, yet undiagnosed, disease in pigs.

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, 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, 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

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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 (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 from 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%.

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).

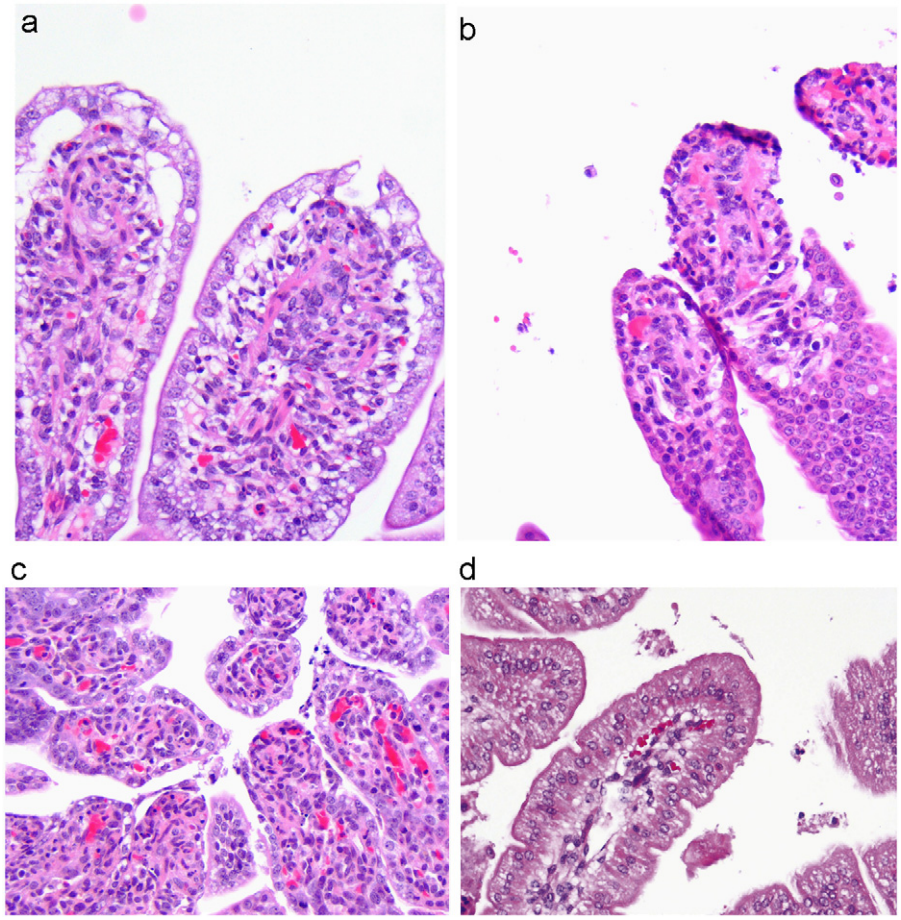


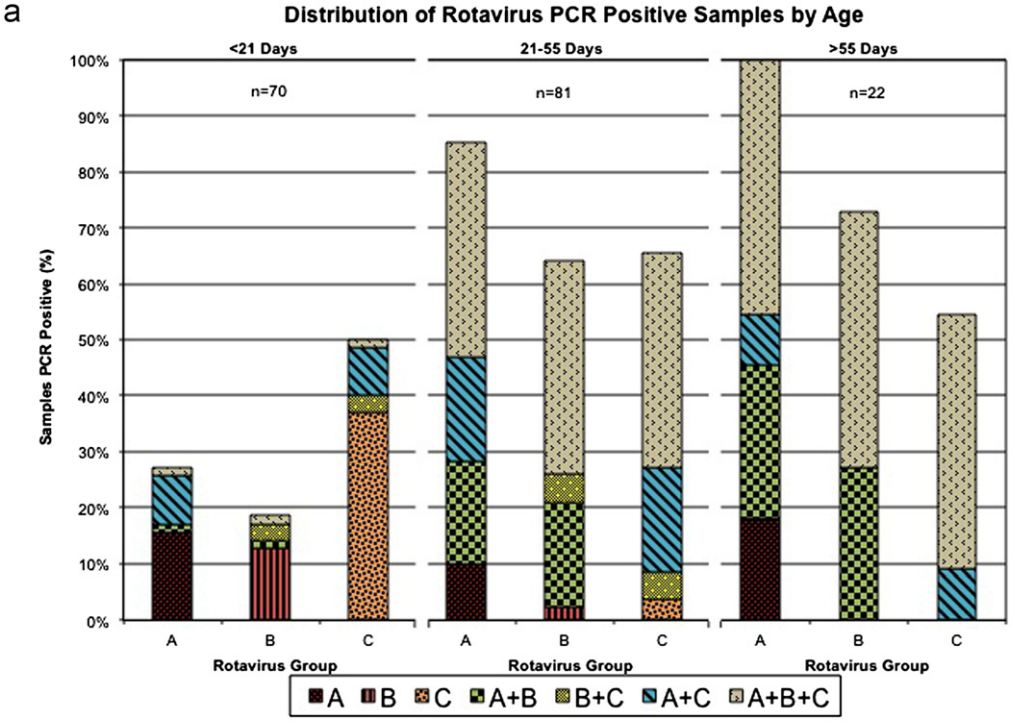
Fig. 1. Sections of jejunum from 2–3 day old pigs with typical lesions of acute RV enteritis by 200× magnification. Villi are characterized by edema, early epithelial necrosis, and sloughing to overt epithelial necrosis with denuded villus tips. (a) RVA infection (b) RVB infection (c) RVC infection (d) Normal villi.

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 (Fig. 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 (70 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 nine pig samples had typical RV lesions, such as those in Fig. 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, 21–55, and >55 days (Fig. 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 percentage of a RVB mixed infection with RVA and RVC was present in the >55 days age group (45.5%). Porcine enteric pathogens *S. enterica* spp., *Escherichia coli*, *Clostridium 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 51 different sites was successfully determined. Table 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 six samples, the presence of diarrhea was unknown. Single RVB G

a



b

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%

Fig. 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) Numeric values of positive samples by age group.

Table 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	Accession number
RVB/Pig-wt/USA/IL09-1/2009/G18P[X]	+	−	−	−	1–10 day	G18	Illinois	1	JQ043748
RVB/Pig-wt/USA/IL09-2/2009/G18P[X]	+	−	−	−	1–10 day	G18	Illinois	1	JQ043749
RVB/Pig-wt/USA/IL09-3/2009/G18P[X]	+	−	−	−	1–10 day	G18	Illinois	1	JQ043750
RVB/Pig-wt/USA/IL09-4/2009/G18P[X]	+	−	−	−	1–10 day	G18	Illinois	1	JQ043751
RVB/Pig-wt/USA/IL09-5/2009/G18P[X]	+	−	−	−	1–10 day	G18	Illinois	1	JQ043752
RVB/Pig-wt/USA/MO09-32/2009/G16P[X]	+	−	−	−	4 day	G16	Missouri	23	JQ043779
RVB/Pig-wt/USA/MN09-42/2009/G20P[X]	+	−	−	−	3 day	G20	Minnesota	31	JQ043789
RVB/Pig-wt/USA/MN09-59/2009/G14P[X]	+	−	−	−	3–10 day	G14	Minnesota	43	JQ043806
RVB/Pig-wt/USA/IL09-25/2009/G16P[X]	+	−	+	−	4 weeks	G16	Illinois	16	JQ043772
RVB/Pig-wt/USA/MN09-68/2009/G12P[X]	+	−	+	−	4 weeks	G12	Minnesota	21	JQ043777
RVB/Pig-wt/USA/MN09-58/2009/G14P[X]	+	−	+	−	3–10 day	G14	Minnesota	43	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	42	JQ043803
RVB/Pig-wt/USA/OH09-60/2009/G11P[X]	+	+	−	−	4 weeks	G11	Ohio	44	JQ043807
RVB/Pig-wt/USA/MN09-64/2009/G8P[X]	+	+	−	−	18 day	G8	Minnesota	48	JQ043811
RVB/Pig-wt/USA/IA09-67/2009/G16P[X]	+	+	−	−	4 weeks	G16	Iowa	50	JQ043814
RVB/Pig-wt/USA/MN09-68/2009/G12P[X]	+	+	−	−	5 weeks	G12	Minnesota	51	JQ043815
RVB/Pig-wt/USA/MN09-20/2009/G11P[X]	+	+	+	−	24 day	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	42	JQ043804
RVB/Pig-wt/USA/NC09-61/2009/G16P[X]	+	+	+	−	4 weeks	G16	North Carolina	45	JQ043808
RVB/Pig-wt/USA/MN09-62/2009/G12P[X]	+	+	+	−	3 weeks	G12	Minnesota	46	JQ043809
RVB/Pig-wt/USA/MN09-65/2009/G8P[X]	+	+	+	−	18 day	G8	Minnesota	48	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	41	JQ043802
RVB/Pig-wt/USA/MN09-63/2009/G18P[X]	−	+	−	−	5 weeks	G18	Minnesota	47	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 day	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 day	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	40	JQ043801
RVB/Pig-wt/USA/OK09-33/2009/G16P[X]	unknown		–	+	–	10 day	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 day	G14	South Dakota	34	JQ043792
RVB/Pig-wt/USA/MN09-66/2009/G8P[X]	unknown		+	–	–	18 day	G8	Missouri	49	JQ043813
RVB/Pig-wt/USA/MO09-31/2009/G18P[X]	unknown		+	+	–	21 day	G18	Missouri	22	JQ043778
RVB/Pig-wt/USA/MO09-36/2009/G8P[X]	unknown		+	+	–	3 weeks	G8	Missouri	27	JQ043783

genotypes were detected in 47 sites while two G genotypes were found to co-circulate in four sites (G8/G16, G12/G16, G12/G17, 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 (Fig. 3) was constructed and a nucleotide sequence based pairwise identity frequency graph was generated (Fig. 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 (Fig. 3, left dashed line), with a limited overlap between the inter- (red) and intra-cluster (orange and green) identities (Fig. 4). 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 (Fig. 3). A second suitable cut-off value of 80% resulted in the identification of 20 G genotypes (Fig. 3, the right dashed line) in the phylogenetic tree, with a very limited overlap between inter- (red and orange) and intra-genotype (green) identities (Fig. 4). 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) (Fig. 3). Table 2 show 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 Fig. 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–G5 and G11–G20. RVB VP7 sequences contained 1–4 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–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 Fig. 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

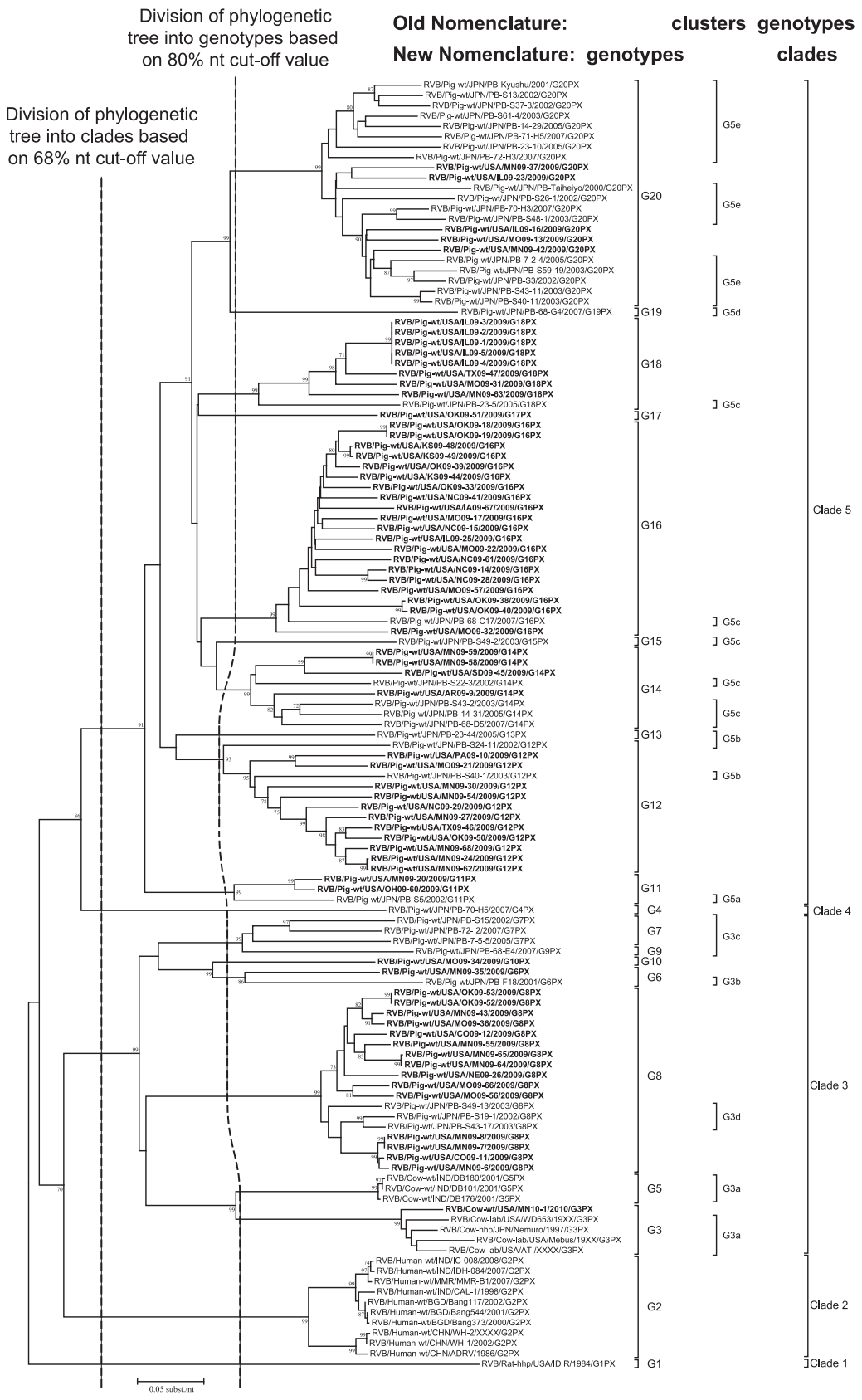


Fig. 3. Phylogenetic tree of RVB VP7 nucleotide sequences. Numbers left of the node characterize the bootstrap (1000 replicates) for each cluster. Bootstrap values less than 50% are not shown. The left dashed line represent the 68% cut-off value, resulting in 5 clades (or genotypes in the old classification 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.

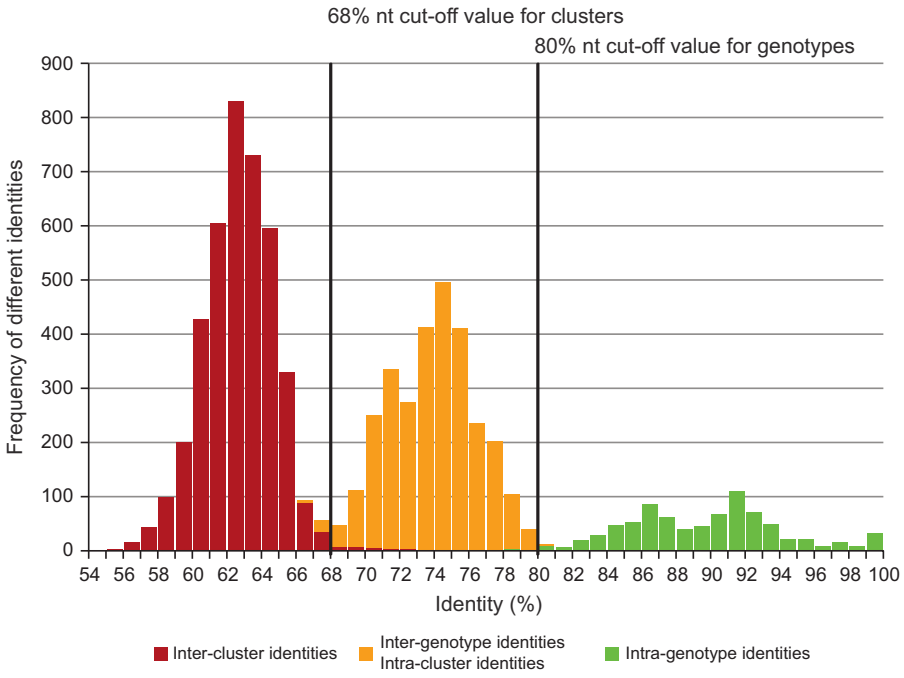


Fig. 4. Pairwise identity frequency graph using 125 available RVB VP7 ORF nucleotide sequences. Two suitable nucleotide cut-off values (68% and 80%) are depicted by vertical solid lines. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

Table 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

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 (*E. coli*, *C. difficile* and *C. perfringens*, coccidian, *L. 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

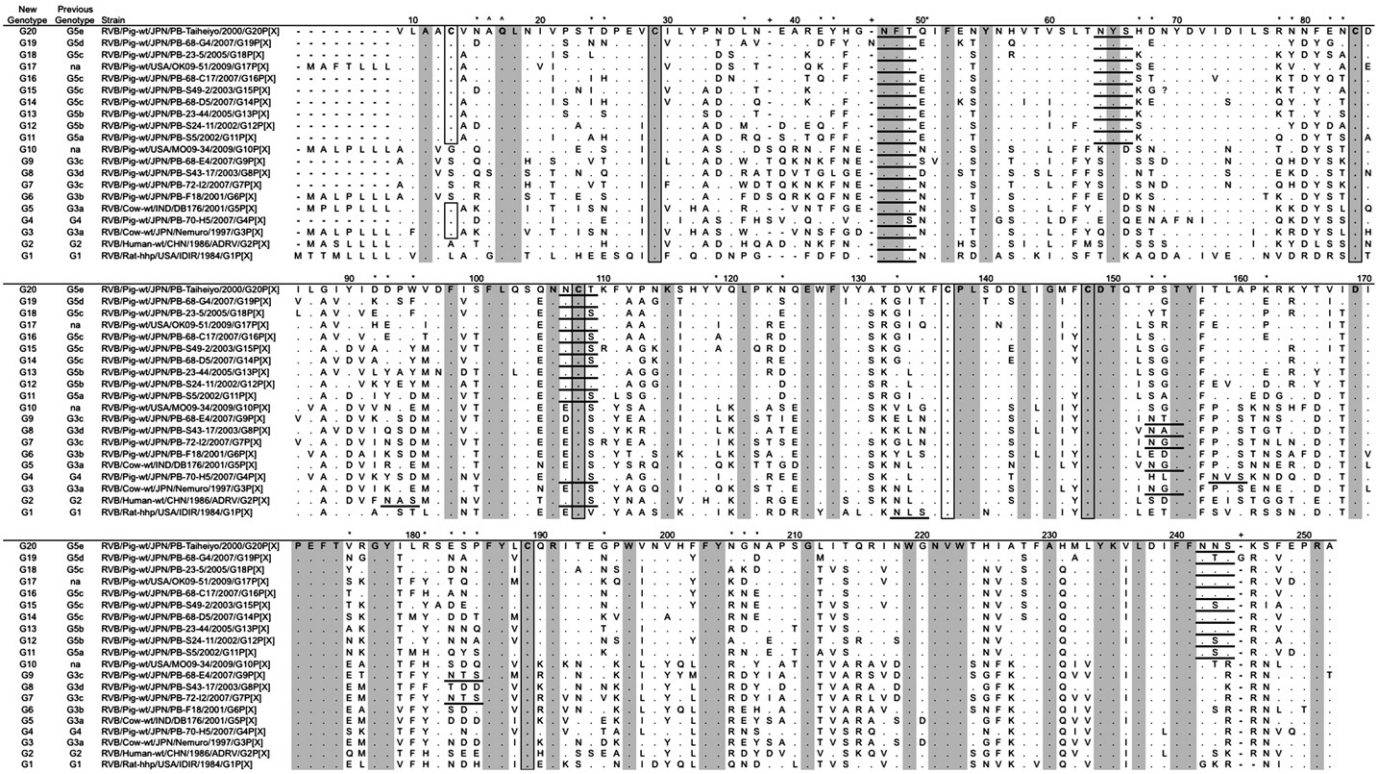


Fig. 5. Multiple amino acid sequence alignment of selected RVB strains including the new and previously described VP7 genotypes. Matching amino acids are indicated with dots; gaps are shown by dashes; potential N-linked glycosylation sites are underlined; cysteine residues are boxed; insertion sites 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.

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 days old age group were positive for all three RV groups tested, indicating that pigs continue to be infected throughout their early life, as suggested by Miyazaki et al. (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 five 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

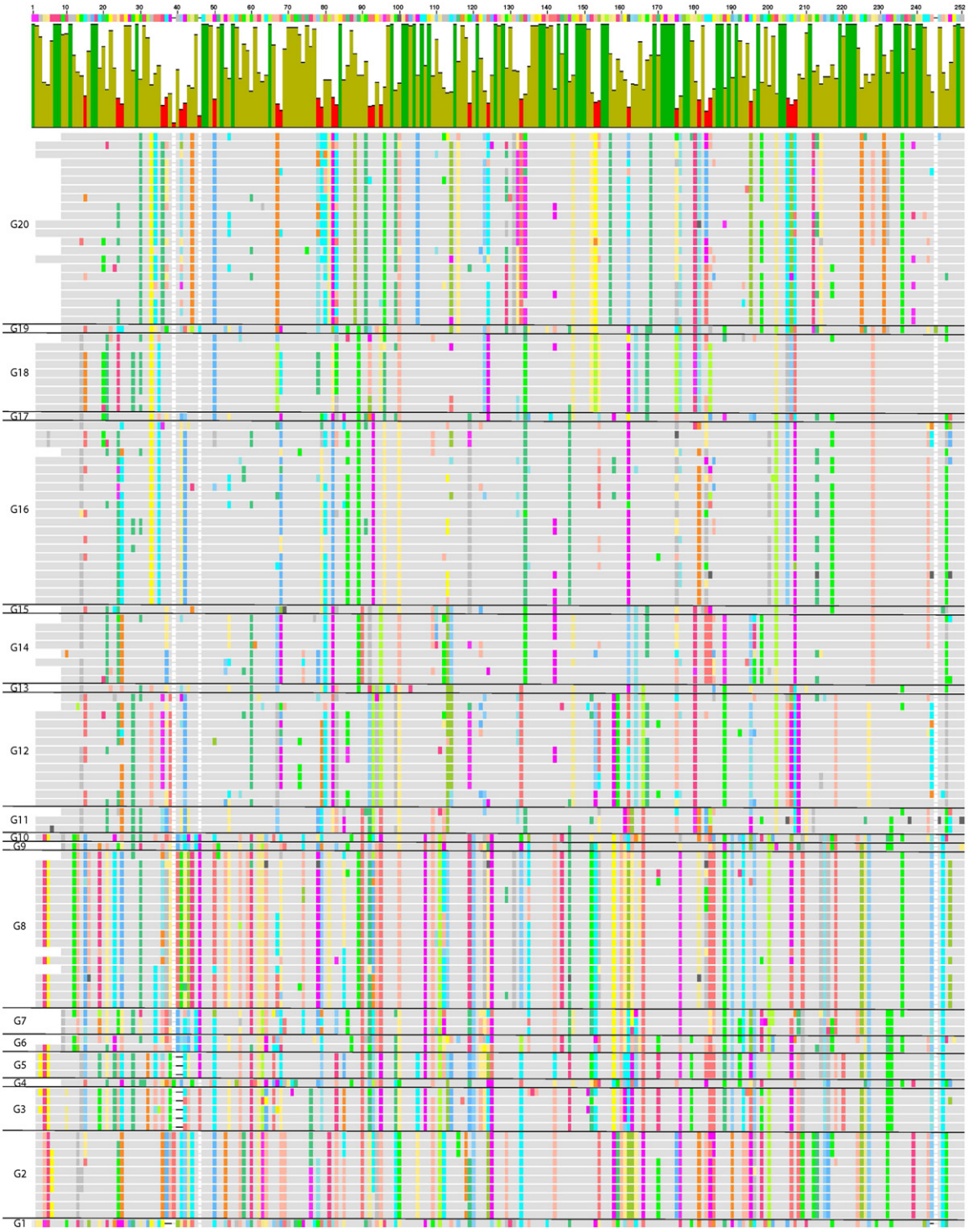


Fig. 6. Visual representation of the alignment for the 125 RVB amino acid sequence divided into the 20 proposed genotypes. The designated G genotype is listed on the left side. The consensus amino acid sequence is listed at the top of the chart where each color represents a specific amino acid. The histogram represents diversity within each amino acid position where peaks (dark green) represent conserved residues while valleys (red) represent divergent amino acid residues.

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) as previously determined for RVA strains to discriminate serotypes (Nishikawa et al., 1989). For a few RVB genotypes (G6, 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.

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 *S. 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, *E. coli*, *C. difficile* and *C. perfringens*; 11–55 days, *E. coli*, coccidia; >55 days, *L. 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 g of each sample was homogenized with 2 mL of gamma-irradiated Hyclone donor equine serum (Thermo Fisher Scientific, Waltham, MA) and centrifuged at 4200 rpm for 1 h. 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 a nd 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 (Supplemental Data 1), as well as the sequences determined in this study (Table 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).

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2012.07.006.

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