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Assessment of porcine Rotavirus-associated virome variations in pigs with enteric disease

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ABSTRACT

Enteric disease is the predominant cause of morbidity and mortality in young mammals including pigs. Viral species involved in porcine enteric disease complex (PEDC) include *rotaviruses, coronaviruses, picornaviruses, astroviruses* and *pestiviruses* among others. The virome of three groups of swine samples submitted to the Kansas State University Veterinary Diagnostic Laboratory for routine testing were assessed, namely, a Rotavirus A positive (RVA) group, a Rotavirus co-infection (RV) group and a Rotavirus Negative (RV Neg) group. All groups were designated by qRT-PCR test results for Porcine Rotavirus A, B, C and H such that samples positive for RVA only went in the RVA group, samples positive for > 1 rotavirus went in the RV group and samples negative for all were grouped in the RVNeg group. All of the animals had clinical enteric disease resulting in scours and swollen joints/lameness, enlarged heart and/or a cough. All samples were metagenomic sequenced and analyzed for viral species composition that identified 14 viral species and eight bacterial viruses/phages. *Sapovirus* and *Escherichia coli* phages were found at a high prevalence in RVA and RV samples but were found at low or no prevalence in the RVNeg samples. *Picobirnavirus* was identified at a high proportion and prevalence in RVNeg and RV samples but at a low prevalence in the RVA group. Non-rotaviral diversity was highest in RVA samples followed by RV then RV Neg samples. A sequence analysis of the possible host of *Picobirnaviruses* revealed fungi as the most likely host. Various sequences were extracted from the sample reads and a phylogenetic update was provided showing a high prevalence of G9 and P[23] RVA genotypes. These data are important for pathogen surveillance and control measures.

1. Introduction

Currently, few surveillance studies assess the virome at the human-animal interface, specifically, for livestock, which are in more frequent contact with humans and can serve as a source of zoonotic pathogens. Zoonotic transmission events involving swine are becoming more common, providing the foundation to surveil and update the incidence of emerging and reemerging pathogens (Miller et al., 2017). Influenza viruses, hepatitis E virus, norovirus and rotaviruses are zoonotic pathogens common in the swine industry (Meslin et al., 2000; Ma et al., 2008; Martella et al., 2010; Meester et al., 2021). Prevalence of these viruses range from 3% to 67% on US swine farms emphasizing the importance of monitoring the swine virome to limit the possibility of future outbreaks (Wang et al., 2006; Chamba Pardo et al., 2017; Vlasova et al., 2017; Sooryanarain et al., 2020).

Zoonotic viruses that cause respiratory and enteric diseases substantially impact the swine industry in the US, particularly, viral diarrheas caused by rotaviruses are known to transmit between swine and humans (or vice versa) or from swine to other mammals (Martella et al., 2010). In children, viral diarrheas caused by rotaviruses remain a leading cause of childhood mortality (Troeger et al., 2018). Similarly, viral diarrhea is a major cause of morbidity and mortality among young pigs in the US (Vlasova et al., 2017; Stuempfig and Seroy, 2021). Common diarrhea-causing pathogens in young pigs include *Escherichia coli*, *Clostridium perfringens*, rotaviruses (RVA, RVB, RVC, RVH) and coronaviruses including *Transmissible gastroenteritis virus* (TGEV), *porcine deltacoronavirus* (PDCoV) (Marthaler et al., 2013, 2014; Wang et al., 2014; Ruiz et al., 2016; Kongsted et al., 2018). More recently, swine enteric diseases have been associated with a complex of viruses from multiple viral families (Shi et al., 2021). Studies of the fecal virome in healthy and diseased pigs have reported on many viral species, namely, *Kobuvirus* (PKV), *Astrovirus* (PAstV), *Sapovirus* (SaV), *Posaviruses,*

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Sapelovirus (PSV), and *Teschovirus* (PTV) (Lauritsen et al., 2015; Goecke et al., 2017; Theuns et al., 2018; Leme et al., 2019). Presently, it is unclear what role these viruses play in disease. For example, *Sapelovirus* and certain *Astrovirus* genotypes have been implicated to cause disease in multiple circumstances including experimental infections (Bak et al., 2017; Kumthip et al., 2018; Ulloa et al., 2019; Li et al., 2021).

Morbidity and mortality related to swine enteric diseases are variably affected by available commercial vaccines, specifically, vaccine efficacy against currently circulating viral genotypes. Commercially available vaccines for rotavirus, one of the only targets of swine enteric vaccines, are all RVA specific and do not offer protection against non-RVA outbreaks of porcine rotavirus. Currently in pigs, 10 species of rotavirus have been identified (A-J) which have been classified based on the VP6 gene sequence, one of 11 segments in the dsRNA genome (King et al., 2011). Within species, viruses are genotyped using VP7 (G) and VP4 (P) gene segments. There are 27 G and 37 P genotypes in pigs (Vlasova et al., 2017). Furthermore, the most prevalent within species genotypes are constantly changing; in 2012, the predominant RVA genotype in the US was G9P[13], but by 2021, G5P[13] is the predominantly circulating strain in North America (Amimo et al., 2013; Naseer et al., 2017). Porcine rotavirus vaccine genotypes are G4P[6], G5P[7] and G9P[7], therefore they do not cover certain circulating strains such as G3, P[13] and P[19] (Naseer et al., 2017). As the development of approved viral vaccine can often take years, the importance of circulating strain genotype analysis cannot be understated.

Beyond Rotavirus, few isolates have been obtained for the aforementioned members of the porcine virome (PKV, PAstV, PSV and PTV) preventing adequate characterization. Those isolates that have been obtained tend to have variable pathogenicity in *in vivo* studies meaning less focus has been placed on surveillance, identification and characterization (Jackova et al., 2017; Matias Ferreyra et al., 2017; Fang et al., 2019; Stäubli et al., 2021). Since little emphasis is placed on defining the role of these pathogens in swine disease, many producers and veterinarians will not test for these viruses. As severe disease has been associated with all of these viruses, it is crucial to maintain an understanding of the prevalence and circulating genotypes on US swine farms and to gain a better understanding of the co-factors involved in the appearance of outbreaks or clinical disease. The virome of 63 diagnostic porcine diarrhea samples submitted to Kansas State University Veterinary Diagnostic Laboratory (KSVDL) were assessed using Illumina sequencing. The samples were grouped by diversity of Rotaviruses in the samples; Rotavirus A only (RVA) samples were only positive by qRT-PCR for RVA, Rotavirus Co-infection (RV) samples were positive for RVA and a second species of rotavirus (RVB, RVC, RVH) by qRT-PCR and Rotavirus Negative samples (RVNeg) were negative for RVA, RVB, RVC and RVH by qRT-PCR. Once grouped, these samples were assessed for viral species breadth and diversity.

2. Methods

2.1. Swine sample acquisition

Swine tissue samples, fecal swabs and feces were submitted to KSVDL for rotavirus diagnostic testing. The clinical signs, sample type, animal age (if known) and co-infecting agents are shown in Table 1. Samples were homogenized in 1 ml of PBS per 1 g of sample on a stomacher 400 (Seward, United Kingdom). All testing was performed under the Institutional Biosafety Committee protocol # 1489.

2.2. Sample preparation and sequencing

Total RNA was extracted from homogenized samples using the Zymo Direct-zol RNA Miniprep kit (Zymo Research) as specified by the manufacturer. Single primer amplification was performed as previously described (Mitra et al., 2016). First strand cDNA was generated using Superscript III first-strand synthesis kit (Invitrogen) per the manufacturer's instructions with previously published primers (Neill et al., 2014). Double-stranded cDNA was produced with primers identical to the first-strand primers but lacking the random hexamer with LA Taq DNA polymerase (TaKaRa). Primers were removed from double-stranded cDNA using 1.2x HighPrep PCR Cleanup kit (Millipore Sigma) following manufacturer's instructions. The concentration of purified cDNA was assessed using the Qubit Fluorometer 4.0, diluted to 0.2 ng/uL and library prepped with Nextera XT v2 Library Preparation kit (Illumina). Libraries were sequenced on a Miseq platform.

2.3. Bioinformatic analysis of deep sequence data

Raw sequence reads were trimmed for quality, *de novo* assembled and contigs were assessed via blastn. Contigs without significant results via Blastn were assessed by Blastx. Trimmed reads were mapped back to the closest reference sequence identified by Blast to yield the most complete and correct sequence. Sequences were used in downstream analyses if they met the following thresholds: $\geq$ 30x coverage at all gene sites, an average read quality of 30 and an average read length of $\geq$ 135 bp. Raw read trimming and mapping to the references (determined by BLAST) was performed in CLC Workbench v 21.0.3 (Qiagen) using default parameters. All sequences were aligned with MAFFT v7.475 (Katoh et al., 2002). Following sequencing, the viral reads were extracted for each of the sample reads for further analysis. The proportion of distinct viruses was calculated by dividing the number of reads for that distinct virus by the total number of viral reads in each sample. The Kruskal-Wallis test, alpha diversity, beta diversity and the DAPC was calculated in R v4.1.1 using the packages stats, entropy, vegan and adegenet, respectively, and was modeled off a previous analysis (Dixon, 2003; Jombart, 2008; Shan et al., 2011; RCore Team, 2019). Statistical significant was calculated using a Wilcoxon test adjusted using the Holm Bonferroni method in R. All analyses in R were performed with Genbank references and the consensus genomes extracted from CLC. A list of Genbank numbers utilized in this study is included in Supplementary Table 1.

2.4. Phylogenetic analysis

Extracted consensus sequences with references were aligned in MAFFT v7.475 (Katoh et al., 2002). Maximum likelihood phylogenetic trees were created from alignments using the most appropriate substitution model and rates as tested in Mega X v10.2.6 (Kumar et al., 2018). The substitution models and parameters used are as follows: RVA VP4, Tamura-Nei with gamma distribution (+*G*) and invariant sites (+*I*); RVA VP7, Tamura 3-parameter model +*G*+*I*; PAstV ORF2, General Time Reversible model (GTR) +*G*; SaV VP2, Kimura 2-parameter model +*G*; and PBV RNA-dependent RNA-polymerase (RDRP), GTR+*G*+*I*.

2.5. Structural analysis

Protein coding sequences were homology modeled on iTasser v5.1 using default parameters (Roy et al., 2010). The model with the lowest C-score for each sequence was selected for visualization on ICM Brower Pro v3.9 (Molsoft).

2.6. Data availability

The consensus sequences were deposited in Genbank under accessions OM366088-OM366152. This study refers to the first version of the sequences.

3. Results

3.1. Porcine sample metadata

A total of 63 porcine diarrhea samples were obtained from available

Table 1
Porcine sample diagnostic and historical data. Information was collated from each submission. Diagnostic data for Rotavirus A, B, C, H (RVA, RVB, RVC and RVH), Porcine deltacoronavrius (PDCoV), Transmissible Gastroenteritis virus (TGEV), Swine Influenza virus (SIV) and Porcine circovirus (PCV) were included. Samples were grouped by rotavirus result as Rotavirus A only (RVA), Rotavirus dual-infected (RV) or Rotavirus Negative (RV Neg).

Group	Sample	Sample Type	RVA	RVB	RVC	RVH	PDCoV	TGEV	SIV	PCV3	Ecoli	Salmonella	Clostridium	Clinical Description				Animal Age (days)
														scours	swollen joints/ lameness	enlarged heart	cough	
RVA1	3	Tissue	24.45								X	X		X			X	21
RVA5	4	Tissue	21.65										X	X				10
RVA6	5	Tissue	26.15		X									X				10
RVA7	6	Tissue	22.88											X				10
RVA8	7	Tissue	19											X				10
RVA9	8	Tissue	22.91										X	X				10
RVA10	9	Tissue	24.16										X	X				10
RVA11	10	Tissue	X											X				
RVA12	11	Feedback Material	19.58															1
RVA13	12	Tissue	21.73								X			X				
RVA14	13	Tissue	20.93								X		X	X				
RVA15	14	Feces	23.67					32.1			X			X				
RVA16	15	Feces	23.82					35.08			X			X	X	X	X	35
RVA17	16	Feces	33								X			X	X	X	X	35
RVA18	17	Tissue	24.88		X				17.1					X			X	21
RVA19	18	Feces	24.25					37.43			X			X	X	X	X	35
RVA20	19	Feces	27.69								X			X				
RVA21	20	Tissue	31.63		X									X				13
RVA22	21	Tissue	31.42											X				13
RVA23	22	Tissue	31.56		X									X				13
RVA24	23	Tissue	27.69								X			X				
RVA25	1	Feedback Material	18.44															1
RVA26	2	Feces	21.81						30					X				10
RV1	27	Feces	31.38											X				16
RV2	28	Feces	23.54	X	36.43									X				17
RV4	29	Feces	22.24	36.37	33.86									X				35
RV5	30	Feces	21.79	31.65	26.65	X								X				35
RV6	31	Feces	22.37	32.31	32.26	X								X				35
RV7	32	Feces	24.57	X	28.18						X		23.18	X				7
RV8	33	Feces	27.32	33.25	27.84		25.31				X			X				
RV9	34	Feces	31.43	X	32.17						X			X	X	X		35
RV10	35	Fecal Swab	26.21		27.12		28.74							X				
RV11	36	Fecal Swab	28.65	X	29.09	X	29.08							X				
RV12	37	Feces	21.12	25.89	26.76		17.04							X				5
RV13	38	Feces	26.28	X	28.28	X	15.42				X			X				5
RV14	39	Feces	21.64		29.41									X				
RV15	40	Feces	25.54	X	24.35	X												
RV16	41	Feces	29.61	X	30.01			35.74						X			X	48
RV17	42	Feces	26.26	X	28.26	X								X				
RV18	43	Tissue	23.67	X	30.86							X		X				48
RV19	44	Fecal Swab	21.37		36.01	X								X				
RV20	45	Fecal Swab	22.14		31.64									X				
RV21	46	Tissue	20.11		25.19									X				5
RV22	47	Feces	19.22		27.74	X								X				5
RV23	48	Feces	27.76	X	26.01									X				5
RV24	49	Feces	22.26		24.06									X				7
RV25	50	Feces	22.88		28.48									X				21

(*continued on next page*)

Table 1 (*continued*)

Group	Sample	Sample Type	RVA	RVB	RVC	RVH	PDCoV	TGEV	SIV	PCV3	Ecoli	Salmonella	Clostridium	Clinical Description				Animal Age (days)
														scours	swollen joints/ lameness	enlarged heart	cough	
RV26	51	Feces	28.33	33.14	35.04					35.03								70
RV27	24	Tissue	20.82		X						X			X				5
RV28	25	Fecal Swab	21.37		X						X			X				5
RV29	26	Tissue	21.99		X	X					X			X				5
RV Neg 1	52	Feces							31					X				5
RV Neg 2	53	Feces												X				5
RV Neg 3	54	Feces												X				15
RV Neg 4	55	Feces		25.33									22.15	X				15
RV Neg 5	56	Feces										25.63		X				
RV Neg 6	57	Feces											32.18					3
RV Neg 7	58	Feces																
RV Neg 8	59	Feces																49
RV Neg 9	60	Feces																3
RV Neg 10	61	Feces																7
RV Neg 12	62	Feces											32.36					
RV Neg 13	63	Feces																168

routine diagnostic submissions represented by three groups, Rotavirus A Positive (RVA; 23), Rotavirus dual-infected samples (RV; 28) and Rotavirus Negative (RV Neg; 12). All samples were represented by four sample types; feces (n = 37), tissue (n = 19), fecal swab (n = 5) or feedback material (n = 2) (Table 1). Feedback material is defined as feces collected from rooms in which piglets are housed that is meant to feed sows in the subsequent farrowing group in order to offer protection from disease. The samples were submitted to the KSVDL between 2019 and 2021, originated from a total of six states (Kansas, 39; Nebraska, 17; Arkansas, 3; Iowa, 2; Massachusetts, 1; Delaware, 1) and 12 distinct farms.

While the experimental groups were differentiated by presence, absence and genotype of rotavirus present, many of the samples were positive for other pathogens via qPCR and/or culture. Although clinical descriptions of the sampled animals were often incomplete, scours was described in all clinical descriptions that were provided. Various testing was performed on each sample as specified by the owners and often did not contain a complete description of the biome, however, the available results correlated to the NGS findings for each sample (Table 1, Fig. 1) with the exception of pathogens at low quantities in the samples (i.e. TGEV and porcine circovirus). A total of 14 viruses (*Rotavirus, Kobuvirus, Astrovirus, Picobirnavirus, Pestivirus, Circovirus, Coronavirus, Orthoreovirus, Influenza virus, Teschovirus, Enterovirus, Sapelovirus, Sapovirus* and *Parvovirus*) and 8 bacteria phages or viruses infecting bacteria (*E.coli* phages, *Pseudomonus* phages, *Gordonia* phages, *Klebsilla* phages, *Salmonella* phages, *Bacteriodes* phages, *Siphoviridae* and *Marinevirus*) were identified in the sample set.

3.2. Porcine Rotavirus

The VP4, VP7 and VP6 sequences were extracted from relevant samples which resulted in 16 VP4s and 12 VP7s. VP4 sequences were largely composed of P[23] (6) and P[13] (5) and to a lesser extent P[5]7 (4) and P[7] (1) while VP7s were composed of only G9 (10) and G4 (2) sequences. Similarly, most of the sequences were G9P[23] (5) or G9P [13] (3). One of the VP7 sequences, RV7, was only 88% identical to any sequence in NCBI (Fig. 2b). As the differentiation for novel G protein classifications is at the 89% level, this sequence could be considered a novel G group (King et al., 2011). At the VP4 and VP6 level, however, the virus did not meet the same threshold as it was 94% similar for both sequences (Fig. 2a and data not shown). Two unique sequences were extracted from RVA5, one of which was a P[13] sequence and the other of which was only 83% similar to the Roteq vaccine sequence (P[5]7), its closest reference (Fig. 2a) (Matthijnssens et al., 2010). Using the same threshold described above, this sequence could be classified as a unique P group. The VP7 and VP6 sequence identified in this sample, however, did not meet the threshold designating a novel genotype as they were 94% similar to the closest available reference (Fig. 2b, Table 2 and data not shown).

Porcine rotavirus A vaccines, the only species with an available vaccine, are becoming increasingly less effective due to genotype mismatches between field and vaccine strains as well as a decreased efficacy due to co-infections (Welter and Welter, 1990; Praharaj et al., 2019). To investigate the potential *in silico* efficacy of the porcine RVA vaccines to currently circulating field strains identified in this study, the RVA VP4 and VP7 sequences generated in this study were homology modeled to produce protein structural models that were compared to the vaccine strains (Fig. 3). Two popular porcine rotavirus vaccine strains include the Gottfried attenuated strain and the OSU 1975 strain (Naseer et al., 2017). These viruses are G4P[6] and G5P[7] genotypes, respectively. In the VP7 protein models, RV7, RVA18, RV5 were the most structurally similar to the OSU strain, however, there was significant structural variability in known epitope regions (red arrows, Fig. 3a) (Shepherd et al., 2020). Proteins RVA15 and RVA16, although more similar to the Gottfried strain than the OSU strain, had a high degree of structural divergence from the Gottfried VP7 model (Fig. 3b). VP4 structural

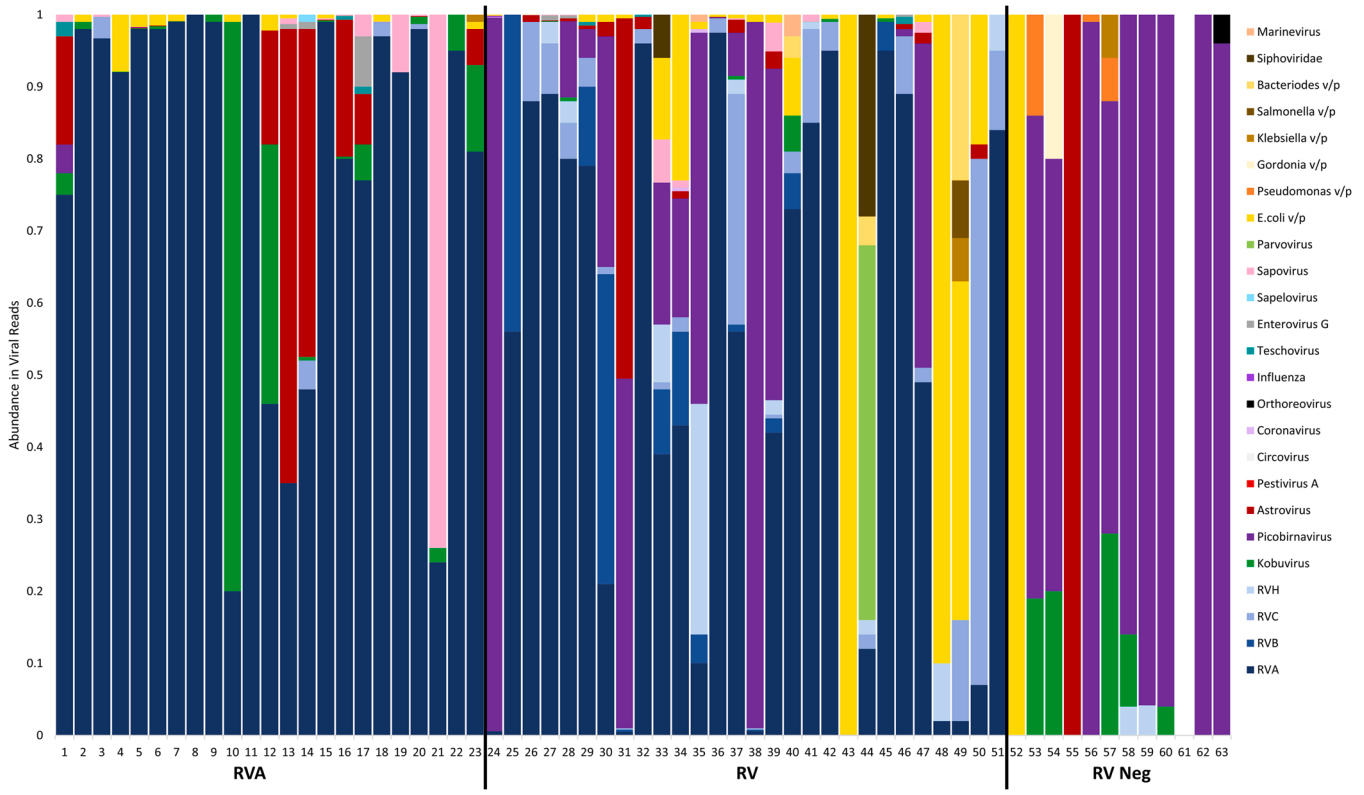

Fig. 1. Abundance of viral read contribution to the virome of pigs with enteric disease. Samples from pigs submitted to Kansas State Veterinary Diagnostic Laboratory with enteric disease were metagenomics sequenced. Viral reads were extracted and the proportion of viral reads of each specified viral species was extracted for analysis. Samples were grouped based on rotavirus result into Rotavirus A (RVA), Rotavirus dual-infected (RV) or Rotavirus Negative (RV Neg) groups. The RV group included reads from Rotaviruses A, B, C, or H (RVA, RVB, RVC, RVH). Bacterial viruses or phages (v/p) reads were included in the analysis.

differences occurred to a lesser degree but were most prominent in the VP5 foot region and the linker region (Fig. 3c, black arrows) (Settembre et al., 2011; Rodríguez et al., 2014).

A VP7 protein alignment including the more divergent structures and porcine rotavirus vaccine sequences showed limited amino acid identities of between 75.5% and 84% as compared to the vaccines. The identity of the protein sequences with known epitope regions, AA87–99, 145–147, 208–214 and 217–221 showed between 4 and 5, 0–2, 0–2 and 2–3 changes, respectively. Two other regions without known epitopes, 1–54 and 198–201 had significant structural divergence as well (Fig. 3a, black arrows). A VP4 protein alignment of the homology modeled samples had amino acid identities of 71.2–77% across the protein sequence and 1, 2–3, 2, 4 and 6–8 amino acid changes in the epitope regions 87–89, 113–116, 131–135, 146–150 and 180–196, respectively.

The relative proportions of RVB, RVC and RVH were noticeably lower than that of RVA, therefore, only one VP4 and VP7 sequences were extracted for each RVC and RVH. The sole RVC sequence was only 93% similar to its closest reference in both the VP4 and VP7 regions, while the RVH sequences were only 88% similar in the VP4 and VP7 regions (Table 2). No complete RVB sequences were extracted from the reads and were not analyzed further.

3.3. Porcine Astrovirus

Of the 63 samples selected for analysis, 28 of the samples contained porcine *astrovirus* sequences (44.4%). Within the sample groups (RV Neg, RVA, RV) the prevalence was 8.3%, 56.5% and 50%, respectively. Average proportion of viral reads within positive samples within each group was 100%, 13.1%, and 4.6%, respectively, but varied widely (100–0.01%). Six complete ORF2 sequences and five complete ORF 1 sequences were extracted from the positive samples. Four of the sequences were phylogenetically classified as PAstV 5 sequences while the remaining two were classified as PAstV 2 sequences (Fig. 2c). All PAstV5 sequences formed a monophylectic clade, closest to two references, one identified from porcine feces in California in 2010 and the other from porcine feces in Iowa in 2011; both sequences were identified during metagenomics studies of porcine feces. The two PAstV2 sequences were members of divergent clades. The closest references were identified from porcine feces in the USA in 2011 or 2016. Interestingly, none of the extracted sequences were similar to *Astrovirus* sequences collected in the last 5 years.

3.4. Porcine Enterovirus

Seven of the samples contained *Enterovirus* reads (11.7%) split between the RVA (17.4%) and the RV (10.7%) groups only. The average proportions of Enterovirus reads in positive samples were 2.2% and 0.4%, respectively. Two of the positive RVA group samples produced complete P1 and 2 C-3D (Partial P2 and complete P3 which contain the unique Papain-like cysteine protease) sequences phylogenetically grouping closest to each other in both analyses (data not shown). The closest historical reference was collected in Kanagawa, Japan in 2019 (Table 2). Samples RVA16 and RVA20 were classified as G1 genotypes. The sequences did contain the unique papain-like cysteine protease that were previously described in US swine samples and is most similar to Nidovirus sequences (Anbalagan et al., 2014).

3.5. Porcine Sapovirus

Among all samples, 27.0% (17/63) contained SaV reads. The RVA and RV groups had a relatively even split of SaV positive samples (39.1% and 28.6%) but the average proportion within positive samples differed greatly (9.7% and 1.7%). No SaV reads were identified in RV Neg samples. Five VP2 sequences were extracted from the reads (RVA only).

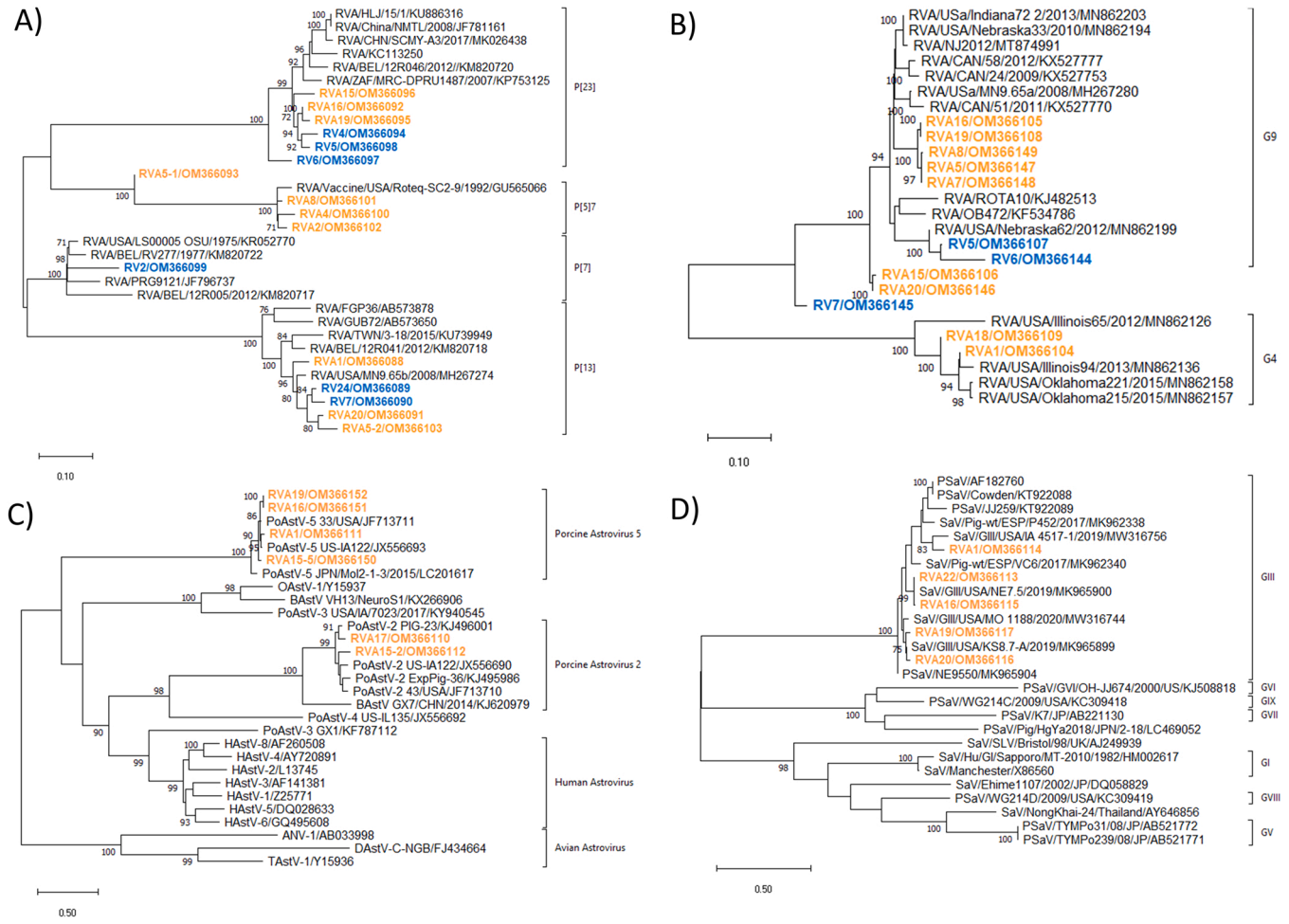

Fig. 2. Phylogenetic analysis of Rotavirus A, Porcine Astrovirus and Porcine Sapovirus from swine with clinical enteric disease. Phylogenies were constructed in Mega X v10.2.6. A) Rotavirus A (RVA) VP4 sequences were run using the Tamura-Nei model with gamma distribution(+*G*) and Invariant sites (+*I*), B) RVA VP7 sequences were run using a Tamura 3-parameter model +*G*+*I*, C) Astrovirus (AstV) ORF2 sequences were run using a General Time reversible model +*G*, D) Sapovirus (SaV) VP2 sequences were run using a Kimura 2-parameter model +*G*. Groups of sequences generated in this study are designated by: Rotavirus A only (RVA; orange), Rotavirus Dual-infected (RV; Blue) or Rotavirus Negative (RV Neg; green).(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Sequence comparison of Rotavirus A (RVA), Rotavirus dual-infected(RV) and Rotavirus Negative(RV Neg) viruses to the closest reference. Viruses identified in the samples include Rotavirus C, H (RVA, RVH), Enterovirus G (EVG), Porcine Teschovirus A (PTVA) and Porcine Kobuvirus (PKV). The nucleotide identity (%) was determined for each of the study samples.

Virus	Segment	Genbank #	Location of collection	Year of collection	Closest sequences	Identity (%)
RVA	VP4	KP753125	South Africa	2007	RVA15, RVA16, RVA19, RV4, RV5, RV6	89.6–93.0
		GU565066	USA	1992	RVA5B, RVA8, RVA4, RVA2	83–96.3
		KM820722	Belgium	1977	RV2	89.4
		MH267274	Minnesota, USA	2008	RVA1, RV24, RV7, RVA20, RVA5A	93.5–96.0
	VP7	MN862199	Nebraska, USA	2012	RVA16, RVA19, RVA8, RVA5, RVA7, RV5, RV6	92.9–98.0
		MN862194	Nebraska, USA	2010	RVA15, RVA20	95.1–95.4
		MT874991	China	2012	RV7	88.2
		MN862126	Illinois, USA	2012	RVA18	89.1
		MN862136	Illinois, USA	2013	RVA1	97.9
RVC	VP4	MG451743	Minnesota, USA	2012	RVA4	92.1
	VP7	MT771542	Minnesota, USA	2012	RVA4	90.6
RVH	VP4	KU254590	Minnesota, USA	2008	RVA17	90.6
	VP7	LC348469	Japan	2014	RVA17	97
EVG	P1	LC549659	Japan	2009	RVA16, RVA20	86.1–86.8
PTVA	VP1	MG875430	China	2014	RVA15	86.4
PKV	VP1	LT898428	Germany	2014	RV Neg2, RVA1	85.4–87.3
		KT266113	Vietnam	2012	RVA15, RVA16, RVA19, RVA20	87.1–90.7

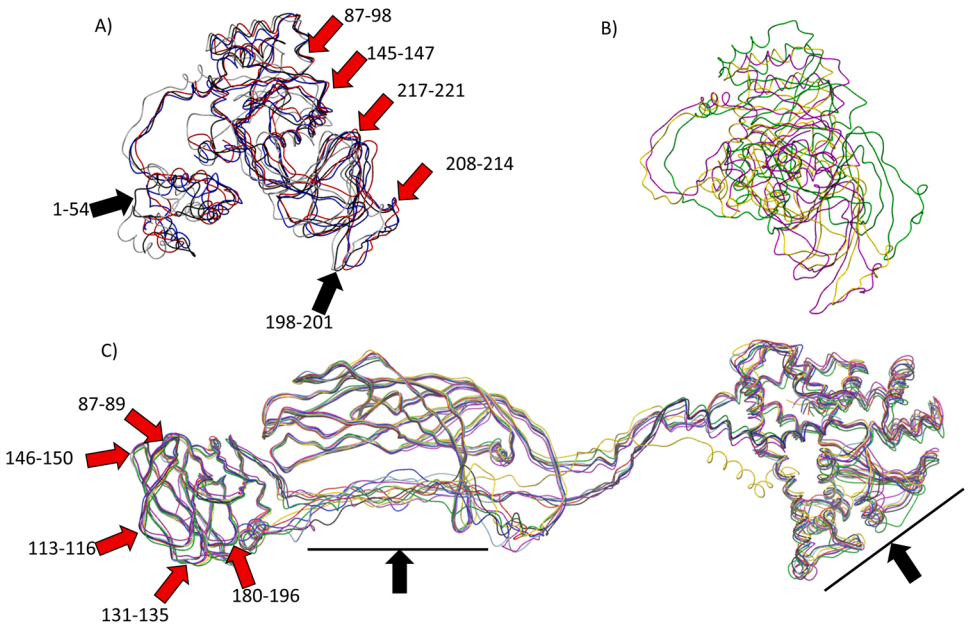

Fig. 3. Rotavirus A VP4 and VP7 homology models of porcine enteric disease samples. Protein sequences were extracted from Rotavirus nucleotide sequences and subjected to homology modeling in iTasser v5.1. Selected rotavirus A A)VP7 including RV7(Red), RVA18 (Blue), RV5 (Gray) and OSU vaccine strain, Genbank # MT025939 (Black) were analyzed. The two sequences similar to the Gottfried VP7 model (green), B) RVA15 (purple) and RVA16 (yellow) were analyzed separately. Selected rotavirus A C)VP4 sequences including RVA5 (Red), RV5 (Blue), RVA2 (Yellow), RV2 (Green), RVA1 (Purple), OSU vaccine strain (Genbank # MT025935; Gray) and Gottfried vaccine strain (Genbank # MT025912; black) were modeled. Red arrows indicate known epitope regions, black arrow indicate regions of structural dissimilarity. Numbers indicate amino acid positions within protein sequences.

These sequences diverged from each other on the phylogenetic tree (Fig. 2d). The closest reference sequences were collected from porcine feces in Kansas, Nebraska or Iowa in 2019 as part of a study collecting samples from diarrheic pigs (Table 2). Using previously defined phylogenetic clades, all viruses in this study were classified as GIII viruses, the predominant type of SaV in the US.

3.6. Porcine Kobuvirus

Twenty-nine of the 63 samples were positive for PKV reads (46.0%). These samples originated from all groups: RV Neg (n = 5; 38.5%), RVA (n = 17; 73.9%) and RV (n = 7; 25.0%). Interestingly, the RV and RV Neg groups had subjectively decreased incidences of PKV infections. Similarly, the average proportions of PKV reads in positive samples was significantly lower in the RV group as compared to the RV Neg group ($p = 0.0016$): RV Neg (16.2%), RVA (8.6%) and RV (1.0%). Previous studies determined an insignificant difference in PKV prevalence in healthy or diarrheic pigs but detected RVA/PKV co-infections at a higher incidence in diarrheic pigs. This finding was not reflected in our results when grouping diarrheic animals by rotavirus genotype. Our results indicate, within the RV/PKV coinfections, PKV/RVA coinfections occur more frequently than RV/PKV co-infections and RVNeg/PKV or RVA/PKV co-infections occur within animals at equivalent frequencies in the virome of diarrheic pigs. A complete VP1 gene sequence was extracted from six of the PKV positive samples (1, RV Neg; 5, RVA). Phylogenetic results indicate the RV Neg sample grouped with RVA1 while the remaining samples formed a monophyletic clade diverging widely from these samples (data not shown). The closest reference to RV Neg and RVA1 is a sequence collected in Ohio, USA in 2011 while the other sequences were most similar to a sequence collected from feces in Vietnam in 2012 as part of surveillance studies (Table 2).

3.7. Porcine Teschovirus

Of the 63 samples, six were positive for PTV (9.5%), three in the RVA group and three in the RV group. Mean proportion of reads within positive samples ineach group did not significantly differ (1.2% and 0.6%, respectively). A complete VP1 sequence was extracted from one of the samples (RVA) which was most similar to a PTV identified in China in 2014, a PTV16 genogroup and has no known association to disease (Table 2) (Yang et al., 2018). No *teschovirus* reads were identified in RV Neg samples.

3.8. Other viruses

One of the 63 samples was positive for PSV (1.6%). This sample was part of the RVA group and occurred at an extremely low proportion in that sample (1%). Consequently, a sequence was not extracted for analysis. *Influenza virus* (3), *Orthoreovirus* (1), *Coronavirus* (2), *Circovirus* (2) and *Pestivirus* (1) was present in a limited number of samples in quantities insufficient to extract genotyping data. Similarly, genotyping sequence was not able to be extracted from the single sample with *Parvovirus*.

3.9. Picobirnavirus

Twenty-four of the samples contained PBV reads (38.1%); RVNeg (n = 9; 69.2%), RVA (n = 1; 4.3%) and RV (n = 14; 50.0%). Similarly, the mean proportion of viral reads in positive samples in the RV Neg samples was greater than any other group ($p = 0.0004$); RVNeg (84.0%); RVA (4.0%) and RV (34.2%). Twelve complete RDRP sequences were obtained from the samples; 3, RVNeg; 2, RVA and 7, RV. PBV RDRP sequences are known to be similar between mammalian hosts. Similarly, the sequences in this study were most similar to Otarine, Porcine or Turkey PBVs but were not similar to each other, even within groups (Fig. 4).

3.10. Putative host association

While the hosts for many of the discussed viruses has previously been determined, viruses such as the novel PBV sequences identified in this study remains undertermined. Furthermore, many of the PBV sequences from this study were phylogenetically most similar to non-porcine PBVs. The lack of viral isolates for many of the viruses identified in the study suggests either an alternative host or additional cofactors are required to sustain infection and replication. We implemented a predictive Discriminate Analysis of Principle Components (DAPC) to 1) suggest a potential host for the novel sequences identified in the study and 2) improve our understanding of the host-virome relationships. The DAPC was constructed using Arthropod, Fungi, Insect, Plant, and Vertebrate viral sequence data. All PBV RDRP sequences generated in this study grouped distinctly within the Fungi group and significantly different

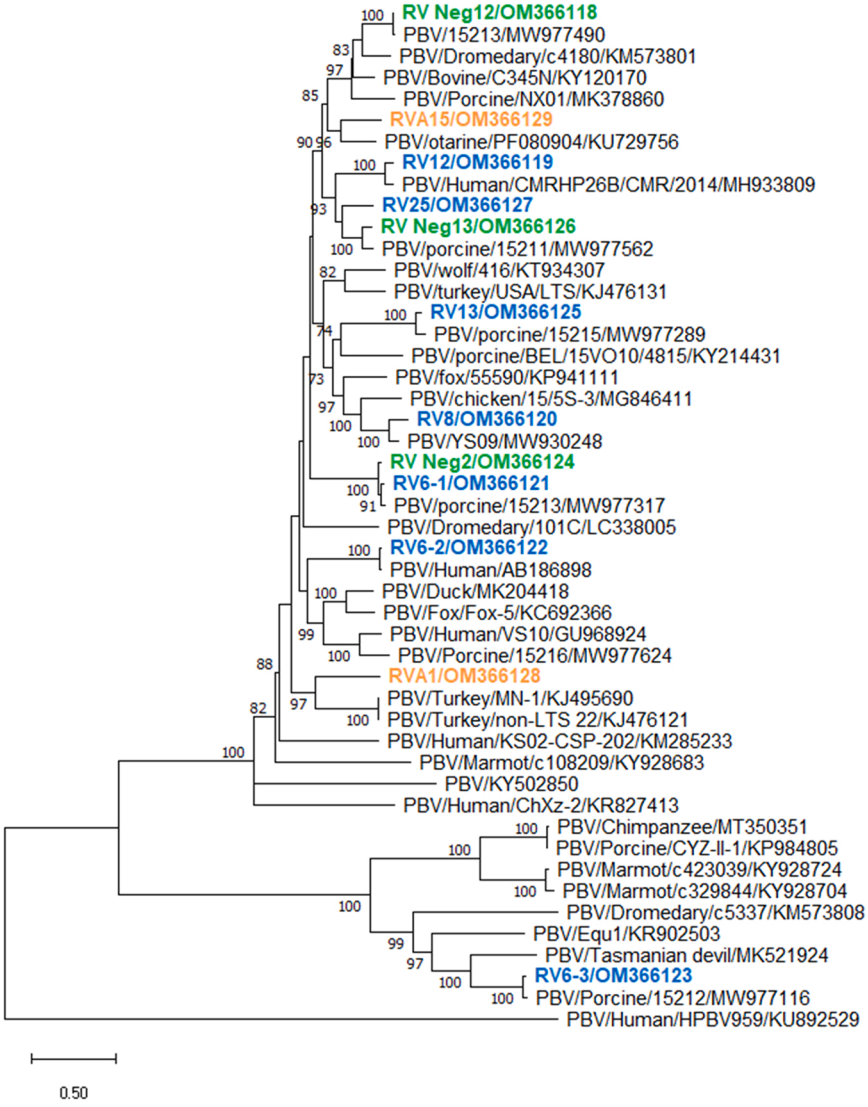

Fig. 4. Phylogenetic analysis of Picobirnavirus sequences from swine with clinical enteric disease. The Picobirnavirus (PBV) phylogeny was constructed in Mega X v10.2.6 using a General Time Reversible model with gamma distribution (+*G*) and Invariant sites (+*I*). Groups of sequences generated in this study are designated by: Rotavirus A only (RVA; orange), Rotavirus Dual-infected (RV; Blue) or Rotavirus Negative (RV Neg; green).(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

from all other included groups (Fig. 5). This suggests the possible hosts of PBV sequences may be Fungi rather than Vertebrates. The associated incidence of PBV in vertebrate hosts could be explained by the presence of various fungi in the gastrointestinal tract of pigs. In a previous iteration of the DAPC (data not shown), bacteria did not overlap with PBV and were removed from subsequent analyses for graph clarity. No other viral sequence from this study grouped outside of the vertebrate ellipses or yielded unexpected results.

3.11. *Viral species diversity within and between groups*

Following the observed variability of viral species diversity within groups, alpha and beta diversity estimates were calculated using Shannon entropy and Bray-Curtis algorithms, respectively (Fig. 6). When rotavirus incidences were included in the analysis, a subjective but non-significant difference was observed in the RV group samples (Fig. 6a). As RVA and RV group samples were overwhelmingly composed of rotavirus sequences, rotavirus sequences were excluded from these groups and alpha diversity was recalculated (Fig. 6b). Alpha diversity of excluded rotavirus groups were significantly different in the viral group diversity ($p = 0.003482$) such that the RV Neg group had a significantly lower non-rotaviral diversity (mainly composed of Picobirnaviral reads) than the RVA or RV groups (Fig. 6b). To confirm the results of the alpha diversity test was associated with the group designations rather than the sample type, the data was regrouped and analyzed by tissue type within groups (Supplementary Figure 2). No significance was determined between sample types within groups except between RV feces and RVNeg feces ($p = 0.0013$) further confirming the results determined between the RV and RVNeg groups is genuine. Beta diversity was tested for the groups by a Bray-Curtis dissimilarity calculation and a Non-Metric Multi-Dimensional Scaling model. When rotavirus was included in the analysis, the RV Neg group does not significantly differ from the RVA group but does differ from the RV group (Fig. 6c). When rotavirus was excluded from the analysis, the RV Neg group significantly differed from the RVA or RV groups although RVA and RV groups did not differ from each other (Fig. 6d), confirming the alpha diversity differences.

4. Discussion

While targeted molecular diagnostic assays often identify disease-causing organisms, they do not assess the pathogen biome within the samples, nor the relatedness of samples to each other or to clinical disease. This study updates the current knowledge of circulating viral species and genotypes on swine farms in the US, suggests that the epitopes of currently circulating porcine rotavirus a strains will not be covered by vaccine strains, illustrates that virome diversity beyond rotaviruses is more diverse if the animal is infected with rotavirus and identifies the viruses (PBV and PKV) that replicate at higher proportions

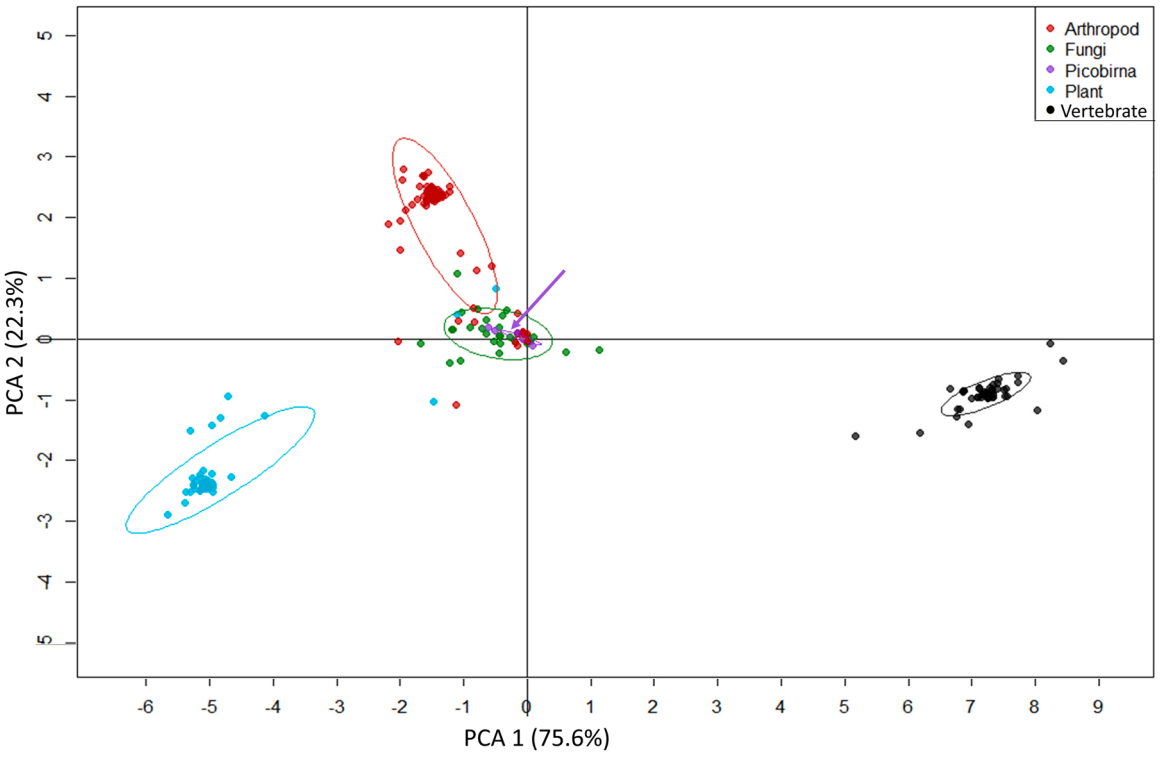

Fig. 5. Scatterplot of Discriminant Analysis of Principle Components (DAPC) comparing various genomic sequences. The two best-fitting principle components (PCA 1, PCA2) describe a total of 97.9% of the observed sequence differences. The analysis was performed in R v4.1.1 using a both sequences generated in this study (Picobirna) as well as Arthropod, Fungi, Plant, and Vertebrate viral sequences.

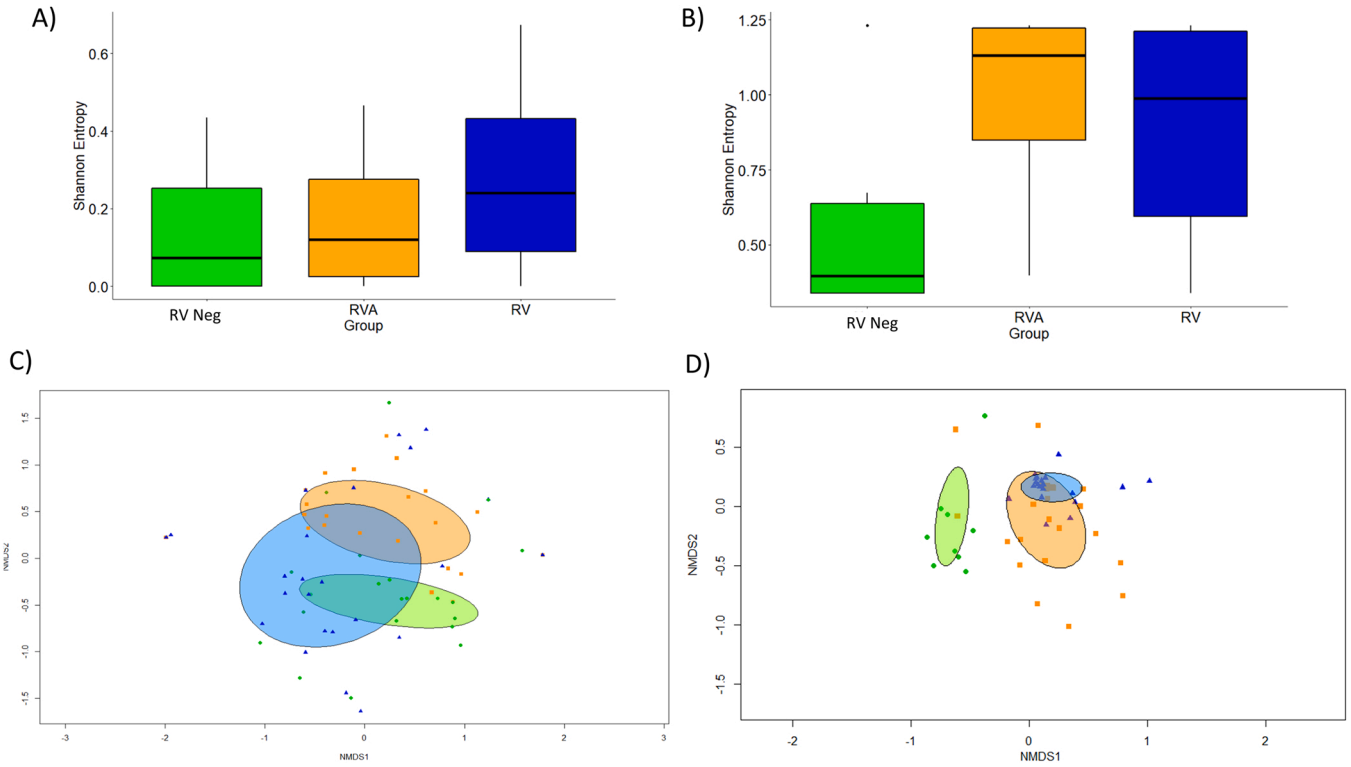

Fig. 6. Alpha and Beta diversity of RVA/RV/RV Neg Porcine virome sequences. Shannon Entropy was used to calculate the Alpha diversity, or diversity within samples, of viral sequence reads and assessed for significance with a Kruskal-Wallis test in R v4.1.1 with either Rotavirus sequences A) included or B) excluded from analysis. Beta diversity was calculated with a Bray-Curtis test using Non-metric Multi-dimensional Scaling (NMDS) in R, C) including or D) excluding rotavirus sequences. Groups are differentiated by color; RV Neg, green; RVA, orange; RV, blue.(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

in the virome of clinically diseased but rotavirus negative pigs. We take a preventative approach to enteric pathogen surveillance in which 63 samples were collected from three distinct pathogen groups (RV Neg, RVA and RV) all sharing the commonality of clinical enteric disease. These groups were defined based on the presence of Rotavirus A (RVA) or multiple rotavirus species (RV) or the absence of any rotavirus (RVNeg) as calculated by qRT-PCR. In these samples, we identified 14 viral species as well as 8 species of bacteriophages, many of which have been identified in pig feces (Shan et al., 2011; Cortey et al., 2019; Giuffrè et al., 2021; Ramesh et al., 2021). In the rotavirus positive groups (RVA and RV), non-rotaviral virome diversity was higher than that of the RVNeg group. Furthermore, the proportion of each virome member in the RVA and RV groups was either significantly or subjectively lower than in the RVNeg group. This finding suggests that 1) rotaviruses may have been the first to sustain infection, 2) rotaviruses are more adept at replicating in the swine hosts or 3) rotavirus infections inhibit (directly or indirectly) the replication of other members of the virome. By defining the most prevalent viruses we can provide insight into potential differential pathogens when animals are demonstrating enteric disease but are Rotavirus negative. Interestingly, we identified a novel group of viruses, *Picobirnaviruses*, that were most prevalent in RVNeg samples suggesting a potential role in clinical disease. To more accurately define the role of PBVs in the manifestation of clinical disease, we sought to first identify the most likely hosts of these viruses. Our results indicate PBVs most likely infect fungi using sequence analyses alone. Taken together, fungi may play a larger role in non-rotaviral disease although more research is needed to evaluate the significance of these findings.

Of the 14 viruses and 8 viruses of bacteria or bacteriophages (v/p), beyond rotavirus, *Kobuvirus, Picobirnavirus, Astrovirus* and *E.coli* v/ps were the most abundant rotaviruses identified in the sample set with a total of 29, 24, 28 and 32 positive samples, respectively. Various sequence data was extracted from the sample set including RVA, RVC, RVH, PKV, PBV, PAstV, SaV, PTV and EVG sequences. These sequences were subjected to genomic analysis. The RVA VP7 sequences were composed of G9 (10) and G4 (2) genotypes and the VP4 were P[23] (6), P[13] (5), P[5]7 (4) and P[7] (1) genotypes. Together, most of the sequences were G9P[23] (5) or G9P[13] (3) genotypes. These findings contradict previous reports that the most prevalent G genotype in the US is G5 and the most prevalent P is P[7] (Vlasova et al., 2017). Of the PAstV sequences, four were PAstV5 and two were PAstV2 genotypes. Although PAstV 5 and 2 are not associated with clinical disease, previous studies determined the prevalence of these viruses to be lower than what was identified here (Mor et al., 2012). Both of the identified EVG (G1) and SaV (GIII) genotypes were consistent with previous studies within and outside the US (Knutson et al., 2017; Tsuchiaka et al., 2018; Mi et al., 2021). Taken together, this information is invaluable to provide accurate diagnostics, monitor viral spread and to prevent future outbreaks.

Rotavirus VP4 and VP7 homology models were compared to two porcine vaccine sequences, the Gottfried strain and OSU 1975. The resulting analysis suggests that our sequences differ in key host-interaction points (Fab connectors on VP7 and linkage with the cell membrane for VP4) on each of the proteins. We suggest these may also correlate to serological data, although additional confirmatory testing is needed. Further analysis of the known epitope regions in the VP7 protein coding sequence revealed significant divergence in amino acid 87–98, 208–214 and 217–221, however, structure modeling only confirmed significant sequence differences between 208 and 214 and 217–221 as well as other, non-epitope regions, 1–54 and 198–201(Settembre et al., 2011; Naseer et al., 2017). Analysis of the more divergent VP4 sequencing using homology modeling and protein alignment resulted in amino acid differences in the VP8 protein region that were not largely transferred to the protein structure. Significant VP4 structural regions were seen in the linker region as well as the VP5 foot region. Vaccine cross-protection has not been thoroughly assessed, however, rotavirus neutralization is generally minimal between serotypes (Kapikian and Hoshino, 2007). In humans, the introduction of the vaccines Rotateq and Rotarix shifted the predominant strains of rotavirus from G1P[8] to G12P[8] and G3P[8]/G2P[4], respectively, suggesting RVA cross-protection is low between serotypes/genotypes (Roczo-Farkas et al., 2018; Hungerford et al., 2019). While our study suggests the predominant porcine VP7 strains are G4 and G9 at least half of these animals would not be protected by RVA vaccination. Furthermore, most of the VP4 sequences identified would not be covered by the P[6] and P[7] vaccine strains. In summary, it is important to provide a framework for future porcine vaccine strategies utilizing genomic and proteomic surveillance data.

In this study, RVA and RV groups had a higher non-rotaviral virome diversity than the RVNeg group. These results were reflected in the alpha and beta diversity tests performed in this study either including or excluding rotavirus sequences. Pigs with diarrhea have been shown to have a higher RNA virus diversity than healthy pigs and Rotaviruses were most commonly associated with clinical disease (Cortey et al., 2019). We build on this finding, showing that pigs with enteric disease, infected with *rotavirus*, have a higher viral diversity than those that lack *rotavirus* infection but are clinically diseased. Additionally, within the RVA and RV groups, non-rotaviral members have a significantly or subjectively lower quantities within the virome than in the RVNeg samples. This increase in viral diversity while decreasing viral quantities associated with rotaviruses could be explained by multiple factors; 1) the rotavirus initially infected the host providing a replication advantage, 2) RVs may replicate more efficiently in the swine host and/or 3) the RV replication interferes with the replication of other viruses. Viral interference has been suggested to occur with both viruses and bacterial species and may be utilized as a therapeutic (Domínguez-Díaz et al., 2019; Kovesdi and Bakacs, 2020; Escobedo-Bonilla, 2021). This idea has been suggested for human rotavirus infections but has not been identified in porcine rotavirus infections (Wang et al., 2012). The concept of viral interference has not been well addressed and may be caused by direct (virus to virus) or indirect (competition of resources, host immune response) interactions. This study is the first to examine, in depth, the occurrence and viruses involved in porcine rotavirus viral interference.

Sample groups (RVA, RV, RVNeg) were used to assess viral associations with the most common cause of swine enteric disease, *Porcine Rotavirus*, resulting in significant differences. In addition to rotavirus, two viral species, *Sapovirus* and *E.coli* phages, were also associated with rotavirus positive samples (RVA or RV) groups but were not found in the RV Neg group. As both SaV and *E.coli* have been associated with enteric disease in young pigs, this finding is not unexpected but it is interesting that these species were only found in the presence of *Rotavirus*. High viral diversity corresponding with porcine rotavirus infection has been seen previously but failed to correlate a specific rotavirus genotype(s) to SaV infection (Li et al., 2017). Both PKV and PAstV species were identified in all tested groups, confirming previous studies linking these viruses to healthy and diseased animals (Jackova et al., 2017; Ulloa et al., 2019). This data suggests PKV and PAstV infections require additional co-factors to cause disease.

The only viral species found at an extremely high prevalence in RV Neg but not other groups was PBV, but these sequences identified were not similar to *Porcine Picobirnaviruses*. *Picobirnavirus* has been associated with enteric disease in a broad range of hosts that include multiple mammals and birds, however, the pathogenicity remains unknown (Ghosh and Malik, 2021). PBV has been detected at a higher incidence in diseased compared to healthy pigs and at a low incidence as a co-infecting agent with RVA, similar to our results (Wilburn et al., 2017). This study is the first to connect Rotavirus presence or absence with PBV occurrence in pigs. The hosts of many of the described viruses have been confirmed as pigs, however, the novel PBV sequences identified in this study were most similar to non-porcine PBVs. We sought to identify a potential host species to assess whether the PBV was infecting the diseased pigs or whether the PBV could have been a result of an infection by a non-viral species. The host of *Picobirnavirus* has been

implicated as a non-mammalian species, most likely a ubiquitous member of the mammalian and avian biome. We sought to assess the most likely host kingdom using sequence-based techniques alone. Initially, many different kingdoms were utilized in the analysis, which excluded bacteria as a likely host. Narrowing the potential hosts resulted in a distinct separation of the host species, grouping *Picobirnavirus* with Fungi. While this is by no means confirmatory, additional factors suggest fungi are the natural hosts of *Picobirnavirus,* including the capsid architecture of PBV as well as the codon usage biases (Shi et al., 2016; Wolf et al., 2018; Yinda et al., 2018; Kleymann et al., 2020). Many fungal species are found in the pig gut biome but are largely composed of *Ascomycota* and *Basidiomycota* phyla (Arfken et al., 2019; Giuffrè et al., 2021). Other fungal phyla identified include *Microsporidia, Chytridomycota* and *Mucoromycota* but these are dynamic through the first 35 days of age (Summers et al., 2019; Giuffrè et al., 2021). The changes to the pig gut mycobiome during illness have not been thoroughly investigated. In this study, we were unable to include fungal analyses due to a lack of adequate sample volume. Further analyses of porcine virome samples including the host association and prevalence of PBV in diseased animals is needed to fully evaluate the study findings.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetmic.2022.109447.

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