SWINE HEALTH INFORMATION CENTER
FINAL RESEARCH GRANT REPORT

Project Title: An underappreciated respiratory pathogen or shifting tropism of porcine rotaviruses
Project Identification Number: 24-082
Principal Investigator: Vlasova, Anastasia
Institution: The Ohio State University
Date Report Submitted: 12/15/2025

Industry Summary:
Rotaviruses, especially A and C, are among the most common causes of diarrhea in young pigs, and new evidence suggests that some strains may also affect porcine respiratory tract. Because these viruses are impossible to keep out of farms, understanding how they spread and cause disease is essential for improving herd health. Thus, the objectives of this study were: **1. To evaluate nasal and fecal rotavirus shedding by suckling and weaned piglets with three health status (respiratory signs, diarrhea, or healthy), and to determine whether RVA or RVC can be associated with respiratory disease/lesions. 2. To genetically characterize RV strains associated with respiratory disease and to define the microbiome composition associated with respiratory RV replication under field conditions.**
We conducted this study in 6 swine farms in Ohio (2 research farms and 4 commercial farms). Nasal and rectal swabs were collected from suckling and weaned piglets that were healthy, experiencing diarrhea, or showing respiratory signs. Samples were tested for rotavirus RT-PCR assays. In addition, we have also analyzed tissues of 16 suckling piglets that died of undefined causes on farm 2 (research farm) to determine whether rotaviruses were present in different organs, including the organs from the respiratory tract.
Our results demonstrated that rotaviruses A and C were present on all 6 farms. While the prevalence of different rotaviruses varied greatly between different farms (A: 67-100; C: 7-56%), overall, 88% and 29% of piglets were positive for rotavirus A and C, respectively. Consistent with prior research, the highest rotavirus A prevalence/viral loads were found in diarrheic weaned piglets on most farms. However, suckling piglets with respiratory signs from farm 6 and diarrheic suckling piglets from farm 5 also had increased rotavirus A loads.
Of importance, suckling piglets with respiratory signs from farm 6 shed either more rotavirus A nasally or the levels were comparable to those shed with feces, while all other piglets on all farms had the levels of the virus shed with feces consistently higher. This can likely be attributed to the genetic diversity of the circulating rotavirus A strains, which is currently being explored further. We have also confirmed rotavirus A presence in various samples, including samples from respiratory tract, of 12 out of 16 dead suckling piglets from farm 2.
Rotavirus C shedding was either at a very low level, suggestive of no ongoing outbreak on farms 2 and 5, or higher in suckling piglets with diarrhea on farms 1 and 4. Farm 6 was an outlier, in which rotavirus C loads were the highest in weaned piglets with diarrhea. Of interest, on farm 3 only, healthy weaned piglets had highest loads of rotavirus A and C. Another interesting observation is that farm 4 had the lowest overall prevalence of rotavirus A, which coincided with the highest prevalence of rotavirus C. It is unclear whether the heightened biosecurity measures in place at the time of sample collection—implemented due to an ongoing outbreak of highly pathogenic influenza A virus in nearby poultry farms—contributed to this outcome.
Screening for other respiratory and enteric pathogens so far did not reveal any strong associations between individual pathogens and increased prevalence of rotavirus infections. However, as we continue with the analysis of our PCR/RT-PCR screening and NGS data, new findings may emerge, which we plan on presenting/publishing promptly.
We have selected representative rotavirus A and C positive (paired nasal and rectal swab) samples from suckling and weaned piglets from different farms for complete genomic sequencing by next generation

sequencing (NGS). We have also selected representative samples from different farms from rotavirus A and C positive piglets with and without diarrhea or respiratory signs to determine whether a specific metagenome composition can protect or predispose piglets from/to the rotavirus-associated illness.
Overall, our data further emphasize the high on-farm prevalence of rotaviruses A and C and their strong association with diarrheal disease. While we do not see any evidence of rotavirus C involvement in the porcine respiratory disease complex, rotavirus A may be emerging as a possible respiratory pathogen of suckling piglets. The remarkably high overall prevalence of rotavirus A on all farms also suggests that it may utilize other transmission routes (e.g. airborne) in addition to fecal-oral/contact.

Contact information:
Anastasia N. Vlasova, DVM, PhD | Associate Professor
Center for Food Animal Health
Department of Animal Sciences
College of Food, Agricultural, and Environmental Sciences
The Ohio State University
1680 Madison Avenue Wooster, OH 44691
330-263-3740 Office
vlasova.1@osu.edu

Talita Resende, DVM, MSc, PhD
Assistant Professor | Food Animal Diagnostician
College of Veterinary Medicine | Veterinary Diagnostic Laboratory
University of Minnesota College of Veterinary Medicine
Phone: 612 626 0693
resen023@umn.edu

Please indicate if you plan to submit this full report for peer-reviewed publication: Yes☒ No☐

Key Producer Outcomes:

- Rotavirus A remains the most widespread rotavirus in U.S. pig farms and is strongly associated with diarrhea in weaned piglets
- RVA may also contribute to respiratory disease, especially in suckling pigs
- Rotavirus C is common but behaves differently between farms, reinforcing the importance of understanding herd-specific patterns
- Farm-specific co-infections and microbial community differences may affect the severity of rotavirus-associated diseases

Keywords: porcine rotavirus; respiratory illness; diarrhea; suckling piglets; weaned piglets; field study

Scientific Abstract:
Rotavirus A (RVA) and C (RVC) cause acute gastroenteritis (AGE) in young children and animals, including piglets. Multiple studies demonstrated RVA/RVC presence in blood and different organs (including lungs) of children and piglets. Additionally, some studies reported on respiratory signs concurrent with RVA/RVC shedding in piglets. However, knowledge on RVA/RVC respiratory tropism and pathogenesis remains limited.
We collected nasal and rectal swabs from suckling and weaned piglets (N=427) (from 6 Ohio Swine farms: 4 commercial and 2 research, F1-F6) that were either healthy, had diarrhea or had respiratory signs. These samples were screened for RVA and RVC using qRT-PCR. Additionally, the samples were screened for various enteric and respiratory pathogens. Swabs collected from different tissues of 16 dead piglets (aged 1-3 weeks, all-cause mortality) from F2 were also tested for RVA and RVC.
Eighty-eight percent and 29% of piglets were positive for RVA and RVC, respectively, and the prevalence varied greatly between different farms (RVA: 67-100%, RVC: 7-56%). Consistent with prior research, the

highest RVA titers were shed by diarrheic weaned piglets on most farms. Of special interest, F6 suckling piglets with respiratory signs had increased RVA titers, suggesting that increased respiratory RVA tropism can be observed in this age group. We confirmed RVA presence in various (including respiratory tract) samples of 12 out of 16 dead piglets. In sharp contrast to RVA, RVC shedding was either at baseline level (suggestive of no ongoing outbreak) or higher in suckling piglets with diarrhea on all farms but one. These data suggest that RVA and RVC remain highly prevalent diarrheagenic pathogens in piglets; however, the mechanisms of RVA and RVC transmission and pathogenesis are distinct.

Introduction:

Rotavirus (RV, *Sedoreoviridae* family) infections are generally associated with acute gastroenteritis in children and animals, including piglets[1]. Besides that, numerous epidemiological, experimental and clinical studies demonstrated that RVA (RV species A) and RVC (RV species C) are frequently detected in various extraintestinal organs (including liver, pancreas, lungs, spleen and salivary glands) and suggested that their replication in respiratory tract may be associated with respiratory illness[2–8]. Of importance, RVA and RVC are detected in humans and pigs more frequently than other RV species and are found in virtually every US swine farm[9–11]. Moreover, RVA presence in respiratory secretions and its airborne spread have been confirmed in clinical settings and in experimental studies in mice[7,12–16]. Consistent with previous findings in children, a recent study that analyzed samples submitted the South Dakota State University Animal Disease Research and Diagnostic Laboratory (2020-2021) and detected RVA in 30.8% of lung specimens obtained from conventionally reared pigs with respiratory signs, but neither did it evaluate whether other respiratory pathogens were present in the lungs of the RVA-positive piglets nor monitored nasal or fecal RVA shedding[12]. Extraintestinal RVA dissemination and pathology are generally reported in some RVA-positive children[7,14]. However, host/viral/environmental factors associated with these contrasting outcomes are not known. Nevertheless, this study and the other findings discussed above highlight RVA as a newly emerging (or previously unrecognized) respiratory/dually tropic swine pathogen. Despite these significant findings, systematic knowledge, and conclusive evidence regarding the RV-associated respiratory/extraintestinal pathology and RV airborne spread are lacking. Our recent study demonstrated that RVA presence in the lungs [evidenced by RNA-in situ hybridization, RT-PCR and immunofluorescence] of most RVA-infected gnotobiotic (Gn) piglets coincided with appearance of characteristic pulmonary gross lesions (multifocal areas of consolidation, localized/multi-lobular hemorrhages) as well as histopathological findings of cellular vacuolar degeneration in epithelial cells of bronchi and bronchioles[17]. Of interest, the lesions and RVA replication in the lungs are detectable as early as post-infection day 1 similar to those observed in the gut[17]. The fact that despite these findings no apparent respiratory illness is observed in RV-inoculated Gn piglets is not surprising because it is well established that in most cases respiratory disease in different hosts is of multietiological nature [referred to as porcine respiratory disease complex (PRDC)[18], bovine respiratory disease complex (BRDC)[19], canine bovine respiratory disease complex (CRDC)[20], etc.]. Moreover, RVA presence and replication in the gut is not always associated with clinical disease in piglets[21]. Additionally, aerosol-based and airborne spread of RVs is not considered in the current control and prevention strategies which results in inadequate biosecurity measures.

Here, we conducted a combination of field studies to comprehensively evaluate if RVA and RVC presence is significantly associated with respiratory illness (concurrently with or in the absence of diarrhea) and extraintestinal pathology. We also compared genomic characteristics of RVA/RVC isolated from respiratory vs. intestinal tract and the rate/severity of respiratory RVA/RVC infections in the presence/absence of other respiratory pathogens. Additionally, we conducted metagenomics analysis to assess specific microbial profiles associated with respiratory illness/pathology in RVA/RVC-infected pigs. These studies will close a significant knowledge gap, improve RVA/RVC-associated disease management in different swine production systems and may lead to updated biosecurity standards to reduce RVA/RVC loads and exposure in nursery facilities (where initial exposure to RV occurs).

Objectives:

Objective 1. ***To evaluate the frequency of nasal and fecal RV shedding by suckling and weaned piglets (with respiratory/gastrointestinal illness/healthy), and to definitively establish whether RVA or RVC can be associated with respiratory disease/lesions in the absence of common respiratory pathogens under field conditions.***

Objective 2. ***To genetically characterize RV strains associated with enteric/respiratory disease and to define enteric/respiratory microbiome composition associated with respiratory RV replication under field conditions.***

Materials & Methods:

Objective. 1. To evaluate the frequency of nasal and fecal RV shedding by suckling and weaned piglets (with respiratory/gastrointestinal illness/healthy), and to definitively establish whether RVA or RVC can be associated with respiratory disease/lesions in the absence of common respiratory pathogens under field conditions. This study was designed to determine whether RV infection (alone or in combination with other pathogens) is significantly associated with respiratory disease on swine farms.

Study design and pig sampling. We conducted a cross-sectional study that involved 6 Ohio swine farms: (2 research: the OSU Western Agricultural Research Facility/Swine Farm and the OSU Agricultural Technical Institute/Swine Farm as well as 4 commercial farms: Hord Family Farms, Cooper Farms, Heimerl Farms A and B). Please see **Table 1/Figure 1** for numbers of samples collected from each farm.

Table 1. Farms and numbers of animals sampled

Farm #	Farm Name	Number of pigs sampled
Farm 1	Hord Family Farms	72
Farm 2	OSU Western Agricultural Research Facility	75
Farm 3	OSU Agricultural Technical Institute	90
Farm 4	Cooper Farms	90
Farm 5	Heimerl Farms A	65
Farm 6	Heimerl Farms B	35

We collected rectal (RS) and nasal (NS) swabs from suckling (≤1 week, peak of RVA/RVC shedding) and weaned (peak of RVA diarrhea) piglets with diarrhea, clinical signs of respiratory infection, and healthy. Thus, the study sampled 6 groups of piglets: 1) suckling diarrheic (with fecal consistency score ≤1; fecal consistency is scored as follows: 0, normal/solid; 1, pasty; 2, semiliquid; 3, liquid/watery; 4, watery with blood or mucus), 2) suckling with respiratory signs (presented with one or more of the following signs: coughing, sneezing, snorting, nasal discharge, epistaxis, dyspnea, fever, retarded growth), 3) suckling healthy (without diarrhea or respiratory signs), 4) weaned diarrheic, 2) weaned with respiratory signs, and 3) weaned healthy. For each group, we targeted to sample 15 piglets/farm; however, some farms did not have suckling piglets with respiratory signs at the time of sampling. The resultant sample size of 427 piglets across 6 different farms **(Table 1)** herds provides ample power (>90%) to identify significant (α=0.05) associations between RV infections and diarrheal/respiratory disease. Additionally, we collected RS

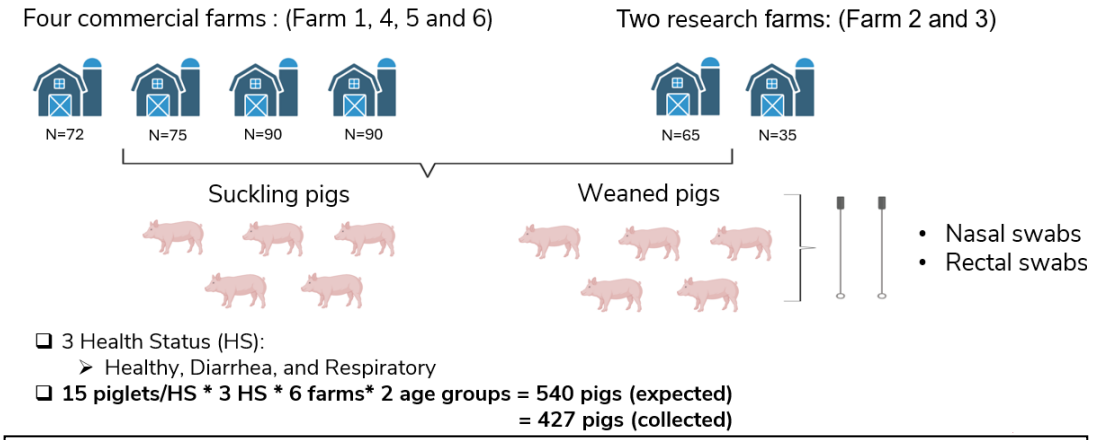


Figure 1. Sampling strategy and the actual numbers of animals sampled on different farms.

and swabs from nasal cavity/turbinates, trachea and lungs from 16 dead/severely ill (with respiratory or enteric disease) and euthanized suckling piglets (were only available for farm 2).

Detection of RVs and common respiratory pathogens in rectal/nasal swabs and different tissues. We have compared several commercial multiplex PCR and RT-PCR kits and selected 1 for PCR and 1 for RT-PCR that showed optimal performance, Platinum® Multiplex PCR Master Mix (Applied Biosystems) and Path-ID™ Multiplex One-Step RT-PCR Kit (Applied Biosystems), respectively. Multiplex real time RT-PCR was conducted to simultaneously detect porcine RVA, RVB, RVC and RVH using the primers shown in **Table 2** and Path-ID™ Multiplex One-Step RT-PCR Kit according to the manufacturer's instructions. The most common viral pathogens associated with PRDC include porcine reproductive and respiratory syndrome virus (PRRSV), swine influenza virus (SIV), pseudorabies virus (PRV), porcine circovirus 2 (PCV2), porcine respiratory coronavirus (PRCV), *Mycoplasma hyopneumoniae*, *Actinobacillus pleuropneumoniae*, *Glaesserella parasuis*, *Streptococcus suis*, *Pasteurella multocida*, and *Bordetella bronchiseptica.* Path-ID™ Multiplex One-Step RT-PCR kit was also used for all RNA viruses listed above and in Table 2. For detection of DNA viruses and bacteria, we used pathogen-specific primers/probes listed in Table 2 and Platinum® Multiplex PCR Master Mix according to the manufacturer's instructions. Most of the primers/probes were from previous publications[22–26]; however, some of them had to be modified from their original design due to suboptimal performance. Universal primers for coronavirus detection were designed in our lab. Pathogen-specific synthetic genes were synthesized (Twist Biosciences or GenScript), cloned into pUC57 vector and used as positive controls.

Table 2. Primers and probes for swine pathogen detection

Pathogen	Primer/Probe	Primer/probe sequence (5'→3')	Amplicon size, bp
BACTERIA			
Bordetella bronchiseptica	F:	AGGCTCCCAAGAGAGAAAGGCTT	128
	R:	AAACCTGCCGTAATCCAGGC	
	Probe:	**HEX**-ACCGGGCAGCTAGGCCGC- **BHQ2**	
Mycoplasma hyopneumoniae	F:	TAAGGGTCAAAGTCAAAGTC	150
	R:	AAATTAAAAGCTGTTCAAATGC	
	Probe:	**FAM**-AACCAGTTTCCACTTCATCGCC-**BHQ1**	
Glaesserella parasuis	F:	CTGCTTTGATTTCGTTTAATAG	169
	R:	GTAGGTGGTATTACGGAAA	
	Probe:	**TexRd**-AACCTTAGCGGCAGCGTCTA -**IB®RQ**	
Pasteurella multocida	F:	GGAAGCCTTCCAAGCAGAATTTG	156
	R:	CGCAATAGCTTTACCCATTACAG	
	Probe:	**HEX**-CAGCAACCCGTTTCGGTTCAGG-**BHQ1**	
Actinobacillus pleuropneumoniae	F:	TGCTTACCGCATGTAGTGGC	92
	R:	TTGGTGCGGACATATCAACCTTA	
	Probe:	**TexRd**-CGGCTCATCGGGTTCATCGTCT -**IB®RQ**	
Streptococcus suis	F:	AGGCAATGATTTATCTGGAGATG	105
	R:	CTGATTGGCTGAGCTGACCT	
	Probe:	**FAM**-TGGAAGTTCAGCTTAAGAACTTAGAGAAAG -**BHQ1**	
Salmonella spp	F:	CGTTTCCTGCGGTACTGTTAATT	129
	R:	GAATTGCCCGAACGTGGCGATAATT	
	Probe:	**FAM**-CCACGCTCTTTCG-MGB-**NFQ**	
Lawsonia intracellularis	F:	CACCTGGACGATAACTGACACT	118
	R:	TAACTCCCCAGCACCTAGCAC	
	Probe:	**HEX**-GAGGTGCGAAAGCGTGGGG-**BHQ2**	
DNA VIRUSES			
Porcine circovirus 2	F:	CTGTTTTCGAACGCAGTGCC	198
	R:	AACTACTCCTCCCGCCATAC	
	Probe:	**FAM**-CCCAGCCCTTCTCCTACCACTCCCG-MGB	
Pseudorabies virus	F:	GCTCCTTCGTGATGACGTGC	141

	R:	GTACACCGGAGAGAGCATGTG	
	Probe:	**Cy5**-CCGCGTCGGCACCCGGAAC-BHQ3	
RNA VIRUSES			
Rotavirus A	F:	AATATGACACCAGCAGTTGCAAA	107
	R:	ACAGATTCACAAACTGCAGATTCAA	
	Probe:	**CY5**-CAAGCACCGCCATTTATATTTCATGCTACA-**BHQ3**	
Rotavirus B	F:	GTGTCYGCRTWTGCTGC	62
	R:	CCTYTCGAAGCACTYCC	
	Probe:	**VIC**-GGRAGCTGACGCCGGATCAGA-**BHQ1**	
Rotavirus C	F:	GTGAAGAGAATGGTGATGTAG	157
	R:	GTTCACATTTCATCCTCCTG	
	Probe:	**FAM**-TAGCATGATTCACGAATGGGTTTAG-**BHQ1**	
Rotavirus H	F:	CCRCCACARYTMGTYCATTGGTC	93
	R:	TCCCAGTGCGTGACCAGAT	
	Probe:	**FAM**-GCATGYTTRATTGCAGCHTATTC-**BHQ1**	
Swine influenza virus	F:	AAGACAAGACCAATYCTGTCACCTCT	80
	R:	TCTACGYTGCAGTCCYCGCT	
	Probe:	**FAM**-TYACGCTCACCGTGCCCAGTG-**BHQ1**	
Porcine reproductive and respiratory syndrome virus	F:	ATRATGRGCTGGCATCT	114
	R:	ACACGGTCGCCCTAATTG	
	Probe:	**HEX**-TGTGGTGAATGGCACTGATTGACA-**BHQ2**	
Coronaviruses*	F:	CHASNAARTTYTAYGGHGGHTGG	450
	F1:	GGGNTGGGAYTAYCCHAARTGYGA	
	R:	ACNSCRTCRTCDSWHARDATCAT	

*Coronaviruses: transmissible gastroenteritis virus, porcine respiratory coronavirus, porcine epidemic diarrhea virus, porcine deltacoronavirus, porcine hemagglutinating encephalomyelitis virus; the primers for universal coronavirus detection were designed for semi-nested RT-PCR (1st round: F-R, 2nd round F1-R)

**Different shading colors highlight pathogen combinations used for different multiplex RT-PCR or PCR assays

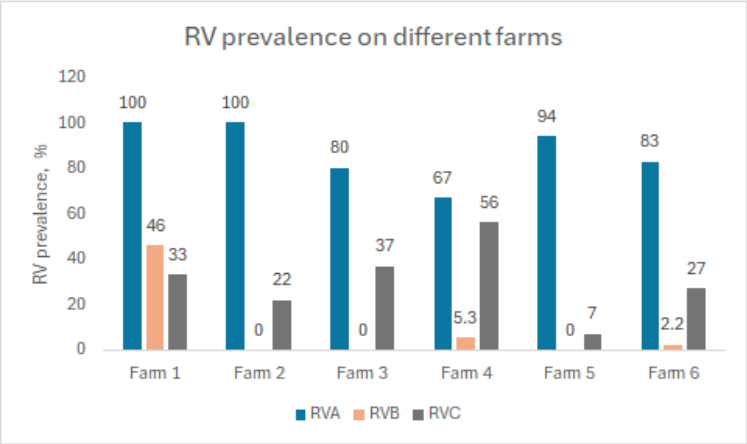
Statistical analysis. Spearman's rank correlation analysis was conducted to evaluate the relationship between the RVA and RVC prevalence. Statistical analysis of RV shedding prevalence and levels was done using t- and one-way ANOVA tests. Statistical significance was assessed at $p \leq 0.05$ for all comparisons. All statistical analyses were performed using GraphPad Prism 7.0c (GraphPad Software Inc. CA, USA).

Objective. 2. To genetically characterize RV strains associated with enteric/respiratory disease and to define enteric/respiratory microbiome composition associated with respiratory RV replication under field conditions. Here, the goal was to identify if certain RV genetic traits and piglet intestinal/respiratory microbiome composition can contribute to enteric vs. respiratory disease in piglets and to establish if different RV genotypes may have variable ability to replicate in the intestinal vs. respiratory tract. We utilized metagenomics analysis to determine if specific microbial profiles in the piglet gut or respiratory tract may contribute to the development of respiratory illness in the presence or absence of other pathogens. We also designed our study to evaluate an alternative hypothesis that certain genetic (intra-host) mutations can lead to differential transcriptional regulation allowing for increased replication in extraintestinal (including respiratory tract) tissues. Metatranscriptomics (=metaviromics) analysis was used to determine complete genome sequences of the identified RVA and RVC strains and to identify any additional pathogens not included in the targeted screening by PCR/RT-PCR described above.

Study design. All RVA-positive RS and NS samples were used to inoculate MA104 cells to amplify RVA present in the samples thus providing better quality samples for NGS. Paired RS (N=10) and NS (N=10) samples [from RVA-positive piglets with respiratory signs or diarrhea (identified in Objective 1)] passaged through MA104 cells that yielded Ct values of ≤27 were selected for complete genome sequencing [metatranscriptomics (=viromics) and submitted to The Ohio State University Infectious Diseases Institute's Genomic and Microbiology Solutions Laboratory (IDI-GEMS). Because RVC does not replicate in any known continuous cell lines, we have selected 8 NS and 8 RS (paired) positive for RVC that yielded Ct values of ≤27 and submitted them to the IDI-GEMS form metatranscriptomics analysis. Additionally, we selected 16 RVA-

or RVC-positive RS samples from piglets with respiratory illness (N=2), with diarrhea (N=10) and 3 healthy piglets for metagenomics analysis to determine what microbial profiles in RV-positive piglets can be associated with healthy or diseased status. As all RNA/DNA samples isolated from NS/RS had lower concentrations than required for NGS, we have used Monarch® Spin RNA Cleanup Kit (Monarch® Spin RNA Cleanup Kit (10 µg) | NEB) and Macherey-Nagel NucleoSpin gDNA Clean-up, Mini kit for DNA clean up and concentration (NucleoSpin-gDNA-Clean-up) for RNA and DNA concentration, respectively.

Materials and methods: Metagenomics: Bacterial composition of the piglet gut and respiratory microbiomes was conducted using shotgun metagenomics as described previously[27]. Briefly, changes in the gut/respiratory



Parameter	Value
Pearson correlation coefficient (r)	**-0.735**
r^2	0.5402
P-value	0.09603
Covariance	-156.7333
Sample size (n)	6
Statistic	-2.1679

Figure 2. RV prevalence on 6 different swine farms in Ohio among suckling and weaned piglets (left graph). RVA and RVC were prevalent on each farm. RVB was only found on 3 out of 6 farms. We found no RVH on any farm. RVA and RVC prevalence appeared to correlate negatively (right table).

microbiota were assessed by monitoring the bacterial species abundance, composition and function in the fecal and respiratory samples by shotgun metagenomics on the Illumina NextSeq2000 platform. Metatranscriptomics (=viromics): complete genomes of the identified RVs were determined using shotgun NGS as previously described[28].

Results:

Objective. 1. To evaluate the frequency of nasal and fecal RV shedding by suckling and weaned piglets (with respiratory/gastrointestinal illness/healthy), and to definitively establish whether RVA or RVC can be associated with respiratory disease/lesions in the absence of common respiratory pathogens under field conditions. Due to the ongoing outbreaks of high path influenza and PRRSV, two out of three commercial farms (Fine Swine and Cooper Farms) that we planned on sampling initially indicated that they were no longer able to allow sampling or provide samples within the study timeframe. Thus, we contacted and enrolled two Heimerl Farms (A and B) instead; however, Cooper Farms were eventually able to contain the outbreak and allow sampling in May. Thus, instead of the originally proposed 3 commercial and 2 research farms, we ended up sampling 4 commercial and 2 research farms. We have also altered our sampling strategy to make it more straightforward and sampled 15 animals/group/farm whenever available. Thus, our sample size increased to 427 animals (instead of proposed 240) or 854 samples (NS and RS) instead of originally proposed 480. While we realize it is a significant increase in sample size (nearly 2-fold) from what we proposed originally, the resultant sample size/sampling strategy yields a more comprehensive understanding of the diversity of RV infections and disease as well as farm-to-farm variability.

Rotavirus screening. Our results demonstrated that rotaviruses A (RVA) and C (RVC) were present on all 6 farms, rotavirus B (RVB) was only found on 3 farms, while there was no evidence of rotavirus H (RVH) or any of the farms surveyed. While the prevalence of different RVs varied greatly between different farms (RVA: 67-100%, RVB: 0-46%, RVC: 7-56%)**(Fig. 2)**, overall, 88%, 9%, and 29% of piglets were positive for RVA, RVB and RVC, respectively. Of interest, there was a weak negative correlation between the frequencies of RVA and RVC detection **(Fig. 2)**. Since currently there is no evidence of cross-protection between different RV species (e.g. RVA and RVC), this finding may indicate that some other (farm-specific) factors may differentially predispose piglets to RVA but not RVC and vice versa.

Table 3. RVA positivity rates in different tissues of suckling piglets dying from all-cause mortality on farm 2.

Tissue/organ	RVA positivity rate
Rectal swabs	75%
Nasal cavity	81%
Nasal turbinates	50%
Trachea	69%
Lungs	69%

Consistent with prior research, the highest RVA prevalence/loads were found in diarrheic weaned piglets on most farms **(Fig. 3)**. However, suckling piglets with respiratory signs from farm 6 and diarrheic suckling piglets from farm 5 also had increased RVA loads. Additionally, all piglets with diarrhea consistently shed more RVA in RS than in NS. However, the

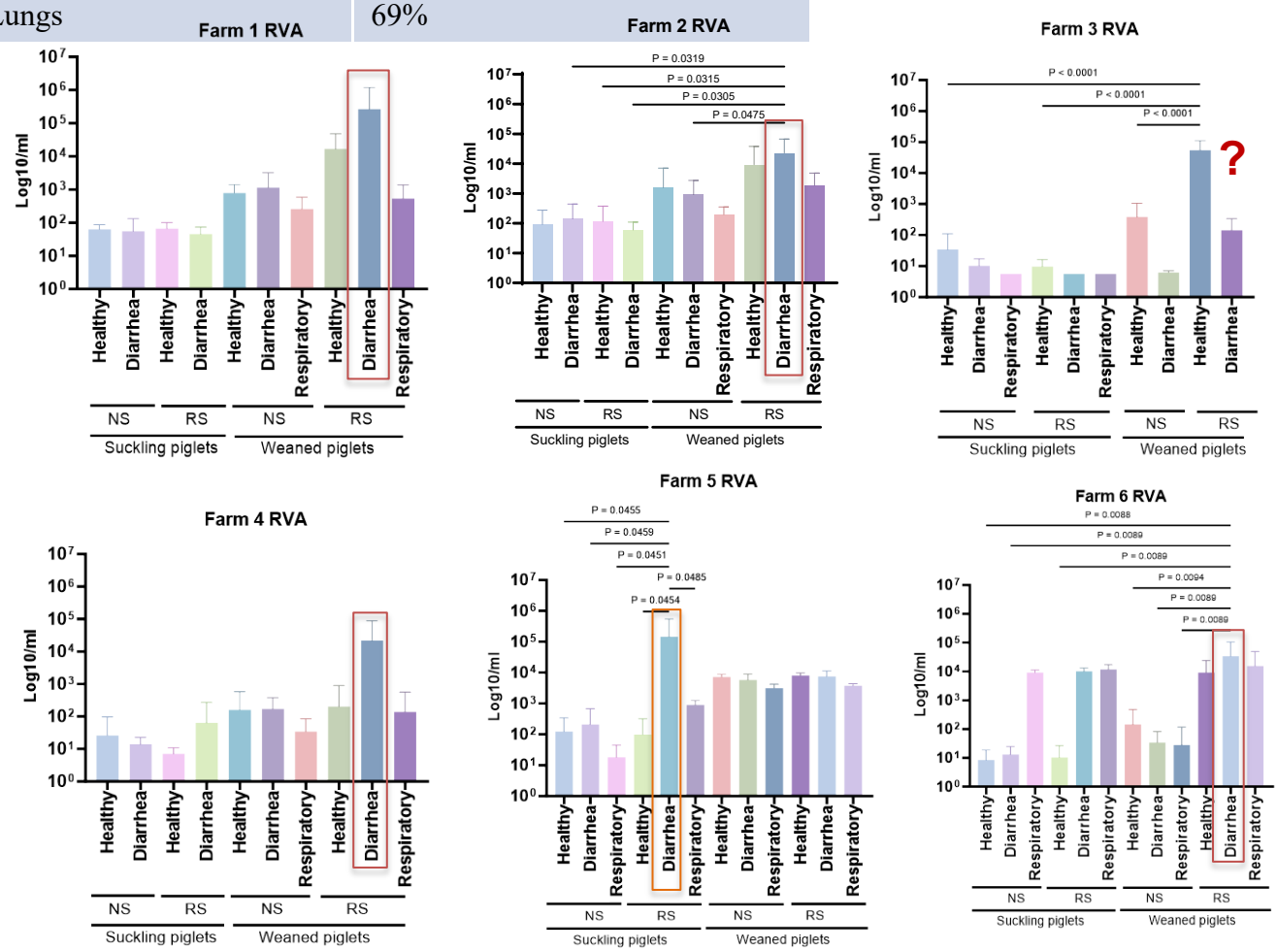


Figure 3. RVA levels in NS and RS of suckling and weaned piglets on 6 different swine farms in Ohio.

farm 6 suckling piglets with respiratory illness shed comparable amounts of RVA in their NS and RS samples, while several piglets from this group shed more RVA in their NS samples. Overall, these data suggest the high genetic diversity of the circulating RVA strains, and we are currently analyzing NGS data to confirm this. We have also confirmed RVA, but not RVC presence in various (including respiratory tract) samples of 12 out of 16 dead suckling piglets from farm 2. It is worth noting that RVA prevalence in various respiratory tract tissues was comparable to that in RS samples collected from the dead/euthanized piglets. Finally, to our surprise, healthy weaned piglets had the highest RVA levels on farm 3. While this further highlights the fact that weaned piglets are highly vulnerable to RVA infections, it also suggests that the overall health status of piglets on this farm could be higher due to the lower animal density.

In general, RVC RNA levels were lower than RVA RNA levels in all groups of piglets **(Figs. 3&4).** In contrast to RVA, RVC shedding was either at a very low level (suggestive of no ongoing outbreak, farms 2 and 5) or higher in suckling

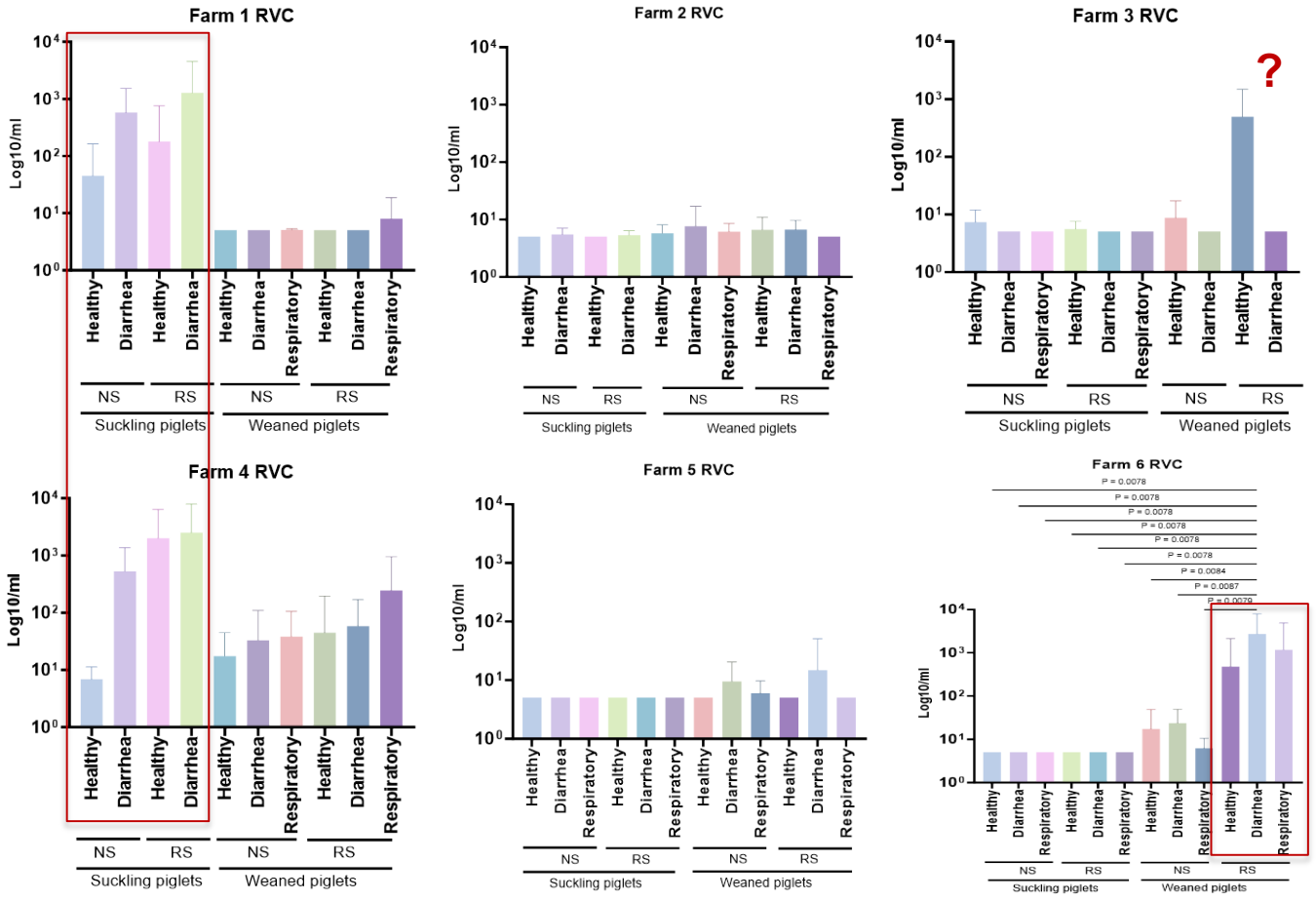


Figure 4. RVC levels in NS and RS of suckling and weaned piglets on 6 different swine farms in Ohio.

piglets with diarrhea on farms 1 and 4 **(Fig. 4)**. Farm 6 was an outlier, in which RVC loads were the highest in weaned piglets with diarrhea. Also, in contrast to our RVA findings, RVC loads have never been found to be increased in NS vs RS and in piglets with respiratory signs in comparison to those with diarrhea. Importantly, high RVC prevalence on farms 1 (suckling piglets), 4 (suckling piglets) and 6 (weaned piglets) was associated with piglet diarrheic status and increased levels of RVC shed **(Fig. 5)**. Overall, these data suggest that RVC infection may be more restricted to the gut than RVA. Of interest and similar to our RVA findings, on farm 3 only, healthy weaned piglets had highest loads of RVC. Another interesting observation is that farm 4 had the lowest overall prevalence of RVA, which coincided with the highest prevalence of RVC. It is not clear whether the fact that this farm at the moment of sample collection was operating under highest biosecurity standards due to the ongoing outbreak of high path influenza A virus contributed to this outcome or not.

Rotavirus B was found on 3 farms: 1, 4, and 6, with most cases clustering on farm 1 and almost exclusively affecting weaned piglets **(Fig. 6)**. Interestingly, 67% of piglets on this farm with respiratory signs were positive for RVB, while only 44% of healthy piglets and 37% of diarrheic piglets were RVB-positive **(Fig. 6)**.

Overall, our data further emphasize the high on-farm prevalence of RVA and RVC and their strong association with diarrheal disease. While we do not see any evidence of RVC involvement in the porcine respiratory disease complex, RVA may be emerging as a possible respiratory pathogen of suckling piglets. The remarkably high overall prevalence of RVA on all farms also suggests that it may utilize other transmission routes (e.g. airborne) in addition to fecal-oral/contact. Rotavirus B prevalence remains lower and more sporadic, and our data is insufficient to link it to enteric disease but suggests there may be a possible association with respiratory illness. Finally, our data suggest that RVH is currently absent in most Ohio swine farms, or it exclusively affects adult swine but not suckling and weaned piglets.

Screening for other pathogens. Screening for other pathogens (respiratory and enteric) so far did not reveal any strong associations between individual pathogens increased prevalence of rotavirus infections or enteric/respiratory illness. However, as we continue with the analysis of our PCR/RT-PCR screening and the Objective 2 NGS data, new findings may emerge, which we plan on presenting/publishing and otherwise sharing promptly. Most additional viral or bacterial pathogens that we screened for were characterized by relatively low prevalence (2-8%) and were clustering on some but

not all farms. Of note, while coronaviruses were almost exclusively found in suckling piglets, all salmonella and most lawsonia cases were among weaned piglets. Farm 3, on which we found the highest prevalence of RVA and RVC in healthy weaned piglets also had the highest prevalence of salmonella shedding. Thus, these findings suggest that different farm management practices and unique microbial combinations may contribute to variable health outcomes irrespective of the presence of ubiquitous RVA and RVC infections.

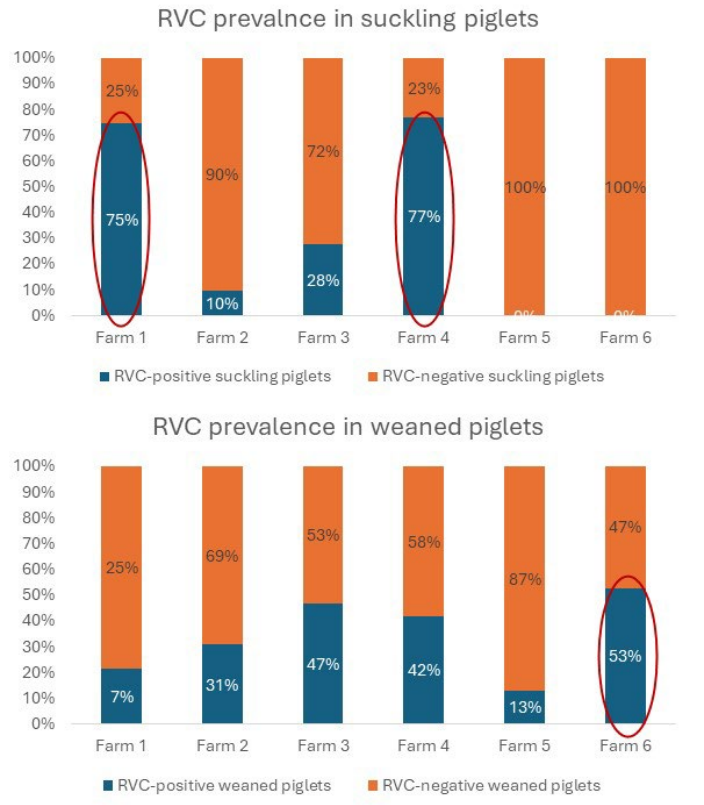


Figure 5. RVC prevalence is increased on commercial farms. RVC prevalence on 6 different swine farms in Ohio (2025). Red ovals indicate that increased RVC prevalence was associated with higher RVC levels and diarrheic piglet status.

<u>Objective. 2. To genetically characterize RV strains associated with enteric/respiratory disease and to define enteric/respiratory microbiome composition associated with respiratory RV replication under field conditions.</u>

Based on the data generated in Objective 1, we have selected representative RVA- and RVC-positive (paired NS and RS) samples from suckling and weaned piglets from different farms for complete genomic sequencing by NGS. The additional selection criterion was that the qRT-PCR Ct values for RVA/RVC-positive samples should have been ≤27. The RVA-positive paired NS and RS samples used for RNA isolation for NGS were passaged through MA104 cells to increase the quality/quantity of RVA RNA. The RNA from the RVA-positive MA104-passaged paired NS/RS samples (from all 6 farms) as well as from the RVC-positive paired NS/RS samples (from farms 1, 4, and 6) was submitted to the IDI-GEMS for NGS sequencing. We have also selected representative samples from different farms from RVA and RVC positive piglets with and without (diarrheal/respiratory) disease and submitted them to the IDI-GEMS for shotgun sequencing and metagenomics analysis to determine whether a specific metagenome composition can protect or predispose piglets from/to the RV-associated illness. All RNA and DNA isolated from the RVA/RVC-positive samples were concentrated using the RNA/DNA

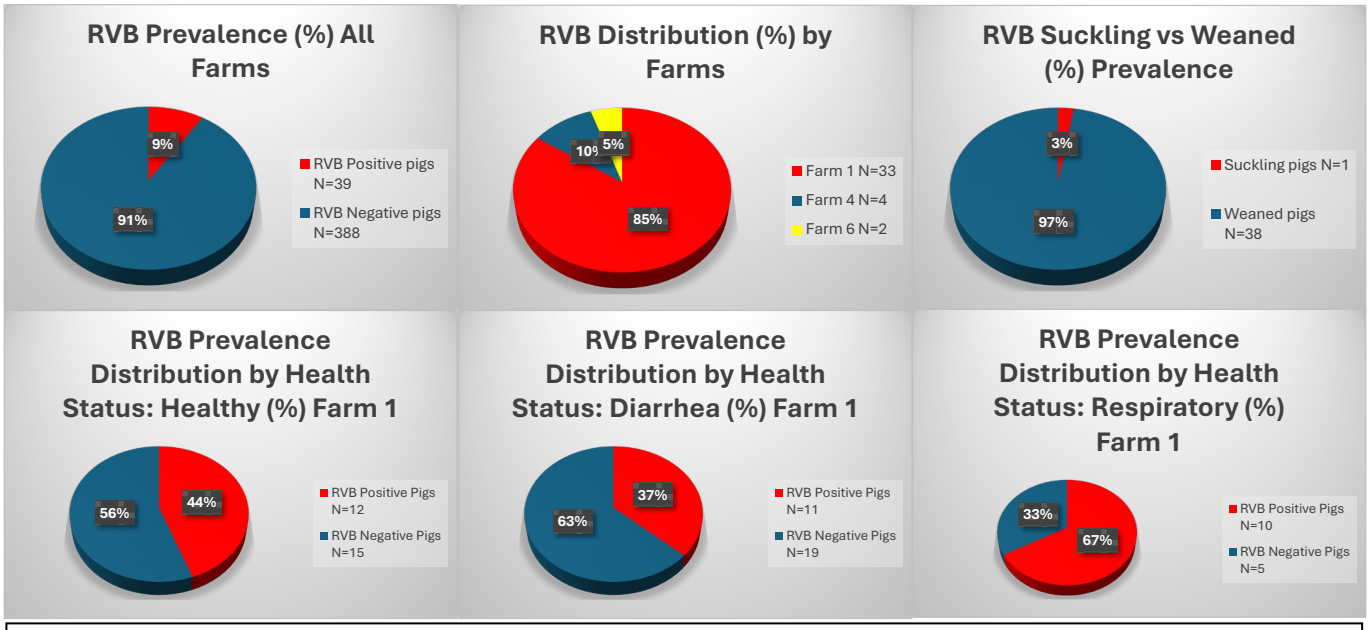


Figure 6. RVB prevalence and distribution among different farms, health status and piglet age groups.

concentration kits. The NGS data (both metagenomics and metatranscriptomics) are currently being analyzed.

Discussion:

The data generated in the current study further emphasize the fundamental differences between porcine RVA and RVC species. First and foremost, our findings show that RVA remains the most prevalent porcine RV species that can be found in association with diarrhea in weaned piglets on most commercial farms at any given moment. This is consistent with the data published previously by us and other research groups[9,29,30]. Despite that, increased levels of RVA can sometimes be found in healthy weaned piglets or in suckling piglets with diarrhea or respiratory illness. This is suggestive of high biological and genetic diversity that is currently being further investigated in our study. The fact that increased RVA levels can be found: a) in suckling piglets with respiratory illness, b) in NS samples, and c) in lower respiratory tract of some suckling piglets dying due to unknown causes, suggests that some RVA strains can contribute to respiratory illness and can be capable of airborne spread. This in turn indicates that the current biosecurity measures implemented on most swine farms for RVA control are inadequate. In contrast to RVA, RVC, while still ubiquitous, is characterized by lower on-farm prevalence and lower shedding levels. It is most frequently found in association with diarrhea in suckling piglets; however, on some farms it can be associated with diarrhea among weaned piglets. This is also consistent with the prior research[9,10]. Further, while RVA is found in association with diarrhea on most (research and commercial) farms, symptomatic RVC infections are mostly observed on commercial farms. Another major difference is that RVC does not seem to be a contributing factor to respiratory illness in either suckling or weaned piglets. It is likely that the intrinsic RVC characteristics restrict its replication to the gut, preventing its extraintestinal spread. Our recent data demonstrated a stronger immune upregulation associated with RVC than RVA infections[31], while prior research by others emphasized the increased ability of RVA strains to suppress various innate immune responses[32]. Thus, the more potent immune response induced by RVC may result in the observed compartmentalization of RVC infections.

Sporadic cases of RVB infections as well as RVB preference for weaned piglets observed in our current study are also consistent with previous observations[9]. However, the highest prevalence of RVB infections among weaned piglets with respiratory signs is somewhat unexpected and requires further examination.

While currently our data do not indicate that other pathogens that we screened for in Objective 1, can contribute to the clinical manifestation and severity of RVA and RVC infections, they suggest that farm-specific microbial signatures may contribute to variable health outcomes in suckling and weaned piglets. Additionally, our current NGS data may demonstrate there are additional pathogenic or commensal microbes contributing to the disease generation in RVA/RVC-positive piglets.

Overall, our current findings highlight that RVA and RVC are ubiquitous pathogens impacting the US swine industry. The contrasting mechanisms associated with their pathogenesis and spread need to be studied in-depth to inform and update the existing control strategies.

References cited.

1. Crawford, S. E. *et al.* Rotavirus infection. *Nat. Rev. Dis. Primer* 3, 17083 (2017).
2. Shao, L. *et al.* Comparative *In Vitro* and *In Vivo* Studies of Porcine Rotavirus G9P[13] and Human Rotavirus Wa G1P[8]. *J. Virol.* 90, 142–151 (2016).
3. Candy, D. C. A. Rotavirus Infection: A Systemic Illness? *PLoS Med.* 4, e117 (2007).
4. Fenaux, M., Cuadras, M. A., Feng, N., Jaimes, M. & Greenberg, H. B. Extraintestinal Spread and Replication of a Homologous EC Rotavirus Strain and a Heterologous Rhesus Rotavirus in BALB/c Mice. *J. Virol.* 80, 5219–5232 (2006).
5. Kim, H.-H. *et al.* Pathogenicity of porcine G9P[23] and G9P[7] rotaviruses in piglets. *Vet. Microbiol.* 166, 123–137 (2013).
6. Marthaler, D. *et al.* Identification, phylogenetic analysis and classification of porcine group C rotavirus VP7 sequences from the United States and Canada. *Virology* 446, 189–198 (2013).
7. Zheng, B. J. *et al.* Rotavirus infection of the oropharynx and respiratory tract in young children. *J. Med. Virol.* 34, 29–37 (1991).
8. Dian, Z. *et al.* Rotavirus-related systemic diseases: clinical manifestation, evidence and pathogenesis. *Crit. Rev. Microbiol.* 47, 580–595 (2021).
9. Marthaler, D. *et al.* Rapid detection and high occurrence of porcine rotavirus A, B, and C by RT-qPCR in diagnostic samples. *J. Virol. Methods* 209, 30–34 (2014).
10. Chepngeno, J., Diaz, A., Paim, F. C., Saif, L. J. & Vlasova, A. N. Rotavirus C: prevalence in suckling piglets and development of virus-like particles to assess the influence of maternal immunity on the disease development. *Vet. Res.* 50, 84 (2019).
11. Zhao, S. *et al.* Global Infection Rate of Rotavirus C during 1980–2022 and Analysis of Critical Factors in the Host Range Restriction of Virus VP4. *Viruses* 14, 2826 (2022).
12. Nelsen, A. *et al.* Identification of Pulmonary Infections With Porcine Rotavirus A in Pigs With Respiratory Disease. *Front. Vet. Sci.* 9, 918736 (2022).
13. Prince, D. S. *et al.* Aerosol transmission of experimental rotavirus infection: *Pediatr. Infect. Dis. J.* 5, 218–222 (1986).
14. Santosham, M. *et al.* Detection of rotavirus in respiratory secretions of children with pneumonia. *J. Pediatr.* 103, 583–585 (1983).
15. Dennehy, P. H., Nelson, S. M., Crowley, B. A. & Saracen, C. L. Detection of Rotavirus RNA in Hospital Air Samples by Polymerase Chain Reaction (PCR) • 828. *Pediatr. Res.* 43, 143–143 (1998).
16. Zhaori, G. T. *et al.* Detection of rotavirus antigen in tracheal aspirates of infants and children with pneumonia. *Chin. Med. J. (Engl.)* 104, 830–833 (1991).
17. Raev, S. A. *et al.* The efficiency of rotavirus A spread to extraintestinal tissues is not determined by the levels of its replication in the gut. *PLOS Pathog.* 21, e1013723 (2025).
18. Burrai, G. P. *et al.* The Synergic Role of Emerging and Endemic Swine Virus in the Porcine Respiratory Disease Complex: Pathological and Biomolecular Analysis. *Vet. Sci.* 10, 595 (2023).
19. Werid, G. M., Miller, D., Hemmatzadeh, F., Messele, Y. E. & Petrovski, K. An overview of the detection of bovine respiratory disease complex pathogens using immunohistochemistry: emerging trends and opportunities. *J. Vet. Diagn. Invest.* 36, 12–23 (2024).
20. Day, M. J. *et al.* Aetiology of Canine Infectious Respiratory Disease Complex and Prevalence of its Pathogens in Europe. *J. Comp. Pathol.* 176, 86–108 (2020).
21. Garcias, B., Migura-Garcia, L., Giler, N., Martín, M. & Darwich, L. Differences in enteric pathogens and intestinal microbiota between diarrheic weaned piglets and healthy penmates. *Vet. Microbiol.* 295, 110162 (2024).
22. Petri, F. A. M. *et al.* Associations between Pleurisy and the Main Bacterial Pathogens of the Porcine Respiratory Diseases Complex (PRDC). *Anim. Open Access J. MDPI* 13, 1493 (2023).
23. Xin, L. *et al.* The establishment and application of a one-step multiplex real-time polymerase chain reaction assay for the detection of *Streptococcus suis* , *Streptococcus suis* serotype 2, and *Glaesserella parasuis*. *Anim. Res. One Health* 2, 59–70 (2024).

24. Shi, K. *et al.* Development of a Quadruplex RT-qPCR for the Detection of Porcine Rotaviruses and the Phylogenetic Analysis of Porcine RVH in China. *Pathog. Basel Switz.* 12, 1091 (2023).
25. Chen, Y. *et al.* Establishment and application of multiplex real-time PCR for simultaneous detection of four viruses associated with porcine reproductive failure. *Front. Microbiol.* 14, 1092273 (2023).
26. Carr, M. J. *et al.* Development of a real-time RT-PCR for the detection of swine-lineage influenza A (H1N1) virus infections. *J. Clin. Virol. Off. Publ. Pan Am. Soc. Clin. Virol.* 45, 196–199 (2009).
27. Srivastava, V. *et al.* Reduced rotavirus vaccine efficacy in protein malnourished human-faecal-microbiota-transplanted gnotobiotic pig model is in part attributed to the gut microbiota. *Benef. Microbes* 11, 733–752 (2020).
28. Amimo, J. O. *et al.* Metagenomic analysis demonstrates the diversity of the fecal virome in asymptomatic pigs in East Africa. *Arch. Virol.* 161, 887–897 (2016).
29. Amimo, J. O., Vlasova, A. N. & Saif, L. J. 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. *J. Clin. Microbiol.* 51, 1142–1151 (2013).
30. Amimo, J. O., Vlasova, A. N. & Saif, L. J. Prevalence and genetic heterogeneity of porcine group C rotaviruses in nursing and weaned piglets in Ohio, USA and identification of a potential new VP4 genotype. *Vet. Microbiol.* 164, 27–38 (2013).
31. Raev, S. A., Raque, M., Kick, M. K., Saif, L. J. & Vlasova, A. N. Differential transcriptome response following infection of porcine ileal enteroids with species A and C rotaviruses. *Virol. J.* 20, 238 (2023).
32. Arnold, M. M. & Patton, J. T. Rotavirus Antagonism of the Innate Immune Response. *Viruses* 1, 1035–1056 (2009).