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Identification, phylogenetic analysis and classification of porcine group C rotavirus VP7 sequences from the United States and Canada

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ABSTRACT

Rotavirus C (RVC) is a major cause of gastroenteritis in swine. Between December 2009 and October 2011, 7520 porcine samples were analyzed from herds in the US and Canada. RVC RNA was detected in 46% of the tested samples. In very young pigs ($\leq$3 days old) and young piglets (4–20 days old), 78% and 65%, respectively, RVC positive samples were negative for RVA and RVB. RVC RNA was also detected in 10% of tested lung tissues. Additionally, we investigated the porcine RVC molecular diversity by sequencing the VP7 gene segment of 65 specimens, yielding 70 VP7 gene sequences. Based on pairwise identity frequency profiles and phylogenetic analyses, an 85% nucleotide classification cut-off value was calculated using the novel sequence data generated in this study (n=70) and previously published RVC VP7 sequences (n=82), which resulted in the identification of 9 VP7 RVC genotypes, G1 to G9.

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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., 2010; 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 (Bohl et al., 1982; Saif et al., 1980). 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 (Chang et al., 1999; Mawatari et al., 2004; Tsunemitsu et al., 1991), dogs (Otto et al., 1999), ferrets (Torres-Medina, 1987), and also humans (Araujo et al., 2011; Banyai et al., 2006; Castello et al., 2009; Mitui et al., 2009; Rahman et al., 2005; Rodger et al., 1982).

Due to difficulties in propagating RVCs in cell culture, a proper serological typing assay has not been developed to analyze RVCs (Fujii et al., 2000; Saif et al., 1988; Tsunemitsu et al., 1991). 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., 2007). 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

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G3 RVC genotype (Tsunemitsu et al., 1992, 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 (Abid et al., 2007; Adah et al., 2002; Araujo et al., 2011; Banyai et al., 2006; Castello et al., 2009; Jiang et al., 1995, 1996; Khamrin et al., 2008, Kuzuya et al., 2007; Martella et al., 2007; Medici et al., 2009; Mitui et al., 2009; Moon et al., 2011; Rahman et al., 2005; Schnagl et al., 2004). 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 (Ciarlet et al., 1997; Nishikawa et al., 1989; Tsunemitsu et al., 1992, 1996).

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., 2008). 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., 2007) 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.

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.*, *Escherichia coli*, *Clostridium difficile* and *Clostridium perfringens*, *Lawsonia intracellularis* and *Brachyspira spp* are also tested by a variety of methods. Among 7520 samples submitted from December 2009 to October 2011, RVC was successfully detected in 3447 samples (46%). From these 3447 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 (Fig. 1). Affected porcine intestinal tissue samples had lesions characterized by villus tip epithelial cell (enterocyte) swelling with cytoplasmic vacuolar change progressing to

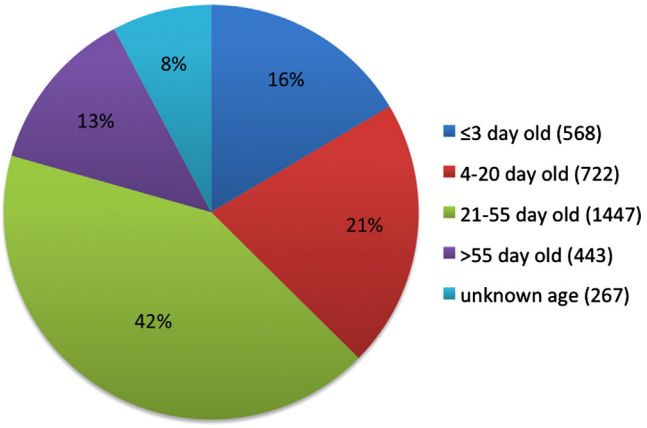

Fig. 1. Age distribution of RVC positive samples, $n=3447$.

enterocyte necrosis and sloughing, resulting in denuded villus tips (Fig. 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 min 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 (Fig. 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 (Fig. 3).

Because RVA has been shown to infect extra intestinal organs (Azevedo et al., 2005; Blutt et al., 2003; Crawford et al., 2006; Kim et al., 2011; Zhao et al., 2005), 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, Mycoplasma hyorhinis, Mycoplasma hyosynovaie,* and *Haemophilus 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 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 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

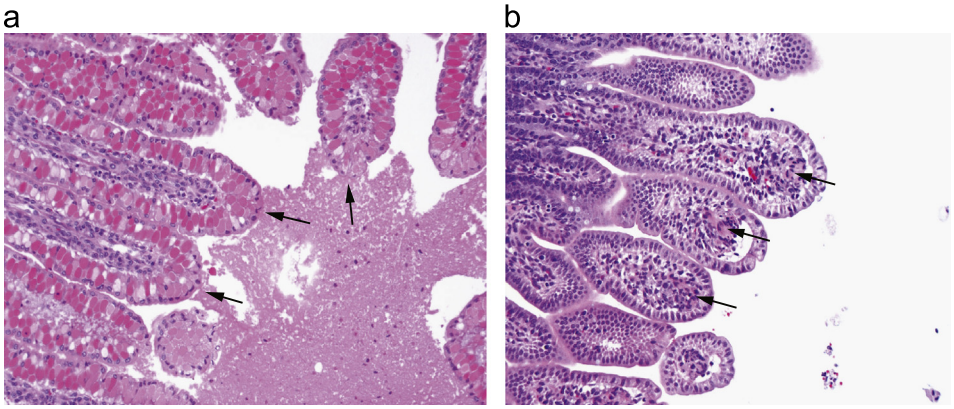

Fig. 2. Hematoxylin and eosin stained jejunum from a 3-day piglet (10× magnification). Tissue architecture was revealed by eosin staining (pinking) while nuclei were stained with hematoxylin staining (blue). (a) Normal villi: The arrows indicate colostrum, which is being absorbed by the villi. (b) Acute enteritis: The arrows indicate enterocyte necrosis in the three center villi.

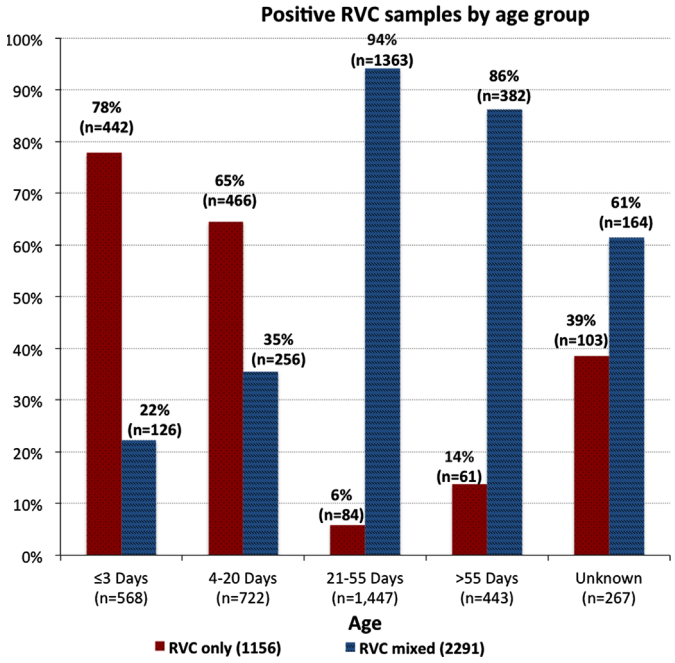

Fig. 3. Histogram illustrating the co-infection rates of RVC with RVA and/or RVB per age group.

Table 1
RV distubution 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%

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; Supplemental data 1), pairwise identity frequency graphs were constructed on the nucleotide and amino acid level (Fig. 4), and a phylogenetic tree was created on the nucleotide level (Fig. 5 and Supplemental data 2). 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) (Fig. 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 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 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 2). In the majority of the herds, only a single RVC genotype was detected, but RVC strains belonging to two distinct G

Table 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	–	–	21–55 days	Intestine	G6	Illinois	21	JX273338
RVC/Pig-wt/USA/IL10-40/2010/G6P[X]	+	na	–	–	21–55 days	Intestine	G6	Illinois	21	JX273337
RVC/Pig-wt/USA/IL10-39/2010/G6P[X]	+	na	–	–	21–55 days	Intestine	G6	Illinois	21	JX273336
RVC/Pig-wt/USA/IL10-38/2010/G6P[X]	+	na	–	–	21–55 days	Intestine	G6	Illinois	21	JX273335
RVC/Pig-wt/USA/IL10-37/2010/G6P[X]	+	na	–	–	21–55 days	Intestine	G6	Illinois	21	JX273334
RVC/Pig-wt/USA/IL10-36/2010/G6P[X]	+	na	–	–	21–55 days	Intestine	G6	Illinois	21	JX273333
RVC/Pig-wt/USA/IL10-35/2010/G6P[X]	+	na	–	–	21–55 days	Intestine	G6	Illinois	21	JX273332
RVC/Pig-wt/USA/IL10-34/2010/G6P[X][c]	+	na	–	–	21–55 days	Intestine	G6	Illinois	21	JX273331
RVC/Pig-wt/USA/IL10-33/2010/G6P[X][c]	+	na	–	–	21–55 days	Lung	G6	Illinois	21	JX273330
RVC/Pig-wt/USA/IL10-32/2010/G6P[X]	+	na	–	–	21–55 days	Intestine	G6	Illinois	21	JX273329
RVC/Pig-wt/USA/IL10-31/2010/G6P[X]	+	na	–	–	21–55 days	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	21-55 days	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	14–28 days	Intestine	G6	North Carolina	3	JX273300
RVC/Pig-wt/USA/NC10-54/2010/G6P[X]	+	+	+	–	42 days	Intestine	G6	North Carolina	24	JX273351
RVC/Pig-wt/USA/TN10-51/2010/G9P[X][d]	+	na	+	–	42 days	Intestine	G9	Tennessee	22	JX273348
RVC/Pig-wt/USA/TN10-50/2010/G5P[X][d]	+	na	+	–	42 days	Intestine	G5	Tennessee	22	JX273347
RVC/Pig-wt/USA/NC10-53/2010/G6P[X][e]	+	na	+	–	42 days	Intestine	G6	North Carolina	23	JX273350
RVC/Pig-wt/USA/NC10-52/2010/G1P[X][e]	+	na	+	–	42 days	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	21-55 days	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	28 days	Intestine	G6	Illinois	9	JX273306

RVC/Pig-wt/USA/IL09-18/2009/G6P[X]	+	na	+	na	28 days	Intestine	G6	Illinois	15	JX273315
RVC/Pig-wt/USA/AR09-17/2009/G6P[X]	+	na	+	na	28 days	Intestine	G6	Arkansas	14	JX273314
RVC/Pig-wt/USA/MN09-23/2009/G1P[X]	+	–	+	na	21-55 days	Intestine	G1	Minnesota	17	JX273320
RVC/Pig-wt/USA/MN11-68/2011/G1P[X]	+	–	+	+	21-55 days	Fecal	G1	Minnesota	27	JX273365
RVC/Pig-wt/USA/MN11-67/2011/G1P[X]	+	–	+	+	21-55 days	Fecal	G1	Minnesota	27	JX273364
RVC/Pig-wt/USA/MN11-66/2011/G1P[X]	+	–	+	+	21-55 days	Intestine	G1	Minnesota	27	JX273363
RVC/Pig-wt/USA/MN10-55/2010/G1P[X]	na	na	–	–	91 days	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	21 days	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.

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 (Fig. 6). The 8 variable regions (VR-1 to VR-8) as described by Tsunemitsu and colleagues are boxed in Fig. 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 (Fig. 6). The 152 RVC VP7 sequences used in the phylogenetic analysis also displayed specific amino acid profiles for the 9 proposed G genotypes (Supplemental data 3). 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 (Bohl et al., 1982; Collins et al., 2008; Saif et al., 1980). 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 (Azevedo et al., 2005; Crawford et al., 2006; Fragoso et al., 1986; Kim et al., 2011). Crawford and colleagues noted an

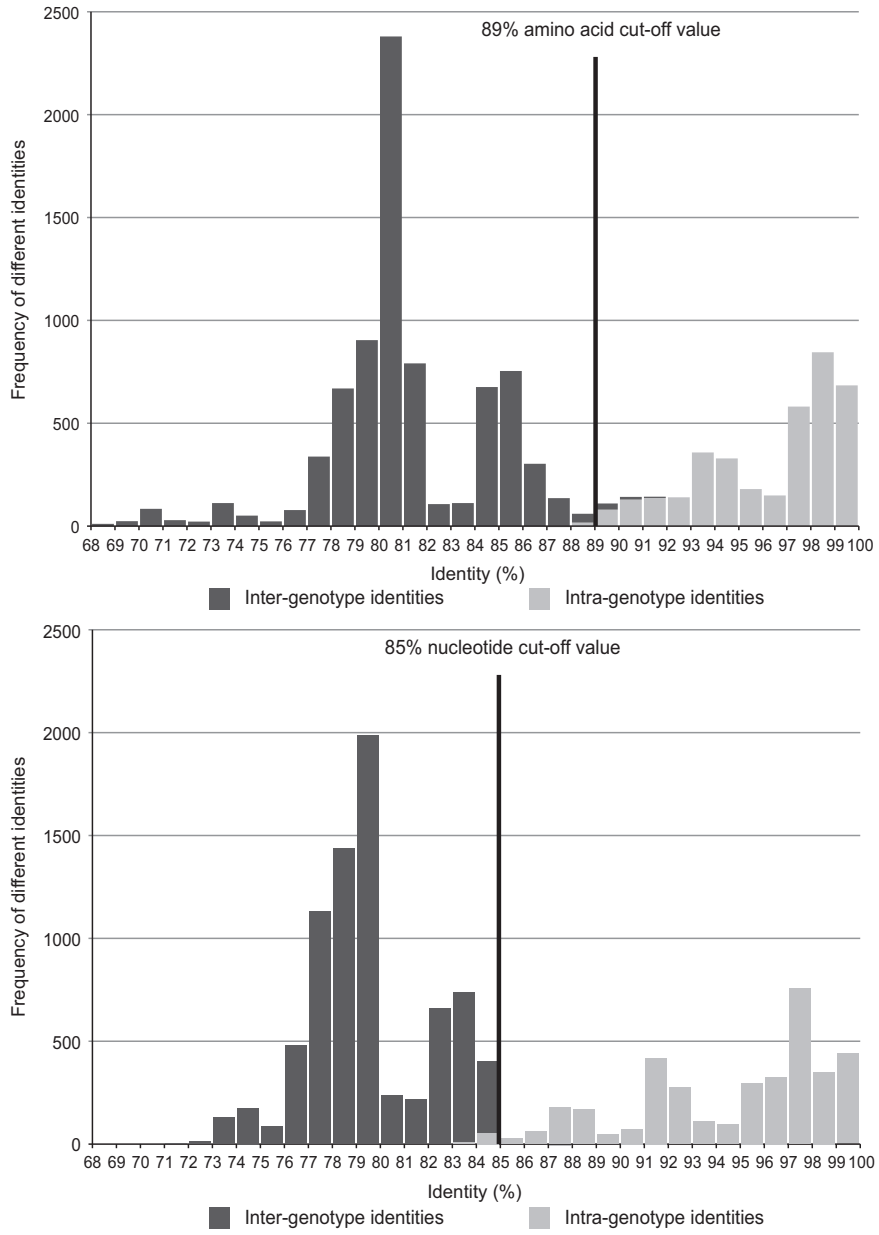

Fig. 4. Nucleotide and amino acid pairwise identity frequency graphs of the VP7 ORF of 152 RVC strains. The proposed 85% nucleotide (bottom) and current 89% amino acid (top) cut-off value are represented by the vertical lines.

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.

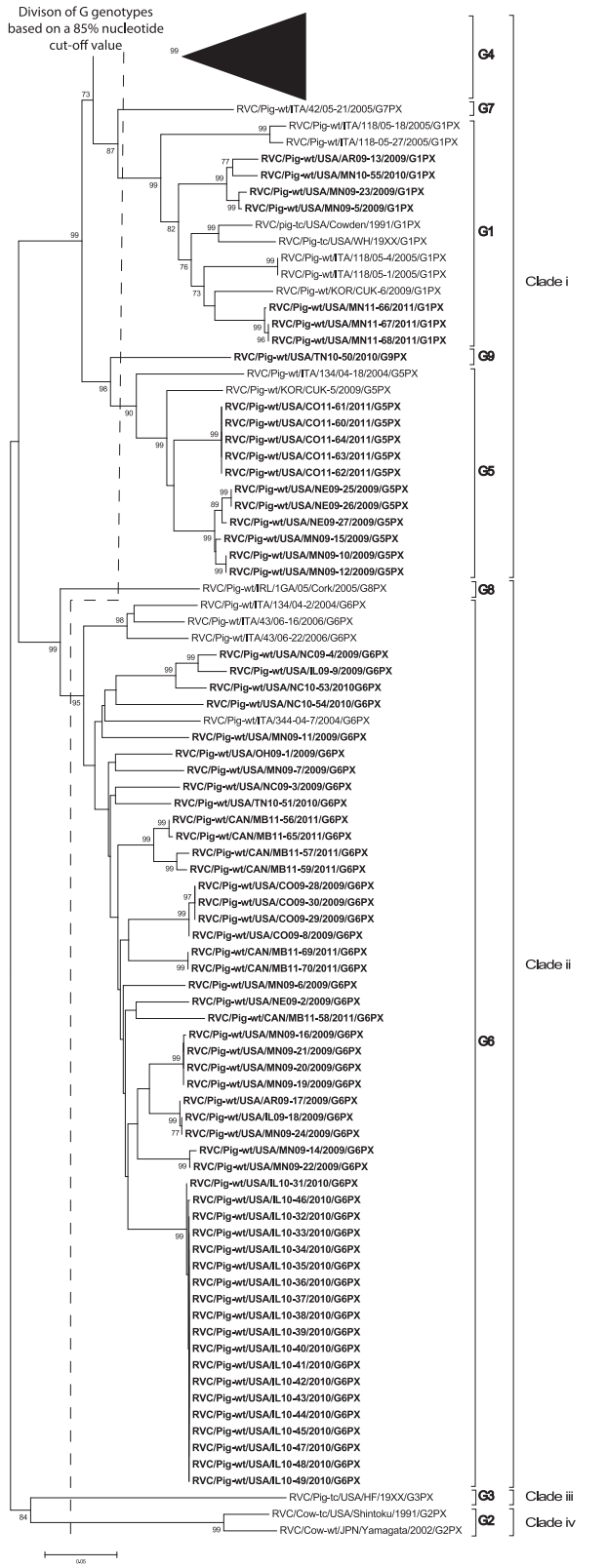

Fig. 5. Phylogenetic trees of nucleotide VP7 sequences from 152 RVC strains. The sequences from this study are bolded. The G4 cluster was replaced by a triangle. The dashed line represents the 85% nucleotide identity cut-off value, establishing 9 G genotypes. Bootstrap values less than 70% are not shown.

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., 2007). 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 (Marthaler et al., 2012; Matthijnssens et al., 2008). 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.

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, 7520 porcine samples from herds in the US and Canada were submitted to our laboratory for analysis, and 3447 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.

Table 3
RVC VP7 genotype nucleotide (bottom) and amino acid (top) percent identities.

	G1	G2	G3	G4	G5	G6	G7	G8	G9
G1	90.1–100	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	91–100 73.2–75.6	96.0	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	93.6 73.2–73.3	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	na 72.4–74.4	94.3–100	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	93.2–100 81.9–84.4	88.6–100	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	87.5–100 75.5–79.6	88.4–100	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	85.3–100 75.9–78.1	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	na 76.1	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	na 77.6	na na

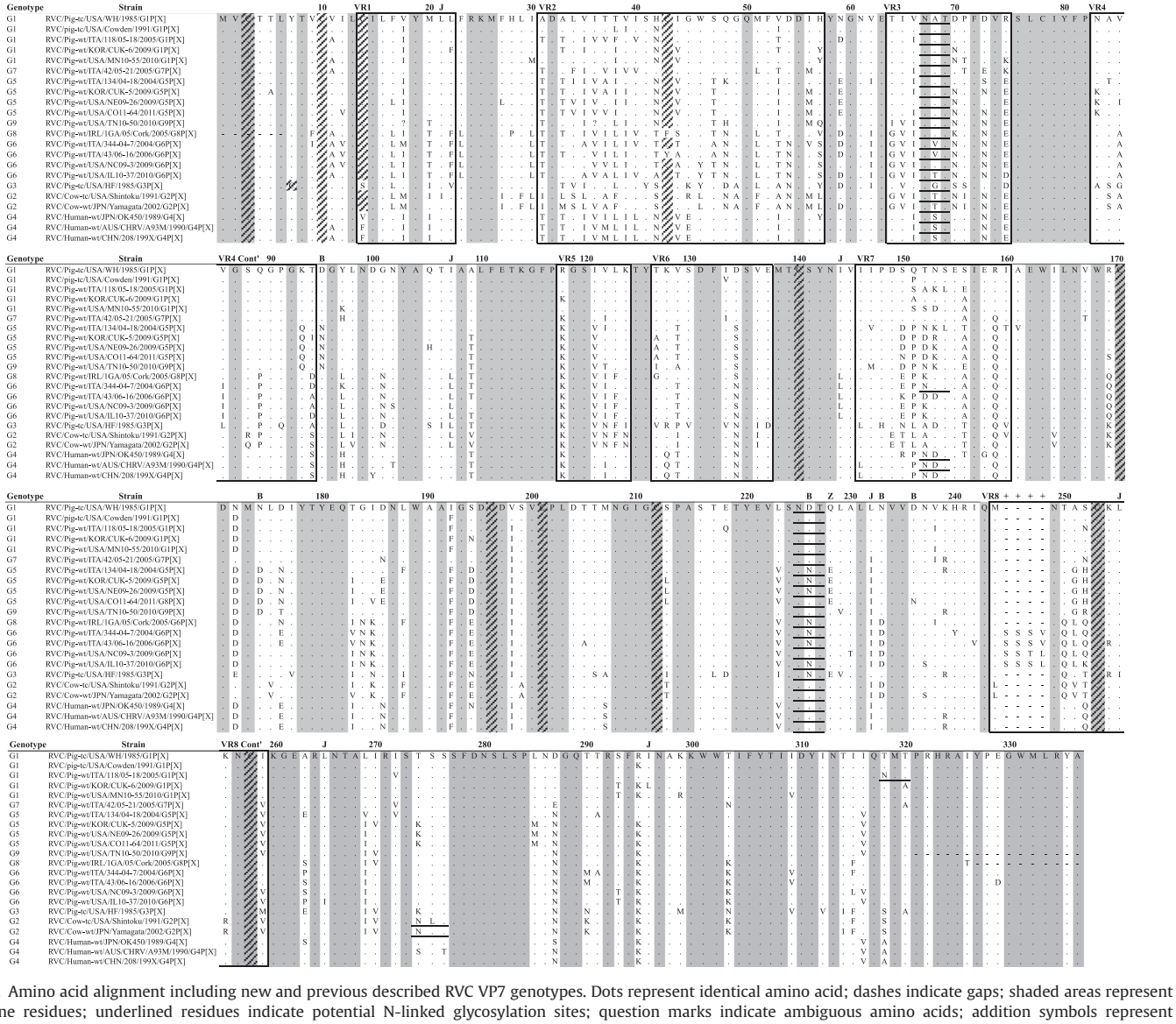

Fig. 6. Amino acid alignment including new and previous described RVC VP7 genotypes. Dots represent identical amino acid; dashes indicate gaps; shaded areas represent cysteine residues; underlined residues indicate potential N-linked glycosylation sites; question marks indicate ambiguous amino acids; 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.

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 g of each sample were emulsified, centrifuged at 4200 rpm for 1 h, 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 (Marthaler et al., 2012; Rahman et al., 2005). 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 min 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., 2008). 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 ($n=82$) (Supplemental Data 1) 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).

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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.2013.08.001.

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