RESEARCH REPORT
SWINE HEALTH

Title: Genogroup A, B and C porcine rotaviruses: Prevalence and implications for effective vaccines – **NPB #12-094**

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Industry Summary: Our study provides critical information on high genetic and antigenic diversity of porcine RVs (PRVs) confirming that the existing PRV vaccines do not include dominant field PRV strains currently circulating in the US. In particular, we have demonstrated that the prevalence of G9 PRV strains that are not included in the current PRV vaccines, is higher than a few decades ago. Molecular analysis based on complete genome sequencing of the current dominant G9 PRVs, indicated that they possibly have emerged as reassortants between the current PRV vaccine, human and bovine RV strains. We have also confirmed high genetic heterogeneity in porcine group C RVs and the concurrent co-circulation of different genotypes. Further, our results confirmed that there is only limited virus neutralizing ability between heterologous vaccines, historic and current PRV strains. **Our research provides novel genetic data for PRV groups A and C that will permit generation of improved/updated PRV vaccines. Such vaccines will be beneficial to the pork industry to prevent group A and non group A PRV diarrhea, improve growth rates and reduce PRV associated morbidity and mortality.** Our results also suggest that swine may be reservoirs for generation of reassortant strains potentially transmissible to humans, thereby posing public health concerns.

Keywords: group A and C porcine rotaviruses, prevalence, genetic diversity, swine, reassortants.

Scientific Abstract:
Fecal samples collected in different seasons of 2004 and 2011 from diarrheic and healthy nursing piglets from 5 selected swine farms in the US were screened for PRV groups A (PRVA) and C (PRVC) using RT-PCR. RVs were identified in 27.4% (65/237) of the samples, with 7.6% and 21.5% positive for Gp A and C RVs, respectively. The highest overall prevalence was in summer (35.2%) followed by winter (27.4%) [2004, spring (23.9%) and summer (19.4%); 2011 winter (61.9%) and summer (43.9%)]. The dominant G-P combination was G9P[13] found in 60.9% of positive samples. The other combinations were G9P[7] (8.7%), G4P[13] (8.7%), G11P[13] (4.3%), and G11P[7] (4.3%). Sequence analysis of partial VP7 genes of selected strains revealed that the G4 strains were closely related to one another (95%) and, to a lesser extent, to human (82 to 84%) and porcine (84 to 86%) G4 strains. Of the 128 samples collected in 2012, 23.5% from nursing piglets and 8.5% from weaned piglets were PRVC positive, with a higher PRVC frequency in diarrheic (28.4%) than in non-diarrheic (6.6%) piglets. Partial sequencing and phylogenetic analysis of the VP6 gene of selected PRVC from different farms (historic and recent) revealed high nucleotide sequence identity with reference human (82.5-86%) and porcine (86.2-97.2%) RVC. For two strains (RVC/Pig-wt/USA/RV0104/2011/G3PX and RVC/Pig-wt/USA/RV0143/2012/G6Px) from two different farms full length sequences of the inner capsid VP6, enterotoxin NSP4 and the outer capsid VP7 and VP4 (partial for RV0104) genes were determined. The VP6 gene of the two strains showed high (99%) nucleotide identity to one another, 84-91% identity to other porcine RVC strains and 81-82% identity to human and bovine RVC strains. The NSP4 gene analysis revealed that RVC/Pig-wt/USA/RV0104/2011/G3PX and RVC/Pig-wt/USA/RV0143/2012/G6Px strains were not closely related to each other (87% identity), but shared higher identity with prototype RVC/Pig-wt/USA/Cowden/1980/G1Px strain (93% and 89%, respectively). The VP7 gene analysis indicated that the two strains were distantly related to one another (72% identity). RVC/Pig-wt/USA/RV0143/2012/G6Px was most closely related to porcine RVC G6 strains (82-86% identity), whereas RVC/Pig-wt/USA/RV0104/2011/G3PX was most closely related to porcine HF (G3) strain (94% identity). Analysis of the full length nucleotide sequence of the VP4 gene revealed that RVC/Pig-wt/USA/RV0143/2012/G6Px was distantly related to porcine (75%), bovine (74%) and human (70%) strains.
Comparative sequence analysis of the full genome constellations of the selected dominant G9P[13] and G9P[7] field PRVA strains revealed that the P[13] strain genome constellation was G9-P[13]-I5-R1-C1-M1-A8-N1-T1-E1-H1, while that of the P[7] strain was G9-P[7]-I5-R1-C1-M1-A8-N1-T7-E1-H1. Remarkably, four (VP2, VP3, NSP2 and NSP4) and five (VP7, VP2, VP3, NSP2 and NSP4) genome segments of the G9P[13] and the G9P[7], respectively, shared the highest nucleotide identity (89.5-95.6%) with the respective genes of PRV OSU. Only NSP1 genes of both G9 strains and NSP3 of the G9P[13] strain shared the highest nucleotide identity (~91-92%) with PRV Gottfried NSP1 and NSP3, respectively. The G9P[7] NSP3 gene was most closely related to RVA/Cow-tc/GBR/UK/1973/G6P5 with 93.4% of similarity. This suggests that both prototype vaccine strains might have contributed some genetic material to the currently circulating field PRV diversity via multiple reassortment events. It is also of interest that VP7 genes of both G9 strains were most closely related to human G9 strains with nucleotide identity of over 92%.
The sera of gnotobiotic (Gn) pigs inoculated with one of the dominant PRVA G9P[13] contained high levels of homologous but very low levels of heterologous virus neutralizing (VN) antibodies (Ab) against several human (porcine-like) and PRVAs. Similarly, reference PRVA OSU and Gottfried (prototype vaccine) strains induced low heterologous VN Ab titers against PRVA G9P[13] and human RV Wa. **This suggests that only limited cross-protection may be conferred by the current PRV vaccines against the circulating wild-type PRVAs**. Consistent with the higher overall nucleotide identity between OSU and the G9 strains, higher levels of cross-neutralizing Abs were observed between the G9P[13] strain and OSU as compared to Gottfried strains. Additionally, when we assessed the one-way cross-reactivity between the historic cell-culture adapted PRVC Cowden (G1) and the newly identified field PRVC (G6), only limited cross-neutralizing Ab titers were detected. The heterologous VN Ab titer for Cowden strain was ~150 lower than for the homologous strain.
Thus, our results demonstrate the high genetic and antigenic diversity and the shift in dominant genotype prevalence among PRVAs and PRVCs, may provide a possible explanation why the current vaccines may lack efficacy. Additionally, our findings suggest that the currently dominant G9 PRVAs may be natural reassortants between RVs from multiple hosts including swine, bovine and human. **Therefore, epidemiological studies on the prevalence and genetic diversity of PRVs are of utmost importance to design updated RV vaccines based on the dominant RV genotypes circulating in swine and to control RV infection in swine and humans.**

Introduction:
Rotaviruses (RVs) are a major cause of viral gastroenteritis in children and young animals worldwide, including pigs. Genogroups (Gp) A, B, and C are associated with acute gastroenteritis in pigs. Within Gp A, 23 G genotypes (structural viral protein [VP] 7 related) and 32 P genotypes (VP4 related) have been identified (De Grazia, Giammanco et al. 2010). Based on the differences in the outer capsid VP7 (G) and VP4 (P) proteins carrying independent epitopes for neutralizing

antibodies, PRVAs are also assigned to respective G and P serotypes. Generally distinct RV G or P genotypes (serotypes) do not induce cross-protection. There are several serotypes presently identified for PRVCs, and there is no recognized genotype/serotype classification for PRVBs. Limited information is available about the prevalence of PRVA genotypes and PRVBs and PRVCs. The current PRV vaccine contains only PRVA genotypes G4 and G5 and likely has reduced efficacy against heterologous PRVA genotypes and no efficacy against antigenically distinct PRVC and PRVBs. A knowledge base of the genetics and epidemiology of PRVs is critical for vaccine development and optimization. **Therefore, studies of the prevalence and in vitro propagation and characterization of the dominant and emerging PRV strains as well as full-genomic sequence analysis of selected strains will allow development of improved PRV vaccines.**

Rotavirus infections seriously impact the pork industry, with mortality rates in piglets ranging from 0 to 50%. Recovered piglets have uneven or reduced growth rates resulting in additional economic losses. Recent data based on molecular assays (RT-PCR) suggests that PRVC is dominant in pigs less than 1 wk of age, whereas PRVAs are prevalent post-weaning (>80% of RV diarrheic cases) and in older pigs (>6 wks of age) (Marthaler, Homwong et al. 2014). Currently, the only RV vaccine commercially available is for PRVAs. Difficulties with in vitro cultivation and lack of comprehensive information on genetic/antigenic variability and prevalence hamper vaccine development for the PRVBs and PRVCs. However, we have successfully adapted the only PRVC to cell culture in our lab (Terrett and Saif 1987; Saif, Terrett et al. 1988). This model strain can be used for in vitro and in vivo studies to provide comprehensive information linking molecular genetic and biological studies of PRVC. Prior experimental pig challenge studies have established that there is no intergroup PRV cross-protection. Previously PRVA, genotypes G3, G4 and G5 and P[6] and P[7] were reported most commonly. However, PRVA vaccines containing only serotypes/genotypes OSU (G4P[6]) and Gottfried (G5P[7]) have low efficacy and do not provide heterotypic protection against distinct PRVA G or P genotypes (Saif and Fernandez 1996). Also, it is unknown whether circulating live vaccine strains contribute to wild-type PRV diversity through genetic reassortment and produce progeny with altered tissue tropism or disease expression (Parks, Latham et al. 2007).

Only a few PRV prevalence studies have been conducted worldwide, including in the US. Information is lacking about the overall genetic and antigenic diversity of PRVs. There are numerous reports on identification of emerging genotypes (G1, G6, G9, G10, G12, P[8], P[23], and P[27]) of PRVA and Gp B and C RVs in pigs (Winiarczyk, Paul et al. 2002; Teodoroff, Tsunemitsu et al. 2005; Kim, Park et al. 2010), but their current prevalence is largely unknown. Emergence of these strains may contribute to RV vaccine failures in pigs. Additionally, an extensive sequence database is lacking for PRVs with just a few complete genome sequences available for only Gp A strains. Without knowing the diversity of PRVs, it is difficult to understand the dynamics of circulating strains and to develop optimal vaccines to prevent PRV infections.

In this study, we used historical (2002-2005) and recent fecal samples (2011-2012) from pigs of various ages (with or without diarrhea) and in different geographical locations (OH, NC and MI) to assess the prevalence of PRVA, PRVB and PRVC groups and PRVA genotypes). Reassortment of all 11 rotavirus genome segments plays a key role in generating RV diversity and defines RV host range. A classification system based on complete genome sequencing has recently been established (Matthijnssens, Ciarlet et al. 2008). Our goals include complete genomic sequencing and comparative sequence analysis of the prevalent field and reference group A and C PRV strains [including prototype vaccine strains Gottfried and OSU (Gp A) and Cowden (Gp C)] to assess their reassortment potential and the molecular basis for emergence of new strains (genotypes). **Such data is critical for PRV vaccine development**. Additionally, full genomic sequencing will allow identification of novel PRVs such as the recently characterized PRV SKA-1 strain closely related to novel human RVs or estimate their zoonotic potential by recognizing genes of human RV origin. Finally, our proposed assessment of the cross-neutralizing potential of the identified dominant PRV strains will allow prediction of their cross-protective potential for future in vivo vaccine studies.

Objectives:

<u>Objective 1.</u> To investigate the prevalence of PRV genogroups (A, B, C) and PRVA genotypes using RT-PCR and partial sequencing of historical (2002-2005) and recent (2011-2012) fecal samples from pigs of various ages collected from swine herds in Ohio (OH), North Carolina (NC) and Michigan (MI).

<u>Objective 2.</u> To perform complete genomic sequencing for selected emerging and endemic PRVA strains, to adapt the dominant strains to cell culture and to assess their cross-neutralizing potential in vitro.

<u>Objective 3.</u> To identify the circulating dominant PRVC genotypes and estimate their prevalence, to adapt the dominant strain to cell culture and assess the cross-neutralizing potential in vitro, and to perform complete genomic sequencing for selected dominant PRVC strains.

Materials & Methods:

Samples. A total of 371 fecal samples were collected in 2004 (n=118), 2011 (n=130) and 2012 (n=123) from 5 selected swine farms in Ohio, USA for detection of RVAs (Table 1).

Table 1: Numbers of historical and recent fecal samples available from different farms.

Swine farm	Nursing (1-3 weeks)	Post-weaning (3-10 weeks)	Finisher (10-24 weeks)	Sow (>1 year)
2002-2005				
OH-A	14	12	22	13
OH-B	61	90	105	30
OH-C	15	12	12	44
OH-D	8	10	NA*	NA
OH Slaughterhouse	NA	NA	83	NA
MI-A	NA	NA	61	NA
NC-A	NA	NA	8	NA
NC-B	NA	NA	21	NA
2011				
OH-B	38	NA	NA	NA
OH-D	34	NA	NA	NA
OH-E	37	NA	NA	NA
Total (621)	207	124	312	87

* NA = not available, OH = Ohio, MI = Michigan, NC = North Carolina

Samples collected in 2004 were all from nursing piglets with no information about diarrheal status (n=44), whereas for the 2011, samples age information was missing for 39 samples from Farm 2 and diarrhea status was not available for 96 samples. However, samples collected in 2012 were from both nursing (n=76) and weaned (n=47) piglets and they were collected from piglets with diarrhea (n=62) and without diarrhea (n=61). Fecal samples (feces or swabs) were collected from individual piglets and placed in labeled sterile plastic tubes and transported to the Laboratory on ice. Ten percent fecal suspensions (wt/vol) were prepared in Minimum Essential Medium (Life Technologies, NY, USA), vortexed and centrifuged at 1,800 x g for 30 min at 40C. The clarified supernatants were used for RNA extraction. None of the farms surveyed used the RVA vaccine (ProSystem Rota, Intervet Inc., Merck Animal Health) licensed for swine in the US.

RNA Extraction. RNA was extracted from 250µl starting volume of centrifuged 10% fecal suspensions using RNeasy mini kit from Qiagen Company (CA, USA) according to the manufacturer's instructions. The total RNA recovered was suspended in 40µl of nuclease-free water and stored at -70 0C until used.

Detection of RVA. Conventional reverse transcription-polymerase chain reaction (RT-PCR) was used for detection of the RVAs with validated primer sets, NSP3F (963–982) and NSP3R (1,049-1,034), (Table 2) using Promega reagents according to the manufacturer's instructions (Promega, CA, USA). Prior to reverse transcription, 2µl of RNA sample was mixed with 0.5µl of Dimethyl sulfoxide (Sigma-Aldrich, MO, USA), heated at 970C for 5 min, then snap chilled on ice, to denature the double stranded segments of the rotavirus genomic RNA. For the RT-PCR reaction the following conditions were applied: incubation for 30 min at 50^0C for RT reaction, preheating the sample at 94^0C for 5 min for initial denaturation followed by 40 PCR cycles at 94^0C for 15 sec, 56^0C for 1 min and 72^0C for 30 sec, then a final extension for 7 minutes at 72^0C and then 4^0C for storage until removed from the machine. The PCR products from the above reaction were analyzed in a 3% Agarose gel after staining with EZ-Vision dye (Amresco) and UV-light transillumination. The amount of PCR inhibitors in the samples were determined to be negligible based upon results obtained by making 10-fold and 100-fold dilutions of each RNA sample.

A chi-square test was used assess the relationship between several factors (farm, year, season, age group and diarrheal status) and the RVA detection using procedure frequency in the SAS computer program.

Detection of RVC.

Conventional RT-PCR was used for detection of the RVCs with validated primer sets (Table 3) using Promega reagents according to the manufacturers' instructions. The amplicons were analyzed in 1.5-3% agarose gel.

Genotyping and genetic analysis of RVA strains. Using the known human, porcine and bovine RVA VP7 gene sequences available in GenBank, we designed genotype specific primers (Table 2) for common G genotypes found in swine (G3, G4, G5, G9 and G11) to genotype the RVA positive samples directly without sequencing. The specificity of these G-typing primers was evaluated using a panel of well characterized laboratory reference strains consisting of human RVA strains Wa (G1), DS-1 (G2), M-strain (G3), 69M (G8) and a characterized human G9P[6] strain obtained from the CDC and the porcine RVA strains Gottfried (G4) and OSU (G5). Selected genotyped samples and the samples that could not be genotyped using the above primers were subjected to RT-PCR and partial sequencing using a universal primer set designed previously for

the VP7 gene (Table 2) to determine the G types for all strains and perform phylogenetic analysis. P types were determined using a universal primer pair (793F/1583R) for RT-PCR and partial sequencing of the RVA VP4 gene (nt 793 to 1604) (this study).

The amplicons were sequenced directly using forward and reverse RT-PCR primers. DNA sequencing was carried out by using BigDye Terminator Cycle chemistry and 3730 DNA Analyzer (Applied Biosystems, Foster, CA). The resulting partial (260bp) VP6 gene sequences of the RVCs were aligned with corresponding fragments of selected reference strains of human, porcine and bovine RVC strains. Distance analysis and phylogenetic inference were conducted using the Mega 5.0 software package. The evolutionary distances were computed using the Maximum Composite Likelihood method [2] and are in the units of the number of base substitutions per site. The P genotypes were assigned to each sample after sequence analysis using either BLAST (blastn) search (http://www.ncbi.nlm.nih.gov/) and/or RotaC2.0 automated genotyping tool for Group A rotaviruses. The DNA sequences were aligned using the ClustalW method. The dendrograms were constructed using the neighbor-joining method supported with a bootstrap test of 1000 replicates for each gene in MEGA 5 software.

Table 2: Primer sets used for RVA detection and genotyping

Genotypes	Name	Sequence	Region	Amplicon (bp)	Reference
Detection primer set	NSP3F	ACCATCTACACATGACCCTC	963–982	87	(Pang, Lee et al. 2004)
	NSP3R	GGTCACATAACGCCCC			
Common VP7 gene	5'comVP7-F	GGCTTTAAAAGAGAGAATTTC	1-1062	1062	(Winiarczyk, Paul et al. 2002)
	3'comVP7-R	GGTCACATCATACAATTCTAA			
G3	G3Fdeg	GAYAATTCRTGGAARGATACACTT	331-782	452	This study
	G3Rdeg	GTACAAGTRTTTGTYGTCACRYTCA			
G4*	JA-AV-G4-Fdeg	ACD TTA GGA RTY GGA TGT CA	655-1062	407	This study
G5 *	JA-AV-G5-Rdeg	YTT WKY RTC YGC RAT TTC	1-339	339	This study
G9	G9-VP7-4F	ACA GCA TAT GCA AAT TCG TCA CAG	241-388	148	This study
	G9-VP7-4R	CAG TTG GCC ACC CCT TAG TCA			
G11	JA-G11Fdeg	GAYGAYAAGTGGARAGATACTCTC	331-589	259	This study
	JA-G11Rdeg	CYTCATYWGTYTGTTGATAATARTAAAGA			
Universal VP4 gene	793F	TGG AAA GAA ATG CAR TAY AA	793-1583	812	This study
	1583R	CCH GAR AAC ATN GAR AAC ATA TC			
Universal VP7 gene	VP7-39F	GCTCYTTTTRATGTATGGTATTGAATATACCAC	39-406	368	(DiStefano, Kraiouchkine et al. 2005)
	VP7-406R	CTT TAA AAT ANA DGA DCC WRT YGG CCA			

*For G4 genotyping, common primer 3'comVP7-R was used as reverse primer, while for G5, common primer 5'comVP7-F was used as forward primer.

Sequencing and molecular analysis of RVC strains.

To confirm the PCR results and to obtain genetic information on virus diversity, the VP6 amplicons of six positive samples were sequenced. The DNA was purified by using a QIAquick gel extraction kit (QIAGEN, Inc.) according to the manufacturer's protocol. Based on results of the partial VP6 gene sequences, two of the RVC strains (RVC/Pig-wt/USA/RV0104/2011/G3PX & RVC/Pig-wt/USA/RV0143/2012) were selected from two different clusters and further characterised using full-length sequences of their NSP4, VP6 and VP7 and VP4 genes (Partial VP4 gene for RVC/Pig-wt/USA/RV0104/2011/G3Px strain) using specific primers for each gene (Table 3). These two samples contained only RVCs (no group A or B RVs or sapoviruses) and were collected from nursing piglets. The RVC/Pig-wt/USA/RV0104/2011/G3PX was from a piglet from Farm 2, 2011, with diarrhea status unknown, and RVC/PIG-WT/USA/RV0143/2012 was from a diarrheic piglet from Farm 6, 2012. cDNA was synthesised using 3µl of the total RNA using SuperScript IIITM first-strand synthesis system for RT-PCR according to manufacturer's protocol (Invitrogen) using gene specific primers for each gene. Two µl of cDNA was used as a template for the PCR reaction, PrimeSTAR GXL DNA Polymerase (Takara, Japan) was used for high fidelity synthesis and PCR was conducted under the following conditions: 35 cycles at 980C for 10s, 500C for 20s and 680C for 2 min. The amplicons were analyzed in 1.5% agarose gel, the DNA was purified using QIAquick gel extraction kit (QIAGEN, Inc.) and sequenced in both directions using the same primers. The nucleotide sequences obtained were compared with those of similar sequences from reference strains available from GenBank using BLAST software (http://www.ncbi.nlm.nih.gov/BLAST/). The DNA sequences were analyzed as described above for RVA.

Table 3: Primers used for detection and full length sequencing of RVCs NSP4, VP4 (VP8* and VP5*), VP6 and VP7 genes designed from human and porcine RVC strains.

Gene	Name	Sequence	Segment	Amplicon (bp)
Diagnostic (partial VP6)	VP6F	ACAGTATTTCAGCCAGGDTTTC	1095-1116	260
	VP6R	AGCCACATAGTTCACATTTCATC	1332-1354	
NSP4	NSP4-F	GGCTTTAAATTTTTCAGATCAC	1-22	613
	NSP4-R	AGCCWCATGAATTTTTCAYATC	592-613	
VP6	5'comVP6-F	GCAWTWAAAATCTCATTCACAATGG	3-27	1352
	3'comVP6-R	AGCCACATAGTTCACATTTCATCC	1331-1354	
VP7	5'comVP7-F	GCTGTCTGACAAACTGGTC	20-38	1043
	3'comVP7-R	GCCACATGATCTTGTTTACGC	1042-1062	
VP4-1 (VP8*)	VP4-17Fdeg	GATCRATGGCGTCYTCAC	17-34	1222
	VP4-1238R	CCTGATGAATGTAATCCWGGAT	1238-1216	
VP4-2 (VP5*)	VP4-1108F	GATTATTGGGACGATTCAG	1108-1126	1179
	VP4-2267F	AGCCACATTTCAAGCTGGTC	2267-2286	

RNA extraction and purification for Illumina® (complete genome) sequencing

Stool from 4 samples available in sufficient quantity was resuspended in 10 vol.of phosphate-buffered saline (PBS) and vigorously vortexed for 5 min. The supernatant was collected after centrifugation (5 min, 15,000 × g) and filtered through a 0.22 μm filter to remove eukaryotic and bacterial cell-sized particles. The filtrates enriched in viral particles were treated with DNase and RNase to digest unprotected nucleic acid at 37 °C for 90 min. Viral RNA was then extracted using the RNeasy kit (Qiagen) following the manufacturer's instructions. The concentration and purity of extracted RNA were measured with a spectrophotometer (ND-1000, Nanodrop Technologies). RNA extracts that presented 260/280 and 260/230 purity indices equal to or greater than 2.0 were selected. Subsequently, Total RNA was used in library construction using the TruSeq RNA Sample Preparation kit v2 (Illumina®, USA). The final size and concentration of each library was estimated using a Bioanalyzer (Agilent, Santa Clara, CA, USA) and the Qubit (Invitrogen, Carlsbad, CA, USA), respectively. Ten nM library pools were prepared by mixing the 12 libraries to achieve an equal molar concentration of each. Pooled libraries were sequenced by the MiSeq (Illumina®) platform using sequenced runs of 2 × 150 paired-end reads at the J. Craig Venter Institute.

The GenBank accession numbers for partial sequences of all field strains RVA and RVC strains mentioned in this study are listed in Amimo et al., 2012, and Amimo et al., 2013. The GenBank accession numbers for 11 complete genome segment sequences of G9P[7] and G9P[13] strains are currently being assigned.

Results:

Objective 1 (Amimo, Vlasova et al. 2013, JCM).

Epidemiological surveillance of PRVA, PRVB and PRVC strains was conducted in 7 swine herds in Ohio, USA using historical (2004) and recent (2011-2012) fecal samples. Rotaviruses (RV) were identified in 28.9% (110/380) of the samples, with 9.2%, 2.9% and 19.5% positive for RVA, RVB and RVC, respectively. Of RV positive samples, 9% (10/110) were RVA, RVB and RVC mixed infections. An increased prevalence of RVA and RVC was observed between 2004 (17.8%), 2011 (33.6%) and 2012 (34.4%), with RVC having the highest increase followed by RVA. RVCs were dominant in all farms, years and seasons. Of the 128 samples collected in 2012, the detection rate of RV was significantly higher in piglets with diarrhea (50.7%, 34 of 67) than in non-diarrheic piglets (16.4%, 10 of 61). RVC (28.4%) was detected more frequently in diarrheic piglets than RVA (16.4%) and RVB (10.4%). However, in non-diarrheic piglets RVA (9.8%) was more frequently detected than RVC (6.6%), while RVB was not detected in non-diarrheic piglets.

A total of 23 positive samples were analyzed for RVA G and P genotypes. The dominant G-P combination was G9P[13] found in 60.9% of positive samples. The other combinations were G9P[7] (8.7%), G4P[13] (8.7%), G11P[13] (4.3%) and G11P[7] (4.3%). Sequence analysis of partial VP7 genes of selected strains revealed that the G4 strains were closely related to one another (95%) and to a lower extent to human (82-84%) and porcine (84-86%) G4 strains. The G11 strains detected shared identical VP7 gene sequence (100%) and were closely related to human (85-86%) and other porcine (83%) G11 strains. The G9 strains identified were closely related to one another, human and other porcine strains (96-97%, 89-91% and 89-91% nucleotide identities, respectively). The VP4 gene analysis revealed that P[7] strains were closely related to

each other and to P[7] strains isolated from porcine, bovine and panda (91-99%, 92-99% and 92-99%, respectively). The P[13] strains showed a higher diversity among themselves and with other porcine P[13] strains ranging from 83% to 99% and 82-97%, respectively. Our results demonstrate broad genetic heterogeneity of the RVA strains and suggest the possibility of genetic reassortment between different RVA genotypes within these farms.

Our findings will aid in the development of more accurate diagnostic tools and provide updated information for RV vaccine development based on the dominant RV genogroups and genotypes circulating in US swine.

Objective 2 (Shao et al., 2014).

Complete genomic sequencing of two selected G9 PRV strains (G9P[7] and G9P[13]) was conducted. BLAST (blastn) search (http://www.ncbi.nlm.nih.gov/) and/or RotaC2.0 automated genotyping tool identified complete genomic constellations as G9-P7-I5-R1-C1-M1-A8-N1-T7-E1-H1 for G9P[7] strain and G9-P13-I5-R1-C1-M1-A8-N1-T1-E1-H1 for G9P[13] strain. Analyses of the nucleotide identity between the two G9, OSU and Gottfried strains demonstrated higher overall similarity between the G9 and OSU strains. Additionally, 1-2 gene segments had highest similarity with Gottfried (NSP1 of both G9 and NSP1 of G9P[13]), human PRV A G9P[8] (VP7 of both G9 strains), and bovine RV A (NSP3 of G9P[7]) (Table 4).

Table 4: Full genome constellations of the selected dominant field PRVA strains G9P[13] and G9P[7], and the prototype vaccine historic PRV strains OSU and Gottfried.

	Strain/Genotype				
Gene	**OSU**	**Gottfried**	**G9P[7]**	**G9P[13]**	**Note**
VP7	G5	G4	G9	G9	Most similar to G9-RVA/Human-wt/BEL/B3458/2003/G9P8 with 92.5% similarity
VP4	P7	P6	P7	P13	**P[7]: Most similar to P7-RVA/Pig-tc/USA/OSU/1977/G5P7 with 92.3% similarity** P[13]: Rotavirus P13- RVA/Pig-wt/JPN/JP40-H4/2006/G9P[13]
VP6	I5	I1	I5	I5	Most similar to I5-RVA/Pig-tc/MEX/YM/1983/G11P7 with 89.9% similarity
VP1	R1	R1	R1	R1	Most similar to R1-RVA/Pig-tc/MEX/YM/1983/G11P7 with 91.8% similarity
VP2	C1	C1	C1	C1	**Most similar to C1-RVA/Pig-tc/USA/OSU/1977/G5P7 with 92.7% similarity**
VP3	M1	M1	M1	M1	**Most similar to M1-RVA/Pig-tc/USA/OSU/1977/G5P7 with 89.5% similarity**
NSP1	A1	A8	A8	A8	Most similar to A8-RVA/Pig-tc/USA/Gottfried/1983/G4P6 with 91.3% similarity
NSP2	N1	N1	N1	N1	**Most similar to N1-RVA/Pig-tc/USA/OSU/1977/G5P7 with 95.6% similarity**
NSP3	T1	T1	T7	T1	P[7]: Most similar to T7-RVA/Cow-tc/GBR/UK/1973/G6P5 with 93.4% similarity P[13]: Most similar to A8-RVA/Pig-tc/USA/Gottfried/1983/G4P6 with 92% similarity
NSP4	E1	E1	E1	E1	**Most similar to E1-RVA/Pig-tc/USA/OSU/1977/G5P7 with 92.7% similarity**
NSP5	H1	H1	H1	H1	Most similar to H1-RVA/Pig-tc/MEX/YM/1983/G11P7 with 97.7% similarity

Up to 20 serial passages were conducted for selected field G9P[13] PRVAs representing the most prevalent current G/P combination. The G9P[13] PRVA (RV0084) replicated in MA-104 monkey kidney cells achieving titers of $2x10^2$-$4.6x10^5$ FFU/ml in the presence of trypsin and $2x10^4$-$5.9x10^7$ FFU/ml in the presence of pancreatin and was, therefore, selected for subsequent experiments.

Using G9P[13] PRVA (RV0084) passaged in Gn pigs we have produced G9P[13] convalescent antiserum in Gn pigs. Using this antiserum against G9P[13] and previously prepared convalescent or hyperimmune antisera against other PRV/HRV, homologous and heterologous virus neutralizing antibody titers were determined and compared for G9P[13] PRVA (RV0084), HRV Wa, PRVAs OSU and Gottfried. Fluorescent foci virus neutralization (FFN) test demonstrated that antisera against PRVA G9P[13] and other HRV/PRV strains contained high homologous but low or no heterologous virus neutralizing (VN) antibody (Ab) titers (see Table 5). Interestingly, heterologous cross-neutralization was higher between OSU and G9P[13] compared to other RVs. It may be due to higher overall nucleotide identity between OSU and G9P[13] or due to the fact that OSU (but not Gottfried or Wa) and G9P[13] share common I5 (VP6) type. Similarly, there were higher levels of cross-neutralizing Abs between HRV Wa and Gottfried that share common I1 (VP6) type unlike G9P[13] and OSU. Possible mechanisms of RV replication inhibition by non-neutralizing Abs against VP6 were previously described by Feng et al. (Feng, Lawton et al. 2002).

Table 5: Virus neutralizing antibody titer (R%) against selected RVs.

Virus	**Convalescent or HI sera**			
	@PRV G9P[13]	**@HRV Wa G1P[8]**	**@PRV OSU G5P[7]**	**@PRV Gottfried G4P[6]**
PRV G9P[13]	**4096 (100)**	5(0.12)	1024(12)	64(3.5)

HRV Wa G1P[8]	8(0.12)	**6856(100)**	37(4.2)	71(15)
PRV OSU G5P[7]	256(12)	1437(4.2)	**4400(100)**	8(1.5)
PRV Gottfried G4P[6]	156(3.5)	4436(15)	254(1.5)	**2000(100)**

R% values are indicated in parentheses in cases where they were possible to calculate, as follows: R%=100(√r1xr2), where r_1 is the heterologous titer of strain 2 divided by the homologous titer of strain 1, and r_2 is the heterologous titer of strain 1 divided by the homologous titer of strain 2.

Objective 3 (Amimo, Vlasova et al. 2013, Vet Microbiol).

Swine fecal samples collected from seven farms were screened for group C rotaviruses (RVCs) using a reverse transcription-polymerase chain reaction assay. A total of 380 samples were tested and 19.5% were positive. Of the 128 samples collected in 2012, 23.5% from nursing piglets and 8.5% from weaned piglets were RVC positive, with a higher RVC frequency in diarrheic (28.4%) than in non-diarrheic (6.6%) piglets. The prevalence of RVCs varied among the farms and between different years within the same farm ranging from 15.6% to 100% (Figure 1). RVC were detected in most farms (except Farms 3 and 5) but not for every sampling: for example, no RVC were detected on Farm 3 in 2004 and Farms 5 and 6 in 2011. It is noteworthy that samples from Farms 6 and 7 were from diarrheic piglets submitted to the laboratory for diagnosis. Yearly differences in the prevalence of RVCs were observed among the 3 years: 2004 (11.9%), 2011 (27.6%) and 2012 (18%). Overall, the RVC incidence was highest in summer (26%) followed by winter (19.5%) and then spring (16.4%). No autumn samples were available. The RVC prevalence differed within each year: in 2004, the highest prevalence occurred in summer, while in 2011, winter had the highest prevalence and in 2012, the detection rate was highest in spring. However, seasonal influences on the prevalence of RVC infections were not evident across the years studied even for Farm 2 that was sampled at least twice a year in different seasons.

Our attempts to adapt representative PRVC field strains to cell culture were unsuccessful. Using G6P[X] PRVC (RV0143) passaged in Gn pigs, we have produced G6P[X] convalescent antiserum in Gn pigs and used it to assess the one-way cross-reactivity between the historic cell-culture adapted PRVC Cowden (G1) and the newly identified field PRVC (G6). As expected, only limited heterologous VN Ab titers were detected ~150 lower than the homologous (data not shown).

Two strains (RVC/Pig-wt/USA/RV0104/2011/G3PX and RVC/Pig-wt/USA/RV0143/2012/G6Px) from two different farms were further characterized genetically to gain information on virus diversity based on complete genome sequencing. Full genome sequence [with the exception of RV0104 VP4 gene, for which only partial (~50%) sequence was obtained] results for the two selected field PRVC (RV0104 - G3P[X] and RV0143 G6P[X]) have confirmed the preliminary results based on VP7, VP4, VP6 and NSP4 gene sequencing and demonstrated that these two PRVC strains from 2011 were only distantly related to one another and to the historic PRVC Cowden strain. For different genes, nucleotide identity between the two strains ranged from 73% (for NSP1) to 98.7% (available segment of VP4) (Table 6). VP1, VP2, VP6, NSP1, NSP2, NSP3 and NSP5 genes were more similar between Cowden and RV0143, whereas VP3, VP4, VP7 and NSP4 shared higher nucleotide identity between Cowden and RV0104 (Table 6). No sequences for other PRVC strains were available for VP1, VP2, VP3, NSP1, NSP2, NSP3 and NSP5 genes besides Cowden. Therefore, the limited analysis conducted for these genes demonstrated an overall higher similarity between the PRVC strains RV0104/RV0143 and human RVC strains (~70-88%) compared to bovine RVC strains (Table 6). Phylogenetic analysis of all the genes demonstrated host-species specific clustering of RVCs, which is different from the observations made for RVA strains (Fig. 1-4). Additionally, our molecular analysis provides evidence that PRVC strains may evolve via multiple reassortment events as observed for PRVA strains.

NSP4 Gene: The sequence analysis of the full length NSP4 gene showed that the two field strains (RV0104 and RV0143) shared 89% nucleotide identity. They were more closely related to prototype Cowden strain with sequence identity of 94% and 90% for RV0104 and RV0143, respectively, while distantly related to human (76-82%) and bovine (74-80%) strains (Table 6). NSP4 based phylogenetic analysis revealed that RV0104 and the prototype Cowden strain belonged to the same cluster while RV0143 formed a different branch indicating increased diversity in porcine RVCs in the NSP4 gene (Fig. 1).

VP4 gene: Remarkably high nucleotide identity of 98.7% was observed between the RV0104 and RV0143 VP4 genes. At the same time, their VP4 genes bore lower similarity (78% and 75%, respectively) to that of Cowden strain compared to other genes (72-94%) (Table 6; Fig. 2). Of all other PRVC strains available in GenBank, the highest nucleotide identity was observed between some recent PRVC strains from Japan and RV0104 (88%) and RV0143 (89%), respectively (Table 6; Fig. 2).

VP6 gene: Full length sequences of the VP6 gene of the RV0104 and RV0143 strains showed ~84% nucleotide identity to each other and 84-94% nucleotide identity to other porcine strains. RV0104 was most closely related to some PRVC strains from South Korea (from 2008-2009), sharing nucleotide identity up to 93%. RV0143 VP6 also shared highest identity of 94% with the latter strains, followed by recent Japanese and Czech PRVC strains sharing nucleotide identity of 92% (Table 6). Phylogenetic analysis based on the full-length VP6 gene revealed that RV0104 clustered with the RV0143 in a

monophyletic branch together with PRVC CA-2 from South Korea (sharing 83 and 88% nucleotide identity, respectively) and separately from historic Cowden (Fig. 3).

VP7 gene: The complete nucleotide sequence of the VP7 gene of the RV0104 (G3PX) and RV0143 (G6PX) strains was compared with complete VP7 gene sequences of other RVC strains available in the GenBank database. Sequence comparison indicated that one strain (RV0143) was most closely related to PRVC G6 strains from the US (IL, CO, NE, OH, NC) from 2009-2010 with sequence identities ranging from 92-97%; while the other strain (RV0104) was most closely related to prototype RVC/Pig-wt/USA/HF/xxxx/G3Px strain (G3) with sequence identity of 95%. The two strains were distantly related to one another with sequence identity of only 75% and they were also distantly related to Cowden (78% and 72%) and to other PRVC G-types, human and bovine strains with sequence identities ranging from 72 to 88% (Table 6). Phylogenetic analysis (Fig. 4) including most recently available full-length VP7 gene sequences of human, porcine and bovine RVCs confirmed that our RV0104 (G3PX) and RV0143 (G6PX) strains belonged to the two distinct porcine RVC genotypes, G3 and G6, respectively.

Our results indicate high genetic heterogeneity in RVCs genes and the concurrent co-circulation of different genotypes. **This provides critical information on PRVC diversity for the development of more accurate diagnostic tools and control strategies including vaccines.**

Discussion: RV is an important animal pathogen due to its economic impact on the swine industry, including mortality in piglets ranging from 0 to 50%, and reduced growth in recovered piglets. Globally common and emerging PRVA genotypes continuously contribute to RV diversity through mutation or reassortment, possibly generating new more virulent, transmissible or zoonotic phenotypes. To better understand the genetic composition and reassortment potential of PRVAs circulating in the US, it is critical to perform a prevalence study using historical and current samples. Studies of RV prevalence in pigs throughout the production system spectrum and from clinical and subclinical infections are critical to permit careful analysis of current vaccines against PRVAs, to define reasons for failures and develop new PRV vaccines as needed. Contribution of PRVBs and PRVCs to overall diarrheal disease and their genetic diversity will be assessed to understand the need for vaccines. PRV strains circulating in Ohio farms are diverse and PRV infections in nursing piglets are common. Our study confirmed that porcine PRVCs are detected more frequently than PRVAs in nursing piglets and should be studied to assess their enteropathogenicity. PRVAs and PRVCs are genetically diverse. This may pose a challenge for the development of prophylactic measures for PRVs in swine.

The effectiveness of future PRV vaccines may differ depending on the predominant genogroups (A, B, C) and genotypes circulating; hence, the most common PRV genogroups and genotypes should be the prime targets for vaccine development. **Detailed molecular epidemiological surveillance is needed for a better understanding of the diversity of PRVs, their role in diarrhea in various age groups and the effectiveness of existing PRVA vaccines.** Our research also demonstrated limited in vitro cross-neutralization between the current dominant G9 PRVAs and prototype vaccine strains, suggesting that only low-level partial protection would be conferred by existing vaccines against dominant emerging PRVA G9 strains.

Table 6: Nucleotide identity between RVC/Pig-wt/USA/RV0104/2011/G3PX and RVC/Pig-wt/USA/RV0143/2012/G6PX and other porcine, human and bovine RVC strains.

	Genes										
Nucleotide indentity (%) between	**VP1**	**VP2**	**VP3**	**VP4**	**VP6**	**VP7**	**NSP1**	**NSP2**	**NSP3**	**NSP4**	**NSP5**
RVC/Pig-wt/USA/RV0104/2011&RVC/Pig-wt/USA/RV0143/2011	88.5	87	89	98.7	84	75	73	97	78	89	86
Cowden&RVC/Pig-wt/USA/RV0104/2011	91	84	88	78	84	78	76	93	77	94	87
Cowden&RVC/Pig-wt/USA/RV0143/2011	92	85	87	75	86	72	93	94	90	90	94
Nucleotide identity (%) between RVC/Pig-wt/USA/RV0104/20114 and:											
Japanese (CJ) PRVC strains (2013-2014)				88	82-83	75-84					
Czech Republic PRVC strains (2009-2013)				82-85	82-84	82-87				89-90	
South Korean PRVC strains (06-146-2 and similar, 2008-2009)					84-93	<75					
PRCV HF G3P[X]						95					
Human PRVC strains	84-86	83	83-86	74-75	82-88	83-88	70-72	86-87	78-79	79-81	<70
Bovine PRVC strains	81	79-80	79-80	74-75	82	82	70-72	81-82	78-79	75-80	79-80
Nucleotide identity (%) between RVC/Pig-wt/USA/RV0143/2011 and:											
Japanese (CJ) PRVC strains				89	92	75-87					
Czech Republic PRVC strains (2009-2013)				<75	92	85-86				86-88	
South Korean PRVC strains (06-146-2 and similar, 2008-2009)					94	<75					
US PRVC G6P[X] strains from IL, CO, MN, NE, OH, NC (2009-2010)						92-97					
Human PRVC strains	84-86	83-84	83-86	74-75	<75	79-80	70-72	86-88	78-80	76-82	76
Bovine PRVC strains	81	80	79-80	74-75	<75	<75	70-72	82-83	78-80	74-80	79

Light pink shading was used to emphasize highest nucleotide identity between RV0104 and RV0143 strains; light blue – between RV0104 and/or RV0143 and Cowden, and light green - between RV0104/RV0143 and other PRVC strains.

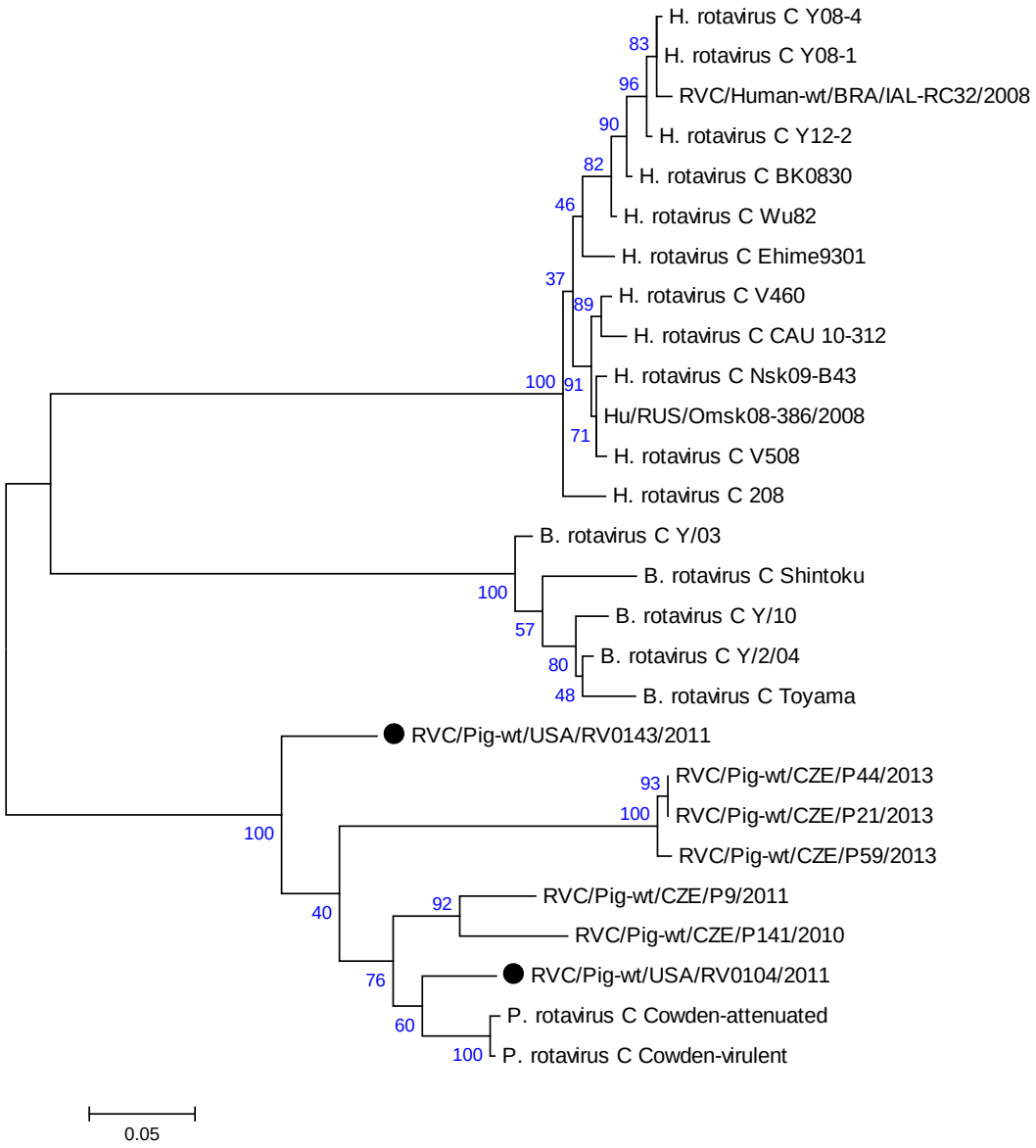

Figure 1: Phylogenetic dendrogram of RVC full-length NSP4 gene of the two field strains (dots) and RVC/Pig-wt/USA/Cowden/1980/G1Px strain compared with NSP4 gene sequences for human, bovine and porcine RVCs available in GenBank.

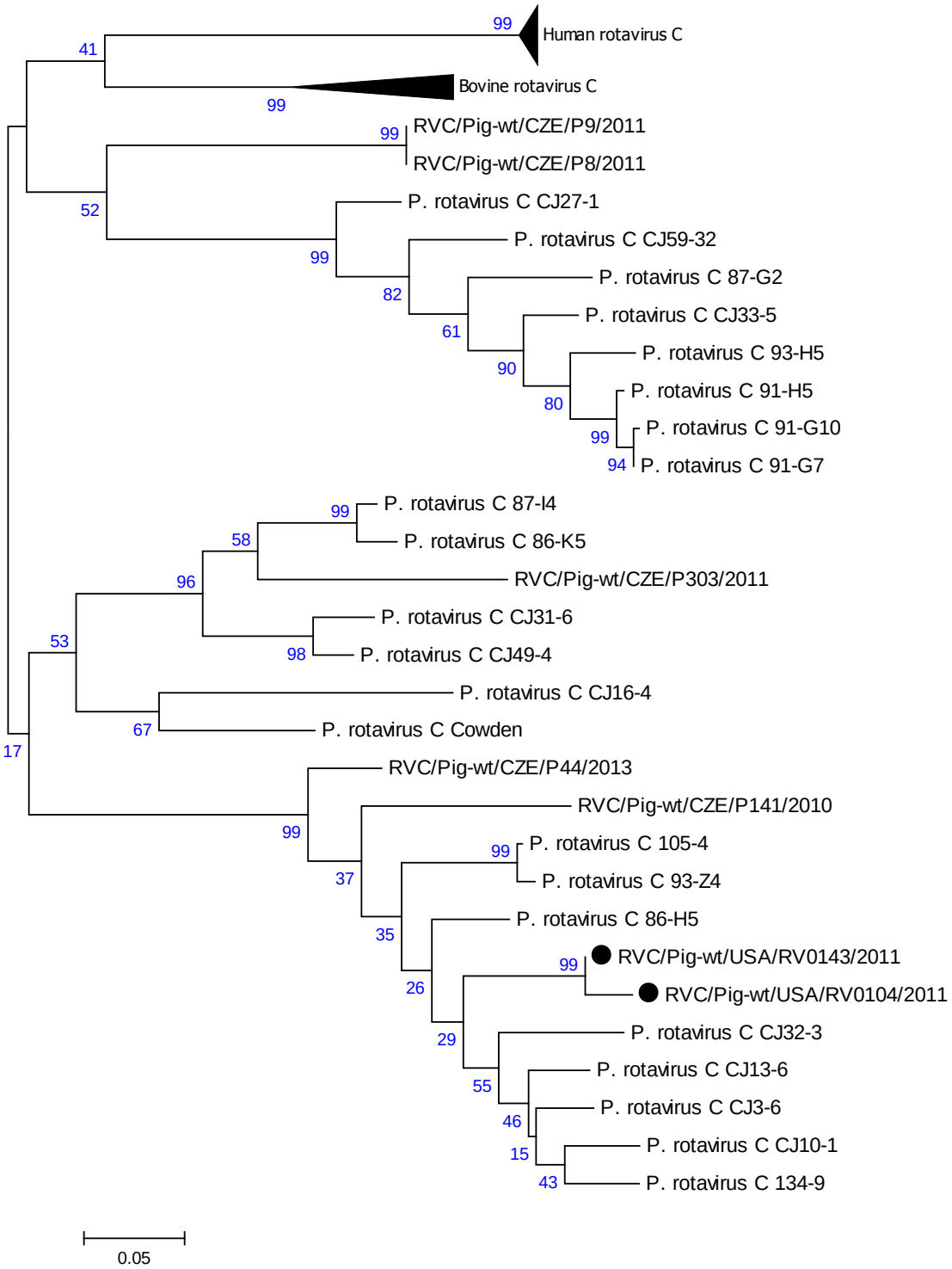

Figure 2: Phylogenetic dendrogram of RVC full-length VP4 gene of the two field strains (dots) and RVC/Pig-wt/USA/Cowden/1980/G1Px strain compared with VP4 gene sequences for human, bovine and porcine RVCs available in GenBank.

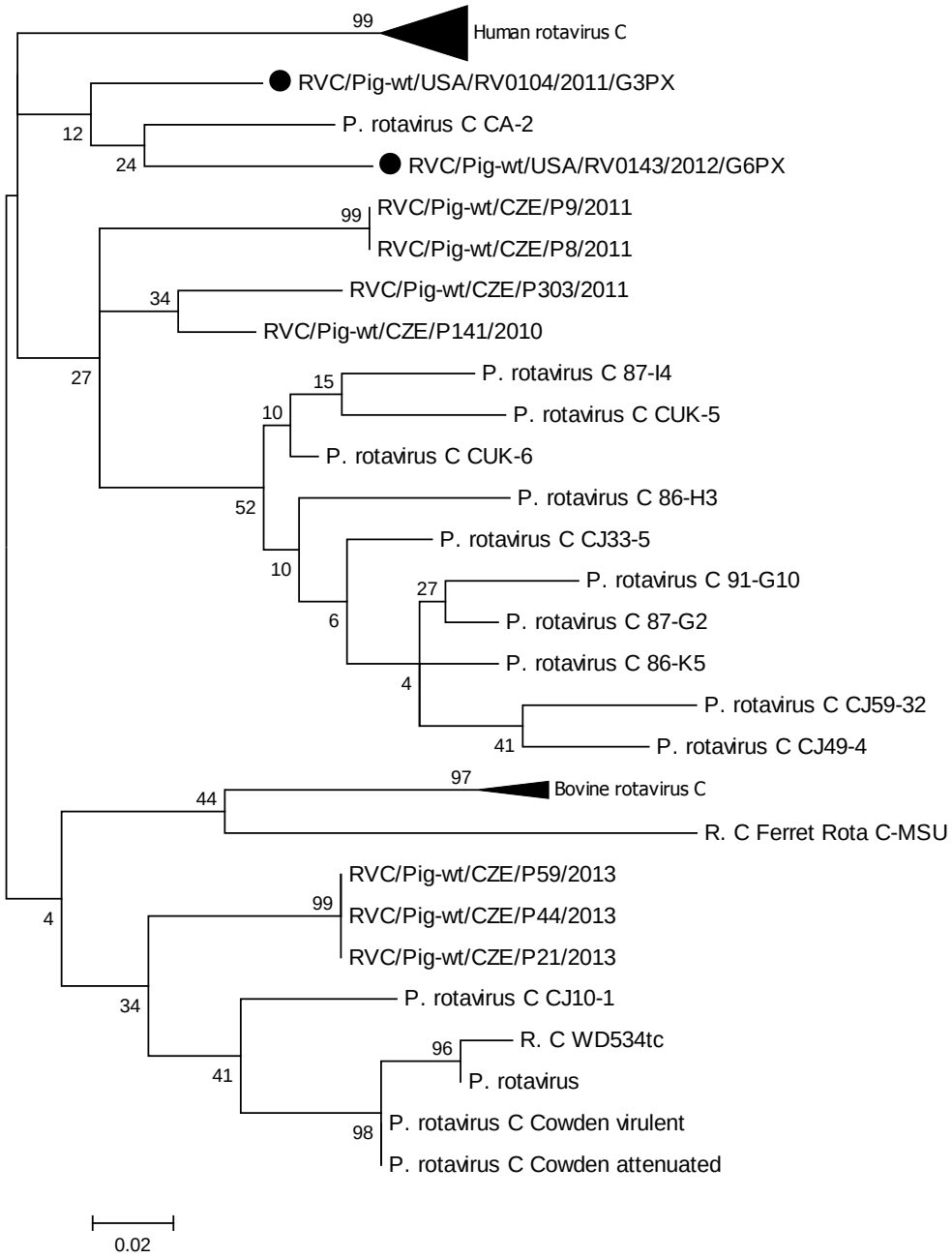

Figure 3: Phylogenetic dendrogram of RVC full-length VP6gene of the two field strains (dots) and RVC/Pig-wt/USA/Cowden/1980/G1Px strain compared with VP6 gene sequences for human, bovine and porcine RVCs available in GenBank.

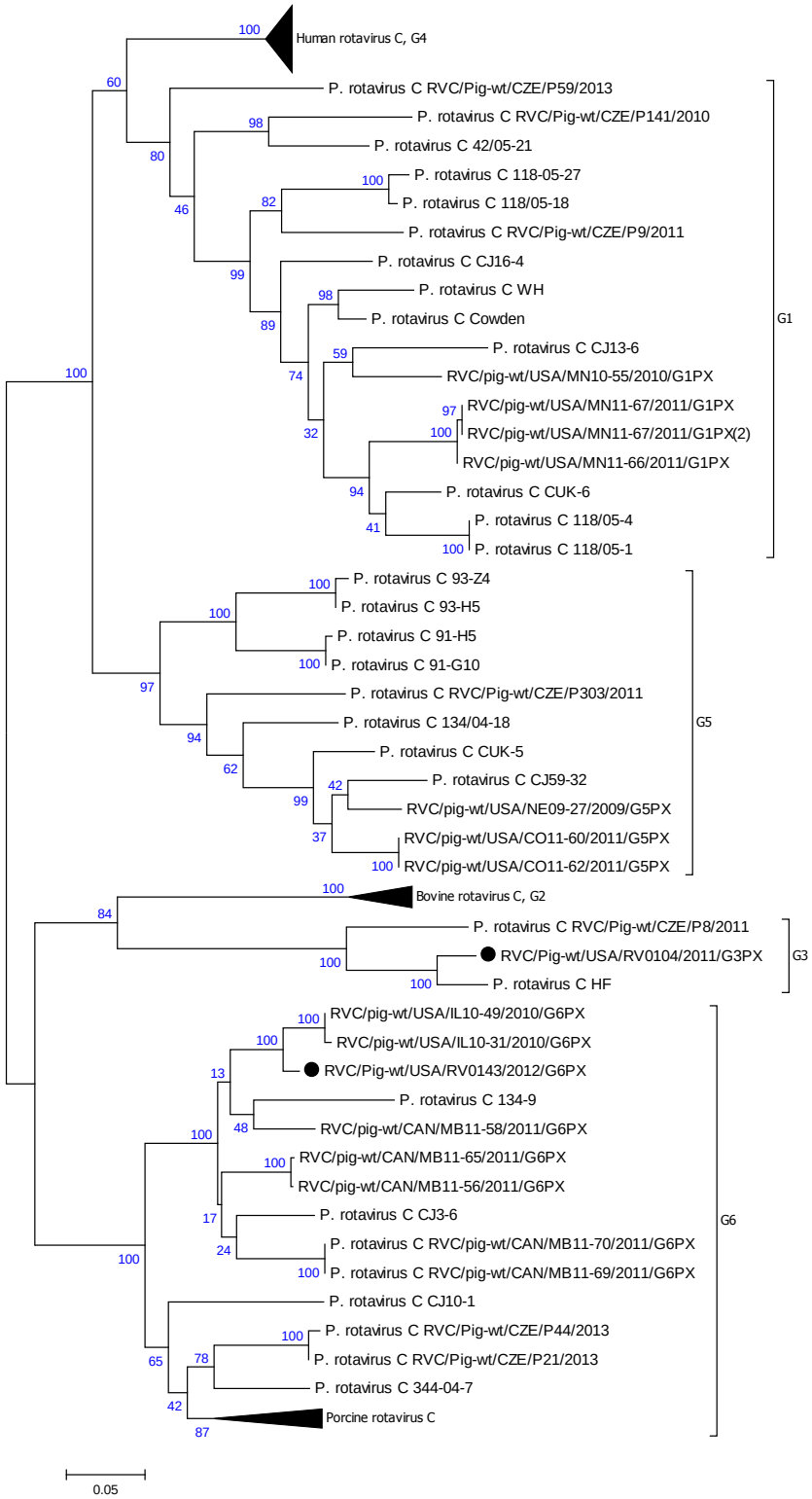

Figure 4: Phylogenetic dendrogram of RVC full-length VP7 gene of the two field strains (dots) and RVC/Pig-wt/USA/Cowden/1980/G1Px strain compared with VP7 gene sequences for human, bovine and porcine RVCs available in GenBank.

Publications:

Abstracts:

1. Amimo, J.O., Vlasova, A.N. and Saif, L.J. 2013. Prevalence and genetic heterogeneity of porcine rotaviruses in nursing and weaned piglets in Ohio, USA. 2nd International Congress on Pathogens at the Human-Animal Interface (ICOPHAI): One Health for Sustainable Development. August 14 - 17, 2013. Porto de Galinhas, Brazil.
2. Amimo, J.O., Vlasova, A.N., Saif, L.J. 2012. Prevalence and diversity of group A and non-group A porcine rotaviruses in selected US farms. 31st Annual Meeting American Society for Virology, 21-25 July, 2012, Madison, Wisconsin.
3. Shao, L., Vlasova, A., Saif, L.J. 2014. In vivo and in vitro characterization of porcine rotavirus G9P[13]. December 6-8, Chicago, Illinois.

Research articles:

1. Amimo, J.O., Vlasova, A.N., Saif, L.J. 2013. Prevalence, genetic heterogeneity and identification of a potential new VP4 genotype of porcine group C rotaviruses in nursing and weaned piglets in Ohio, USA. Veterinary Microbiology, 164(1-2):27-38.
2. Amimo, J.O., Vlasova, A.N., Saif, L.J. 2013. Detection and Genetic Diversity of Porcine Group A Rotaviruses in Historic (2004) and Recent (2011 and 2012) Swine Fecal Samples in Ohio: Predominance of the G9P[13] Genotype in Nursing Piglets. Journal of Clinical Microbiology, 51(4):1142-51.

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