

# Compendium | 2020

A collection of NA PRRS Symposium extended abstracts and NC-229 station reports.



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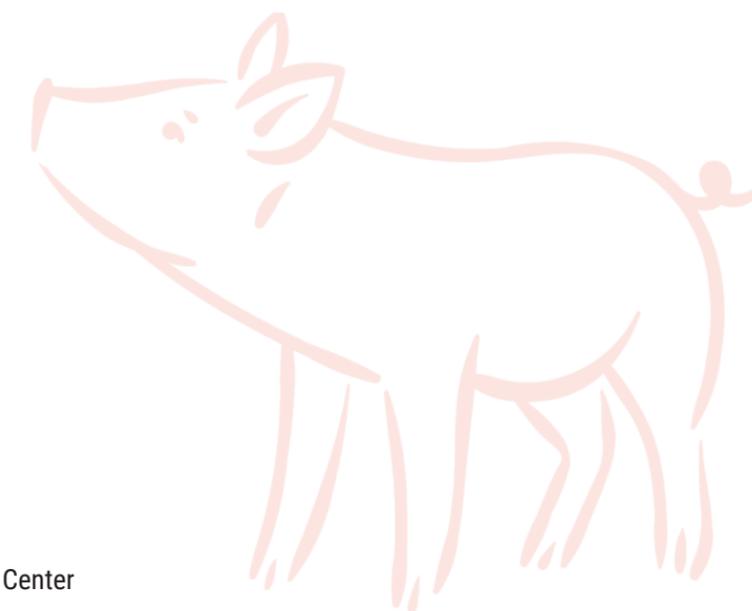
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# Association of the Blood Transcriptome of Healthy Piglets with Response to Natural Polymicrobial Disease, Include PRRS

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<b>Key Words</b>	Pigs, Disease Resilience, Disease Challenge, Blood, Transcriptomics
<b>Objectives</b>	Our objective was to investigate population-level gene expression profiles in the blood of 912 healthy nursery pigs for associations with performance and health before and after exposure to the natural disease challenge.
<b>Methods</b>	We applied a natural polymicrobial disease challenge model, including PRRS, to collect detailed data on disease resilience on F1 barrows. Blood samples were collected at ~27 days of age prior to exposure to disease, and gene expression profiles were quantified using 3' mRNA-sequencing.
<b>Results</b>	The most significant ( $q < 0.20$ ) associations were identified among the level of expression of individual genes in blood of young healthy pigs and resilience phenotypes including growth rate and health scores prior to the challenge, and feed conversion rate, mortality, and a combined mortality-treatment trait after the challenge. The expression of <i>CD163</i> was associated with both pre- and post-challenge phenotypes. Gene set enrichment analyses revealed three groups of gene ontology biological process terms in the blood of young healthy piglets that were associated with their performance and health traits, before and after the disease challenge. First, greater expression of genes related to immune/stress response tended to be associated with a decrease in performance and health traits both pre- and post-challenge. Second, greater expression of genes related to heme metabolism tended to be associated with an increase in some performance and health traits, both pre- and post-challenge. Third, greater expression of genes associated with

protein localization and viral gene expression tended to be associated with reduced performance and health traits after but not before challenge.

## Conclusion

In conclusion, gene expression profiles in blood from young healthy piglets provide insight into their performance when exposed to disease. This includes the level of expression of genes that appear to respond to the various stressors that piglets are exposed to even without major disease, of genes related to heme metabolism, as well as the baseline expression of host genes related to virus propagation.

## Financial Support

Funding from USDA-NIFA, Genome Canada, Genome Alberta, and PigGen Canada.

# Attenuation of a PCV2B Vaccine Candidate by Directed Suicidal Replication

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**Key Words** Vaccine, Porcine Circovirus, PCV2, Attenuated PCV2, Antibody, Virus Neutralization

**Abstract** Postweaning multisystemic wasting syndrome (PMWS), is caused by porcine circovirus type 2 (PCV2), an economically important swine virus which affects production worldwide. Although a DNA virus, PCV2 has a high rate of mutation and continues to evolve into new subtypes. The aim of this study is to take the advantage of the high mutation rate of PCV2 to target the premature termination of the gene expression as a strategy for rapid attenuation and vaccine development (suicidal PCV2 vaccine or sPCV2-Vac) by altering serine and leucine codons of the capsid gene to increase the probability of accumulating stop mutations during viral replication. The sPCV2-Vac candidate developed was successfully rescued by transfection and showed a reduction in fluorescence over serial passages when compared to wild type virus, indicating attenuation of the virus in vitro. Vaccination of pigs with the sPCV2-Vac showed that the binding antibody response in test group was higher than the unvaccinated control in pre-challenged sera. As hypothesized, the sPCV2-Vac virus was cleared in vaccinated pigs within 2 weeks of exposure but elicited strong binding antibody and virus neutralization responses. Vaccinated pigs were protected against heterologous challenge with a PCV2d virus, as vaccinated pigs were protected against viremia and the development of lesions due to challenge. Thus, the described strategy has potential application as a rapid attenuation method for newly emerging viruses, including DNA viruses.

**Introduction** Post-Weaning Multi-Systemic Wasting Syndrome (PMWS) is an important manifestation of porcine circovirus type2 (PCV2) infection in weanling pigs. [1,2] Porcine circovirus associated diseases (PCVAD) continue to cause a huge economic losses to the swine industry [3] each year, even though commercial vaccines are able to reduce the clinical signs of PCV2. Over time, new subtypes of PCV2 have been emerged periodically, and currently PCV2d is the most

predominant one. [4-6]. Although a DNA virus, PCV2 has a high mutation rate, which is close to a single stranded RNA viruses [7, 8]. The frequent evolution of PCV2 despite the availability of standard vaccines indicates that there is room for the improvement of current vaccines.

In this study, the high mutation rates of PCV2 were used to redesign the serine and leucine codons of the capsid gene in a way that the chances of accumulation of stop codons will increase during viral replication to eventually attenuate and eliminate the virus from vaccinated hosts. For example, if the serine codon UCU is changed to UCA in the vaccine virus, a mutation of C to A during vaccine viral replication in the host would result in a sequence change to UAA, a stop codon. Under selection pressure in vitro or the immune system in vivo, the likelihood that rapidly mutating viruses with modified serine and leucine codons will acquire mutations which result in stop codons is high for viruses with high mutation rates. Although this strategy was successfully applied for the RNA viruses [9] it has not been for DNA viruses yet. Further, with the increasing number of newly emerging viruses, necessitates the availability of tools for rapid response vaccine development. The strategy developed in this study is both elegant and highly effective for the rapid attenuation of viruses for application as vaccine candidates.

## Methods and Materials

**Cloning and attenuation of the virus:** An infectious clone of PCV2b subtype 41513 (accession number KR816332) [10] was used as backbone for the vaccine virus. The serine and leucine codons of the ORF2 were redesigned as described above and commercially synthesized (Eurofin genomics). The ORF2 of PCV2b 41513 was replaced with the redesigned ORF2 gene (sPCV2-Vac). sPCV2-Vac was rescued by transfection of PK-15 cells and viral replication in infected cells visualized with an immunofluorescence assay (IFA) [11].

**Immunization of pigs:** Approximately 3-week-old piglets from a PCV2 PCR negative herd were administered treatments as follows: Group I- unvaccinated control (N=9), Group II – one 2.0 ml-i/m dose of an inactivated commercial PCV2 vaccine (N=9), Group III - sPCV2-Vac (N=9), 10<sup>4</sup> TCID<sub>50</sub>, 2ml i/n, 2ml i/m. Serum was collected on day 0,14 and 28 to assesses antibody responses. The animal study was conducted at South Dakota State University, Animal Resource Wing, following the guidelines of the Institutional Animal Care and Use Committee.

**Read-outs:** Serum antibody responses against PCV2 in vaccinated pigs was achieved with a commercial PCV2 ELISA kit (Ingezim Circovirus IgG kit,

# Attenuation of a PCV2B Vaccine Candidate by Directed Suicidal Replication (Continued)

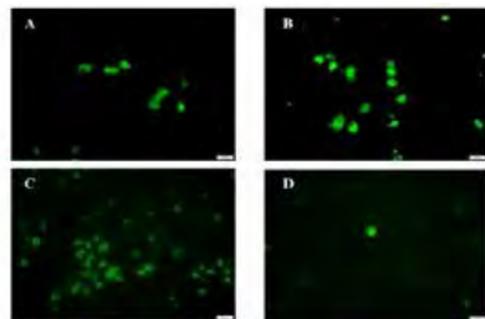
## Results and Discussions

Ingenasa, Madrid, Spain). The titer of neutralizing antibody (NA) against PCV2a, 2b, and 2d were assessed by the fluorescence focus neutralization (FFN) assay essentially as described before [12]. The challenge virus replication was measured by a PCV2d specific qPCR from post challenged sera. The pathological scores were measured by a board-certified veterinarian.

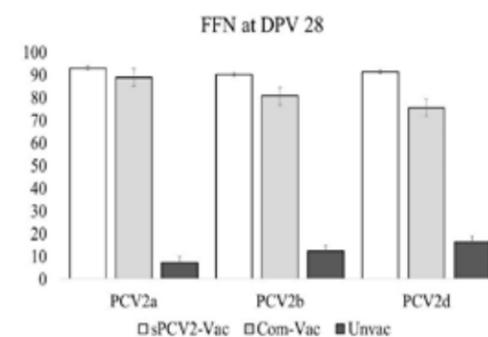
**Rescue of the sPCV2-Vac construct:** The vaccine virus was successfully rescued by transfection of PK-15 cells, at titers comparable to the wild type virus (fig. 1, A and B). However, the titer of sPCV2-Vac decreased over serial passages when detected with an immune fluorescence assay (fig. 1, C and D), indicating possible attenuation in vitro.

**sPCV2-Vac induces heterologous virus neutralization (V/N) responses:** Unlike the commercial vaccine, the test vaccine is not dose optimized or adjuvanted. However, testing of serum from day post vaccine 28 (DPV 28) for homologous and heterologous V/N titers showed that robust responses against all three circulating PCV2 subtypes.

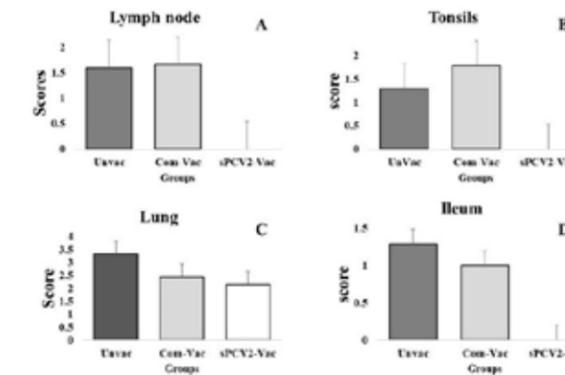
**sPCV2-Vac protects against heterologous PCV2d challenge:** As hypothesized, the sPCV2-Vac was cleared in vaccinated pigs within 2 weeks of vaccination, indicating that the rapid-attenuation strategy was successful. In addition, the PCV2d challenge virus was completely cleared in vaccinated pigs, which remained PCR negative until DPC 21 (data not shown). Pigs vaccinated with the sPCV2 had significantly lower lesions scores in lymph nodes, tonsils, ileum and lungs (fig. 2), compared to unvaccinated control and commercial vaccine.



**Figure 1.** Immune fluorescence assay of transfection and infection with the wild type PCV2b 41513 and sPCV2-Vac construct and rescued virus on PK-15 cells. Viable viruses have stained with a PCV2 specific polyclonal antibody. A. transfected wildtype PCV2b, B. Transfected sPCV2-Vac, C. Passage 5 of wildtype PCV2b, D. Passage 5 of sPCV2-Vac.



**Figure 2.** Virus neutralization assay: Virus neutralizing antibodies measured by a fluorescent focus neutralization assay using days post vaccination 28 pre-challenge sera. X-axis— PCV2 subtypes used in the assay. Y axis mean % reduction in fluorescent foci compared to the untreated virus culture.



**Figure 3.** Microscopic lesions scores of pig tissues resulting from PCV2d challenge. Bar shows the average lesions score of respective tissues. A, B, C, and D represents lesions scores of Lymph node, Tonsils, Lung and Ileum respectively.

## Conclusion

Thus, the strategy described for rapid attenuation of viruses has significant application to animal health, considering the increasing emergence of new viruses and the long lag time with current strategies for producing attenuated vaccine candidates.

## Acknowledgement

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## Conflict of Interest

The authors declare no financial conflict of interest.

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# Co-vaccination and Schedule of Immunization with Attenuated Porcine Reproductive and Respiratory Syndrome (PRRS) Vaccine Could Potentially Affect Efficacy of Subunit Classical Swine Fever Vaccine

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<b>Key Words</b>	Classical Swine Fever (CSF), E2, Vaccine, PRRS, Subunit
<b>Introduction</b>	Commercial pigs have been routinely injected with multiple vaccines that are either administered separately or co-administered at the same time for convenience, and to minimize pig stress. However, viruses, including attenuated and modified live virus (MLV) vaccines, can modulate host immune responses that could potentially impact the efficacy of co-administered vaccines.
<b>Methods</b>	Here we report the effects of pre- and co-administered Chinese highly pathogenic porcine reproductive and respiratory syndrome (PRRS) virus MLV, JXA1-R, on the efficacy of an emulsion-based classical swine fever virus (CSFV) subunit vaccine, KNB-E2. Immune responses to the CSFV and JXA-1R vaccines were evaluated by testing CSFV-specific and PRRSV-specific sera antibodies and then challenged with CSFV at 4 weeks post KNB-E2 vaccination.
<b>Results</b>	Pigs co-administered with JXA1-R vaccine exhibited slightly lower levels of PRRSV-specific antibodies than pigs vaccinated with JXA1-R two weeks before KNB-E2 vaccination. On the other hand, both JXA1-R/KNB-E2 vaccinated pig groups had slightly lower CSFV-specific antibodies than pigs vaccinated with KNB-E2 alone at 3 weeks post KNB-E2 vaccination.
<b>Discussion</b>	These observed differences imply an effect of live MLV vaccination on other vaccines, and that should be considered in multiple swine vaccination schedules. In this study, both groups of JXA-1R/KNB-E2 vaccinated pigs were amply protected from CSF clinical symptoms upon challenge. However, the observed differences in the pre- and co-administered JXA1-R/KNB-E2 vaccinated pig groups compared with single KNB-E2 vaccination pig group indicate an unintended effect of PRRS MLV on the elicited immune response to the CSF KNB-E2 vaccine. The immunological responses affected by

## Acknowledgements

various multiple vaccination combinations in swine would be an interesting aspect for future investigations.

This research is supported by awards from the National Bio and Agro-Defense Facility Transition Fund, the USDA NIFA Hatch-Multistate project 1021491, the USDA ARS Non-Assistance Cooperative Agreements (58-8064-8-011, 58-8064-9-007, 58-3020-9-020, and 59-0208-9-222), and National Pork Board Grant #18-059. Authors declare no financial conflict of interest.

# Cytokine Response in Cells Co-Infected with Type I Interferon Suppression-Negative and NF-κB Activation-Negative PRRS Virus and Bacterial Pathogen

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<b>Key Words</b>	PRRSV, nsp1-beta, Nucleocapsid, Co-Infection, NF-κB, Cytokine.
<b>Abstract</b>	<p>Porcine reproductive and respiratory syndrome virus (PRRSV) suppresses innate immunity, namely, type I interferon responses, and activates NF-κB signaling during infection. The NF-κB activation by PRRSV may predispose to the secondary bacterial infections and increase the clinical severity. Among the PRRSV proteins, non-structure protein 1β (nsp1β) has been identified as the potent interferon antagonist, and leucine at position 135 in the SAP motif is determined as the active residue for nsp1β-mediated IFN suppression. The viral nucleocapsid (N) protein has been found as the effector protein for the NF-κB activation, and the nuclear localization signal (NLS) overlaps the NF-κB activation domain. In the present study, SAP motif-deleted and NLS motif-deleted double mutant virus was generated by reverse genetics. The double mutant PRRSV was characterized in cells and examined for its immunological phenotypes. The results demonstrated that the SAP motif-deleted and NLS motif-deleted double mutant PRRSV attenuated the expression of proinflammatory cytokines in macrophages compared to wild-type PRRSV. While TNF-α and IL-1β were significantly upregulated in cells co-infected with wild-type PRRSV and <i>Streptococcus suis</i> (<i>S. suis</i>), the double mutant PRRSV and <i>S. suis</i> co-infection did not enhance the TNF-α and IL-1β productions. This study suggests that the intervention of nsp1β-mediated IFN suppression and N protein-mediated NF-κB activation relieves the clinical severity that may be caused by co-infection of PRRSV and other swine pathogens. Our findings pave a way to developing a novel vaccine candidate that may reduce a risk of developing clinical severity which may be induced by current vaccines in swine farms.</p>
<b>Introduction</b>	<p>Porcine reproductive and respiratory syndrome virus (PRRSV) suppresses type I interferon (IFNs-α/β) response and also activates NF-κB signaling during infection and predisposes infected hosts to secondary pathogen infection. In swine farms, pigs are commonly co-infected with PRRSV and other pathogens, which will trigger enhanced expression of proinflammatory cytokines. PRRSV predisposes a secondary infection caused by other infectious agents, including <i>Mycoplasma hyopneumoniae</i> (<i>M. hyo</i>) and <i>Streptococcus suis</i> (<i>S. suis</i>), leading to severe porcine respiratory disease complex (PRDC) and increased morbidity and mortality in pigs (1, 2). An <i>in vitro</i> study showed that PRRSV and <i>S. suis</i> co-infection upregulated</p>

IL-1β, IL-6, IL-8, TNF-α, CCL4, IL-10, and INF-β in pulmonary alveolar macrophages (PAMs) (3). Several pieces of evidence indicate that the co-infection of PRRSV and other pathogens would enhance proinflammatory cytokines response and further cause a severe outcome.

PRRSV non-structure protein 1β (nsp1β) has been demonstrated as an IFN antagonist, and a highly conserved SAP (SAF-A/B, Acinus, and PIAS) motif inhibits cytoplasmic translation of host mRNAs, further suppresses type I IFNs (4, 5). Previously, we show that the mutation at position 135 in the SAP motif was attenuated and released a higher concentration of IFN in pigs during infection (4). Furthermore, the nuclear localization signal (NLS) of the PRRSV nucleocapsid (N) protein was identified as the NF-κB activation domain (6). We hypothesize that PRRSV mutant in which both the SAP motif in nsp1β protein and the NLS motif in N protein may exhibit both type I IFN suppression-negative and NF-κB activation-negative phenotypes and may lead to relieving the severity of PRDC causing by co-infection. In the present study, a double-mutant PRRSV was generated by reverse genetics to eliminate both IFN suppression and NF-κB activation functions. The immunological phenotype was examined in cells during co-infection of PRRSV and a bacteria pathogen.

## Materials and Methods

Baby hamster kidney-21 (BHK-21) cells were cultivated in modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (FBS). MARC-145 cells were grown in Dulbecco's modified Eagle's medium containing 10% heat-inactivated FBS. Extralong inverse PCR was conducted using full-length infectious clones to delete L135 in nsp1β and NLS in N from PRRSV. A series of mutant PRRSVs were then rescued by reverse genetics in BHK-21 cells and MARC-145 cells. Mutations were confirmed by sequencing 'passage 4' virus. RT-qPCR was used to determine mRNA expressions for cytokines and chemokines in infection with individual mutant PRRSV at different times post-infection. For co-infection, MARC-145 cells were inoculated with P129 wild type PRRSV or mutant PRRSV. After 48 h of infection, the cells were infected with 1 multiplicity of infection (MOI) of *Streptococcus suis* for four hours. After incubation, supernatants were removed, and penicillin G (5 μg/ml) and gentamicin (100 μg/ml) were added to each well for two hours to kill extracellular bacteria. NF-κB response was examined by luciferase reporter assay, and pro-inflammatory cytokines were determined by RT-qPCR. Statistical analyses were performed using Student t-tests, and statistical significance was expressed as P < 0.05.

## Results

**Generation of L135-deleted and NLS motif-deleted double mutant virus:** Deletion of L135 in the SAP motif of nsp1β and deletion of NLS motif in N were

# Cytokine Response in Cells Co-Infected with Type I Interferon Suppression-Negative and NF- $\kappa$ B Activation-Negative PRRS Virus and Bacterial Pathogen (Continued)

hypothesized to confer the mutant PRRSV to lose the type I IFN suppression and NF- $\kappa$ B activation functions. To determine this possibility, three mutant viruses were generated by the reverse genetics approach using infectious clones. PN- $\Delta$ NLS was first generated to delete NSL from the N gene as described previously (7, 8), and P1 $\beta$ - $\Delta$ 135 was generated by introducing a specific deletion in the SAP motif of nsp1 $\beta$ . (7, 8). Then using PN- $\Delta$ NLS, the double mutant virus was generated by introducing a deletion in the SAP motif. The double mutant was named PD- $\Delta$ 135-NLS. After transfection with wild-type P129 infectious clone, P1 $\beta$ - $\Delta$ 135, PN- $\Delta$ NLS, and PD- $\Delta$ 135-NLS full-length genomic mutant clones in BHK-21 cells, infectious PRRSVs were rescued. The viruses were subsequently amplified in MARC-145 cells. The viral infectivity was determined by CPE and IFA for nsp1 $\beta$  and N protein expressions. The multi-step growth kinetics revealed that P1 $\beta$ - $\Delta$ 135 and PD- $\Delta$ 135-NLS exhibited decreased growth rates compared to that of wild-type P129 PRRSV. These results showed that mutant PRRSVs were successfully generated.

**Cytokine responses by nsp1 $\beta$ -mediated IFN-suppression negative and N-mediated NF- $\kappa$ B-activation negative mutant viruses:** To determine the NF- $\kappa$ B directed proinflammatory cytokine expressions regulated by mutant PRRSV, MARC-145 cells were infected with wild-type P129 PRRSV or PD- $\Delta$ 135-NLS, and RT-qPCR were conducted. The expressions of IL-6, IL-8, and TNF- $\alpha$  were lower at 12 h postinfection in MARC-145 by the double mutant PRRSV. The expression of IL-1 $\beta$  was also significantly lower at 24 h postinfection by double mutant PRRSV compared to wild-type p129 PRRSV. These results indicated that the double deletion of both NLS and SAP motif attenuate NF- $\kappa$ B directed proinflammatory cytokine productions.

**Cytokine responses to co-infection of double mutant PRRSV and *S. suis*:** A previous study showed that co-infection of PRRSV and *S. suis* activated NF- $\kappa$ B and enhanced the expression of proinflammatory cytokines (3). Since the double mutant PRRS virus decreased the expression of NF- $\kappa$ B directed proinflammatory cytokines, this mutant was expected to lose the NF- $\kappa$ B activation during co-infection. To test this hypothesis, MARC-145 cells or macrophages were coinfecting with mutant PRRSVs and *S. suis*, and NF- $\kappa$ B luciferase assay and RT-qPCR were conducted. Co-infection with wild-type PRRSV and *S. suis* significantly activated NF- $\kappa$ B reporter expression compared to that of wild-type PRRSV alone. However, co-infection with mutant PRRSV and *S. suis* showed no significant differences in NF- $\kappa$ B reporter expression compared to mutant PRRSV infection alone. Compared to the co-infection of wild type PRRSV and *S. suis*, expressions of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  were decreased in co-infection with the double mutant virus and *S. suis*. These results indicate that the SAP motif-deleted and NLS motif-deleted double-

mutant PRRSV has attenuated activation of NF- $\kappa$ B and thus reduced cytokine productions during co-infection with other pathogens.

## Discussion

The SAP motif-deleted and NLS motif-deleted double mutant PRRSV was generated in this study. This virus represents type I IFN suppression-negative and NF- $\kappa$ B activation-negative in its phenotype. This double-deletion mutant virus attenuates proinflammatory cytokine productions in cells. During co-infection, wild-type PRRSV can activate NF- $\kappa$ B and induces higher proinflammatory cytokine responses compared to those of single infection, but our double-deletion mutant PRRSV shows noticeable attenuation in the NF- $\kappa$ B activation and cytokines responses. This double mutant PRRSV may lessen the clinical severity and lead to attenuation of the clinical outcome of secondary pathogen infections compared to those of wild-type PRRSV. Our study provides a viable platform for development of a better vaccine candidate to reduce the clinical severity of PRDC.

## Acknowledgements

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# Development and Characterization of Monoclonal Antibodies Against African Swine Fever Virus

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<b>Key Words</b>	ASFV, Monoclonal Antibody, Diagnostic Reagent
<b>Abstract</b>	This study generated a panel of specific monoclonal antibodies (mAbs) against selected immunogenic ASFV proteins, including p10, p14.5, p22, p49, p54, p72, and CD2v. These mAbs were initially screened by immunofluorescent assay using <i>in vitro</i> expression system. The antibody reactivity was confirmed in virus-infected cells. Their application in the detection of ASFV infection was further tested using the methods of Western blotting, immunoprecipitation and ELISA. The availability of these mAbs provides an important tool in aid of ASFV diagnostics and research.
<b>Introduction</b>	African swine fever virus (ASFV) is a large double-stranded DNA virus that belongs to family <i>Asfarviridae</i> , genus <i>Asfivirus</i> (1). The virus is enveloped with two membranes at its inner and outer sides, wrapped around an icosahedral capsid. The viral genome varies in size between 170 and 190 kb, which encodes over 170 proteins. Among all the ASFV proteins that have been analyzed as far, p30 protein is determined to be a highly immunogenic protein, which is capable to stimulate the highest level of antibody response during the virus infection (2). Besides p72 and p54 were also reported to be highly immunogenic (3). In a recent study (4), a total of 47 ASFV proteins were screened in order to identify the immunogenic and protective antigens for vaccine development. Among these proteins, 8 antigens including p10, p14.5, p22, p30, p49, p54, p72, and CD2v, were tested to be able to induce certain levels of antibody responses in immunized pigs. These antigens were selected based on their (known or predicted) properties of being present on the surface of the intracellular mature virion or extracellular viral particles (Table 1). They are potentially important for induction of protective antibody

responses, which could be implicated in vaccine development as well as in diagnostic assays for detection of ASFV infection.

ASFV causes high morbidity and mortality in domestic pigs (5). Currently, the only strategy to control the disease is to quarantine and eliminate the infected animals; therefore, highly sensitive and specific diagnostic reagents are urgently needed for rapid detection and isolation of the ASFV-infected pigs. Monoclonal antibody (mAb) is a key reagent for the detection of viral infection. In our previous study, we generated and characterized specific mAbs against p30 (6). Here, we applied our technology to generate mAbs against additional viral proteins based on their immunogenic nature and functional importance in viral replication as described above (Table 1). This panel of mAbs were further characterized in various assays for future use in ASFV diagnostics and research.

## Methods

ASFV genomic region encoding antigenic domain of each viral protein was amplified by PCR and then cloned into the protein expression vector pET 28a (Novagen) as a fusion protein containing an amino-terminal 6-His tag for facilitating protein purification. Recombinant proteins were expressed in *E. coli* BL21 (DE3) cells and purified by nickel-affinity chromatography using the method described in our previous publications (7). The purified antigens were used for mice immunization, and spleenocytes from immunized mouse were fused with mouse myeloma cells to generate hybridomas as we described previously (8). ASFV specific mAbs from hybridoma cell culture were initially screened by immunofluorescent assay (IFA) using MARC145 cells transfected by a plasmid DNA expressing each individual ASFV protein. The antibody reactivity was confirmed by IFA using Vero cells infected by ASFV strain BA71 V. Specificity of each mAb was further determined by Western blot and immunoprecipitation using the methods as we described previously (6). In addition, indirect ELISA was performed using the previous described method (9).

## Results and Discussions

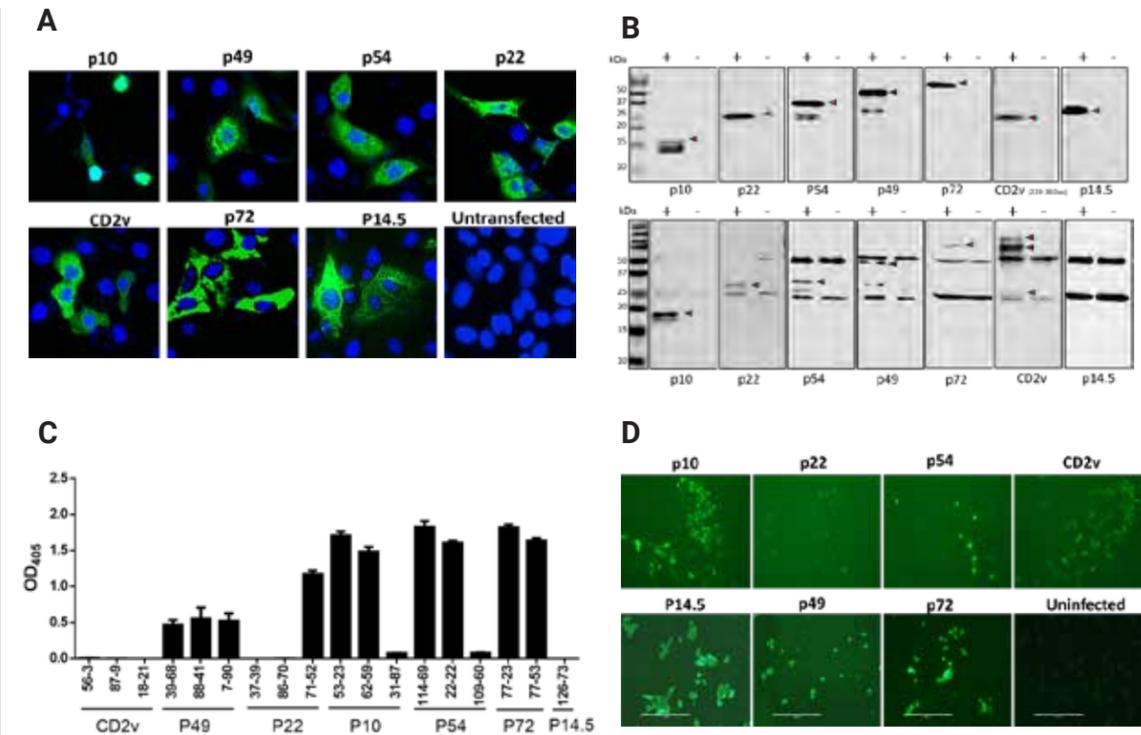
Initial hybridoma screening by IFA using MARC-145 cells that express each individual ASFV protein resulted in a total of 62 mAbs against ASFV p10, p22, CD2v, p49, p14.5, p72, and p54 proteins (Figure 1A). Two to three hybridoma clones from each antigen were expanded for further characterization (Table 1).

# Development and Characterization of Monoclonal Antibodies Against African Swine Fever Virus (Continued)

Antigen	Protein Function	mAb	Assays			
			IFA	WB	IP	ELISA
CD2v	Glycoprotein inserted into virus' external envelope; host cell CD2-like protein, function as viral hemagglutinin to mediate hemadsorption by ASFV-infected cells	#87-9, #56-3, #18-21	+	+	+	-
p54	Structural protein binds to LC8 chain of dynein, involved in virus entry; required for recruitment of envelope precursors to the factory for generating viral particles	#114-69, #22-22, #109-60	+	+	+	+
p10	Structural protein involved in virus adsorption and has DNA binding capacity	#31-87, #53-23, #62-59	+	+	+	+
p49	Structural protein required to form the vertices of the icosahedral capsid	#7-90, #88-41, #39-68	+	+	+	+
p22	Envelope protein	#37-39, #71-52, #86-70	+	+	+	+
p14.5	DNA-binding protein on the surface of intracellular virions; required for movement of virions to plasma membrane	#126-73	+	+	-	-
p72	Major capsid protein, involved in virus entry	#77-23, #77-53	+	+	+	+

**Table 1.** Characterization of ASFV specific monoclonal antibodies\*  
\*Refer to Petrovan et al (2) for the information about mAbs against p30.

Western blot (WB) and immunoprecipitation (IP) were performed using the lysate of transfected 293T cells expressing a specific viral protein. In western blot analysis (Fig. 1B, top panel), all mAbs specifically detected protein bands for p10, p49, p54, CD2v, p14.5, p72, and p22. As expected, these bands were not detected in mock-transfected cells. MAb against p54, p22, p10, p49, CD2v, and p72 also detected corresponding proteins in immunoprecipitated proteins from transfected cells (Fig. 1B, bottom panel). This panel of mAbs were further tested on indirect ELISA using recombinant proteins as the coating antigens. As is shown in Figure 1C, mAbs against p72, p54 (#114-69, #22-22), p10 (#53-23, #62-59), and p22 (#71-52) showed high reactivity with OD405 value above 1.0, while low reactivity was observed for mAbs against p49 (#39-68, #88-41, and #7-90). No reactivity was detected for mAbs against CD2v and p14.5. Optimization of the ELISA conditions for these two antigens are in the process.



**Figure 1.** Characterization of mAbs against ASFV. (A) IFA detection of specific ASFV antigens expressed in transfected MARC145 cells. Fixed cells were stained by the corresponding mAb and FITC-conjugated goat anti-mouse IgG was used as the secondary antibody. Nuclei were counterstained with DAPI (blue). (B) MAb reactivity in Western blot and immunoprecipitation analysis. Lysates from transfected 293T cells expressing a specific viral protein were harvested and utilized for WB and IP. Red arrows point to specific viral proteins detected by WB or IP. (C) MAb reactivity tested on indirect ELISA. Immunolon 2HB plates were coated with a recombinant viral protein and further incubated with a corresponding mAb. HRP-conjugated goat anti-mouse IgG was used as the secondary antibody. (D) IFA detection of ASFV antigens using Vero cells infected with ASFV BA71 V strain (top panel) or Vietnam virus (bottom panel).

Specificity of these mAbs were further confirmed by IFA test using ASFV-infected Vero cells. As shown in Figure 1D, anti-p10, anti-p54, anti-p14.5, anti-p49, and anti-p72 mAbs showed strongly reactivity with the viral proteins, while anti-p22 and anti-CD2v showed weak reactivity. The weak binding activity could be due to the lower expression levels of the specific viral protein in infected cells. Another reason is that original protein structure on the virion may be different from that in the *in vitro* expression system.

# Development and Characterization of Monoclonal Antibodies Against African Swine Fever Virus (Continued)

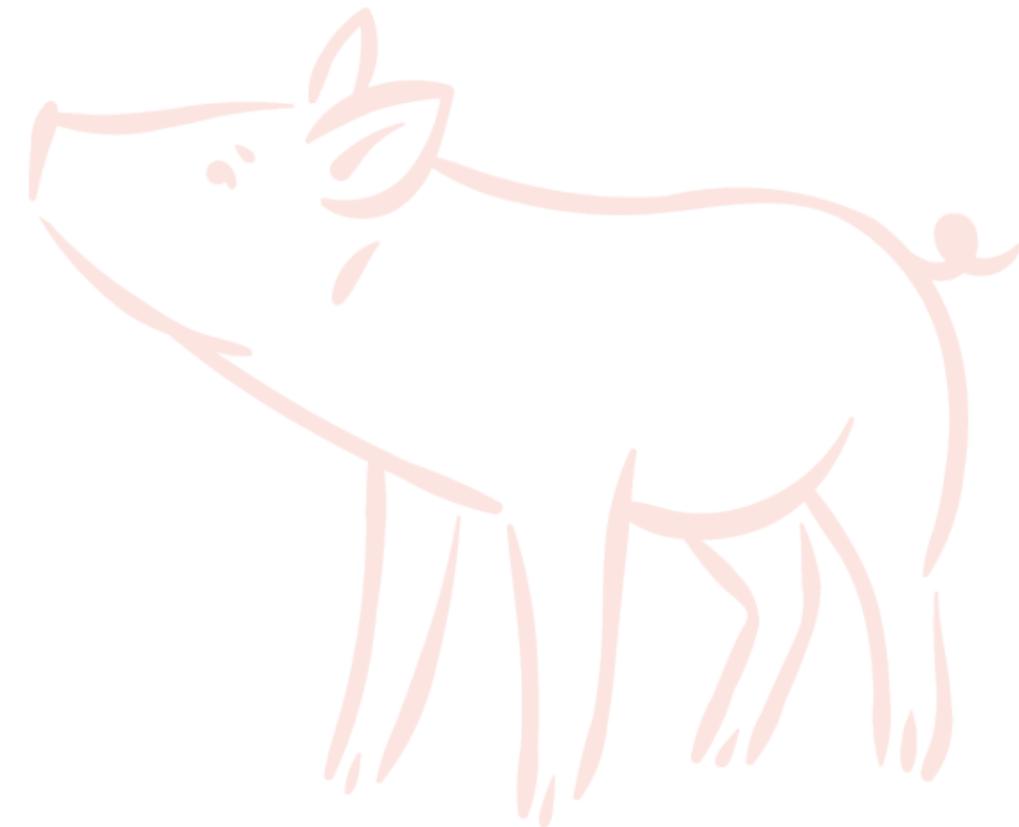
Taken together, we have generated a total of 18 mAbs against 8 ASFV antigens. This panel of mAbs provides a valuable tool for ASFV diagnostics. They are also important reagent for basic mechanism studies toward developing vaccines and antiviral agents.

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# Development of an Infectious Clone for Porcine Deltacoronavirus Strain USA/IL/2014/026

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<b>Key Words</b>	Porcine Deltacoronavirus, Infectious Clone, Enteric, Pathogenesis, Piglets
<b>Abstract</b>	We report the generation of a full-length infectious cDNA clone for porcine deltacoronavirus strain USA/IL/2014/026. The rescued virus, designated icPDCoV, replicates as efficiently as the parental strain in cell culture. Both parental and icPDCoV had comparable growth kinetics and formed similar sizes of plaques in PK1 cells. To evaluate the replication and pathogenesis of the infectious clone, 7-day-old conventional piglets were inoculated orally with either the parental strain or icPDCoV. Although only mild clinical symptoms were observed, we detected similar amounts of viral RNA in rectal swabs and comparable virus-specific IgG titers in the serum of both groups of pigs. Immunohistochemistry analysis and histological examination indicate that both viruses infect the jejunum and ileum epithelial cells and disrupt the integrity of the epithelium. Taken together, these results collectively indicate that the infectious clone PDCoV replicates as efficiently as the parental strain in cell culture and in piglets.
<b>Introduction</b>	<p>Porcine deltacoronavirus (PDCoV) was first detected in 2012 and later identified as an enteric pathogen of swine (1). PDCoV infection causes an age-dependent gastroenteritis with symptoms of acute diarrhea and dehydration seen in neonatal pigs (reviewed in (2)). It primarily spreads in the swine population but can also experimentally infect calves and chickens, possibly due to its broad receptor usage (3–5). This highlights the potential of diverse cross-species transmission.</p> <p>Coronaviruses (CoVs) are infamous for their ability to transmit across species barriers and can cause significant economic impact. CoVs belong to</p>

the family *Coronaviridae* of the order *Nidovirales* and have been genetically grouped into four genera: alpha-, beta-, gamma-, and deltacoronavirus. To date, six porcine CoVs have been identified: four alphacoronaviruses [Transmissible gastroenteritis virus (TGEV), porcine respiratory coronavirus (PRCoV), porcine epidemic diarrhea virus (PEDV), and swine acute diarrhea syndrome coronavirus (SADS-CoV, also known as swine enteric alphacoronavirus)], one betacoronavirus [porcine haemagglutinating encephalomyelitis virus (PHEV)], and one deltacoronavirus [porcine deltacoronavirus (PDCoV)] [reviewed in (6)].

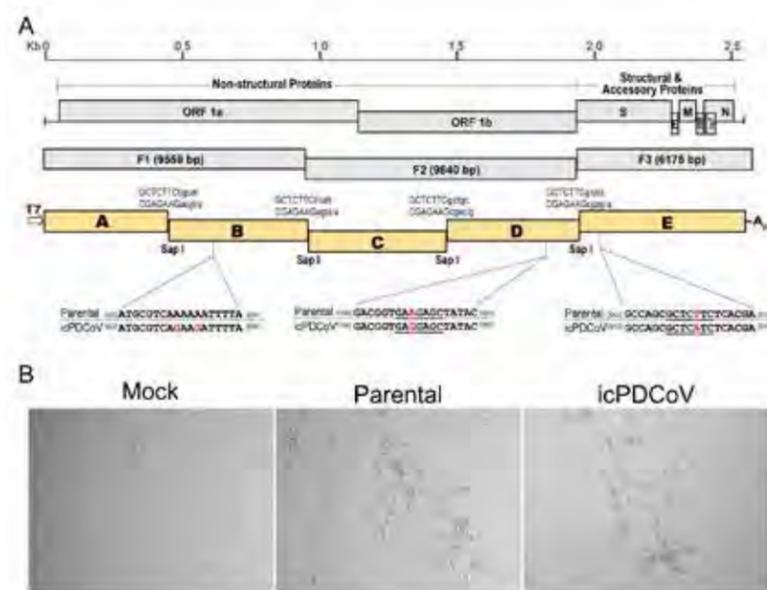
PDCoV has been circulating in the US swine population since 2013 and there is no vaccine commercially available (7, 8). Generating a reverse genetic tool for PDCoV is pivotal for understanding the molecular signatures of its pathogenesis and developing genetically modified live-attenuated vaccines.

## Results

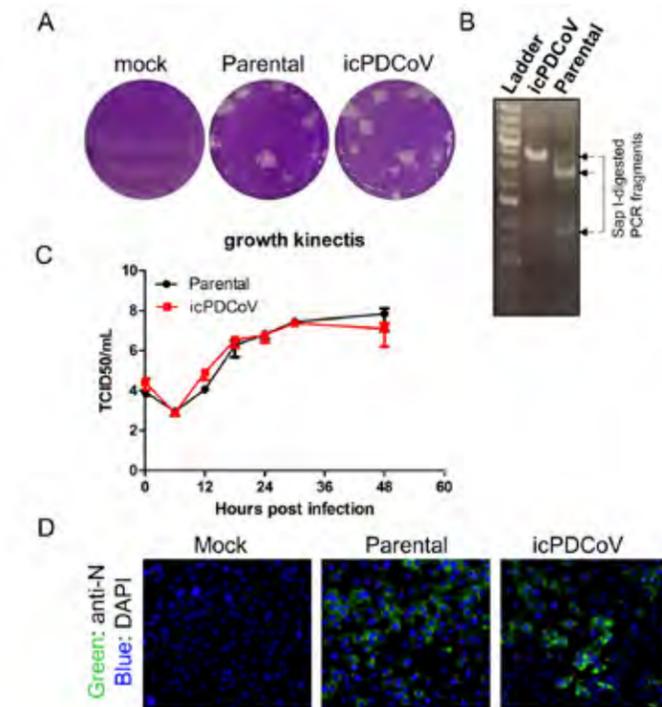
**Designing an infectious PDCoV clone and rescuing recombinant virus.** We used a previously published strategy to develop an infectious clone of a US strain of PDCoV (9). Briefly, three DNA fragments (F1 to F3) composing the complete genomic sequence of PDCoV strain USA/IL/2014/026 (GenBank accession number KP981395) were synthesized. These synthetic DNAs were used as the template for PCR amplification of five segments that were then cloned into plasmid vector backbones, designated PDCoV subclones A to E (Figure 1A). All subclones were joined by a unique Sap I restriction endonuclease cleavage site that allowed for directional assembly into a full-length cDNA without alteration of the viral amino acid sequence. A T7 RNA polymerase promoter sequence and a poly(A) tail (23 As) were added to the 5' and 3' ends of subclones A and E, respectively, allowing for the generation of capped and polyadenylated full-length transcripts using *in vitro* RNA transcription. In subclones D and E, two naturally occurring Sap I sites were removed by introducing silent mutations at positions 18001 and 20143, respectively. In subclone B, two silent mutations (A6545G and A6548G) were introduced to disrupt a stretch of six A nucleotides that might interfere with *in vitro* RNA transcription. In addition, the coding sequence of the N gene was cloned into a pcDNA3.1 vector that carries a T7 promoter sequence. To recover infectious virus from the full-length cDNA clone, the five PDCoV DNA subclones were ligated and *in vitro* transcribed into viral RNA. A linearized N gene plasmid was used to generate N gene transcripts. These RNA transcripts were electroporated into LLC-PK1 cells. 24 hours post electroporation, PDCoV-specific cytopathic effect (CPE) was observed (Figure 1B), indicating an infectious PDCoV was rescued. This recombinant PDCoV was designated icPDCoV.

# Development of an Infectious Clone for Porcine Deltacoronavirus Strain USA/IL/2014/026 (Continued)

**Characterization of recombinant PDCoV.** To characterize the recombinant virus, we first conducted a plaque assay and found that both the parental and icPDCoV formed plaques with a similar size in LLC-PK1 cells (Figure 2A). To verify the sequence of icPDCoV, a genomic region that contains the removal of naturally occurring Sap I site (T20143A) was amplified and the PCR fragment was subjected to Sap I digestion. As expected, the PCR fragment amplified from the parental virus was digested by the Sap I enzyme, while the PCR product derived from icPDCoV was resistant to Sap I-digestion (Figure 2B). We further performed whole-genome sequencing on a plaque-purified icPDCoV clone and confirmed the fidelity of the genomic sequence. To characterize viral replication in cell culture, both the parental and recombinant PDCoVs were used to infect LLC-PK1 cells and the extracellular titer in the cell culture supernatant was determined. As shown in Figure 2C, the growth kinetics of icPDCoV was similar to that of the parental virus and both viruses replicated to peak titers at 48 hours post-infection. In addition, we detected the expression of the nucleocapsid (N) protein in PK1 cells that were infected with either parental or recombinant PDCoV using a specific N protein monoclonal antibody (Figure 2D) (10). These results together demonstrate that the rescued icPDCoV replicates as efficiently as the parental strain in LLC-PK1 cells.



**Figure 1.** Schematic diagram of a PDCoV infectious clone and CPE induced by PDCoV infection. (A) The gene order of PDCoV is 5'-UTR-ORF1a/1b-S-E-M-NS6-NS7-3'-UTR. Two thirds of the genome encodes a non-structural polyprotein while the rest of the genome encodes structural and accessory proteins. Three DNA fragments (F1-F3) composing the entire genome were synthesized and used as template for the amplification of five DNA segments (A-E). Restriction enzyme sites used for directional ligation and the intentional nucleotide substitutions described in this study are depicted. (B) Cytopathic effect induced by parental PDCoV and recombinant icPDCoV infection.

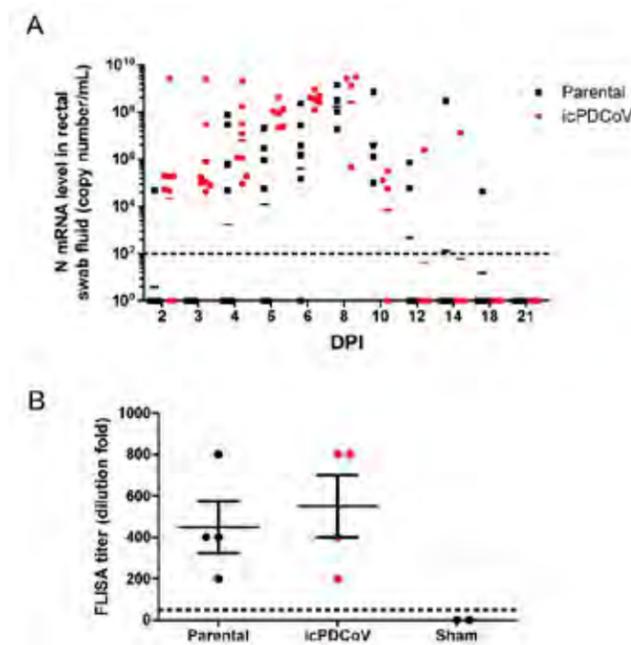


**Figure 2.** Characterization of the rescued PDCoV from infectious clone. (A) Plaque assay results show that both the parental and rescued PDCoV form plaques in PK1 cells with similar size. (B) The PCR product derived from icPDCoV was resistant to Sap I-digestion, while the PCR fragment amplified from the parental virus was digested by the Sap I enzyme. (C) Growth kinetics of the parental strain (Parental) and infectious clone strain (icPDCoV) in PK1 cells. (D) Detection of N protein expression in parental- or icPDCoV-infected PK1 cells using an immunofluorescence assay with a mouse anti-N monoclonal antibody.

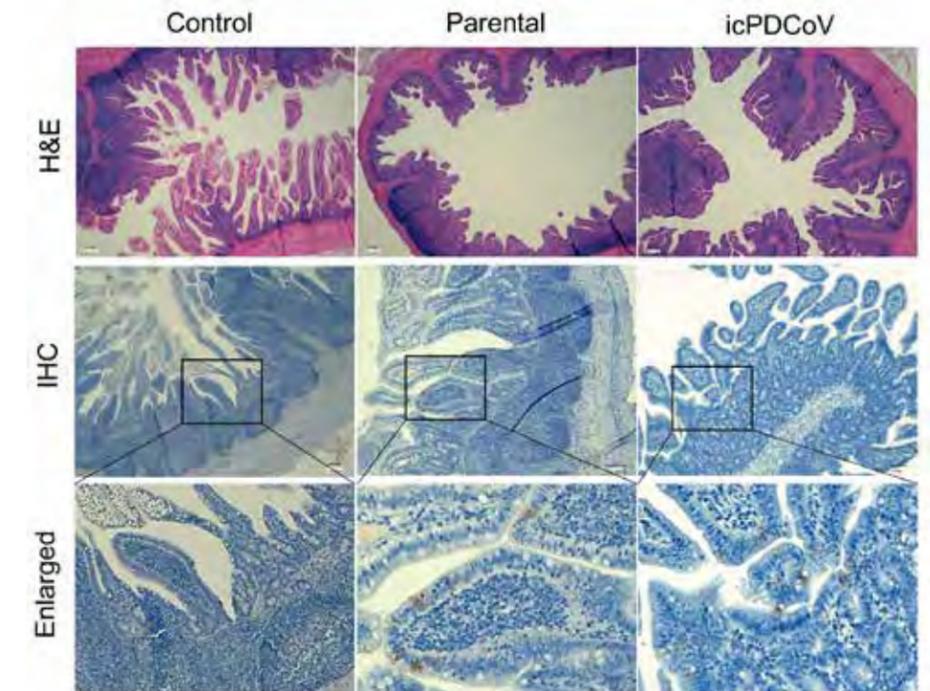
**Evaluating the replication and pathogenesis of the parental strain and recombinant PDCoV in conventional piglets.** Eight 7-day-old piglets were orally inoculated with either parental strain or icPDCoV at a dose of  $10^5$  TCID<sub>50</sub> per animal. Both groups of infected piglets exhibited negligible or mild clinical symptoms, while control animals were healthy and showed no sign of disease through the entire 21 day infection course. Although only mild clinical symptoms were observed, high and comparable levels of viral RNA in the rectal swabs taken from both infected groups were detected (Figure 3A). The peak viral RNA level ( $\sim 10^8$  copy number) of fecal shedding was detected between 6-8 days post infection (dpi). To assess the antibody response to infection, sera were collected at 21 dpi and subject to a fluorescence-linked immunosorbent assay (FLISA) to detect virus-specific IgG titer. As shown

## Development of an Infectious Clone for Porcine Deltacoronavirus Strain USA/IL/2014/026 (Continued)

in Figure 3B, both virus-infected groups had high titers of virus-specific IgG with a similar mean of 400~500-fold dilution. We further performed histopathological examination on the sections of the small intestines from two pigs from each group that were euthanized on both 4 and 7 dpi. At 4 dpi, all examined tissue sections exhibited healthy histology and no lesion was identified. At 7 dpi, lesions consisted of villus atrophy and fusion, contraction of superficial villus lamina with necrotic cells, vacuolar degeneration of villus tip, and necrotic, attenuation and sloughing of enterocytes were observed in the ileum and jejunum sections of both infected groups but not of the control animals. We report that lesion severity was not significantly different in affected pigs between the two virus-infected groups; however, individual variation was observed in both groups. Representative images of ileum sections from control piglet and from one piglet per virus-infected group are shown in Figure 4 (upper panel). To determine the sites of virus replication, immunohistochemistry analysis was performed to detect the nucleocapsid (N) protein. Similar to previous studies (11–13), N protein was mainly detected in epithelial cells, and no apparent difference in the sites of replication between challenge groups was observed (Figure 4, middle and lower panels). These results collectively indicate that the infectious clone PDCoV replicates efficiently as the parental strain in piglets.



**Figure 3.** Evaluating viral replication and virus-specific IgG response to PDCoV infection. 5~7-day-old piglets were orally inoculated with either the parental strain or infectious clone PDCoV at a dose of 10<sup>5</sup> TCID<sub>50</sub> per pig. (A) RT-qPCR was performed on fecal swab samples that were taken at the indicated time points in a 21-day infection course. The bars represent the geometric means of N gene copy numbers of the animal group on a specific day. (B) The titer of PDCoV-specific IgG in serum samples was measured using FLISA (see details in Materials and Methods). Error bars represent mean ± SEM.



**Figure 4.** Histology and immunohistochemistry staining of uninfected control, parental PDCoV, and icPDCoV-infected piglet ileum. (Upper panel) Representative images of H&E stained histological sections of ileum specimens collected at day 7 post-infection (magnification, 4x). (Middle and lower panels) Immunohistochemistry (IHC) staining of ileum specimens collected at day 4 post-infection (middle panel, 4x; lower panel, enlarged) using a mouse anti-PDCoV-N antibody.

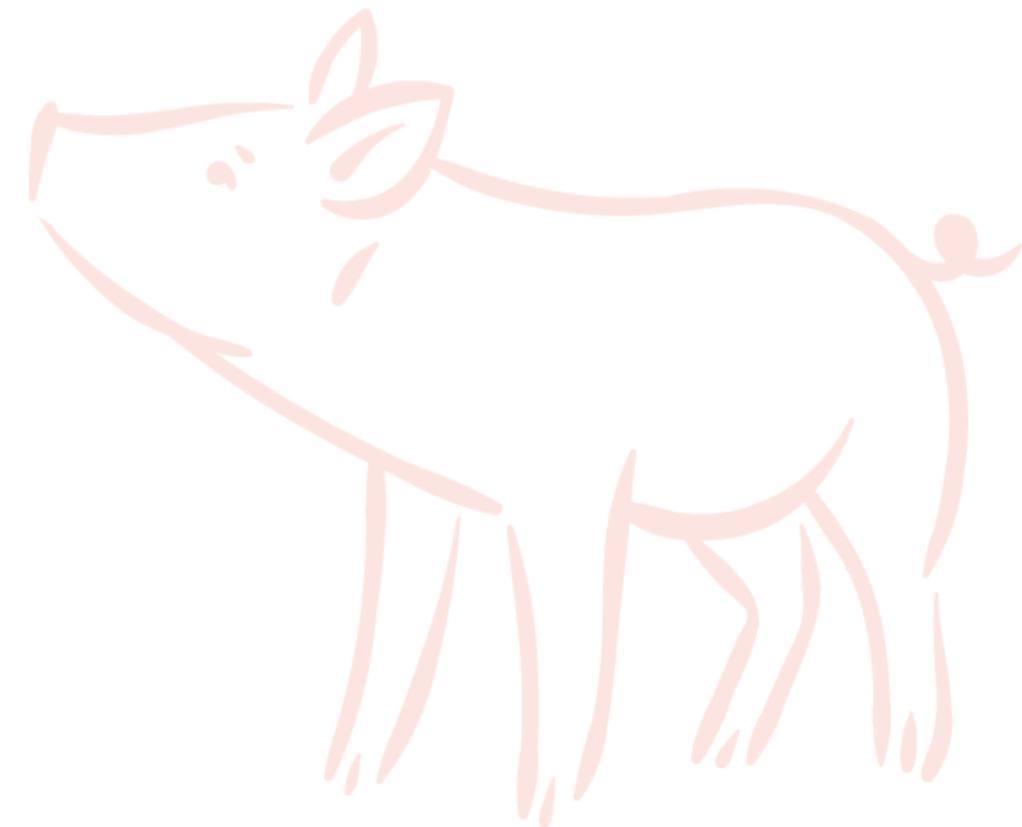
### Conclusion

In this study, we describe the construction of a full-length cDNA infectious clone for PDCoV strain USA/IL/2014/026. With this clone, a recombinant PDCoV was rescued and assessed to have similar replication in cell culture and animals as the parental strain. This infectious clone will be useful tool for understanding the function of viral components of PDCoV and developing genetically modified live-attenuated vaccines.

# Development of an Infectious Clone for Porcine Deltacoronavirus Strain USA/IL/2014/026 (Continued)

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# Diagnostic Performance vs Surveillance Performance – The Case of PRRSV Oral Fluid ELISA

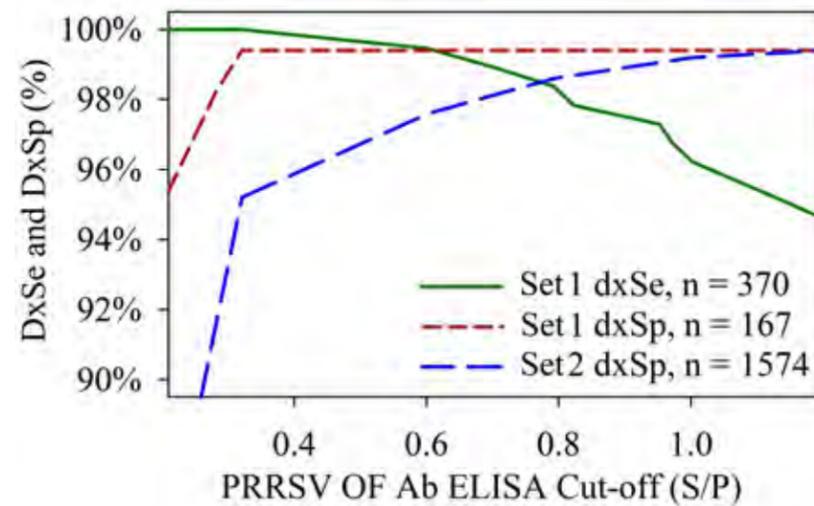
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<b>Key Words</b>	Diagnostic performance, ELISA, Oral fluids, PRRSV, Surveillance performance
<b>Abstract</b>	<p>The PRRS OF Ab ELISA Test has demonstrated a good diagnostic performance, with balance between diagnostic sensitivity (dxSe) and specificity (dxSp). However, rather than a balanced diagnostic performance, a higher diagnostic specificity and, thus the absence of false positives, is the major consideration for routine surveillance. Herein, we evaluated the use of an alternative cut-off S/P <math>\geq 1.0</math> instead of the manufacturer cut-off S/P <math>\geq 0.4</math> and it's potential to provide for both, diagnostic performance and surveillance performance.</p> <p>Two set of samples were used in this study, Set 1 included 596 oral fluids of known positive and negative PRRSV status and Set 2 included 1574 oral fluids from PRRSV-negative sites. Using the alternative cut-off S/P <math>\geq 1.0</math> on Set 1 resulted in a dxSe - dxSp of 96.2% - 99.4%. In set 2 the dxSp was improved from 95.2% to 99.2% and the number of false positives reduced from 76 to 12. Interestingly, from 76 samples classified as false positives by the manufacturer cut-off (S/P <math>\geq 0.4</math>), 46 were identified as "Extreme S/P outliers" and most of them associated with specific pig age (9, 24, 25 week) or gestation.</p> <p>The manufacturer cut-off offers a good diagnostic performance, but using the alternative cut-off S/P <math>\geq 1.0</math> to interpret the test results provides also for an enhanced surveillance performance by reducing the occurrence of false positives.</p>

<b>Introduction</b>	<p>Oral fluids are a diagnostic specimen easy to collect that provide for the sufficient and accurate epidemiological data necessary for surveillance and control of PRRSV(1, 2). For this reason, this specimen has been adapted to the main assays routinely used in veterinary diagnostic laboratories (VDL), i.e., RT-PCR and ELISA. PRRSV oral fluids RT-PCR detects PRRSV-RNA during the short viremia period and PRRSV oral fluids ELISA detects the specific antibody response against the virus for at least 6 months post-infection(3). One of the best assays to test PRRSV-antibody is the commercial PRRS OF Ab ELISA Test (IDEXX Laboratories, Inc., Westbrook, ME). This test has demonstrated an excellent diagnostic performance(4) and the interpretation of the ELISA results using the manufacturer cut-off (S/P <math>\geq 0.4</math>) offers a balance between diagnostic sensitivity (dxSe) and specificity (dxSp). However, rather than a balance, the major consideration for routine surveillance is an almost perfect diagnostic specificity because false positive results disrupt the work flow in the farms.</p> <p>Herein, we evaluated the effect of using an alternative cut-off on the PRRS OF Ab ELISA test diagnostic performance and surveillance performance using oral fluids of known and unknown PRRSV status.</p> <p>Two sets of oral fluids (OFs) were used: <b>Set 1:</b> 596 experimental OFs of known PRRSV positive and negative status. <b>Set 2:</b> 1574 field OFs of known PRRSV-negative status.</p> <p>Set 1 was collected from 12 experimental vaccinated pigs (PRRSV MLV) at -7 to 42 days post vaccination(4). Set 2 consisted of samples submitted for routine testing at the Iowa State University VDL from known PRRSV-negative status farms. All samples were tested on the PRRS OF Ab ELISA (IDEXX Laboratories Inc.) and the data analyzed using non-parametrical statistical procedures:</p> <ul style="list-style-type: none"><li>• The test diagnostic performance (dxSe, dxSp) was analyzed by receiver operating characteristic curve (ROC) (Set 1);</li><li>• The effect of an alternative cut-off on proportion of samples classified as positives analyzed using Cochran's Q (Sets 1, 2);</li><li>• The relationship between ELISA results and pig age was described using linear regression and Tukey's box plot (Set 2);</li></ul>
<b>Materials and Methods</b>	

# Diagnostic Performance vs Surveillance Performance – The Case of PRRSV Oral Fluid ELISA (Continued)

## Results

As shown in Figure 1, using the manufacturer cut-off (S/P ≥ 0.4), the dxSp estimated for Set 1 and Set 2 results were 99.4% and 95.2%, respectively. The alternative cut-off S/P ≥ 1.0 did not improve Set 1 dxSp because of the limited number of negative samples (n = 167), but significantly improved the Set 2 dxSp to 99.2% reducing the rate of false positives from 4.8% to 0.8% (Cochran Q test, p > 0.05) (Table 1).



**Figure 1.** PRRS OF Ab ELISA diagnostic performance by cut-off. Set 1 corresponds to experimental oral fluids of known PRRSV positive (n = 370) and negative (n = 167) status and Set 2 corresponds to field oral fluids of known PRRSV-negative (n = 1574) status.

Cut-off (S/P ≥)	Set 2 <sup>1</sup> (n = 1574 OF)		Set 2 (n = 1528 OF) <sup>2</sup>	
	False Pos	dxSp (%)	False Pos	dxSp (%)
0.4 <sup>3</sup>	76 <sup>a</sup>	95.2	30 <sup>a</sup>	98.1
1.0	12 <sup>b</sup>	99.2	0 <sup>b</sup>	100
True	0 <sup>b</sup>	100	0 <sup>b</sup>	100

**Table 1.** Effect of “extreme S/P outliers” on PRRS OF Ab ELISA diagnostic specificity (dxSp)

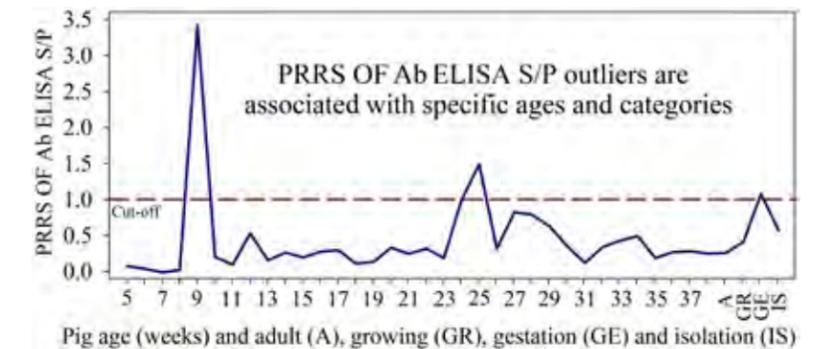
<sup>1</sup> Set 2 consisted on 1574 field known PRRSV negative oral fluids samples

<sup>2</sup> 46 extreme S/P outliers (Tukey’s box plot) not included.

<sup>3</sup> Cut-off recommended by the assay manufacturer

<sup>a b</sup> Different letters indicate statistical differences in the proportion of positives within the column (Cochran’s Q, p < 0.05).

Figure 2 shows the 99th percentile S/P response of Set 2 by pig age or production category. 46 “Extreme S/P outliers” were identified and the majority (~70%) were associated with few specific categories, i.e., 9, 24, 25 weeks of age and gestation (Linear regression, p < 0.05, Tukey’s box plot).



**Figure 2.** Set 2 PRRS OF Ab ELISA results (99th percentile) by age or production category. Set 2 corresponds to oral fluids (n = 1574) from PRRSV-negative sites.

## Conclusion and Discussion

Using the alternative cut-off S/P ≥ 1.0 to interpret PRRS OF Ab ELISA Test results showed a similar dxSp for both experimental and field samples. This test adaptation minimally affected the test dxSe (Figure 1) estimated on experimental samples but significantly reduced the occurrence of false positives in a -84% (from 76 to 12) in field samples (Table 1). These results support the use of cut-off S/P ≥ 1.0 for improved test surveillance performance when testing samples from presumed negative or low prevalence populations(5). Notably, extreme S/P outliers were associated with specific ages or in gestation (Figure 2) suggesting an undetermined common cause for unspecific ELISA results. After excluding the extreme outliers from Set 2 data, the cut-off S/P ≥ 1.0 classified all remaining weak S/P responses as negatives and showed a dxSp of 100%. That is, no false positives were detected. Work in progress is expected to identify the cause(s) of this non-specific responses observed at specific pig ages.

## Acknowledgements

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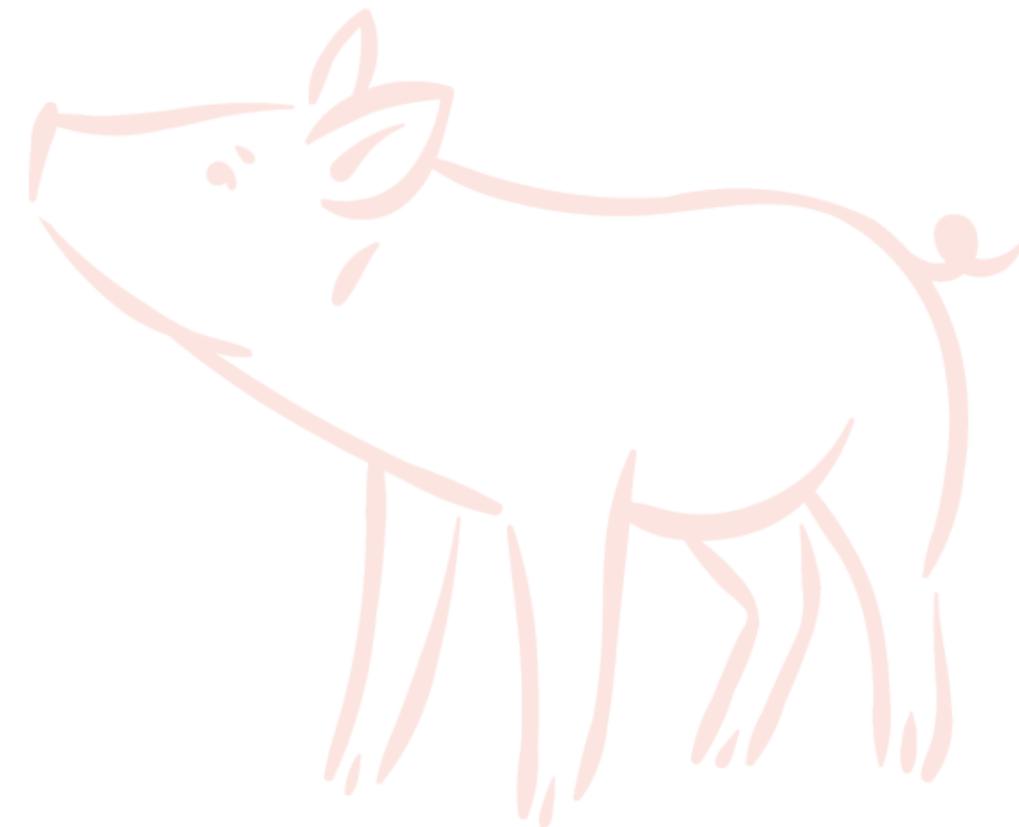
## Diagnostic Performance vs Surveillance Performance – The Case of PRRSV Oral Fluid ELISA (Continued)

### Conflict of Interest

Authors declare no conflicts of interest (COI). JZ has consulted with IDEXX Laboratories, Inc. independent of this research. Consulting has been reviewed and approved by ISU in accordance with its COI policies.

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# Effects of Immunodominance on Porcine Circovirus Type 2 (PCV2) Vaccine Design

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<b>Key Words</b>	Vaccine, Porcine Circovirus, PCV2, Decoy Epitope, Antibody, Virus Neutralization, Mutation
<b>Abstract</b>	Porcine circovirus type 2 (PCV2) is an economically important swine virus which continues to evolve into new strains despite the availability of commercial vaccines which prevent clinical signs. At least 4 immunodominant but non-protective, linear B cell epitopes have been previously identified in the PCV2 capsid antigen and a majority of vaccine derived antibody responses were found to map to the non-protective epitopes. To determine if mutation of the identified epitopes would improve the quality of the neutralizing antibody responses to PCV2 vaccination, two of the identified decoy epitopes were mutated in the backbone of a PCV2 infectious clone. As hypothesized, vaccination of piglets with the re-designed PCV2 modified live vaccine resulted in a broadening of the virus neutralization responses and an overall reduction in binding antibody responses. The use of surface plasmon resonance to determine whether the mutations abrogated the immunodominance hierarchy showed that serum from the pigs administered the experimental vaccine did not recognize the selected immunodominant epitopes while serum from the pigs administered the wildtype virus strong responses to the decoy epitopes. Thus, as hypothesized, rational epitope-modification resulted in improved virus neutralization responses due to vaccination. The results from this study have important implications for improving PCV2 vaccine efficacy.
<b>Introduction</b>	PCV2 is a very small (17 nm in diameter) icosahedral, non-enveloped, virus having single-stranded DNA genome [1]. It was first identified as a cause for post-weaning multi-systemic wasting syndrome (PMWS) in a swine herd [2]. It is also responsible for a variety type of diseases, collectively called porcine circovirus associate disease (PCVAD); and also causes a huge economical losses at swine industry [3]. Current PCV2 vaccines were introduced around

2006 and are based on PCV2a, the first-discovered subtype. Although, commercial vaccines are able to effectively reduce the clinical signs of PCV2, new PCV2 subtypes including PCV2b and PCV2d have emerged and replaced PCV2a as the predominant subtype. So, it's highly probable that the vaccine selection pressure might influence PCV2 viral evolution.[4-6]. Immunodominance confounds vaccine design stimulating the preferential recognition of selected epitopes in antigen, which many times are not protective [7, 8] In natural PCV2 infections strong binding antibody responses are detected at day 7 of infection but neutralizing antibody responses are delayed [9-11]. Since the PCV2 capsid antigen is both necessary and sufficient for protection against PCV2, in a previous study, we hypothesized that early antibody responses to in PCV2 infected pigs will map to the immunodominant but non-protective epitopes of the PCV2 capsid protein [12]. Using sequentially collected sera and pep-scan assays we identified three new putative decoy epitopes and confirmed the presence of a previously described immunodominant decoy epitope [10, 12, 13]. In this study, we hypothesize that mutation of selected non-protective immunodominant epitopes alter the natural pattern of immunodominance to result in a broadening of the neutralization response and have important implications for rational vaccine design.

## Methods

**Cloning and rescue to the re-designed PCV2 vaccine virus (rPCV2-MLV):** An infectious clone of PCV2b strain 41513 (accession number KR816332) [14] was used as backbone for the vaccine virus. Two of the previously identified immunodominant decoy epitopes were mutated by Q5 site directed mutagenesis kit as follows: Epitope A124 ILDDNFVTKATALTYDPY 141 was modified to 124 ILDDNFVNKSTALTYDPY 141 and epitope B 166 VLDSTIDYFQPNNKR 180 was modified to 166 VLDSTIDYFNPNNR 180. The rPCV2-MLV was rescued by transfection of PK-15 cells and viral replication in infected cells visualized with an immunofluorescence assay (IFA) [15].

**Immunization of pigs:** Approximately 3-week-old piglets from a PCV2 PCR negative herd were administered treatments as follows: Group I- unvaccinated control (N=9), Group II – one 2.0 ml-i/m dose Merial (N=9), Group III -Expt PCV2-MLV- I (N=9) ,10<sup>4</sup> TCID<sub>50</sub> , 2ml i/n, 2ml i/m. Serum was collected on day 0,14 and 28 to assesses antibody responses.

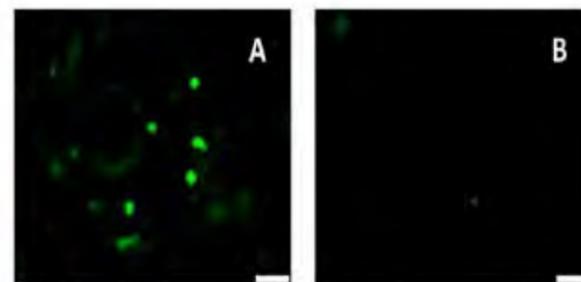
**PCV2 serology:** The measurement of binding IgG responses to PCV2 in vaccinated pigs was achieved with a commercial PCV2 ELISA kit (Ingezim Circovirus IgG kit, Ingenasa, Madrid, Spain), at the Iowa State University

## Effects of Immunodominance on Porcine Circovirus Type 2 (PCV2) Vaccine Design (Continued)

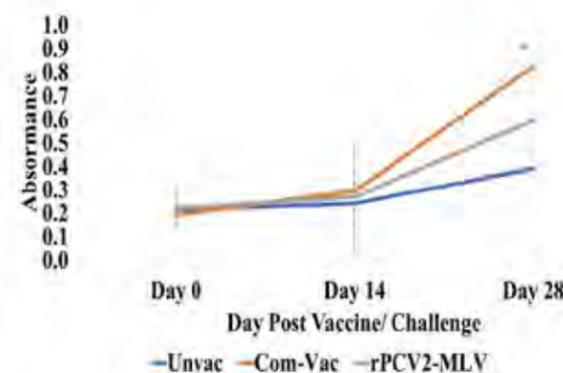
Veterinary Diagnostic Laboratory. The titer of neutralizing antibody (NA) against PCV2a, 2b, and 2d were assessed by the fluorescence focus neutralization (FFN) assay essentially as described before [16]. The abrogation of antibody responses to the selected epitopes in vaccinated pigs was assessed by surface plasmon resonance (Reichert 4SPR, Reichert Technologies, USA), using biotinylated, peptides encoding the wildtype peptide sequences of epitopes A and B and pooled sera from pigs infected with PCV2b wildtype virus, or the DPV 28 serum from the three treatment groups. The reactivity between the IgGs and peptides was measured as the response in  $\mu$  response units ( $\mu$ RU).

### Results and Discussions

**Rescue of rPCV2-MLV:** Refocusing of immune responses to protective epitopes has been previously achieved by alteration to charge, glycosylation patterns or deleting amino acid residues [17-19]. In this study, we elected to replace residues with other amino acids with a low penalty score for mutations as PCV2 does not tolerate large insertions or deletions. As expected, the re-designed vaccine virus was successfully rescued by transfection, as the IFA showed that the MLV virus was infective after passaging three times (Fig 1).

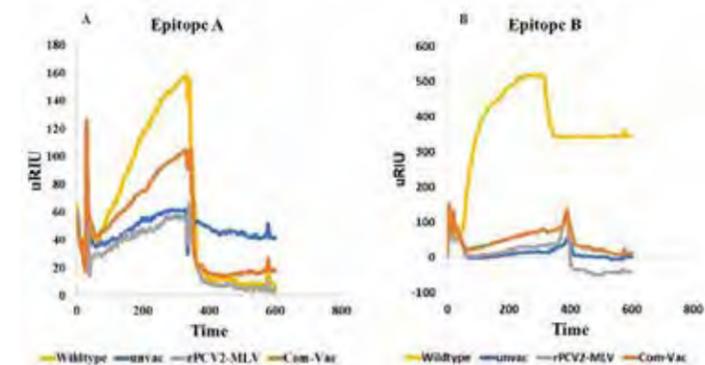


**Figure 1:** Rescue of rPCV2-MLV. A. PK-15 cells transfected with the MLV construct and stained with a PCV2 specific polyclonal antibody. B. Untransfected PK-15 cells stained with the PCV2. Apple green fluorescence is indicative of a specific signal.



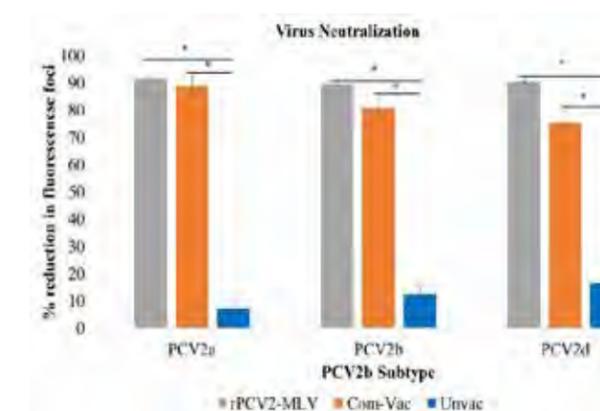
**Figure 2:** Anti-capsid IgG ELISA from study serum. The vertical red line indicates the time of Challenge. The X axis represents the days of vaccination and infection whereas Y axis shows the OD value as a measure of antibody. The blue, orange and gray line represents the unvaccinated, commercial and MLV as test vaccine respectively. The symbols mean statistically significant at  $p < 0.01$ .

**Immunodominant binding antibody responses are diminished in vaccinated pigs:** While detectable antibody responses were noted in rPCV2-vaccinated pigs, and direct comparison is avoided due to differences in the formulation and adjuvants used, as expected, the commercial vaccine induced a stronger binding but not neutralizing antibody response than the rPCV2-MLV.



**Figure 3:** Loss of immunodominant effects due to mutation of epitopes A and B as qualitatively assessed by surface plasmon resonance. A. Responses to a peptide encoding the wildtype epitope A, B.

**Mutation results in loss of immunodominance:** Antibody responses to the mutated epitopes were not detected in rPCV2-MLV vaccinated pigs by SPR whereas, serum from pigs infected with wild type PCV2 showed a strong interaction with peptides encoding wildtype epitope sequences. Pigs administered the commercial vaccine showed responses to epitope A but not B and the responses were of a lesser magnitude than the pigs infected with the wildtype virus. It has been previously suggested that strong responses to epitope B are induced by the monomeric form of the capsid protein subunits but not the assembled virus like particle [10].



**Figure 4:** Virus neutralization assay: Virus neutralizing antibodies measured by a fluorescent focus neutralization assay using days post vaccination 28 pre-challenge sera. X-axis– PCV2 subtypes used in the assay. Y axis mean % reduction in fluorescent foci compared to the untreated virus culture.

## Effects of Immunodominance on Porcine Circovirus Type 2 (PCV2) Vaccine Design (Continued)

<b>Conclusion</b>	<p><b>rPCV2-MLV broadens virus neutralization responses:</b> Sera collected at DPV 28 from test groups showed that test vaccine showed over 90% reduction of the fluorescence foci for the heterologous and currently predominant PCV2d subtype, which was significantly different from the commercial vaccine group despite the fact that the rPCV2-MLV is currently not dose optimized or adjuvanted. Neutralization responses against the homologous PCV2b and heterologous PCV2a subtypes were equally effective.</p> <p>Overall, mutation of immunodominant epitopes altered the immunodominance hierarchy of the host antibody response to PCV2 vaccination, a corresponding improvement in virus neutralization activity and a clear abrogation of responses to the mutated epitopes. The approaches and findings described have positive implications for the improvement of current PCV2 vaccines and can potentially be applied to other pathogens where immunodominance impairs the stimulation of adequate vaccine mediate immunity.</p>
<b>Acknowledgements</b>	<p>We thank Dr. Michele Mucciante and Ms. Amanda Zubke from SDSU for help with the animal experimentation.</p>
<b>Conflict of Interest</b>	<p>The authors declare no financial conflict of interest. The submitted work is protected by a provisional patent application, in compliance with the regulations of North Dakota State University.</p>
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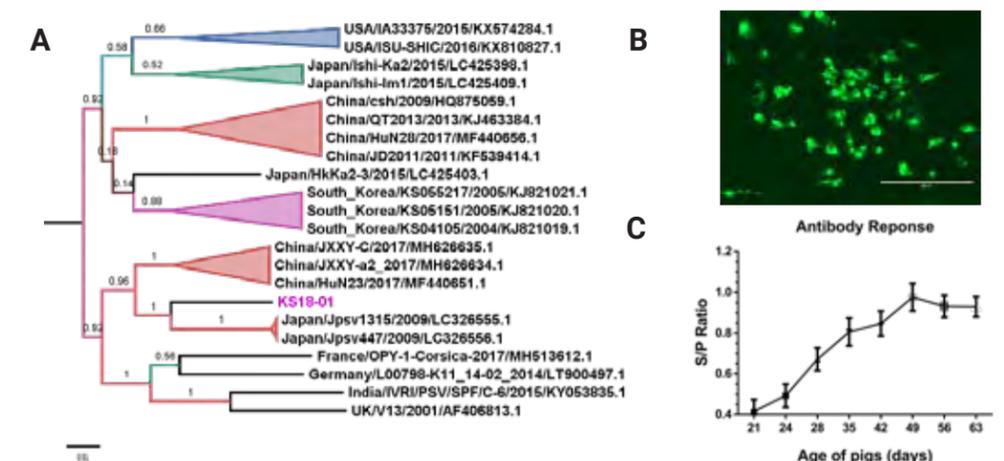
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# An Emerging Porcine Sapelovirus in the United States: Genetic Characterization and Diagnostic Tool Development

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<b>Key Words</b>	Porcine Sapelovirus, Emerging Swine Virus, PSV Monoclonal Antibodies, PSV ELISA
<b>Abstract</b>	<p>An emerging porcine sapelovirus was isolated in a diagnostic specimen from a US swine farm, designated as PSV KS18-01. Full-length genome sequence was obtained through next-generation sequencing. Phylogenetic analysis showed that the virus is more closely related to two Japanese strains but is distantly related to two known US strains. PSV specific diagnostic tools were developed, including the monoclonal antibodies against VP1 and VP2, and a VP1-VP2 antigen-based indirect ELISA. Using this assay, the dynamic response of PSV antibody was investigated in a group of post-weaned pigs that naturally exposed with PSV. The availability of the PSV isolate (KS18-01) and the specific diagnostic reagents and assays provide important tools for PSV control and prevention.</p> <p>Porcine Sapelovirus (PSV), previously named as porcine enterovirus 8, belongs to the genus <i>Sapelovirus</i> in the family <i>Picornaviridae</i> (1). PSV is a non-enveloped, positive-sense single-stranded RNA virus. It has a genome of approximately 7.5 kb, with a typical genomic organization of 5' untranslated region -Leader-polyprotein (P1-P2-P3)-3'UTR. The polyprotein is synthesized and proteolytically cleaved into seven non-structural proteins (2A, 2B, 2C, 3A, 3B, 3C, 3D) and four structural proteins (VP4, VP2, VP3, VP1) (2). The PSV capsid is icosahedral arrangement with VP1, VP2, and VP3 on the surface, while VP4 embedded in the internal side of the virion. Host antibodies are mainly directed toward the viral capsid proteins, in which VP1 was reported having the ability to stimulate strong antibody response during the infection (2).</p> <p>PSV infection is commonly asymptomatic, but clinical disease of respiratory failure, diarrhea, reproductive disorder, and polioencephalomyelitis have been reported in swine farms from many countries (3-5). Neurotropic strains have</p>

been reported from China, Indian, the United Kingdom, and the United States (US). However, little is known about the disease prevalence, morbidity and mortality caused by PSV infection. PSV isolates and diagnostic tools are need for the studies of PSV pathogenesis and epidemiology.

In this study, we isolated an emerging PSV in a fecal sample from the US swine farm. The virus isolation was performed on ST cells using the method as described previously (6). The new isolate was designated as PSV KS18-01. The full-length genome sequence was obtained through metagenomic sequencing (7). The genome size of KS18-01 was 7,453 bp, encoding a polyprotein of 2,316 amino acids (aa). Compared to other PSV strains, KS18-01 genome has 79.88-87.91% nucleotide (nt) identity. The 5'-untranslated region (5'-UTR) is the most conserved region of the genome, while the most variable region is the VP1 gene with 72.13-88.8% nt identity (78.2-97.5% aa identity) to other strains. Phylogenetic tree was constructed with all available PSV sequences in GenBank using Maximum-likelihood method (MEGA 7). KS18-01 were grouped together with two Japanese strains, Jpsv1315 and Jpsv447, but is distantly related to two known US strains with 85.5% nucleotide (nt) identity to ISU-SHIC strain, and 85.7% nt identity to USA/IA33375/2015 strain (Fig. 1A). This suggests that KS18-01 emerged as a novel strain in the US.



**Figure 1.** Phylogenetic analysis and diagnostic detection of emerging porcine sapelovirus, KS18-01. (A) Phylogenetic tree constructed with PSV full-length genome sequences. Phylogenetic analysis was performed by the maximum likelihood method. The numbers on branches represent bootstrap values. (B) IFA detection of PSV infection. ST cells were infected by KS18-01 virus. Fixed cells were stained by anti-VP1 mAb, and FITC-conjugated goat anti-mouse IgG was used as secondary antibody. (C) Investigation of dynamic antibody response against PSV infection using indirect ELISA. The serum samples were collected weekly up to 7 weeks post-weaning. Results were shown as mean values for each time-point (n=10).

## An Emerging Porcine Sapelovirus in the United States: Genetic Characterization and Diagnostic Tool Development (Continued)

Next, we developed specific diagnostic reagents and assays for detection of PSV infection. Initially, recombinant proteins of VP1, VP2, and VP1-VP2 were generated by cloning and expression of corresponding genes of KS18-01 isolate using the methods described previously (8). The recombinant VP1-VP2 protein was expressed as a linked protein with GGGGS linker between VP1-VP2. Individual VP1 or VP2 antigen was used to immunize BALB/c mice for monoclonal antibody (mAb) production using the methods as we described previously (9). A total of nine mAbs were generated for VP1 and VP2. This panel of mAbs were tested in IFA using the ST cells infected with KS18-01 virus. All mAbs showed strong reactivity with the virus (Fig. 1B). Specificity of these mAbs were further confirmed in Western blot analysis using the lysate of infected cells. VP1 was specifically detected as a 28.3 kDa protein band, while VP2 was specifically detected as 26.1 kDa protein band.

Subsequently, we developed indirect ELISA for serological detection of pig antibody response against PSV infection. When comparing VP1, VP2 and VP1-VP2 as the coating antigen for ELISA, the linked protein VP1-VP2 showed the highest optical density (OD) reading. Thus, VP1-VP2 protein was used for ELISA validation. A total of 604 serum samples (503 positives and 101 negatives) from experimental animals were used, and their infection status was verified by IFA using KS18-01 infected ST cells. Receiver Operating Characteristic (ROC) analysis (MedCalc) determined the optimal cutoff of 0.6 with maximal diagnostic sensitivity of 99.2% and diagnostic specificity of 97.0%. Using a single lot of internal control serum, PSV ELISA exhibits a within-plate CV of 5.13%, within-run CV of 5.96%, and between-runs CV of 7.73%, indicating this assay is highly repeatable.

The PSV ELISA was further applied to investigate the kinetics of host antibody response in a group of commercial piglets after natural exposure of PSV infection. A total of ten post-weaning piglets were monitored from three-nine weeks old. Blood samples were collected weekly. Results showed that during the first week post-weaning (three-week-old), antibody response was very weak; after that, an increased pattern of antibody response was observed with the antibody level peaked at 28 days post-weaning (49 days of age) (Fig. 1C). This result correlates with the amount of viral RNA detected in fecal samples.

Clinical signs were monitored daily for this group of pigs. One of the pigs showed neurological signs of circling and abnormal gait for 2 days during the six weeks post-weaning. Rest of the pigs did not exhibit any clinical signs. It is unclear whether the neurological signs were directly related to PSV infection, and whether immune response had protected rest of the pigs from the disease. PSV has been reported to be associated with multiple diseases, including

diarrhea, reproductive failure, neurological disorder, but most of the pigs are commonly asymptomatic (10). The pathogenic outcomes resulted from the infection of different PSV strains are unknown. So far, only one Korean study fulfilled the Koch's postulates of PSV caused enteritis and diarrhea in piglets (11). Additional pathogenesis studies are required for in depth characterization of different PSV strains, especially the newly emerging strains. The virus isolate, diagnostic reagents and assays generated in this study will be important tools in aid of future pathogenesis studies as well as development of vaccines and therapeutics against PSV infection.

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# Evaluation of Antibody Response Directed Against Porcine Reproductive and Respiratory Syndrome Virus Structural Proteins

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<b>Key Words</b>	Swine viruses, PRRSV, Humoral Immunity, Luciferase-Immunoprecipitation System, Antibody Profile
<b>Abstract</b>	Luciferase-immunoprecipitation system (LIPS), a liquid phase immunoassay, was used to evaluate antibody responses directed against the structural proteins of PRRSV in pigs that were experimentally infected with virulent PRRSV strains. First, the viral N protein was first used as a model antigen to validate the assay. The LIPS results were highly comparable to that of the commercial IDEXX PRRS X3 ELISA, both with sera obtained from experimentally infected animals and clinical sera obtained from the field. Subsequently, the assay was applied to simultaneously measure antibody reactivity against all eight structural protein of PRRSV. The highest immunoreactivities were against GP3, M and N proteins while the lowest reactivity was against ORF5a protein. The levels of antibody reactivities against GP2, E, GP4 and GP5 were intermediate and were not significantly different among each other. Collectively, the results provide additional information about the host antibody response to PRRSV infection.
<b>Introduction</b>	Pigs infected with virulent PRRSV strains develop a robust antibody response that can be detected at 5 days post-infection (dpi) (1). By 14 dpi, all pigs exposed to PRRSV have seroconverted and antibodies can be detected for up to 300 dpi (1, 2). Significant effort has been made to characterize the ontogeny of swine humoral immune response to PRRSV infection. Immunoblotting analysis, using PRRSV-infected cell lysate as the target antigens, revealed that PRRSV-infected pigs developed antibodies against 3 viral major proteins: N (15 KDa), M (19 KDa) and GP5 (25 KDa) (1-3). Antibodies against N protein were consistently detected from pigs from 7 dpi and continued to be detected up to at least 300 dpi (1, 2). On the other hand, antibodies against M and GP5 varied among infected pigs and were not be detected until 14 or 35 dpi (1). Protein-specific antibody ELISAs were developed to study antibody responses to 5' and 3' termini of GP5 and M protein as well as the chimeric protein containing GP5 and M ectodomain

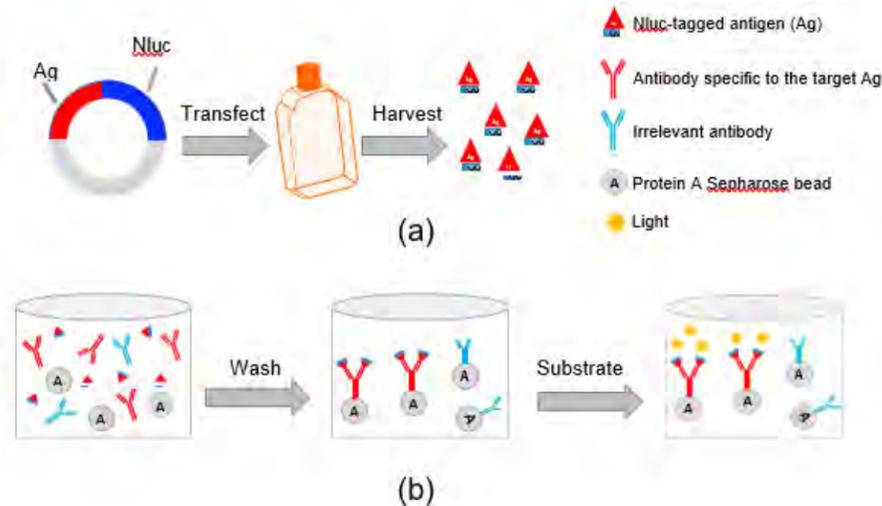
(4). The results indicated that antibodies directed against these two proteins were detected between 28 and 42 dpi. Pepscan ELISA was used to identify B cell epitopes located in the nsp2 and all viral structural proteins (5). It was found that nsp2 contained higher frequency of immunodominant epitopes than structural proteins. Of the structural proteins studied, only GP3 and M protein possess peptides that were recognized by 100% (n=15) infected pigs (5).

While there is a large body of the literature describing the overall humoral immune responses to PRRSV infection, information about antibody response to the viral minor glycoproteins is scarce. Thus, the primary objective of this study was to comparatively evaluate immunogenicity of the PRRSV structural proteins.

## Methods

Luciferase-immunoprecipitation system (LIPS), a liquid phase immunoassay, was used to measure antibody reactivities against the structural proteins of PRRSV in serum samples collected from pigs experimentally infected with virulent PRRSV strains and in clinical serum sample collected from a veterinary diagnostic laboratory. The LIPS utilizes a luciferase-fusion antigen as the bait to capture antigen-specific antibodies. Specifically, the target antigen is cloned in-frame with a luciferase reporter gene and expressed in mammalian cells. A crude cell extract containing the luciferase-tagged antigen is mixed with a test serum sample in the presence of protein A Sepharose beads. If the test serum samples contain antibodies specific to the luciferase-tagged antigen, the antigen will be immobilized on the beads. The amount of antigen-specific antibody present in the test serum will be quantified by adding a luciferase substrate, followed by measuring light production (Figure 1).

# Evaluation of Antibody Response Directed Against Porcine Reproductive and Respiratory Syndrome Virus Structural Proteins (Continued)



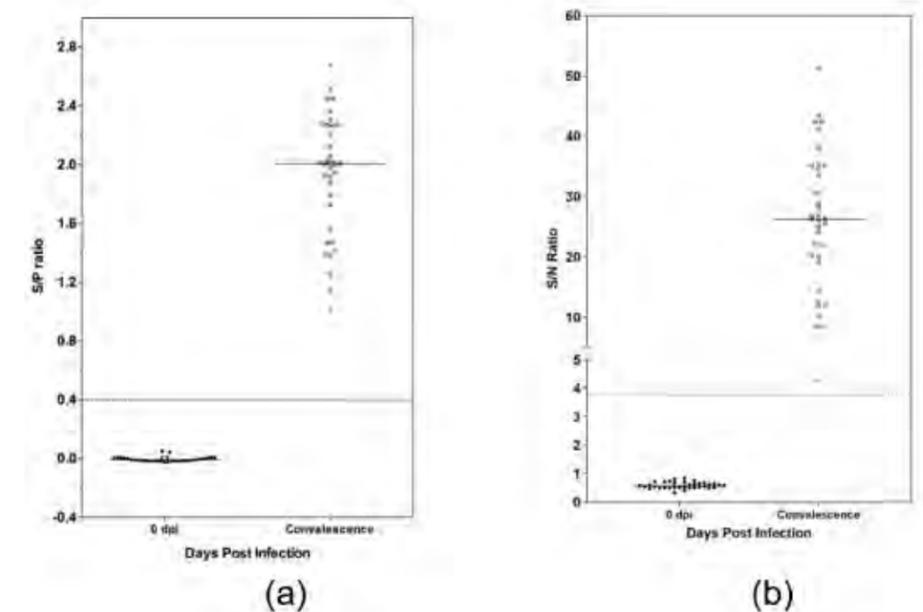
**Figure 1.** Schematic representation of the Luciferase immunoprecipitation system (LIPS). (a) Generation of luciferase-tagged antigens (Ag). Each individual PRRSV structural protein antigen is cloned in-frame to the 5' terminus of the nanoluc luciferase gene (Nluc). The resulting plasmid is transfected into 293-T cells. At 60h after transfection, cell lysate containing Nluc-tagged antigen is harvested and used for the LIPS. (b) Evaluation of immunoreactivities against the Nluc-tagged antigens. Cell lysate containing Nluc-tagged antigen is incubated with test serum samples together with protein A Sepharose beads in a filter 96 well plate. If the test samples contain antibody (IgG) specific to the Nluc-tagged antigen, the antigen-antibody complexes are formed, which are captured by the protein A Sepharose beads and retained in the well. Unbound Nluc-tagged antigen is washed away. Once the luciferase substrate is added to the well, the Nluc-tagged antigen bound to the bead will react with the substrate and produce luminescence. The light units produced by the Nluc-tagged antigen is proportional to the amount of antigen-specific antibody present in the test serum samples.

## Results

### Establishment of a luciferase immunoprecipitation system to detect antibodies against PRRSV

To establish the assay, we used a set of antisera collected from pigs before (0 dpi) and at 42 days after they were experimentally infected with different PRRSV strains. The commercial IDEXX ELISA was used as a reference test to determine the serological status of these antisera. As expected, all antisera collected before experimental infection (0 dpi) tested negative while all antisera collected at 42 dpi tested positive by the IDEXX ELISA (Figure 2a). The LIPS results show that 42 dpi antisera reacted strongly to the Nluc-tagged N protein, with the S/N ratio of  $26.1 \pm 11.5$  (Figure 2b). On the other hand, the 0 dpi antisera did not react significantly with the Nluc-tagged N protein (S/N  $0.6 \pm 0.1$ ). A cutoff value was calculated based on the S/N ratio of 0 dpi antisera. Based on this cutoff, all convalescent sera tested positive

while all 0 dpi sera test negative by LIPS. The LIPS results were highly comparable to the IDEXX ELISA results, with a kappa value of 1.00.



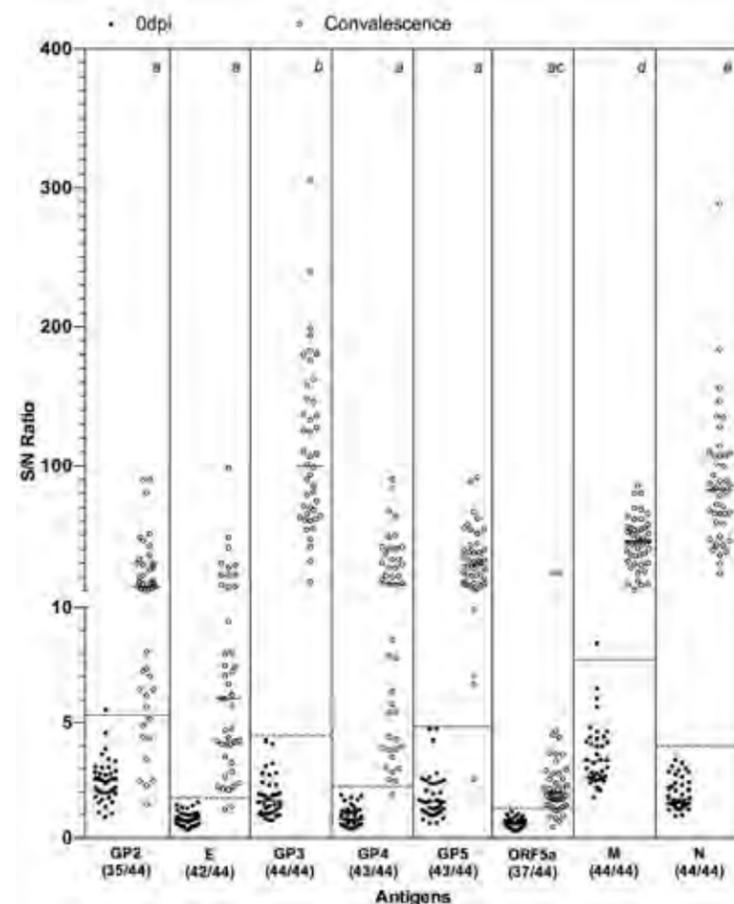
**Figure 2.** Optimization of the LIPS with N protein. (a) Serum samples collected from 35 pigs experimentally infected with the PRRSV-01 and FL12 and their derivative mutants at 0 dpi and 42 dpi. Serological status of the serum samples as determined by the IDEXX ELISA. Data are expressed as the sample to positive (S/P) ratios. The horizontal dotted line at S/P = 0.4 indicates the cutoff of this assay. (b) Immunoreactivity of the serum samples were measured against Nluc-tagged N protein as described in Material and Method. Fetal bovine serum (FBS) was used as negative control. Data are expressed as sample to negative (S/N) ratios. The dotted line at S/N = 4.02 indicates the cutoff of the assay.

### Swine antibody response to PRRSV structural proteins

Next, we simultaneously measured antibody reactivities against these eight structural proteins using a new set of antisera collected from 44 pigs experimentally infected with the PRRSV strain FL12 between 42 and 63 dpi. The serology status of these antisera was evaluated using the IDEXX ELISA test which confirmed that all serum samples collected at 0 dpi were seronegative while all convalescent antisera were seropositive (data not shown). Of the eight structural proteins, GP3 had the highest S/N ratio ( $111.7 \pm 59.6$ ), followed by N ( $87.9 \pm 47.2$ ) and M ( $44.9 \pm 18.6$ ) (Figure 3). The S/N ratios for the GP2 ( $21.2 \pm 22.32$ ), E ( $11.9 \pm 17.1$ ), GP4 ( $22.6 \pm 22.8$ ) and GP5 ( $31.9 \pm 20.7$ ) were not statistically different from each other. The

## Evaluation of Antibody Response Directed Against Porcine Reproductive and Respiratory Syndrome Virus Structural Proteins (Continued)

S/N ratio for ORF5a-protein (3.1  $\pm$  4.5) was the lowest. Cutoff values were separately calculated for each of these eight proteins. All convalescent antisera tested positive for GP3, M and N. The percentage of convalescent antisera tested positive for GP2, E, GP4, GP5 and ORF5a-protein were 79.6%, 95.5%, 97.7%, 97.7%, and 84.1% respectively (Figure 3).



**Figure 3.** Swine antibody reactivities against PRRSV structural proteins. Thirty-eight serum sample collected before infection (0 dpi) and 44 samples collected at between 42 and 63 dpi from 44 pigs experimentally infected with the PRRSV strain FL12. The antibody reactivities of these serum samples were simultaneous measured against eight PRRSV structural proteins. Fetal bovine serum (FBS) was used as negative control. Data are expressed as sample to negative (S/N) ratios. The dotted lines indicate the cutoffs of the respective assays. Number within parenthesis below the antigen names indicate the proportion of convalescent serum samples tested positive for that respective antigens. The superscript letters at the top of the graph denote the statistical comparison of the S/N ratios among the eight antigens. Means sharing the same superscript are not significantly different from each other ( $P > 0.05$ ).

### Discussion

The LIPS has been widely used for profiling humoral immune responses in autoimmune and infectious diseases owing to its several advantages as compared to other serological assays such as ELISA or protein microarray (6, 7). Briefly, target antigens used for the LIPS are produced in mammalian cells, allowing them to undergo necessary post-translational modifications and proper folding and exposure of the antigenic epitopes. In addition, the LIPS can be performed with crude cell extracts; thus, significantly reducing the time and effort required to produce the target antigens. Renilla luciferase (Rluc) has been widely used as the reporter gene for the LIPS (6, 7). In this study, we chose to use nanoluc luciferase (Nluc) instead of Rluc because Nluc is smaller, brighter and displays lower background activity than Rluc (8).

We first used PRRSV N protein as the model antigen to validate the LIPS because its immunogenicity has been well characterized (9-12). We then applied the LIPS to simultaneously measure antibody against all viral structural proteins derived from the PRRSV strain FL12. The serum samples used in this study were collected from pigs experimentally infected with the wild-type PRRSV strain FL12. Thus, the antibody response against each structural protein measured in this experiment should be considered a homologous antibody response. Equivalent amount of each of the eight Nluc-tagged antigens was used to measure immunoreactivity. Therefore, the amount of light produced should be proportional to the amount of soluble Nluc-tagged antigens captured by the antibody-bound beads which allows us to directly compare the immunoreactivities of convalescent antisera directed against these eight structural proteins. The results show that GP3, M and N display the highest degree of immunogenicity. GP2, E, GP4 and GP5 have intermediate levels of immunogenicity while ORF5a protein has the lowest level of immunogenicity.

Although GP5 is a major viral envelop glycoprotein, the intensity of antibody reactivity against GP5 is not significantly different from the antibody reactivities against the minor proteins GP2, E and GP4. In an effort to identify potential linear B cell epitopes, de Lima et al. screened the reactivity of convalescent sera collected from FL12-infected pigs against a library of overlapping peptides encompassing the viral structural proteins (5). The authors reported that the frequency and magnitude of antibody reactivity against GP2, GP4 and GP5 peptides were similar. Thus, the results obtained in this current study corroborate the results previous reported by de Lima et al. (5).

# Evaluation of Antibody Response Directed Against Porcine Reproductive and Respiratory Syndrome Virus Structural Proteins (Continued)

ORF5a-protein is a newly discovered viral protein. It is the smallest among the eight structural protein and is expressed in significantly low levels in PRRSV infected cells (13). Its immunogenicity has not been well characterized. It was reported previously that antibodies specific to ORF5a-protein slowly appeared in pigs infected and remained at lower levels as compared to antibodies against N protein (13). In the present study, we observed that immunoreactivity against ORF5a-protein was the lowest among the eight structural proteins (Figure 3). We suggest that the low immunogenicity of ORF5a-protein might be due to its small molecular weight in combination with its low expression levels.

The finding that GP3 also has high degree of immunogenicity as compared to M, and N protein is interesting. The relative abundance of GP3 on the viral virion is significantly lower than GP5, M and N (14). However, it was reported that a portion of GP3 is secreted into culture medium in a soluble membrane-free form (14, 15). It is believed that both virion-associated and soluble-forms of GP3 can elicit immune responses in the infected pigs, which might explain for its high immunogenicity (15).

In summary, we report here the application of LIPS to simultaneously measure antibody responses against the structural proteins of PRRSV. The results of this study indicate that the levels of immunogenicity are highest with GP3, M and N, intermediate with GP2, E, GP4 and GP5 and lowest with ORF5a-protein. This study expands our knowledge on the humoral immune response against PRRSV infection.

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The authors declare no conflict of interest.

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## Acknowledgements

## Conflict of Interest

# Evaluation for Genetic Stability of a US Commercially Licensed Modified Live Porcine Reproductive and Respiratory Syndrome Virus Vaccine

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**Key Words** Genome, Stability, Virulence, Sentinel, PRRSV

**Abstract** The assessment of genetic stability is important in any modified live vaccine (MLV). Demonstration of genetic stability of porcine reproductive and respiratory syndrome virus (PRRSV) MLV is no exception in that genetic diversity has been quite problematic in PRRS management and control in swine herds. Two studies are presented to demonstrate Prevacent® PRRS vaccine master seed virus (MSV) genetic stability. A reversion to virulence study involving infection, isolation and reinfection of successive PRRSV naïve piglets was performed over a total of four passages [Back Passage (BP) 1, BP 2, BP 3, BP 4]. Each group of twenty 14-15 day old piglets were infected, observed for 14 days, then humanely euthanized and PRRSV diagnostics and isolation were performed. BP 4 was a blind passage which observed for 21 days because virus isolation on BP 3 were negative but RTqPCR positive. BP 4 was negative for both PRRSV isolation and RTqPCR. The inability to isolate the PRRSV from BP 3 and BP 4 demonstrated stability and freedom from reversion to virulence. The second study was an *in vitro* assessment of Prevacent® PRRS vaccine MSV stability in which Prevacent® PRRS MSV (X) was continuously passed over 10 cell culture passages and whole genomic sequences were assessed at passages 1 (X+1), 5 (X+5), and 10 (X+10). The analysis demonstrates that X+10 genome sequences were well conserved in which only 4 sequence variants ( $\leq 0.03\%$ ) were identified within the viral protein coding genes resulting in 99.97% sequence similarity relative to X. Taken together Prevacent® PRRS vaccine virus is genetically stable, but behavior in the field with multiple diseases and circulating wild type PRRSV may complicate this assessment.

**Introduction** Genetic variability of PRRSV is a hallmark of RNA viruses. PRRSV has a high frequency of mutation (Hanada et al.; Paploski et al.; Figlerowicz et al.). Genetic variability is caused by mutations during replication, as well as, heterologous and homologous recombination of the viral genome (Yuan, Nelsen, et al.; Murtaugh). Recombination of many types have been described (Nagy and Simon). The replicase and transcription complex (RTC) is involved in the replication, the immunogenicity of the virus, and may

modulate viral recombination (Yun and Lee; Fang and Snijder). To evaluate the genetic stability of a recently commercially licensed PRRSV vaccine several evaluations have been performed. This report includes two such studies. One to determine the vaccine's reversion to virulence (Study 1) and the other to analyze the genetic stability (Study 2). Both studies demonstrate a high degree of stability. The studies are well controlled and may not reflect the behavior in the field, as it is well known that PRRSV has a high genetic variability.

## Methods

**Study 1:** A total of 80 clinically healthy, PRRSV free (negative by both serology and RTqPCR) 14-15 day old weaned piglets were enrolled over 4 passages. Pigs were placed in a biosafety level (BSL) 2 facility for each passage consisting of 20 randomly allotted pigs [10 control injected with PBS and 10 administered with the vaccine or bronchoalveolar lavage fluid (BALF) from the previous group]. BP 1 inoculum was Prevacent® PRRS (PRRSV SD11-21) MSV X+1 ( $8.2 \log_{10}$  TCID<sub>50</sub>/ml), which was the first cell passage of the X for vaccine production. The BP 2 through 4 were given BALF from the previous passages. BALF was collected and subjected to RTqPCR (EZ-PRRSV™ MPX 4.0 RT-PCR, Tetracore) analysis and virus isolation. For virus isolation, 500µl of BALF sample was inoculated in one well of a 24 well plate seeded with approximately  $4.0-4.5 \times 10^4$  cells/ml within 2 to 4 days and incubated for up to 7 days 37°C and 5% CO<sub>2</sub> incubator to observe PRRSV specific cytopathic effect (CPE) on MARC-145 cells and confirmed by indirect immunofluorescent assay (IFA) with PRRSV specific antibody. BALF positive for PRRSV by RTqPCR were concentrated over a sucrose cushion and pooled. The pooled materials were again tested for PRRSV by RTqPCR and the titer was determined by CPE as a 50% tissue culture infectious dose (TCID<sub>50</sub>) using the Reed-Muench method. Clinical observations were made daily for 14 days and the final blind passage was monitored for 21 days. A pig was considered clinically affected by PRRSV if it exhibited pyrexia and typical clinical signs of PRRS or pig died or was removed due to a confirmed PRRS diagnostic, or had gross lung lesions attributable to PRRS. A blind passage was considered as negative for culture or RTqPCR on BALF if the last passage was tested negative. Other diagnostic tests were performed at Iowa State Diagnostic Veterinary Lab in accordance with established procedures.

**Study 2:** Prevacent® PRRS vaccine MSV was propagated in Opti-MEM® I (Life Technologies) supplemented with 5% Fetal bovine serum and 50 µg gentamycin on MARC-145 cells derived from an approved cell lines used for vaccine production. Next Generation Sequencing (NGS) was performed by

## Evaluation for Genetic Stability of a US Commercially Licensed Modified Live Porcine Reproductive and Respiratory Syndrome Virus Vaccine (Continued)

BioReliance, Rockville, MD, USA. Previously NGS was completed on X, and was used as the reference to compare with passages X+1, X+5 and X+10 to determine genetic variability. Sequence characterization consists of standard procedures briefly described as follows:

1. Nucleic acid extraction and conversion to sequence compatible double stranded DNA library
2. Library concentration using SYBR Green based quantitative PCR
3. Sequencing and Cluster generation by Illumina® MiSeq® NGS platform
  - a. MiSeq utilizes sequencing by synthesis to determine the nucleotide order of millions of DNA fragments
  - b. Variant detection, genome identification and characterization, was performed by BioReliance's proprietary bioinformatics and analyses
4. Sequences of X, X+1, X+5 and X+10 were aligned and analyzed by Geneious' ClustalW pair wise/multiple alignment tools

### Results and Discussions

**Study 1:** BP 1 BALF collected was RTqPCR positive and culture positive with a titer of 2.1 log<sub>10</sub> TCID<sub>50</sub>/ml. BP 2 BALF collected was RTqPCR positive and culture positive with a titer of 2.6 log<sub>10</sub> TCID<sub>50</sub>/ml. BP 3 BALF collected was RTqPCR positive and culture negative. BP 4 BALF collected was both RTqPCR and culture negative. Pooled BALF from the last PRRSV positive (BP 2) was analyzed by CPE and IFA with PRRSV specific antibody along with ORF5 gene sequencing. It was shown to be phenotypically similar to the inoculum used in BP 1 with a 100% genotypic match on the ORF5 gene.

The inability to isolate the PRRSV from BP 3 and BP 4 demonstrated stability and freedom from reversion to virulence satisfying the requirements for US licensure.

**Study 2:** After completion of the described analysis steps, full genome consensus sequences were generated for all three passages with various lengths and fidelities. Table 1 shows the summary of consensus sequences and variants analysis.

The primary goal was to confirm genetic stability of Prevacent® PRRS MSV over the passage levels up to 10 (X+10). Even though only 14,866bps (98.98% of 15,019 bps' full genome) were well matched and aligned with the reference sequences (X) due to the nature of NGS, and this genetic analysis tool limits fidelity readings of genome ends without the Rapid Amplification of cDNA Ends (RACE) amplification, we were able to detect various sequence variants throughout the genome in X+10.

Overall X+10 genome sequences were well conserved and only 4 sequence variants (≤0.03%) were identified within the viral protein coding genes resulting in 99.97% sequence similarity relative to X.

Position	Consensus sequences*				Region	Amino acid changes**	Consequences to coding sequences	Type of changes
	x	x+1	x+5	x+10				
38	T	-	R	T	5'UTR	-	-	-
69	C	C	A	C	5'UTR	-	-	-
94	C	C	Y	C	5'UTR	-	-	-
111	T	G	T	T	5'UTR	-	-	-
185	T	C	T	T	5'UTR	-	-	-
3,667	T	G	T	T	NSP2 (ORF1a)	Serine	Synonymous	-
4,526	C	C	Y	T	NSP3 (ORF1a)	Proline to Serine	Nonsynonymous	Transition
6072	C	Y	C	C	NSP5 (ORF1a)	Serine to Leucine	Nonsynonymous	Transition
12,314	G	A	R	A	ORF2a ORF3	Alanine to Threonine Serine to Asparagine	Nonsynonymous	Transition
12,911	C	Y	Y	T	ORF3 ORF4	Serine to Leucine Phenylalanine	Nonsynonymous Synonymous	Transition
12,914	C	Y	C	C	ORF3 ORF4	Proline to Leucine Alanine	Nonsynonymous Synonymous	Transition
14,528	A	A	W	T	ORF7	Asparagine to Isoleucine	Nonsynonymous	Transversion

**Table 1.** Consensus sequences and variant analysis

\*Ambiguous nucleotides indicate Y = C or T, R = A or G and W = A or T

\*\*Any amino acid (AA) changes observed during passages

Prevacent® PRRS vaccine MSV is genetically very stable and only limited number of mutations were found throughout the genome during passages from X to X+10. Overall total 4 nucleotides changes resulted in AA changes in NSP3, ORF2a, ORF3 and ORF7 and most mutations were relatively conservative in which 3 transition mutations in NSP3, ORF2a and ORF3 coding sequences and only one transversion mutation in ORF7 coding sequence were found.

PRRSV is shown to mutate and recombine naturally and reports of recombination have been described (Zhao et al.; Zhang et al.; Yuan,

# Evaluation for Genetic Stability of a US Commercially Licensed Modified Live Porcine Reproductive and Respiratory Syndrome Virus Vaccine (Continued)

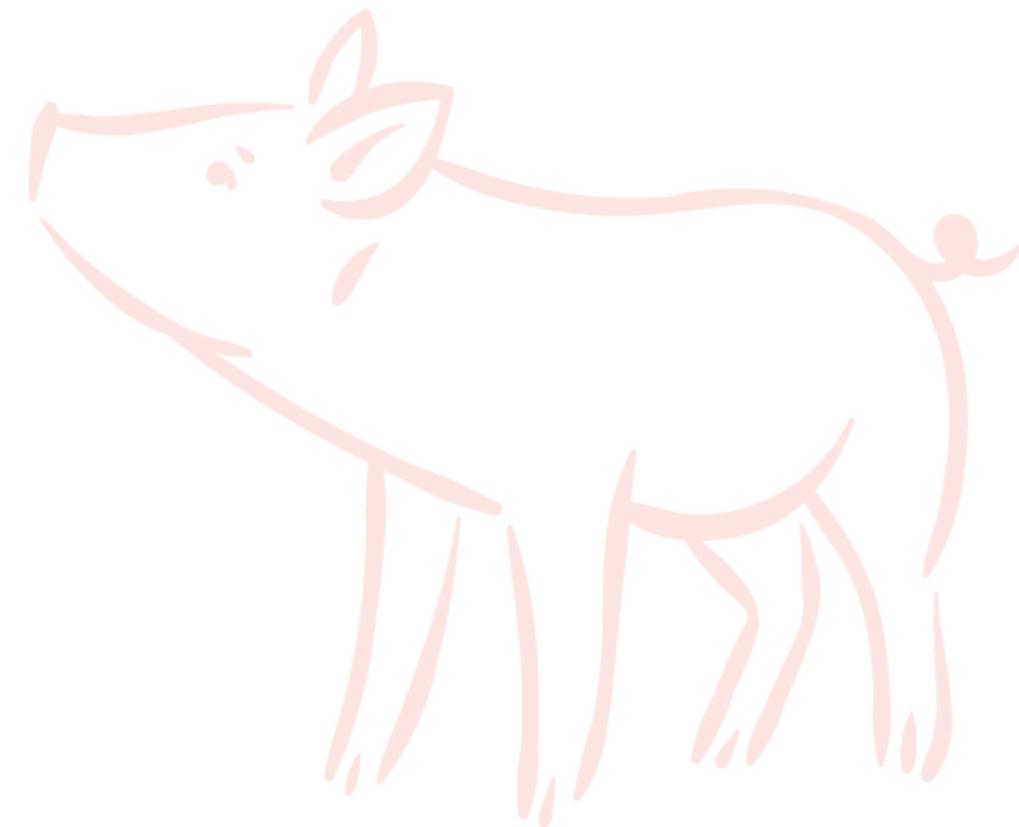
Murtaugh, et al.; Liu et al.; Yuan, Nelsen, et al.; Li et al.; Bian et al.). The studies presented here demonstrate genetic stability of Prevacent® PRRS vaccine. Coinfections of Prevacent® PRRS vaccine with other wild-type or vaccine-like strains may affect this stability. Vaccination timing, frequency of administration, and dosing may alter this genetic stability.

## Acknowledgements

JMH, BJK, and SW are employed by Elanco and have been involved in the development, licensing, and post marketing development of Prevacent®.

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# Host Transcriptional Response to Persistent Infection with a Live-Attenuated Porcine Reproductive and Respiratory Syndrome Virus Strain

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<b>Key Words</b>	Persistence, Transcriptome, Inguinal lymphnode (ILN), Apoptosis, T-cell Exhaustion
<b>Objectives</b>	Both virulent and live-attenuated PRRSV strains can persist in lymphoid tissues of infected pigs for longer period (up to 5 months), suggesting that the host immune system does not effectively clear the virus. To investigate the mechanisms of PRRSV persistence, we performed a transcriptional analysis of inguinal lymphoid tissue (ILN) from pigs experimentally infected with a live-attenuated PRRSV strain at 46 days post-infection (dpi).
<b>Methods</b>	Ten three-week-old PRRSV naïve pigs were divided into two groups: group 1 was injected intramuscularly (IM) with DMEM to serve as the negative control, while group 2 was inoculated IM with 10 <sup>5</sup> TCID <sub>50</sub> of a live attenuated PRRSV strain CON90. Blood samples were collected weekly to determine antibody (Ab) and T cell responses. T-cell responses from blood and ILN was evaluated using ELISPOT and flowcytometric assay. Lymph node biopsy was collected at 46 dpi for RNA sequencing to study host transcriptome signatures associated with persistent infection.
<b>Results</b>	A total of 6,404 differentially expressed genes (DEGs) were detected of which 3,960 DEGs were upregulated and 2,444 DEGs were downregulated. Specifically, genes involved in innate immune responses and chemokine and receptors associated with T-cell homing to lymphoid tissues were downregulated. As a result, homing of virus-specific T-cells to lymphoid tissues seems to be ineffective, evidenced by the lower frequencies of virus-specific T-cells in lymphoid tissue than in peripheral blood. Genes associated with T-cell exhaustion were upregulated. Likewise, genes involved in the anti-apoptotic pathway were upregulated.

**Conclusion** Collectively, the data suggested that PRRSV establishes a pro-survival microenvironment in lymphoid tissue by suppressing innate immune responses, T-cell homing and preventing cell apoptosis.

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**Introduction** Persistent infection is a common phenomenon of arteriviruses. Lactate dehydrogenase-elevating virus (LDV) (e.g., Gammaarterivirus lacdeh) and Simian hemorrhagic fever virus (SHFV) (e.g., Deltaarterivirus hemfev) establishes an asymptomatic, lifetime persistent infection in their respective natural host (1, 2). Likewise, Equine Arteritis virus (EAV) (e.g., Alphaarterivirus equid) establishes long-term persistent infection in a small portion of horses (Reviewed in (3)). PRRSV mainly persists in lymphoid tissue of infected pigs longer than 200 days (4, 5). The mechanisms behind PRRSV persistence is not yet understood. A genome-wide association study (GWAS) was conducted to identify host factors associated with PRRSV persistence. While no quantitative trait loci (QTL) with major effects on PRRSV persistence were identified. Recently, a study of experimental infection in pigs with PRRSV-1 revealed that during persistent infection, viral proteins are minimally expressed, and viral genomes exist predominantly in the form of double stranded RNA (dsRNA). The study of mechanism behind PRRSV persistence can provide us with important factors that are limiting control of PRRS infection and development of effective vaccine.

The goal of this study is to elucidate the putative mechanisms by which PRRSV can establish persistent infection. We performed a genome-wide transcriptomic analysis of RNA collected from the inguinal lymph node (ILN) of pigs persistently infected with a live-attenuated PRRSV strain. Analysis of RNAseq data revealed large number of differentially expressed genes (DEGs). Among them, genes involved in innate immunity, chemokine signaling, and T-cell homing and trafficking were downregulated. On the other hand, genes involved in the anti-apoptotic pathway and T-cell exhaustion were upregulated. Functional studies revealed that the frequencies of virus-specific IFN-γ-secreting cells are lower in lymphoid tissue than in peripheral blood mononucleated cells (PBMCs). Collectively, the results shed important insight into the mechanisms of PRRSV persistence in the host.

# Host Transcriptional Response to Persistent Infection with a Live-Attenuated Porcine Reproductive and Respiratory Syndrome Virus Strain (Continued)

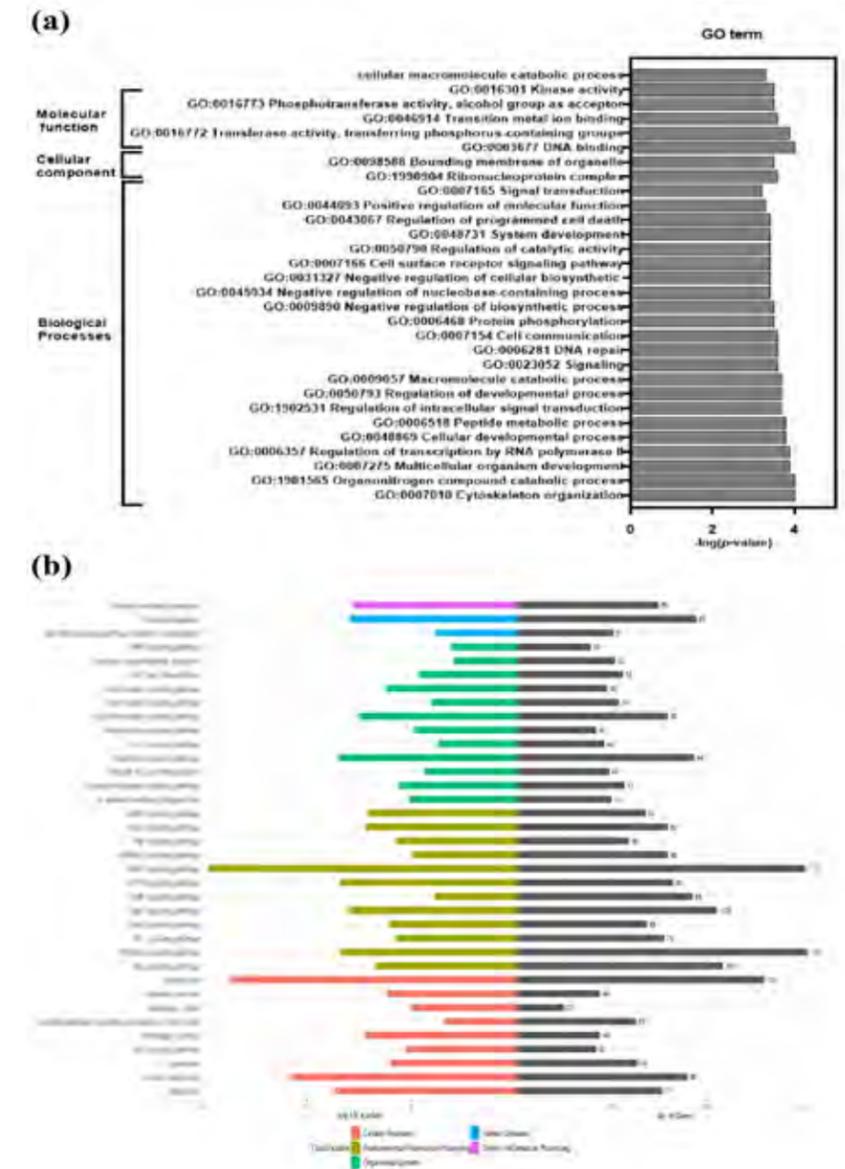
## Methods

The attenuated PRRSV strain designated CON90 used in this study was recovered from an infectious cDNA clone constructed using viral RNA extracted from the attenuated CON-P90 (6). Ten 3-week-old PRRSV, porcine circovirus type 2 (PCV2), and SIV negative pigs were purchased from the Midwest Research Swine. Pigs in group 1 were injected with DMEM to serve as negative controls, whereas pigs in groups 2 were inoculated intramuscularly with 105.0 TCID<sub>50</sub> of live-attenuated PRRSV strain CON90. Whole blood samples with anticoagulant EDTA were collected to obtain plasma and PBMCs. A portion of freshly isolated PBMCs were used for measurement of IFN- $\gamma$ -secreting cells responses using ELISPOT and flowcytometry. At 46 dpi, sample of ILN was aseptically collected from the pigs under anesthesia. One half of the INL was used for lymphocyte isolation while the other half was utilized for RNA extraction. The libraries were sequenced on an Illumina platform Hiseq 2000 and approximately 40 million raw 125 bp/150 bp paired end) reads were generated.

## Results and Discussions

### DEGs and KEGG analysis for CON90 persistent infection

RNA reads were mapped to the reference pig genome (Sscrofa11.1; GCF\_000003025.6). Out of 17553 genes in the annotated porcine genome, there were 6404 DEGs (FDR < 5%,  $|\log_2|$  fold change  $\geq 1$ ), of which 3960 DEGs were upregulated and 2444 DEGs were downregulated. Thirty highly enriched GO terms in CON90-infected pigs are shown (Figure 1a). KEGG pathway analysis revealed multiple enriched pathways including apoptosis, chemokine signaling, cellular, senescence, mitophagy, lysosome, endocytosis, and MAPK (Figure 1b).



# Host Transcriptional Response to Persistent Infection with a Live-Attenuated Porcine Reproductive and Respiratory Syndrome Virus Strain (Continued)

## Expression of Genes Involved in the Innate Immune Response

The innate immune system is the first line of defense against invading pathogens, with the major influence on the development of strong adaptive immune responses. In the current study, we detected suppression of innate immune response. The crucial innate immune molecules like IFN or interferon stimulated genes (ISG) RNA transcripts showed no differential expression in ILN of CON-infected pigs at 46 dpi. In addition, we observed an upregulation of TLR10 transcripts, an anti-inflammatory receptor which can suppress TLR3-induced IFN- $\beta$  production (7). We also observed an increased expression of CD200 (OX2) and CD200R, an inhibitory immune receptor on B- and T- cells. Collectively, the data suggest that the CON90 virus suppresses an inflammatory response at the site of persistent infection.

## Regulation of Apoptosis During Persistence PRRSV infection

Apoptosis is a powerful innate immunity mechanism to curtail viral spread through eliminating virally infected cells. In this study, expression of TNFRSF1A gene that contains a death domain (8), was downregulated in the ILN of infected pigs. Similarly, expression of pro-apoptotic genes including AIFM2, CHAC1, and OSR1, was downregulated. On the other hand, expression of BIRC3 and BCL2A1, two apoptosis inhibitors (9), was upregulated. Furthermore, expression of several anti-apoptotic genes including BAK1, DDIAS, XIAP, MCL1, API, BNIP2, and FAIM was upregulated. Together, these results suggest that the pro-apoptotic signaling pathway was suppressed in ILN tissue of pigs persistently infected with CON90.

## T cell Transcriptional and Functional analysis

Chemokines, and receptors play a central role in regulating T-cell migration (10). In this study, expression of several important chemokine ligands (CCL19, CCL21, CCL24, CCL22, CX3CL1, and CCL14) and chemokine receptors (CCR6 and CCR10) which play an essential role in migration and localization of lymphocytes and antigen-presenting cells (APCs) to the lymphoid tissues was down regulated in ILN of CON90-infected pigs. On the other hand, expression of CD274 (PD-1), a marker of T-cell exhaustion, was upregulated. Likewise, expression of inhibitory receptors HAVCR2 and TGIT, which transmit the inhibitory signals for T-cell differentiation and effector activities (11), was upregulated. We also found increased expression of other co-inhibitory molecules (BTLA, FASLG, FAS, and IDO1) that are associated with the T-cell exhaustion during chronic viral infection. Together, the results suggest that T-cell migration to ILN, might be affected because of the down regulation of important chemokines and receptors, and that the T-cells in ILN might be exhausted.

We sought to compare the frequencies of virus-specific T-cells in PBMCs and in ILN using the IFN- $\gamma$  SC Elispot assay. The number of spots was similar when PBMCs and ILN cells were stimulated with non-specific T-cell activator PHA/Ionomycin. However, the number of spots was significantly lower in ILN than in PBMCs when the cells were stimulated with whole PRRSV antigen. The results clearly indicate that the frequencies of PRRSV-specific IFN- $\gamma$  SCs were significantly lower in ILN than in PBMCs. Similar to the Elispot assay, the flow cytometric assay also indicated that the percentage of T-cell secreting IFN- $\gamma$  was comparatively lower in ILN than in PBMCs.

## Transcriptional Regulation of Humoral Immune Response

Genes associated with humoral response were upregulated in the ILN of CON90-infected pigs. IL-21, RGS13, and NUGGC are involved in the development of germinal center (GC) and activation of B-cell follicles (12). TNFSF13B and TNFSF8 are potent activators of B-cell lineage and Ig class switching, respectively (13). B-cell surface antigens MS4A1 (CD20), AICDA (14), and RFTN2 are associated with B-cell receptor signaling. The upregulated expression of these genes in ILN of infected animals suggested that the humoral immune response to CON90-infection was not affected.

In summary, we identified a robust host transcriptional response in the ILN tissue of pigs persistently infected with the attenuated PRRSV strain CON90. Genes involved in the innate immune responses, chemokines and receptors associated with T-cell homing to the lymphoid tissue are downregulated. Additionally, the genes involved in the anti-apoptotic pathways are upregulated. Collectively, the data suggest that PRRSV can create a pro-survival microenvironment at the lymphoid tissue which allows the virus to persist for an extended period.

We would like to thank Dirk Anderson at Flow Cytometry Core Facility, Nebraska Center for Biotechnology for assistance on flow cytometric analysis and the staff members of UNL Life Sciences Annex and Veterinary Diagnostic Center for the care of animals.

The authors declare no financial conflict of interest.

## Conclusion

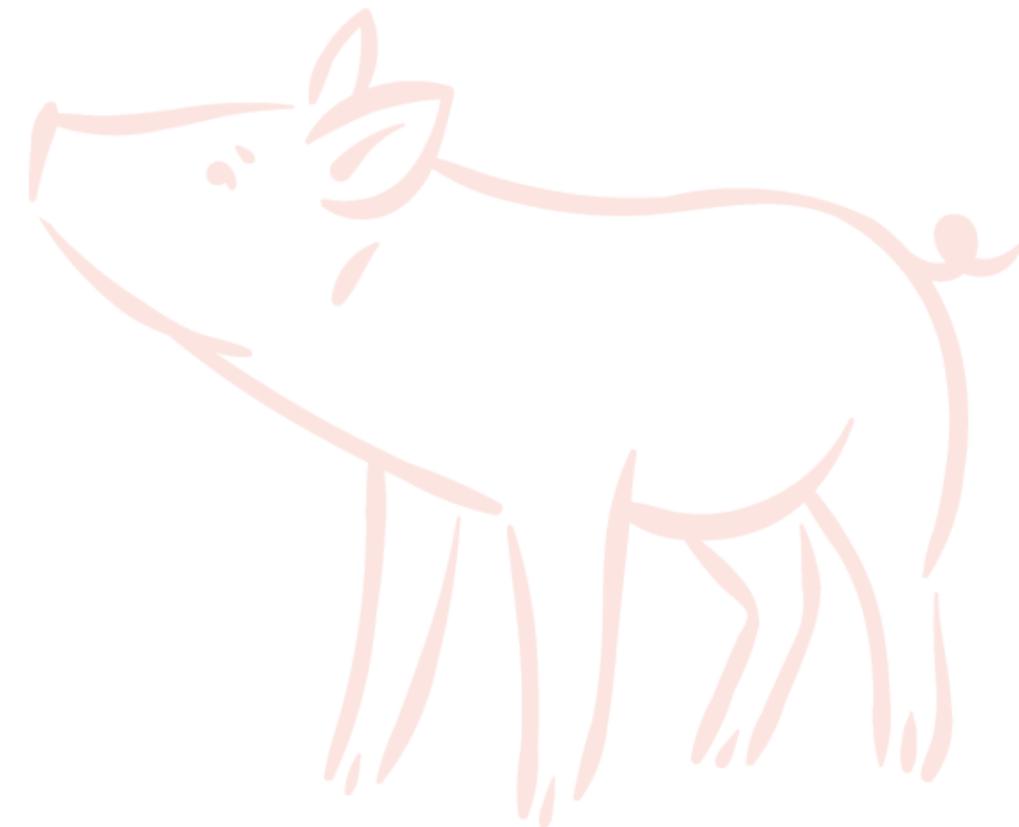
## Acknowledgement

## Conflict of Interest

# Host Transcriptional Response to Persistent Infection with a Live-Attenuated Porcine Reproductive and Respiratory Syndrome Virus Strain (Continued)

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# How to Best Protect Pigs Against PRRSV Based on Our Understanding of the Adaptive Immune Response to PRRSV

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<b>Key Words</b>	Porcine Reproductive and Respiratory Syndrome Virus, PRRSV, T Cells, Antibodies, Immunological Memory, Vaccines
<b>Abstract</b>	<p>Despite the availability of a variety of vaccines, the porcine reproductive and respiratory syndrome virus (PRRSV) continues to cause tremendous losses in the global swine industry. Besides vaccination, swine producers also use live virus inoculations (LVIs) of low-dose PRRSV from the PRRSV strain present on the farm. These LVIs aim to generate protection against a homologous circulating strain.</p> <p>While the humoral immune response to PRRSV vaccination and infection is well described, insights on the T-cell immune response have been rather rudimentary. Therefore, we performed two animal trials to provide insight into the immunogenicity and/ or efficacy of two vaccination strategies: The first trial assessed the effect of autogenous inactivated virus (AIV) vaccination of sows on the disease resilience of their offspring when challenged at 2 weeks of age; the second trial determined a timeline for the antibody and T-cell response after PRRSV vaccination or infection. The combination of these trials allows us to discuss the roles of an antibody and T-cell response against both homologous and heterologous PRRSV strains. The following shall provide an overview of our results as well as recommendations for the use of vaccination and/or LVI based on our current understanding of the development of immunity to PRRSV.</p>
<b>Introduction</b>	There are two main strategies to control PRRSV infection: i) Biosecurity measures and all-in-all-out swine production systems aim to minimize PRRSV transmission between and within farms (1); and ii) vaccination with mainly

modified live virus (MLV) or autogenous inactivated virus (AIV) vaccines or live virus inoculation (LVI) to prevent infection or manage PRRSV stability on a farm (2). While both strategies contribute to minimize the impact of PRRSV on swine production, it continues to be one of the most detrimental pig diseases, currently challenged by African swine fever. Rigorous biosecurity is the first line of defense against PRRSV infection of a farm; but once this line of defense is breached, a strong immune response against PRRSV is required to prevent infection of the pigs or at least curtail the losses. In addition to the different stages of pig production, pig farms can be separated into classified into five categories according to their PRRSV status (3) including PRRSV-negative, PRRSV-stable, and PRRSV-unstable. Each of these categories has its own goals for PRRSV management. PRRSV-negative farms try to prevent PRRSV exposure by any means, including the prevention of MLV vaccination. A PRRSV-stable farm tries to minimize the impact PRRSV has on the farm, e.g. by using vaccination or LVI. A PRRSV-unstable farm needs to improve its status by any means. These categories create limitations to swine producers: For example, piglets from a PRRSV-negative sow farm should optimally be fed into a PRRSV-negative grow/finisher farm since introducing PRRSV-naïve weaners into a PRRSV-positive herd might risk the stable status of the PRRSV-stable farm or lead to major losses in the PRRSV-naïve weaners based on the high exposure in a PRRSV-unstable farm. This complicated situation has also major implications on the vaccination strategies against PRRSV: Swine producers must not only consider the highly mutative and immunosuppressive characteristics of PRRSV (4) but also the pig production stage and PRRSV category of all farms in the pig flow. The diversity of considerations prevents a one-fits-all vaccination strategy. In the following, we will address how our studies of the development of immunological memory and protection following PRRSV vaccination and infection can guide the decision-making process for vaccination strategies against PRRSV.

## Methods

The presented data are based on two animal trials. In the first animal trial, 24 weaners from a PRRSV-negative herd were randomly distributed into four groups and either infected with MOCK, one of two PRRSV strains (NC134 and NC174; TCID<sub>50</sub> 10<sup>6</sup>), or MLV vaccinated. Infection, clinical signs, and immune response were followed for a total of nine weeks. At the end, the pigs were sacrificed to study the local viral loads as well as the T-cell and antibody response in BAL, lung, and tracheobronchial lymph nodes (tbLN) (5).

In the second animal trial, 19 gilts from a NC farm with an industry standard MLV vaccination program were allocated into three groups, each of them

# How to Best Protect Pigs Against PRRSV Based on Our Understanding of the Adaptive Immune Response to PRRSV (Continued)

receiving a total of four additional vaccinations during gestation – TRT0: MOCK; TRT1: AIV1, and TRT2: AIV2. Each of the AIVs was based on the circulating NC174 strain but different in their adjuvant formulation.

The gilts were monitored for their development of anti-PRRSV neutralizing antibodies (nAbs) against both the MLV strain (VR2332) and the AIV strain (NC174). A total of 36 piglets were weaned early at 2 weeks of age (four piglets from three representative gilts from each group) were selected and moved to the NC State Laboratory Animal Resources (LAR) for intranasal PRRSV challenge using the homologous NC174 strain (TCID<sub>50</sub> 10<sup>5.5</sup>). Fifty percent of these piglets were sacrificed 2 weeks post infection (wpi) and 50% at four wpi. At both time points, the systemic immune response and viral loads were analyzed in blood and the local immune response in nasal swabs. In addition, the sacrificed animals were analyzed for their local immune response and viral loads in bronchoalveolar lavage (BAL), lung tissue and tLN (manuscript in preparation).

For both animal trials, viral loads and nAbs were determined in nasal swabs and serum throughout the trial and at necropsy in BAL. The T-cell response was analyzed by multi-color flow cytometry in PBMC throughout the trial and in BAL, lung tissue and lymph nodes at necropsy. Analysis was focused on the homo- and/or heterologous proliferative and interferon- $\gamma$  (IFN- $\gamma$ ) response of the CD4+, CD8+, and TCR- $\gamma\delta$  T-cell subsets.

Our first animal trial characterized the immune response of weaners to PRRSV vaccination or infection. This study shows that a local IgA, systemic IgG and a low systemic T-cell response starts at ~2 weeks post infection or vaccination (5). Our two PRRSV strains (NC134 and NC174) also generated a system nAb response within 1-2 weeks; but it took ~4 weeks to generate this systemic nAbs by MLV vaccination (manuscript in preparation). At 4 weeks, the T-cell response peaked and the nAb levels reached a strong level of neutralization with suppressing >90% of PRRSV infection.

A comparison to previous results revealed that the lower levels of nAb at 1-3 weeks post infection will probably not provide full protection to pigs (6). Furthermore, based on the low T-cell response at such early states, it is unlikely that germinal centers have formed to facilitate antibody affinity maturation, isotype switching and the generation of memory B cells or long-lived plasma cells. Therefore, these early detected nAbs were likely low-affinity IgM molecules without contribution to long-term immunological

## Results and Discussions

**memory. In conclusion, vaccination or infection with PRRSV induces a strong T-cell and nAb response and potential protection at ~4 weeks.**

Our second animal trial studied the ability of AIVs to boost maternal transfer of immunity to PRRSV. The most outstanding results were obtained by comparing the effect of AIV1 (=TRT1) with TRT0 (no AIV) in regards to viremia, lung pathology and weight gain: TRT1 significantly decreased viremia at 1-2 wpi, lung gross- and histopathology at 2 wpi and weight gain at 4 wpi (manuscript in preparation). The most noticeable transfer of immunity was seen by studying the strain-specific neutralizing antibody levels: While all gilts had nAbs against the MLV parent strain VR2332, only the gilts from the AIV groups (TRT1+2) had nAbs against the circulating NC174 strain. In a high correlation, these gilts from TRT1+2 transferred these nAbs to the piglets with median FFN titers in piglet serum at 0 wpi (=pre-challenge) of FFN=64 for TRT1 and FFN=16 for TRT2. In contrast, piglets from TRT0 which only received the MLV did not have any nAbs against the NC174 strain. Regarding the T-cell response to PRRSV challenge in these piglets, neither the systemic nor the local responses revealed striking differences with one exception: While lung TCR- $\gamma\delta$  T cells from TRT0 piglets did not show any IFN- $\gamma$  production upon restimulation with NC174, the lung TCR- $\gamma\delta$  T cells from nearly all piglets in the TRT1+2 groups showed a strong IFN- $\gamma$  production. Taken together, these data demonstrate that **AIV vaccination of gilts or sows can lead to an increased resilience of their offspring**; this improved disease resilience is likely based on the transfer of nAbs with a potential involvement of **lung-residing TCR- $\gamma\delta$  T cells**.

## Conclusion

**Sow/ gilt vaccination and transfer of immunity:** Our data indicate that standard MLV vaccination of gilts/ sows does not lead to the transfer of probably protective nAbs or lung TCR- $\gamma\delta$  cells to piglets against currently circulating NC174 strains. Therefore, AIVs are recommended either in conjunction with MLVs or alone if the gilt/ sow needs to stay PRRSV negative. To ensure that the vaccine does not result in a positive PRRSV serum test, AIV vaccination could be administered intranasally. This intranasal administration could further boost a lung T-cell response (7) including TCR- $\gamma\delta$  T cells that might be involved in the protection of piglets.

**Protection of older piglets and weaners:** The long duration to establish protection against PRRSV by vaccination or even infection (e.g. using LVI) creates issues for the protection of older piglets in which maternal immunity is fading as well as weaners upon transfer into non-PRRSV free farms:

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Vaccination has to occur as early as possible to provide a protective level of immunity. With a weaning age of 3.5 weeks, even one vaccination at processing is probably too late to protect the vulnerable weaners against PRRSV. Therefore, our recommendation is to administer a prime vaccination to young piglets as early as processing and boost this vaccination between processing and weaning. If the piglets come from PRRSV-positive gilts or sows, our recommendation is to use intranasal prime vaccination of an AIV based on the better performance of intranasal AIVs in the presence of pre-existing immunity (8) as well as the higher safety profile of AIVs (9). It remains to be determined if a boost vaccination with an MLV or an AIV provides better protection. **In conclusion, these data provide evidence for the immunogenicity of both AIVs and MLVs; but it takes ~4 weeks that this immune response provides protection. On top, both heterologous immunogenicity and efficacy highly depend on the circulating PRRSV strain.**

## Acknowledgements

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# A Neutralizing Monoclonal Antibody-Based Competitive ELISA with the Emphasis on the Replacement of Virus Neutralization Test for Classical Swine Fever C-Strain Post-Vaccination Monitoring

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<b>Key Words</b>	Classical Swine Fever (CSF), Monoclonal Antibody, Competitive ELISA (cELISA), Virus Neutralization Test
<b>Introduction</b>	Virus neutralization test (VNT) is widely used to survey the efficacy of classical swine fever (CSF) vaccines and infection of classical swine fever virus (CSFV). However, VNT is a work-intensive and time-consuming procedure that requires cell culture and high-containment laboratory able to handle CSFV. In addition, it cannot be automated, thus it is not suitable for mass analysis of samples. Here we present a neutralizing monoclonal antibody (mAb) based competitive enzyme-linked immunosorbent assay (cELISA) with an emphasis on the replacement of VNT for CSF vaccine C-strain post-vaccination monitoring.
<b>Methods</b>	For generating suitable capture recombinant antigens, E2 protein of C-strain was expressed in insect cells using the Bac-to-Bac® Baculovirus Expression System. For generating suitable competitive mAbs, Balb/c mice were immunized with purified E2 of C-strain. The specificity and neutralizing activity of generated mAbs were assessed by ELISA, western blot and indirect fluorescent antibody assay (IFA). cELISA was established based on the strategy that neutralizing mAbs can compete with C-strain induced neutralizing antibodies in pig serum to bind the capture antigen (C-strain E2).

## Results

The cELISA was optimized and was further evaluated by testing different panels of pig sera.

i) C-strain E2 protein was successfully expressed. The purified native C-strain E2 protein existed mainly as homodimer; ii) mAb 6B211 showed potent neutralizing activity against C-strain CSFV and bound to a specific conformational epitope on C-strain E2 protein; iii) The 6B211 based cELISA was established and optimized, it showed 100% sensitivity (95% confidence interval: 94.87 to 100%) and 100% specificity (95% confidence interval: 100 to 100%) by testing C-strain VNT negative pig sera (n = 445) and C-strain VNT positive pig sera (n = 70); iv) The cELISA showed excellent agreement (Kappa = 0.957) with VNT by testing pig sera (n = 139) in parallel. The inhibition rate of serum samples in the cELISA is highly correlated with their titers in VNT (r<sup>2</sup>=0.903, p<0.001).

## Discussion and Conclusions

cELISA has been proved to be nearly as sensitive and specific as the VNT while being simpler and more rapid for detection and titration of antibodies against different viruses. In this study, C-strain envelope glycoprotein E2 was used as the capture antigen and the neutralizing 6B211mAb was used as the competitive antibody for establishing the cELISA. The 6B211 based cELISA can effectively differentiate the C-strain VNT positive and C-strain VNT negative samples, which indicate that 6B211 can efficiently compete with C-strain induced neutralizing antibodies from pigs. The excellent agreement (Kappa=0.957) between cELISA and VNT, and the high correlation VNT (r<sup>2</sup>=0.903) between inhibition rate in cELISA and VNT titers of tested samples indicate that the 6B211 based cELISA can replace the VNT for C-strain post-vaccination monitoring. In addition, the intra-assay CVs and the inter-assay CVs of the 6B211 based cELISA are lower than 10% when tested with the negative and E2 antibody positive pig serum samples, which indicate that the established cELISA is repeatable with acceptable variations. Through the aforementioned experiments and analysis, we concluded that this cELISA is a reliable, rapid, simple, safe, and cost-effective tool for sero-monitoring of C-strain vaccination at a population level. We believe that the cELISA presented in this study can be used to assist in CSF control and eradication.

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# A Risk-Free In Situ Non-Animal (RISNA) Surrogate Assay for ASFV

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**Abstract** African swine fever (ASF) causes high mortality in swine and is currently a significant and urgent threat to the U.S. pork industry if ASFV, the etiological viral agent of ASF, were to be introduced. The ASFV and other viruses have been shown to survive in soybean meal for extended periods of time (months) and be capable of infecting pigs at very low concentrations. However, there is very limited information on ASFV survival in feed ingredients, inactivation kinetics required for conducting risk assessments, and the effectiveness of various mitigation strategies to inactivate ASFV. This lack of scientific data is due to regulatory restrictions for using ASFV in research laboratories, and the inappropriateness of using other RNA-based viral surrogates for determining permanent inactivation of ASFV. Therefore, it is imperative that a megavirus surrogate assay for ASFV be developed and validated for use in implementing effective biosecurity and mitigation procedures to prevent transmission of ASFV under real-world scenarios. Here we show data that an algal megavirus EhV is a suitable surrogate for ASFV.

**Introduction** The continual spread of ASFV in Asia (Vergne *et al.*, 2020) and Europe (recent discovery of ASFV in wild boar in Germany) is a significant threat to the U.S. pork, soybean, and corn industries. The most current estimate of the total economic impact of an introduction of ASFV to the U.S. pork industry is \$57 billion over a 10-year period (Hayes *et al.* 2011), which represents the decreased revenue from increased pig mortality and decreased pork exports. Consequently, consensus among pork producers attending the 2019 Pork Industry Forum was that keeping the U.S. pork industry ASF-free is an extremely high priority and immediate action must be taken. It is also important to realize that the economic impact of an ASFV outbreak in the U.S. would also have dramatic effects on the U.S. corn and soybean industries because the prices and revenue from production of corn (\$ 28.4 billion) and soybeans (\$16.8 billion) would also significantly decrease as consequence of decreased utilization of these ingredients in swine diets (Hayes *et al.*, 2011). Results from a study by Dee *et al.* (2018) showed that ASFV survives

in soybean meal and choline chloride under conditions of trans-Pacific and trans-Atlantic transport scenarios. Therefore, it is imperative that everyone involved in the feed industry, including corn and soybean growers, work together to keep the U.S. free of ASFV.

Because of the high infectivity and risk of introduction of ASFV into the U.S., there are very strict regulatory requirements (BSL Level 3) to work with ASFV in research laboratories. In addition, because of the high cost of conducting ASFV research under these strict biosecurity and regulatory conditions, and limited work load capacity of this facility, the capability of this lab to conduct experiments necessary to quickly provide essential information urgently needed by the feed and pork industry is limited. The USDA is not currently conducting ASFV research. Efforts by the APHIS Center for Epidemiology and Animal Health (CEAH) are focused on expert solicitation to identify gaps in understanding of risk for ASFV introduction through the importation of contaminated plant-based feed ingredients, with pending projects on epidemiology and economic modeling. None of the CEAH projects involve risk analysis or evaluating mitigation strategies for ASFV. Therefore, because of the high level of biosecurity needed to investigate ASFV survival and inactivation in research laboratories, the urgent need to conduct experiments and data to conduct risk assessments to improve biosecurity, and the need to identify effective mitigation procedures for feed ingredient supply chains, a suitable and safe non-pathogenic surrogate virus assay is essential to move research ahead more quickly.

There is tremendous industry support for developing, validating, and using a surrogate virus assay for research studies to understand ASFV survival and inactivation in feed ingredients. This concept was first introduced during the ASFV-Vitamin Supply Chain Workshop (April 26, 2019) and the ASF-Soy Supply Chain Workshop (July 10, 2019), which were organized and co-sponsored by the University of Minnesota and Swine Health Information Center. One of the action items identified by pork and feed industry representatives in these workshops was to develop and use a surrogate assay for ASFV which could be used to conduct research to understand ASFV survival and inactivation under various conditions. After these workshops, several multi-national companies in the feed industry inquired about developing research collaborations with our U of M African Swine Fever Response Team to use this assay for evaluating various mitigation strategies for ASFV. Because of the high value of this research, some of these companies have provided initial partial funding commitments to support

# A Risk-Free In Situ Non-Animal (RISNA) Surrogate Assay for ASFV (Continued)

this proposal. The Swine Health Information Center also endorses the research described in this proposal and has also provided a letter of support. Therefore, there is significant industry interest and commitment to work with us on this research project.

African Swine Fever Virus is a complex enveloped virus that belongs to a group of viruses known as megaviruses, which replicate completely or partially in the cytoplasm of eukaryotic cells (Colson et al., 2013; Schroeder et al., 2002). Our overarching objective is to develop and use a risk-free *in situ* non-animal (RISNA) megavirus surrogate model assay based on an algal megavirus *Emiliana huxleyi* virus (EhV) (Schroeder et al., 2002, Mackinder et al., 2009) to test conditions that will effectively eliminate ASFV and related animal megaviruses from contaminated feed or feed ingredients in the supply chain. More specifically, our immediate objective is to determine whether EhV has a similar temperature sensitivity range as ASFV.

## Methods

A stock culture of *E. huxleyi* was maintained, infected with an EhV stock, lysed and filtered. The EhV filtered lysate (106 viruses/ml) served as the inoculum for the inactivation assays. Time and temperature combinations were used as the inactivation method to validate whether EhV is a suitable surrogate for ASFV. Experiments were designed to obtain inactivation kinetics data of the virus as affected by temperature (4°C to 60°C) over a time course ranging from seconds to minutes. A minimum of 5 experimental data points per treatment were obtained. Algal cultures were inoculated with the different temperature treated viruses and inhibition of cell growth was monitored daily over eight days and kill curve plots will be generated. The mechanism of inactivation was determined by confocal microscopy.

## Results

We evaluated different time and temperature combinations to determine the effects of exposure on EhV inactivation which included replicating the conditions known to inactivate ASFV (60°C/140°F for 20 min). We showed that EhV is similarly sensitive to temperatures greater than 50°C (120°F). EhV is still alive if treated at 37°C (98°F) and inactivation occurs at either 50°C or 60°C occurs in minutes.

## Conclusion

EhV is a suitable surrogate for ASFV. Subsequent studies will involve using RISNA to evaluate the effectiveness of chemical mitigants to eliminate ASFV if present in feed ingredients and complete feed. Therefore, this research benefits multiple segments of the global swine and feed industry including the U.S. soybean industry.

## Acknowledgements

US Soybean Board & private sector food industry.

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# Role of Host Restriction Factors in Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) Replication

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<b>Key Words</b>	Restriction Factors, Interferon Stimulated Genes, ZMPSTE24, Porcine Reproductive and Respiratory Syndrome Virus (PRRSV)
<b>Abstract</b>	<p>The main aim of this study was to elucidate the role of host restriction factor- ZMPSTE24 in Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) replication. Exogenously expressed ZMPSTE24 reduced PRRSV replication, confirming the antiviral role of this protein. In addition, siRNA induced knockdown of endogenous ZMPSTE24 slightly affected PRRSV replication. Confocal microscopy showed that over-expression of ZMPSTE24 did not significantly inhibit PRRSV entry at 3 hour post infection. Cells over-expressing ZMPSTE24 showed little co-localization with PRRSV at 24 h post infection. Amphotericin B did not restore the replication of PRRSV in cells over-expressing ZMPSTE24. These findings suggest that ZMPSTE24 restricts PRRSV infection at the post-entry step. In future studies, the mechanism by which ZMPSTE24 restricts PRRSV infection will be investigated. These findings may be useful to improve our understanding on PRRSV and host restriction factor interaction.</p>
<b>Introduction</b>	<p>Host's restriction factors such as the interferon (IFN) stimulated genes (ISGs) block viruses at various stages of their replication cycle (1-3). The zinc metalloprotease ZMPSTE24 is a host's natural restriction factor that has been shown to broadly inhibit the replication of a number of enveloped RNA and DNA viruses (4, 5). The ZMPSTE24 protein is important in the processing of the precursor protein prelamin A to lamin A in the nuclear lamina (6). ZMPSTE24 acts as a downstream effector of the interferon-induced transmembrane protein 3 (IFITM3) and has been shown to be important in the restriction of Influenza A virus (IAV) and several other enveloped viruses (4, 5). Porcine reproductive and respiratory syndrome virus (PRRSV) is an enveloped RNA virus. Following attachment to the cell surface</p>

## Materials and Methods

receptor CD163, PRRSV enters cells by receptor mediated endocytosis and undergoes uncoating within endosomes at low pH (7). Little is known as to how ZMPSTE24 affect PRRSV replication.

MARC-145 cells were transfected with either pCMV-3Tag-8 control vector, or pCMV-3Tag-8 ZMPSTE24 (ZMPSTE24-FLAG) plasmid using Lipofectamine 3000 (Invitrogen) and then infected with PRRSV SD-23983 at an MOI of 1 for 24 hr. Western blot and TCID<sub>50</sub> assays were performed to confirm the expression of the desired proteins and to determine the virus titer. For western blot, the following primary antibodies were used: anti-Flag 1o Ab against ZMPSTE24 at 1:1000 dilution, anti-PRRSV SR-30 1o monoclonal Ab at 1:300 dilution, and mouse beta actin Ab at 1:5000 dilution. The membrane was then incubated with anti-mouse IRD 2o Ab diluted to 1:5000 in 1xPBST. The specific proteins were detected as follows: beta actin -42 KD, Flag tagged ZMPSTE24-55KD, and PRRSV Nucleocapsid (N) protein-13 KD.

Next, siRNA induced knockdown of ZMPSTE24 was performed to study the roles of the endogenous restriction factor on PRRSV replication. For siRNA induced knockdown study, MARC145 cells were transfected with either negative control siRNA or ZMPSTE24 siRNA at 50 uM concentration using Lipofectamine RNAi max reagent (Invitrogen) and then infected with PRRSV SD-23983 at an MOI of 1 for 24 hr. Silencing of ZMPSTE24 gene was confirmed by RT-PCR. Quantification of PRRSV RNA copies was also done by RT-PCR. Primers specific to mouse beta actin housekeeping gene, PRRSV N and ZMPSTE24 were used for gene amplification.

Immunofluorescence assay and flow cytometry was performed to test if Amphotericin B can restore PRRSV replication in MARC145 cells overexpressing ZMPSTE24. To each of the three wells transfected with vector control ZMPSTE24 or ZMPSTE24-Flag, cells were treated with Amphotericin B and incubated at 37oC for 1 hr. Cells were infected with PRRSV SD-23983 at an MOI of 1 for 24 hr, then fixed, stained with FITC conjugated PRRSV Ab specific to N protein (SDOW-17) and then observed by immunofluorescence microscopy. MARC145 cells were harvested and then analyzed by flow cytometry to estimate the mean fluorescence intensity.

The role of ZMPSTE24 in restricting PRRSV entry was studied. Briefly, MARC145 cells cultured in an 8-chamber slide were transfected with either vector control ZMPSTE24 or ZMPSTE24-Flag. For 3 hr post infection (p.i.) study, PRRSV SD-23983 at an MOI of 4 was added to each well. For 24 p.i.

# Role of Host Restriction Factors in Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) Replication (Continued)

study, MARC145 cells were infected with PRRSV at an MOI of 1. The two slides were fixed in 3.8% formaldehyde and 0.2% Triton x-100 and then incubated with 5% goat serum for blocking non-specific antibody binding. Cells were incubated with mouse monoclonal Anti-Flag primary antibody against ZMPSTE24 (9A3) (Cell Signaling Technology) at a dilution of 1:1600. Then the slides were washed in 1xPBS and then incubated for 1 hr at 37°C with the secondary antibody. Alexa fluor 647 conjugated goat anti-mouse IgG 2<sup>o</sup> Ab (abcam) at 1:200 dilution and anti-PRRSV FITC conjugated Ab (SDOW17) at 1:80 dilution were added to each well, then counterstained with DAPI and mounted. Colocalization of PRRSV and ZMPSTE24 at 3 hr or 24 hr p.i was studied using confocal microscopy.

## Results

Over-expression of ZMPSTE24 reduced PRRSV replication, as confirmed by western blot and TCID<sub>50</sub> assays. RT-PCR analysis showed that silencing of endogenously expressed ZMPSTE24 slightly affected PRRSV replication. To elucidate the role of ZMPSTE24 in restricting PRRSV entry, co-localization study was performed using confocal microscopy. At 3 h post infection, there was no significant difference in the percentage of PRRSV positive cells in ZMPSTE24 overexpressing cells as compared to the vector control transfected cells. Similarly, there was no significant difference in the fold change in PRRSV RNA between vector control and ZMPSTE4 transfected cells as confirmed by RT-PCR. Further, cells over-expressing ZMPSTE24 showed little or no co-localization with PRRSV at 24 h post infection. Amphotericin B did not restore the replication of PRRSV in cells over-expressing ZMPSTE24 as confirmed by flow cytometry. Cytotoxicity assay confirmed that ZMPSTE24 overexpression or knockdown did not affect cell viability.

## Discussion

In this study, we confirmed the antiviral role of ZMPSTE24 in PRRSV replication. Our findings suggest that ZMPSTE24 does not affect PRRSV entry into MARC-145 cells. ZMPSTE24 blocked virus infection at the post entry step as PRRSV infection was significantly reduced at 24 hour post infection. Amphotericin B is an antifungal drug known to restore membrane fluidity, thereby reversing the effects of interferon-induced transmembrane protein 3 (IFITM3) mediated virus restriction (8, 9). In one study, ZMPSTE24 was shown to act as a downstream effector of IFITM3 (4, 5). Therefore, we tested if Amphotericin B can also reverse the antiviral effect of ZMPSTE24. However, in this study, Amphotericin B treatment did not restore PRRSV replication, suggesting that ZMPSTE24 may utilize a different mechanism to restrict PRRSV infection. There are only limited studies demonstrating

the role of ZMPSTE24 in restricting virus replication. In an earlier study, ZMPSTE24 was shown to restrict nuclear entry of IAV by trapping the virus within the highly acidic endolysosomal compartment (4). In future studies, it will be useful to test if ZMPSTE24 restricts PRRSV by blocking its release from the endosome. Studying PRRSV colocalization with early or late endosomes at different time points may reveal more information on this. Previous studies have demonstrated that PRRSV uses tunneling nanotubes (TNTs) for intercellular spread (10). It will be useful to test if ZMPSTE24 restricts PRRSV through its effect on TNTs and if it inhibits PRRSV by limiting intercellular spread. In future studies, investigating the role of ZMPSTE24 in PRRSV replication at the post-entry step may reveal more knowledge on this restriction factor. These studies may improve our understanding of PRRSV infectivity and host-pathogen interaction.

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# Shedding and Transmission of a Lineage One Modified Live Porcine Reproductive and Respiratory Syndrome Virus Vaccine

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**Key Words** Shedding, Transmission, Viremia, Sentinels, PRRSV

**Abstract** Porcine reproductive and respiratory syndrome virus (PRRSV) causes annually about \$1 billion in the US swine industry. PRRSV control has been inadequate because of the genetic variability, lack of broad cross protection, and continuous shedding and transmission of PRRSV vaccine-like strains. Two shedding and transmission studies are summarized to characterize potential shedding and transmission of a recently USDA licensed vaccine. Understanding shedding and transmission may help veterinarians, researchers and producers make more informed decisions when evaluating modified live (MLV) PRRS vaccine. The two studies are similar but serve for different goals: Study A is a 21 day study of vaccinated and sentinel pigs observed clinically and diagnostically; the results indicated a limited shedding without a transmission event. To better evaluate the shedding and transmission, a 70-day study was conducted to define when vaccine induced viremia would cease and if shedding and transmission would be observed. In this study a competitor's vaccine was used to evaluate the difference in the shedding and transmission of vaccine virus. The 21 day study demonstrated Prevacent did not transmit to sentinel pigs. The 70 day study demonstrated Prevacent sheds for 28 days but the trend would indicate up to 50 days is possible. The competitor's MLV vaccine sheds for at least 45 days, but the full extent of transmission could not be determined because all sentinel pigs were infected and removed by day 45. These results demonstrate difference between the vaccines which may be important in limiting vaccine like viral shedding.

**Introduction** Porcine reproductive and respiratory syndrome virus (PRRSV) causes annual losses of approximately \$1 billion in the US swine industry (Holtkamp et al.). To date the control of PRRSV has been inadequate because of the virus's inherent genetic variability, lack of broad cross protection, and continuous shedding and transmission of PRRSV vaccine-like strains. Genetic evaluations of PRRSV2 ORF 5 into ancestral groups show 9 families or lineages circulating worldwide (Shi et al.). USDA requires a disclaimer

that the potential for transmission exists with PRRSV modified live vaccine. Elanco has developed a PRRSV modified live vaccine which is licensed for the control of the respiratory and reproductive phases of PRRSV. The experimental PRRSV modified live vaccines underwent a 21 day study to determine the extent of shedding and transmission of the modified live vaccine strain. Herein is reported a 21 day shedding and transmission study (study A) and a seventy-day shedding and transmission study (study B) to better define the period of transmission and shedding of Prevacent® PRRS.

**Methods** **Study A:** Twenty 14-day old PRRSV seronegative and reverse transcriptase quantitative PCR (RTqPCR) negative piglets from a PRRSV naïve sow herd were randomly allocated to vaccine or sentinel groups on day -1 of the study. Two pigs from the vaccine group were placed with two pigs from the non-vaccinated sentinel group in one of five 4'x 5' pens in the Biosafety Label (BSL) 2 facility on day -1. On day 0 the vaccine group received 1 ml intramuscular injection of 8.2 log<sub>10</sub> TCID<sub>50</sub>/ml experimental vaccine derived via cell passage from strain PRRSV SD 11-21. Blinding was accomplished through separation of the personnel administering the vaccine from those collecting the following samples. Nasal swabs and serum were collected on day -1, day 3, day 5, day 7, day 10, day 14, day 17, and day 21. Iowa State Veterinary Diagnostic Laboratory performed RT-qPCR, on both specimens. On day 21 all pigs were humanely euthanized and necropsied. The lungs were harvested, and an aseptic sample of bronchial alveolar lavage fluid (BALF) was collected and tested via PRRSV RTqPCR from each pig. In addition, tissues samples consisting of lung, spleen, tonsil, right and left tracheobronchial lymph nodes were collected and tested via RTqPCR for PRRSV. Any positive sample was genetically sequenced using the ORF5 region and compared to the ORF5 region of the experimental vaccine.

The statistical analysis was descriptive only. The case definition for shedding in the vaccinated group was an RTqPCR positive on nasal swab. The case definition of transmission was if a sentinel pig tested RTqPCR positive on nasal swab, serum, and either BALF or tissue.

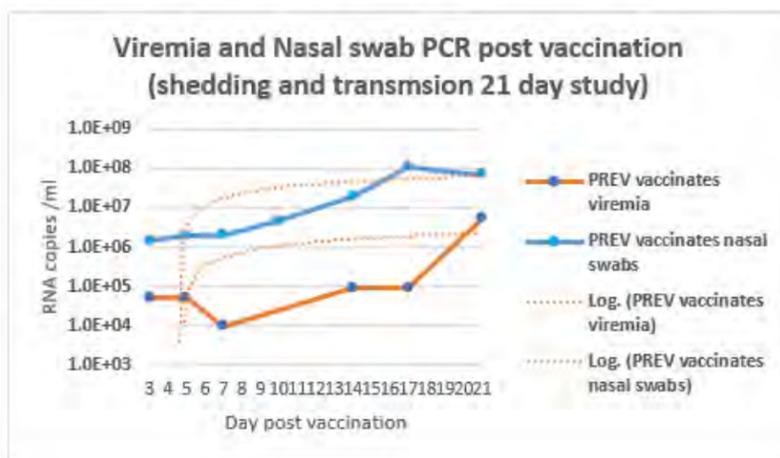
**Study B:** Forty 17 to 18 day old weaned PRRSV seronegative and RTqPCR negative from a PRRSV naïve sow herd allotted to two BSL 2 rooms using a randomized complete block design for 4 treatment groups: group 1) Unvaccinated controls (sentinel) placed with vaccine CV (Competitor's vaccine), group 2) Vaccinated with vaccine CV placed together in room 1, group 3) Unvaccinated controls (sentinel) placed with vaccine PREV

# Shedding and Transmission of a Lineage One Modified Live Porcine Reproductive and Respiratory Syndrome Virus Vaccine (Continued)

(Prevacent® PRRS, Elanco AH), group 4) Vaccinated with vaccine PREV placed together in room 2. Each room had 2 pens of 10 pigs (5vaccinated and 5 sentinels per pen) with vaccines separated by room. At placement (day 0) all vaccinated pigs were vaccinated according to label directions. Sentinel piglets received 1 ml of sterile saline as a placebo. Serum and nasal swabs were collected weekly (11 collections) through the study end on day 70 for serological (Idexx PRRSX# Ab) and/or RTqPCR. Oral fluids were collected every two weeks starting on day 0 through the ending day 70 (6 collections). Individual rectal temperature was collected on days 0, 1, 3, 7 and 10 and individual pigs were weighted weekly. Sentinel pigs were removed from the study when collected samples for PRRSV analysis demonstrated one of the following criteria: RTqPCR positive for two consecutive nasal swab samples or any single serum sample. Removal was within 3 to 4 days, with one exception of 10 days of one pig in group 3.

**Study A:** Nine out of 10 (90%) of the vaccinated pigs had at least one nasal swab positive during the 21 day study. Only one nasal swab was positive in the sentinel group and no sera tested positive during the 21 day study.

All vaccinated pigs were RTqPCR positive in sera (viremic) on at least one sampling day from day 3 to day 21 (Figure 1). All vaccinated pigs were RTqPCR positive via nasal swabs (shedding) on day 21 (Figure 1). The overall average estimated viremic load (standard deviation) of all vaccinated pigs on day 21 was  $6.8 \times 10^7$  RNA copies/ml ( $1.8 \times 10^8$  RNA copies/ml).



**Figure 1.** All PREV vaccinates were RTqPCR positive (viremic) during the 21 day study. All were viremic on day 21. Nasal swabs were intermittently positive with 44.4% positive (shedding) on day 21.

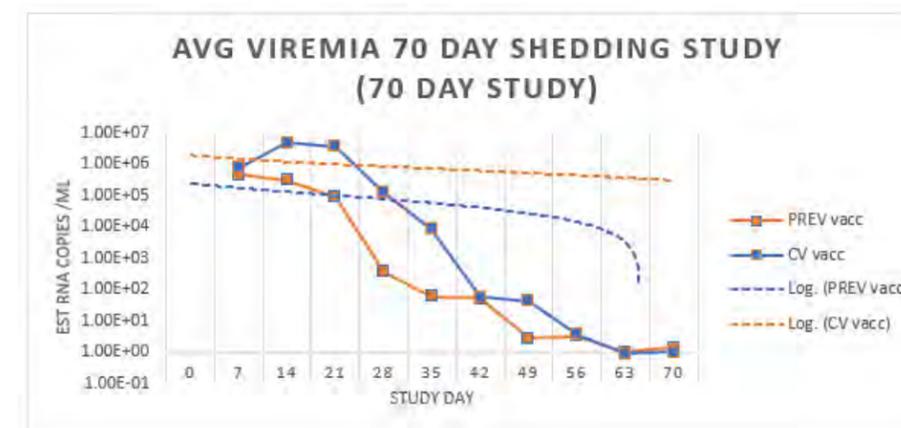
## Results and Discussions

All BALF and tissues were RTqPCR positive in the vaccinated group on day 21.

The ORF5 sequence was 99.7 to 100% identical to the experimental vaccine on 60 tissue samples from the vaccinated group. No tissue or BALF was RTqPCR positive in the sentinel group.

Although the vaccinated group was viremic and nasal swabs were intermittently RTqPCR positive throughout the study, no evidence of transmission was identified in the sentinel group. No evidence of genetic variation was found in the 21-day study as demonstrated through the high degree of homology found in the ORF5 genetic sequence.

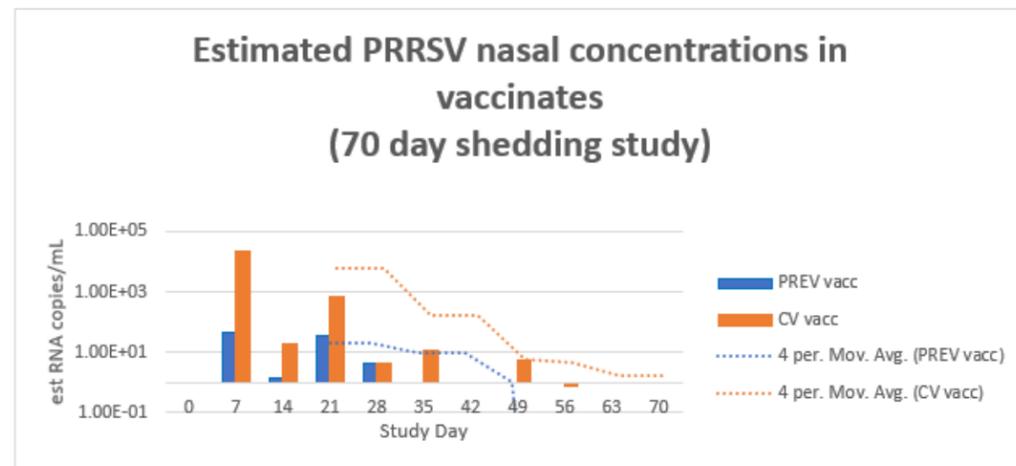
**Study B:** All vaccinated pigs became viremic. The average viral loads (RNA copies/ml) of serum in the PREV vaccine group were significantly lower ( $P < 0.05$ ) compared to CV vaccinated group on study days 14, 21, 28 and 35 (Figure 2).



**Figure 2.** Prev Vaccinated pigs had significantly lower RNA copies/ml compared to the CVvaccinated pigs on days 14, 21, 28, and 35. Logarithmic trend lines were calculated in Excel. The trendlines estimate expected viremia of each vaccinated group.

# Shedding and Transmission of a Lineage One Modified Live Porcine Reproductive and Respiratory Syndrome Virus Vaccine (Continued)

RTqPCR with nasal swabs were sporadically positive in both vaccinated groups as indicated in Figure 3.



**Figure 3.** Estimated RNA copies/ml in nasal swabs. The 4 period moving average was calculated in Excel and estimated decay of RNA copies/ml over the 4 period moving average.

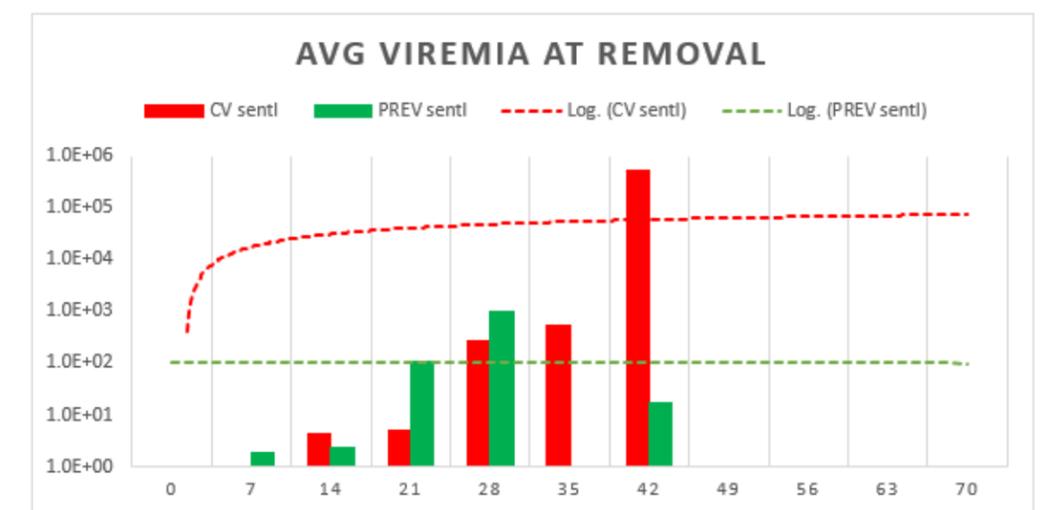
The 4 period moving average demonstrates decay of estimated nasal RNA copies/ml of each group. The PREV vaccinated group goes below zero while the CV vaccinated group does not at day 70 (end of study) although at trend line estimates very low levels.

Sentinel pig removal is illustrated in figure 4. Sentinel pigs housed with vaccine PREV or vaccine CV had 90% and 100% removal in each group, respectively. Merging the shedding data (ie nasal swab result) with the transmission data (ie removals) indicates vaccine PREV stopped shedding at around day 42 as no further transmission events were detected and one sentinel remained throughout the 70-day study. Because vaccine CV had all sentinels removed by day 42 no further transmission events could be detected but the shedding data would indicate further transmission events would have been possible. A more robust comparison would have been possible if replacement of sentinel pigs were added upon removal. Field experience and a literature review indicated extended shedding with vaccine CV.



**Figure 4.** Cumulative sum of removals over the 70 day study. All CV sentinels were all removed by day 42. One Prev sentinel remained until day 70 (end of study).

Figure 5 depicts average estimated RNA copies/ml of pigs removed by day of removal. As time progressed viremia in removed sentinels increase within both groups although the duration and peak appear differences. This is substantiated by the logarithmic trend lines generated for the data. Again, the extent and duration of this observation was truncated because no replacement sentinels were used in the study. The use of replacement sentinels (ie replacement of a sentinel upon removed because of a defined transmission event) would have better defined the observations.



**Figure 5.** Average estimated RNA copies/ml at removal for each sentinel group. All removed sentinels were removed because of serum RTqPCR positive (viremia).

# Shedding and Transmission of a Lineage One Modified Live Porcine Reproductive and Respiratory Syndrome Virus Vaccine (Continued)

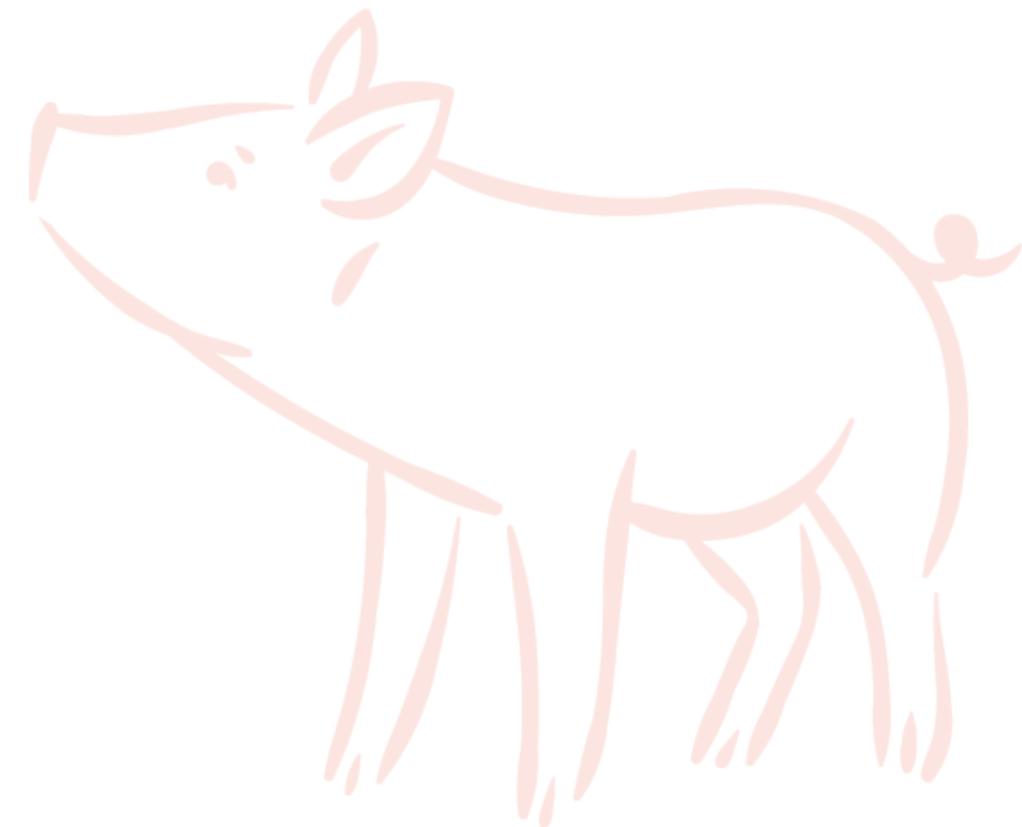
Although Study A showed minimal transmission, an interesting finding is that all PREV vaccinated pigs were still viremia and shedding at day 21 or the end of the study. Although limited by study design, these studies may demonstrate differences between two vaccines.

## Acknowledgements

JMH, BJK, and SW are employed by Elanco and have been involved in the development, licensing, and post marketing development of Prevacent® PRRS.

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# Annual Station Report: Project NC-229

## Iowa State University

<b>Institution/Station</b>	Iowa State University, Ames, IA 50011
<b>Period Covered</b>	November 30, 2019 to November 1, 2020
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- Zhiyi Zheng, Graduate Student, zyzheng@iastate.edu

### Objective 1. Control of PRRSV

#### 1.1. PRRS immunology/vaccinology:

ISU evaluate the effect of PRRS on different immune-tissues

#### 1.2. PRRS epidemiology:

- ISU evaluate potential NSp2 recombination patten in US and China
- ISU evaluated on the prediction of seasonal patten PRRSV detection
- ISU evaluate vaccination traits to predict reproductive performance.
- ISU evaluate the correlation of PRRSV exposure in sow herd with shedding in nursery

#### 1.3. PRRS Surveillance and Diagnostics:

- ISU worked on guidelines for the use of oral fluid for surveillance
- ISU worked in the field application of oral fluid ELISA
- ISU evaluate the application of PRRSV detection on processing fluids
- ISU evaluate different cell lines to improve PRRSV isolation
- ISU development of a bead-based assay for detection and differentiation of field and vaccine strains

### Objective 2. Developing effective and efficient approaches for detection, prevention and control of pressing viral diseases of swine of recent emergence

#### 2.1. ASFV:

## Annual Station Report: Project NC-229 Iowa State University (Continued)

### 2.2. Swine Influenza Virus:

ISU collected evidence of reassortment with vaccine and field strains

### 2.3. Porcine Circovirus:

- ISU Develop animal model to understand the pathogenicity of PCV3
- ISU collaborate with detection of PCV3 on other swine producing countries

### 2.4. Swine Pestiviruses:

### 2.5. Senecavirus:

- ISU evaluate UV light to deactivate SVA on swine farms
- ISU evaluated potential risks factor for the emergency of SVA clinical signs in packing plants

### 2.6. Sapelovirus:

### 2.7. Viruses with potential interest to Xeno-transplantation science:

1. PRRSV: ISU was able to evaluate the seasonal pattern of PRRSV detection. Importantly for producer ISU is providing a monthly report presenting actual disease trends and forecasting potential disease outbreaks. In addition, ISU was also able to generate information related to the effect of vaccination and reproductive performance and well as the impact of sow herd exposure on nursery pigs shedding. ISU has been working intensively in the application of oral fluids in field conditions as well as developing guidelines for its use for routine surveillance. Finally, ISU also worked in the development of a bead-base assay to differentiate field and vaccine strains.
2. IAV: Investigate and provide information regarding the potential reassortment of current commercial vaccine and ongoing field strains.
3. PCV2: ISU has been working to provide more information regarding the role of PCV3 in clinical disease. Multiple animal models were evaluated to help producers understand the role of PCV3 infection and the interpretation of current field diagnostic results.
4. SVA: Important information for producers has been generated related to viral inactivation on swine farms using direct UV light. In addition, risk factors that can trigger potential SVA breaks in packing plants have been investigated that can provide new strategies to prevent the economical burden of breaks of vesicular disease.

### Pertinent (Swine Virology) Publications Issued or "In Press"

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### Impact and Value of Research to Stakeholder

## Annual Station Report: Project NC-229 Iowa State University (Continued)

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#### Non-refereed Articles

1. PCV2 update: Pathogen, the disease and the diagnosis. Piñeyro P, Zhang J, Harmon K, and Gauger P. December 2020. *National Hog Farmer* ([www.nationalhogfarmer.com](http://www.nationalhogfarmer.com)).
2. Gauger P/ Emerging diseases in swine: past, present, and the future. April 2020. *National Hog Farmer* ([www.nationalhogfarmer.com](http://www.nationalhogfarmer.com)).
3. Chen T, Zimmerman J, Giménez -Lirola L. Detection of pseudorabies virus antibody in swine oral fluid. April 2020. *National Hog Farmer* ([www.nationalhogfarmer.com](http://www.nationalhogfarmer.com)).
4. Henao-Diaz A, Zhang M, Giménez-Lirola L, Gauger P, Baum D, Clavijo MJ, Rotolo M, Ramirez E, Main R, Zimmerman J. November 2020. Adapting the PRRS oral fluid ELISA to routine surveillance. *National Hog Farmer* ([www.nationalhogfarmer.com](http://www.nationalhogfarmer.com)).
5. Henao-Diaz A, Baum D, Giménez-Lirola L, Zimmerman J. November 2020. The invisible life of PRRS virus and what it means for disease diagnosis <Невидимая жизнь вируса РРСС и что это означает для диагностики заболевания>. *АПК Эксперт <АПК Эксперт>* 11:34-35.
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## Annual Station Report: Project NC-229 Iowa State University (Continued)

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  10. Trevisan G. et al. Swine Disease Reporting System – Report #24. Swine Health Information Center. February, 2020; Available at <https://www.swinehealth.org/domestic-disease-surveillance-reports>
  11. Trevisan G. et al. Swine Disease Reporting System – Report #25. Swine Health Information Center. March, 2020; Available at <https://www.swinehealth.org/domestic-disease-surveillance-reports>
  12. Trevisan G. et al. Swine Disease Reporting System – Report #26. Swine Health Information Center. April, 2020; Available at <https://www.swinehealth.org/domestic-disease-surveillance-reports>
  13. Trevisan G. et al. Swine Disease Reporting System – Report #27. Swine Health Information Center. March, 2020; Available at <https://www.swinehealth.org/domestic-disease-surveillance-reports>
  14. Trevisan G. et al. Swine Disease Reporting System – Report #28. Swine Health Information Center. May, 2020; Available at <https://www.swinehealth.org/domestic-disease-surveillance-reports>
  15. Trevisan G. et al. Swine Disease Reporting System – Report #29. Swine Health Information Center. June, 2020; Available at <https://www.swinehealth.org/domestic-disease-surveillance-reports>
  16. Trevisan G. et al. Swine Disease Reporting System – Report #30. Swine Health Information Center. July, 2020; Available at <https://www.swinehealth.org/domestic-disease-surveillance-reports>
  17. Trevisan G. et al. Swine Disease Reporting System – Report #31. Swine Health Information Center. August, 2020; Available at <https://www.swinehealth.org/domestic-disease-surveillance-reports>
  18. Trevisan G. et al. Swine Disease Reporting System – Report #32 Swine Health Information Center. September, 2020; Available at <https://www.swinehealth.org/domestic-disease-surveillance-reports>
  19. Trevisan G. et al. Swine Disease Reporting System – Report #33 Swine Health Information Center. October, 2020; Available at <https://www.swinehealth.org/domestic-disease-surveillance-reports>
  20. Trevisan G. et al. Swine Disease Reporting System – Report #34 Swine

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### Funding Sources

1. USDA Hatch/Multi State
2. USDA/NIFA
3. USDA/ARS
4. Swine Health Information Center
5. National Pork Board

### Work Planned for Next Year

### Changes/ Problems Due to COVID-19

# Annual Station Report: Project NC-229

## Kansas State University

<b>Institution/Station</b>	Kansas State University, Manhattan, KS 66506
<b>Period Covered</b>	November 30, 2019 to November 1, 2020
<b>Personnel</b>	<p><b>NC-229 Station Representative:</b></p> <ul style="list-style-type: none"><li>Jishu Shi, Professor, <a href="mailto:jshi@vet.k-state.edu">jshi@vet.k-state.edu</a></li></ul> <p><b>Other Principal Leaders Associated With the Projects:</b></p> <ul style="list-style-type: none"><li>Megan Niederwerder, Assistant Professor, <a href="mailto:mniederwerder@vet.k-state.edu">mniederwerder@vet.k-state.edu</a></li></ul> <p><b>Staff and Students:</b></p> <ul style="list-style-type: none"><li>Rachel Madera, Senior Scientist, <a href="mailto:rachelmadera@vet.k-state.edu">rachelmadera@vet.k-state.edu</a></li><li>Lihua Wang, Research Assistant Professor, <a href="mailto:lihua@vet.k-state.edu">lihua@vet.k-state.edu</a></li><li>Yuzhen Li, Research Assistant, <a href="mailto:yuzhen@vet.k-state.edu">yuzhen@vet.k-state.edu</a></li><li>Travis Foland, Research Assistant, <a href="mailto:tfoland@vet.k-state.edu">tfoland@vet.k-state.edu</a></li><li>Pratiksha Khanal, Graduate Research Assistant, <a href="mailto:pratiksha@vet.k-state.edu">pratiksha@vet.k-state.edu</a></li></ul>
<b>Progress of Work and Principal Accomplishments</b>	<p><b>Objective 1. Control of PRRSV</b></p> <p><b>1.1. PRRS immunology/vaccinology:</b> We have conducted one PRRS vaccine animal challenge study to evaluate the efficacy of an experimental vaccine, the study was terminated prematurely due to COVID-19 related university operation shutdown. We have done one in vitro anti-viral compound screening study and identified a small molecule compound that can block the replication of PRRSV in MARC-145 cells. We also evaluated the therapeutic efficacy of an anti-inflammatory compound in pigs challenged with high path PRRS virus. This study is still ongoing. Results will be available in 2021.</p> <p><b>1.2. PRRS epidemiology:</b></p> <p><b>1.3. PRRS Surveillance and Diagnostics:</b> We have identified beneficial gut microorganisms associated with improved health and growth outcomes in pigs co-infected with PRRSV and PCV2, such as increased weight gain, reduced virus replication, less antimicrobial treatment, and decreased pathology. Fecal microbiota transplantation has been utilized as a preventative medicine tool to improve subsequent outcome during PRRS-associated polymicrobial respiratory disease. Further, we have identified microbiome characteristics associated with improved PRRS vaccine response.</p>

### Objective 2. Developing effective and efficient approaches for detection, prevention and control of pressing viral diseases of swine of recent emergence

#### 2.1. ASFV:

Using ASF Georgia 2007 and Vietnam 2019, we have created three gene-deleted mutants as experimental vaccines against ASF. We will test the efficacy and safety of these vaccines in 2021. In addition, we are developing monoclonal antibodies against various ASFV proteins with the intention to develop DIVA assays for the ASF vaccine developed by USDA ARS and our own lab. We have received some serum samples from pigs infected with ASFV in Vietnam. Immunological analysis of these samples will be conducted in 2021.

We have characterized ASFV decay during transoceanic shipment conditions in 9 feed ingredients commonly imported into the U.S. From the ASFV decay curves, we have calculated half-life estimates with SE and CI for recommended feed ingredient storage time. We have investigated chemical mitigation strategies, such as the use of formaldehyde and medium chain fatty acid based feed additives, for inactivating ASFV in feed ingredients. Further, we defined the dose-dependent ASFV inactivation curves for each feed additive in cell culture.

#### 2.2. Swine Influenza Virus:

#### 2.3. Porcine Circovirus:

#### 2.4. Swine Pestiviruses:

We have developed a competitive ELISA that can differentiate pigs vaccinated with the C-strain CSF vaccine from pigs infected with wildtype CSF viruses or other swine viruses.

Using a transoceanic shipment model, we identified what feed ingredients support CSFV stability during environmental import conditions from Asia.

#### 2.5. Senecavirus:

#### 2.6. Sapelovirus:

#### 2.7. Viruses with potential interest to Xeno-transplantation science:

## Annual Station Report: Project NC-229 Kansas State University (Continued)

### Impact and Value of Research to Stakeholder

1. Novel PRRS vaccine is being evaluated as new tools for PRRS prevention and control in regions with highly pathogenic PRRSV.
2. The anti-PRRSV and anti-inflammatory compounds may be used as experimental tools in studies employing PRRSV infection as a model for human COVID-19.
3. DIVA assays are CSF and ASF vaccines so that they can be used on swine farm to differentiate vaccinated pigs from animal infected with the wildtype CSFV or ASFV.
4. The swine microbiome provides an opportunity to improve the growth and health outcomes of pigs with respiratory and other viral diseases. Our work is aimed at identifying beneficial organisms from the gut microbiomes of pigs with improved health outcomes after viral disease challenge. Microbiome therapeutics may be used as preventative medicine tools to reduce the effects of viral infections and decrease the need for antimicrobials in swine.
5. Defining the risk and mitigation of African swine fever virus and other emerging viral diseases of swine in feed provides the opportunity to implement interventions for preventing introduction and spread of foreign viral diseases into the U.S. herd. We are identifying high-risk ingredients commonly imported in the U.S. that support virus stability, and investigating physical and chemical mitigation strategies, such as feed additives with antimicrobial activity, to inactivate viruses in feed.

### Refereed Publications

1. Shi, J., L. Wang, and D.S. McVey. 2020. Of pigs and men: the best laid plans for prevention and control of swine fevers. *Animal Frontiers* (Invited review and accepted for publication).
2. Wang, L., R. Madera, Y. Li, D.S. McVey, B.S. Drolet, and J. Shi. 2020. Recent advances in diagnosis of classical swine fever and future perspectives. *Pathogens*. 9(8), 658; <https://doi.org/10.3390/pathogens9080658>
3. Wang, L., S. Mi, R. Madera, L. Ganges, M.V. Borca, J. Ren; C. Cunningham, A.G. Cino-Ozuna, H. Li, C. Tu, W. Gong; and J. Shi. 2020. A neutralizing monoclonal antibody-based competitive ELISA for classical swine fever C-strain post-vaccination monitoring. *BMC Veterinary Research*. Open Access, Published online Jan. 14, 2020. <https://link.springer.com/article/10.1186/s12917-020-2237-6>
4. Borca, M.V., V. O'Donnell, L.G. Holinka, G.R. Risatti, E. Ramirez-Medina, E.A. Vuono, J. Shi, S. Pruitt, A. Rai, E. Silva, L. Velazquez Salinas, and D.P. Gladue. 2020. Deletion of CD2-like gene from the genome of African

swine fever virus strain Georgia does not attenuate virulence in swine. *Scientific Reports*. 10:494. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6965178/>

5. Evans, T.S., Z. Shi, M. Boots, W. Liu, K. Olival, X. Xiao, S. Vandewoude, H. Brown, J. Chen, D. Civitello, L. Escobar, Y. Grohn, H. Li, K. Lips, Q. Liu, J. Lu, B. Martinez-Lopez, J. Shi, X. Shi, B. Xu, L. Yuan, G. Zhu, and W. Getz. 2020. Synergistic China-US Ecological Research is Essential for Global EID Preparedness. *EcoHealth*, Open Access, Published online Feb. 3, 2020. <https://link.springer.com/article/10.1007/s10393-020-01471-2>
6. Zhang, H., Y. Wang, E. Porter, N. Lu, Y. Li, F. Yuan, M. Lohman, L. Noll, W. Zheng, C. Stoy, Y. Lang, V. Huber, W. Ma, L. Peddireddi, Y. Fang, J. Shi, G. Anderson, X. Liu, J. Bai. 2019. Development of a multiplex real-time RT-PCR assay for simultaneous detection and differentiation of influenza A, B, C, and D viruses. *Diagnostic Microbiology and Infectious Disease*. 95(1):59-66.
7. Patterson G, Niederwerder MC, Spronk G, Dee SA. Quantification of soya-based feed ingredient entry from ASFV-positive countries to the United States by ocean freight shipping and associated seaports. *Transbound Emerg Dis*. 2020 Oct 16. doi: 10.1111/tbed.13881. Epub ahead of print. PMID: 33064921.
8. Sanglard LP, Fernando RL, Gray KA, Linhares DCL, Dekkers JCM, Niederwerder MC, Serão NVL. Genetic Analysis of Antibody Response to Porcine Reproductive and Respiratory Syndrome Vaccination as an Indicator Trait for Reproductive Performance in Commercial Sows. *Front Genet*. 2020 Sep 11;11:1011. doi:10.3389/fgene.2020.01011. PMID: 33024439; PMCID: PMC7516203.
9. Dee SA, Niederwerder MC, Edler R, Hanson D, Singrey A, Cochrane R, Spronk G, Nelson E. An evaluation of additives for mitigating the risk of virus-contaminated feed using an ice-block challenge model. *Transbound Emerg Dis*. 2020 Jul 24. doi: 10.1111/tbed.13749. Epub ahead of print. PMID: 32706431.
10. Niederwerder MC, Dee S, Diel DG, Stoian AMM, Constance LA, Olcha M, Petrovan V, Patterson G, Cino-Ozuna AG, Rowland RRR. Mitigating the risk of African swine fever virus in feed with anti-viral chemical additives. *Transbound Emerg Dis*. 2020 Jul 2. doi: 10.1111/tbed.13699. Epub ahead of print. PMID: 32613713.
11. Dee S, Shah A, Cochrane R, Clement T, Singrey A, Edler R, Spronk G, Niederwerder M, Nelson E. Use of a demonstration project to evaluate viralsurvival in feed: Proof of concept. *Transbound Emerg Dis*. 2020 Jun 14. doi:10.1111/tbed.13682. Epub ahead of print. PMID: 32536022.

## Annual Station Report: Project NC-229 Kansas State University (Continued)

12. Dee SA, Niederwerder MC, Patterson G, Cochrane R, Jones C, Diel D, Brockhoff E, Nelson E, Spronk G, Sundberg P. The risk of viral transmission in feed: What do we know, what do we do? *Transbound Emerg Dis.* 2020 May 2. doi:10.1111/tbed.13606. Epub ahead of print. PMID: 32359207.
13. Sanglard LP, Schmitz-Esser S, Gray KA, Linhares DCL, Yeoman CJ, Dekkers JCM, Niederwerder MC, Serão NVL. Vaginal microbiota diverges in sows with low and high reproductive performance after porcine reproductive and respiratory syndrome vaccination. *Sci Rep.* 2020 Feb 20;10(1):3046. doi:10.1038/s41598-020-59955-8. PMID: 32080317; PMCID: PMC7033195.
14. Stoian AMM, Petrovan V, Constance LA, Olcha M, Dee S, Diel DG, Sheahan MA, Rowland RRR, Patterson G, Niederwerder MC. Stability of classical swine fever virus and pseudorabies virus in animal feed ingredients exposed to transpacific shipping conditions. *Transbound Emerg Dis.* 2020 Jul;67(4):1623-1632. doi:10.1111/tbed.13498. Epub 2020 Feb 9. PMID: 31999072.
15. Wang Y, Noll L, Lu N, Porter E, Stoy C, Zheng W, Liu X, Peddireddi L, Niederwerder M, Bai J. Genetic diversity and prevalence of porcine circovirus type 3 (PCV3) and type 2 (PCV2) in the Midwest of the USA during 2016-2018. *Transbound Emerg Dis.* 2020 May;67(3):1284-1294. doi: 10.1111/tbed.13467. Epub 2020 Jan 16. PMID: 31886622.
16. Sanglard LP, Schmitz-Esser S, Gray KA, Linhares DCL, Yeoman CJ, Dekkers JCM, Niederwerder MC, Serão NVL. Investigating the relationship between vaginal microbiota and host genetics and their impact on immune response and farrowing traits in commercial gilts. *J Anim Breed Genet.* 2020 Jan;137(1):84-102. doi:10.1111/jbg.12456. Epub 2019 Nov 25. PMID: 31762123.
17. Gebhardt JT, Thomson KA, Woodworth JC, Dritz SS, Tokach MD, DeRouchey JM, Goodband RD, Jones CK, Cochrane RA, Niederwerder MC, Fernando S, Abbas W, Burkey TE. Effect of dietary medium-chain fatty acids on nursery pig growth performance, fecal microbial composition, and mitigation properties against porcine epidemic diarrhea virus following storage. *J Anim Sci.* 2020 Jan 1;98(1):skz358. doi: 10.1093/jas/skz358. PMID: 31758795; PMCID: PMC6978897.
18. Stoian AMM, Zimmerman J, Ji J, Hefley TJ, Dee S, Diel DG, Rowland RRR, Niederwerder MC. Half-Life of African Swine Fever Virus in Shipped Feed. *Emerg Infect Dis.* 2019 Dec;25(12):2261-2263. doi: 10.3201/eid2512.191002. Epub 2019 Dec 17. PMID: 31524583; PMCID: PMC6874236.

### Abstracts or Proceedings

1. Sanglard, L.P., R.L. Fernando, K.A. Gray, D.C.L. Linhares, J.C.M. Dekkers, M.C. Niederwerder and N.V.L. Serão. 2020. Genomic relationship between antibody response to porcine reproductive and respiratory syndrome virus vaccination and reproductive performance in commercial sows. ASAS-CSAS-WSASAS Virtual Annual Meeting.
2. Dee, S., M. Niederwerder, G. Patterson, D. Diel, E. Nelson. 2020. Update on feed mitigation research. 51st Annual Meeting of the American Association of Swine Veterinarians, Atlanta, GA.
3. Niederwerder, M.C. 2020. Survival and transmission of foreign animal diseases in feed. 51st Annual Meeting of the American Association of Swine Veterinarians, Atlanta, GA.
4. Sanglard, L.P., R.L. Fernando, K.A. Gray, D.C.L. Linhares, J.C.M. Dekkers, M.C. Niederwerder, and N.V.L. Serão. 2020. Genomic basis of antibody response to porcine reproductive and respiratory syndrome virus vaccination. Midwest Animal Science Meeting, Omaha, NE.
5. Niederwerder, M.C. 2019. Role of the gut microbiome in porcine respiratory disease complex. Annual Finnish Veterinary Congress, Helsinki, Finland.
6. Niederwerder, M.C. 2019. Risk of feed in the introduction and transmission of viral diseases. Annual Finnish Veterinary Congress, Helsinki, Finland.
7. Sanglard, L.P., S. Schmitz-Esser, K.A. Gray, D.C.L. Linhares, C.J. Yeoman, J.C.M. Dekkers, M.C. Niederwerder, N.V.L. Serão. 2019. Association between vaginal microbiome and antibody response to PRRS vaccination in commercial gilts. Conference for Research Workers in Animal Disease, Chicago, IL.
8. Sanglard, L.P., S. Schmitz-Esser, K.A. Gray, D.C.L. Linhares, C.J. Yeoman, J.C.M. Dekkers, M.C. Niederwerder, N.V.L. Serão. 2019. Vaginal microbiome of PRRS-vaccinated gilts differs between animals with high and low farrowing performance. North American PRRS Symposium, Chicago, IL.
9. Sanglard, L.P., S. Schmitz-Esser, K.A. Gray, D.C.L. Linhares, C.J. Yeoman, J.C. Dekkers, M.C. Niederwerder, N.V.L. Serão. 2019. Association between vaginal microbiome and antibody response to PRRS vaccination in commercial gilts. North American PRRS Symposium, Chicago, IL.
10. Wang, Y., L. Noll, N. Lu, E.G. Porter, C.P.A. Stoy, W. Zheng, X. Liu, L. Peddireddi, M. Niederwerder, T.G. Nagaraja, J. Bai. 2019. Genotyping and Prevalence of Porcine Circovirus Type 3 (PCV3) and Type 2 (PCV2) in the Midwest of the USA during 2016-2018. USAHA-AAVLD Annual Meeting, Providence, RI.

## Annual Station Report: Project NC-229 Kansas State University (Continued)

11. Dee, S., M. Niederwerder, G. Patterson, D. Diel. 2019. Risk of ASFV and PEDV transmission in feed ingredients. USAHA Foreign and Emerging Diseases committee meeting, USAHA-AAVLD Annual Meeting, Providence, RI.

### Book Chapters or Monographs

### Non-refereed Articles

1. Niederwerder, M.C. 2020. Feed additives mitigate for ASFV. Invited author contribution, National Hog Farmer Blueprint. <https://www.nationalhogfarmer.com/nutrition/feed-additives-mitigate-asfv>
2. Niederwerder, M.C. 2020. Foreign animal disease: The risk in feed. Invited author contribution, National Hog Farmer Blueprint. <https://www.nationalhogfarmer.com/feed/foreign-animal-disease-risk-feed>
3. Niederwerder, M.C. 2019. Using gut microbes for respiratory relief. Invited author contribution, National Hog Farmer Blueprint. <https://www.nationalhogfarmer.com/animal-health/using-gut-microbes-respiratory-relief>

### Funding Sources

1. 2020-2021 Evaluation of Recombinant African swine fever (ASF) vaccines. Principal Investigator, Private company.
2. 2020-2021 Deconstructing The Role of Syngr2 In Viral Disease Susceptibility In Livestock. Principal Investigator, sub-award # 25-6226-0633-002 from University of Nebraska-Lincoln. USDA NIFA Award #2020-67015-31415 (PI: Daniel Ciobanu)
3. 2020-2021 Efficacy of NDT-30249 as an anti-inflammatory drug for LPS and PRRS virus-induced swine inflammation models. Principal Investigator, Private company.
4. 2020-2021 Evaluation of PRRSV compounds. Principal Investigator, Private company.
5. 2020-2021 Recombinant Adeno-associated virus as a long-acting passive antibody vaccine against foreign animal diseases. DHS Science and Technology. Co-Investigator with PI-Dr. Michael McIntosh (University of Florida).
6. 2020-2022 VAP Vector-enhanced Arboviral Vaccines, USDA ARS, #58-3030-9-020, Award #AR9786, Principle Investigator.
7. 2019-2020 Testing of a Novel Vaccine for PRRS. Principle Investigator, Private company.
8. 2019-2022 Novel Vaccines for African Swine Fever (ASF). An award

from the National Bio and Agro-Defense Facility (NBAF) Transition Fund. Principle Investigator.

9. 2019-2022 Actions Supporting the Development of an African Swine Fever Virus Live Attenuated DIVA Vaccine. USDA ARS, #58-8064-9-007, Award #AR9775, Principle Investigator.
10. 2019-2022 VAP Vector-enhanced Arboviral Vaccines, USDA ARS, #58-3030-9-020, Award #AR9786, Principle Investigator.
11. DeLong, Robert, Santosh Aryal, Kartik Ghosh, Megan C. Niederwerder. July 1, 2020 – June 30, 2021. RAPID – Impact of Coronaviridae lipid, protein and RNA interaction on copper, zinc, and their derivatives coated personal protective equipment surfaces and viral infectivity. National Science Foundation Nanoscale Interactions Program.
12. Dee, Scott, Roger Cochrane, Apoorva Shah, Megan Niederwerder. January 1, 2020 – December 31, 2020. Using demonstration projects to validate laboratory-based viral survival in feed. Swine Health Information Center.
13. Niederwerder, Megan. July 1, 2020 – June 30, 2023. Assessing The Microbiome As A Tool For The Mitigation Of Viral Disease In Nursery Pigs. USDA NIFA Agriculture and Food Research Initiative Foundational grant.
14. Diel, Diego, Megan Niederwerder, Bob Rowland, Jianfa Bai, Jianqiang Zhang. December 1, 2019 – February 1, 2021. Validating pathogen nucleic acid extraction from animal feed and feed ingredients. National Pork Board and Foundation for Food and Agriculture Research.
15. Niederwerder, Megan. May 1, 2019 – October 31, 2021. Investigating viral mitigation in feed. Private company and the State of Kansas National Bio and Agro-defense Facility Fund.
16. Jones, Cassandra, Steve Dritz, Jason Woodworth, Megan Niederwerder. March 1, 2019 – February 29, 2020. Assessing distribution and mitigation of Senecavirus A, a foot and mouth disease surrogate, in a swine feed mill. Swine Health Information Center.
17. Fang, Ying, Raymond Rowland, Daniel Rock, Megan Niederwerder, Yanhua Li, Bonto Faburay, Biao He. April 1, 2019 – March 31, 2020. Development of ASFV-specific monoclonal antibodies and mAb-based blocking ELISA. National Pork Board and Kansas National Bio and Agro-defense Facility Fund.
18. Niederwerder, Megan, Raymond Rowland. April 1, 2019 – March 31, 2021. Understanding the survivability and infectivity of African swine fever virus in various environments. National Pork Board, Foundation for Food and Agriculture Research, Private companies, State of Kansas National Bio and Agro-defense Facility Fund.

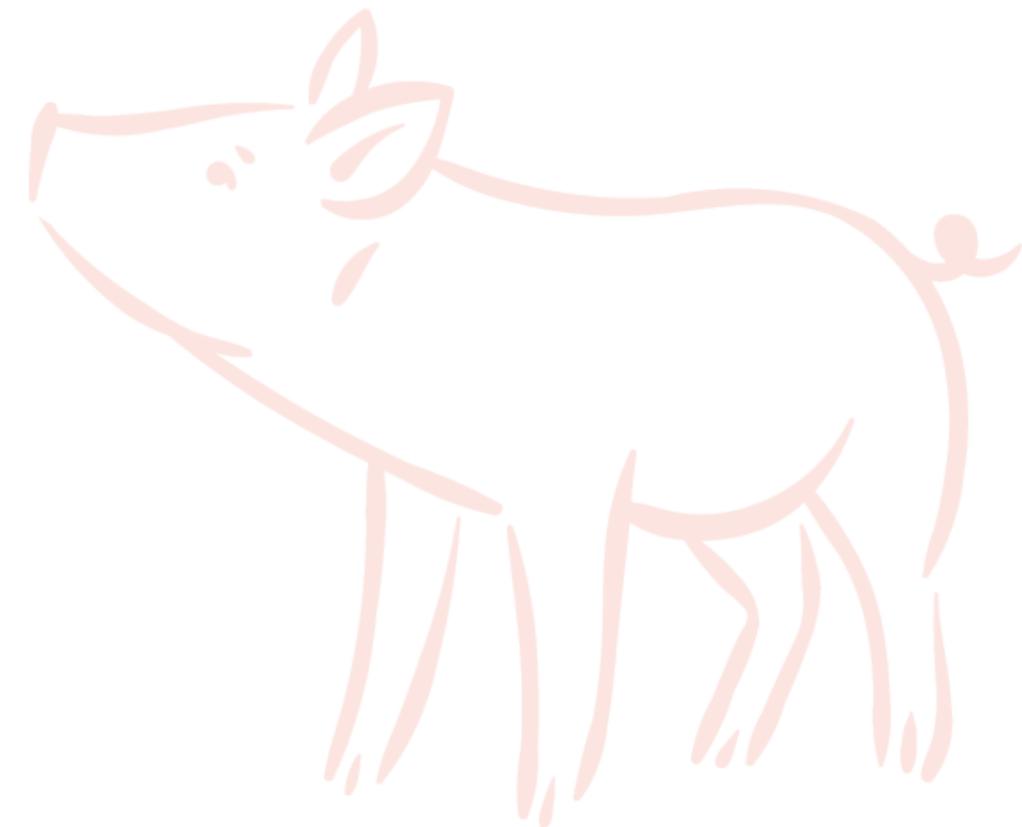
## Annual Station Report: Project NC-229 Kansas State University (Continued)

### Work Planned for Next Year

- Develop validate novel ASF vaccines and companion DIVA diagnostic assays.
- Develop and validate the cELISA that can differentiate pigs vaccinated with the C-strain vaccine from pigs infected with wildtype CSF viruses.
- Evaluate novel vaccines and therapeutic compounds against PRRSV.
- Investigate physical and chemical mitigation strategies for reducing the risk of foreign animal disease entry and spread through feed. Investigate the use of microbiome modulation in preventing respiratory disease in weaned pigs.

### Changes/Problems Due to COVID-19

We had to terminate an animal vaccine study on HP-PRRSV in the BSL-3 facility in mid-March 2020 because of limited university operation in response to COVID-19. We will repeat the study in 2021.



## Annual Station Report: Project NC-229 North Dakota State University

<b>Institution/Station</b>	North Dakota State University, Fargo, ND 58102	
<b>Period Covered</b>	November 30, 2019 to November 30, 2020	
<b>Personnel</b>	<p><b>NC-229 Station Representative:</b></p> <ul style="list-style-type: none"> <li>Sheela Ramamoorthy, Associate Professor, sheela.ramamoorthy@ndsu.edu</li> </ul> <p><b>Other Principal Leaders Associated With the Projects:</b></p> <p><b>Staff and Students:</b></p>	<b>Impact and Value of Research to Stakeholder</b>
<b>Progress of Work and Principal Accomplishments</b>	<p><b>Objective 1. Control of PRRSV</b></p> <p>1.1. PRRS immunology/vaccinology:</p> <p>1.2. PRRS epidemiology:</p> <p>1.3. PRRS Surveillance and Diagnostics:</p> <p><b>Objective 2. Developing effective and efficient approaches for detection, prevention and control of pressing viral diseases of swine of recent emergence</b></p> <p>2.1. ASFV:</p> <p>2.2. Swine Influenza Virus:</p> <p>2.3. Porcine Circovirus:</p> <ul style="list-style-type: none"> <li>Potentially new linear immunodominant epitopes were identified and functionally characterized.</li> <li>Efficacy of current PCV2a vaccines against PCV2d was assessed.</li> </ul> <p>2.4. Swine Pestiviruses:</p> <p>2.5. Senecavirus:</p> <p>2.6. Sapelovirus:</p> <p>2.7. Viruses with potential interest to Xeno-transplantation science:</p>	<b>Pertinent (Swine Virology) Publications Issued or "In Press"</b>
		<p><b>2.8. Swine coronaviruses:</b></p> <p>PEDV was used as a surrogate for SARS-CoV2 to optimize protocols for environmental decontamination using heat and UV sources.</p> <ol style="list-style-type: none"> <li>New neutralizing and non-neutralizing epitopes of the PCV2 capsid protein were identified. Findings will help further vaccine design for PCV2. Current PCV2a vaccines were determined to be effective in preventing clinical signs of PCV2d.</li> <li>A unique polymeric adjuvant was determined to be an effective delivery system for synthetic peptide vaccines which can be rapidly developed in emergency situations</li> <li>A novel, first generation, rapid-response vaccine platform was validated for PEDV and SIV in a weanling pig model.</li> <li>Provisional patent applications were filed for the emergency vaccine platform and PCV2 epitope based vaccine.</li> </ol> <p><b>Refereed Publications</b></p> <ol style="list-style-type: none"> <li>Rakibuzzaman A, Kolyvushko O, Singh G, Nara P, Piñeyro P, Leclerc E, Pillatzki A, Ramamoorthy S. Targeted Alteration of Antibody-Based Immunodominance Enhances the Heterosubtypic Immunity of an Experimental PCV2Vaccine. <i>Vaccines (Basel)</i>. 2020 Sep 4;8(3):506. doi: 10.3390/vaccines8030506.PMID: 32899842; PMCID: PMC7563983.</li> <li>Webb B, Rakibuzzaman A, Ramamoorthy S. Torque teno viruses in health and disease. <i>Virus Res</i>. 2020 Aug;285:198013. doi: 10.1016/j.virusres.2020.198013.Epub 2020 May 11. PMID: 32404273.</li> <li>Benfield D, Lunney JK, Murtaugh M, Nelson E, Osorio F, Pogranichniy R, Ramamoorthy S, Rowland RRR, Zimmerman JJ, Zuckermann FA. The NC229 multi-station research consortium on emerging viral diseases of swine: Solving stakeholder problems through innovative science and research. <i>Virus Res</i>. 2020 Apr 15;280:197898.</li> </ol> <p><b>Abstracts or Proceedings</b></p> <ol style="list-style-type: none"> <li>G. Singh and S. Ramamoorthy. Rapid response vaccines for emergency management. in 96th Annual Meeting of the Council of Research Workers in Animal Diseases. 2019. Chicago, IL.</li> </ol>

# Annual Station Report: Project NC-229 North Dakota State University (Continued)

## Book Chapters or Monographs

## Non-refereed Articles

1. Advances in veterinary vaccines: Changing Perspectives. International conference on The Current Scenario and Future Strategies of disease control for Augmenting Livestock and Poultry Productivity Under Changing Climatic Conditions. Nov 2019. Tamil Nadu, India.

## Ongoing:

1. USDA NIFA - First response vaccines for emergency preparedness. Agency- USDA-NIFA, Sheela Ramamoorthy, Angela Pillatzki, Brett Webb. (June 2018-June 2021)
2. NIH NIAID - A porcine model for Torque Teno Virus (TTV) infections. Agency - NIH-NIAID R21, Sheela Ramamoorthy, Angela Pillatzki, Brett Webb (Jan 2018-Dec 2021)

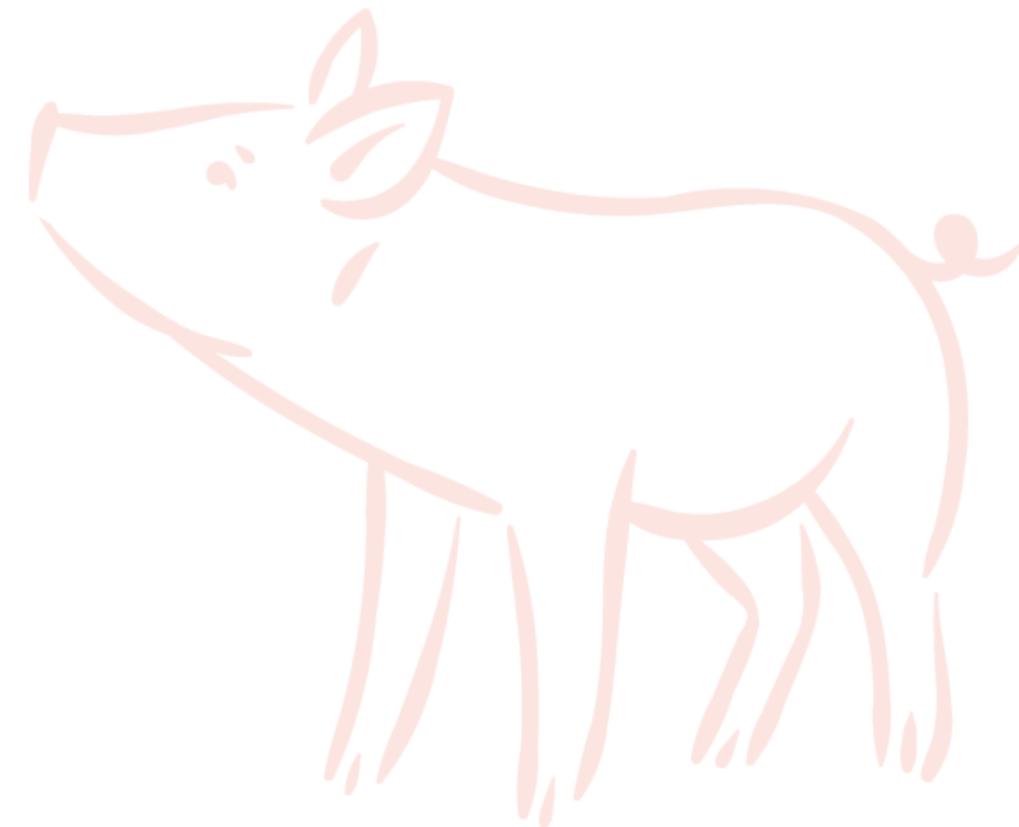
- We will continue to work on understanding the biology of torque teno viruses, develop animal models to study pathogenesis and immune regulation.
- We will continue to work on the development of novel emergency vaccines and oral delivery systems using PEDV as a model.

- Progress on a USDA-NIFA funded project (2018-2020) pertaining to novel delivery methods for rapid-response vaccines against RNA viruses was significantly delayed due to closure of facilities, turnover of research staff and delays in hiring due to closed borders.

## Funding Sources

## Work Planned for Next Year

## Changes/ Problems Due to COVID-19



## Annual Station Report: Project NC-229 Purdue University

<b>Institution/Station</b>	Purdue University, West Lafayette, IN 47906
<b>Period Covered</b>	November 30, 2019 to November 1, 2020
<b>Personnel</b>	<p><b>NC-229 Station Representative:</b></p> <ul style="list-style-type: none"> <li>Jonathan (Alex) Pasternak, Assistant Professor, jpastern@purdue.edu</li> </ul> <p><b>Other Principal Leaders Associated With the Projects:</b></p> <ul style="list-style-type: none"> <li>John Harding, Professor, University of Saskatchewan, Saskatoon, SK, Canada, john.harding@usask.ca</li> <li>Joan K. Lunney, Supervisory Research Scientist, USDA ARS NEA BARC, joan.lunney@usda.gov</li> </ul> <p><b>Staff and Students:</b></p> <ul style="list-style-type: none"> <li>Erin Ison, MSc Student, eison@purdue.edu</li> <li>Leah Jenkins, MSc Student, jenki155@purdue.edu</li> <li>Jocelyn Kleiman, Undergraduate Research Assistant, jkleima@purdue.edu</li> <li>Margaret Mulligan, Undergraduate Research Assistant, mmullig@purdue.edu</li> <li>Andrew Caldemeyer, Undergraduate Research Assistant, andycc414@aol.com</li> <li>Elizabeth Montgomery, Undergraduate Research Assistant, montgo62@purdue.edu</li> <li>Kylee Lindsey, Undergraduate Research Assistant, lindseyk@purdue.edu</li> </ul>
<b>Progress of Work and Principal Accomplishments</b>	<p><b>Objective 1. Control of PRRSV</b></p> <p>We are a newly established lab in the animal sciences department at Purdue University, and recent addition to NC-229 project. This year we initiated work designed to improve our understanding of the host response to PRRSV and other key porcine pathogens. Our current focus is on a Non-thyroidal illness syndrome (NTIS) like effect, which is characterized by a significant decrease in circulating thyroid hormones. We have previously demonstrated a decrease in circulating Thyroxin (T4) and Triiodothyronine (T3) following disease challenge in large sample sets obtained from the PRRS Host Genetics Consortium (PHGC), Natural Disease Challenge Barn (NDCB), and Pregnant Gilt models. More critically, we have shown that the degree of thyroid hormone suppression in each of these challenge experiments is associated with a resilient phenotype in the form of average daily gain or fetal survival. Our work now focuses on probing the molecular and physiological aspects of this response in an effort to identify potential interventions.</p>

<p><b>1.1. PRRS immunology/vaccinology:</b></p> <ul style="list-style-type: none"> <li>We have shown that the fetal immune response is not a primary factor in fetal resilience following PRRSV infection</li> <li>We have demonstrated a combination of maternal and fetal NTIS like effects following PRRSV2 challenge</li> <li>We have evaluated the PRRSV2 induced disruption in cell cycle progression across seven fetal tissues (heart, kidney, lung, liver, spleen, thymus and muscle) derived from the latest iteration of the Pregnant Gilt Model (University of Saskatchewan) We have assessed the involvement of the TGFβ and TP53 pathway in regulating PRRSV2 disruption in cell cycle progression</li> <li>We evaluated the impact of PRRSV2 infection on the intestinal development weaned pigs, measuring villi height and crypt depth at Days 10 and 28 post infection, and are now following up with an investigation is associated gene expression.</li> <li>We have initiated studies evaluating the impact of PRRSV infection of fetal deiodinase activity</li> <li>We have initiated studies on the distribution of virus across fetal organs of highly infected fetus and the association with resilience phenotypes.</li> <li>We have been involved in the evaluation of a genetic marker on SSC7 for its value in selection of fetal resilience during PRRSV2 infection and the association with potentially causative mutations.</li> </ul> <p><b>1.2. PRRS epidemiology:</b></p> <p><b>1.3. PRRS Surveillance and Diagnostics:</b></p> <p><b>Objective 2. Developing effective and efficient approaches for detection, prevention and control of pressing viral diseases of swine of recent emergence</b></p> <p><b>2.1. ASFV:</b></p> <p><b>2.2. Swine Influenza Virus:</b></p> <p><b>2.3. Porcine Circovirus:</b></p> <p>We have demonstrated that the scale of the NTIS like response following PCV infection is associated with average daily gain.</p>
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## Annual Station Report: Project NC-229 Purdue University (Continued)

### 2.4. Swine Pestiviruses:

### 2.5. Senecavirus:

### 2.6. Sapelovirus:

### 2.7. Viruses with potential interest to Xeno-transplantation science:

1. We have identified the thyroid hormone systems as a component of the host response to PRRSV2 and other diseases and believe this system could be manipulated to improve post infection performance
2. We have identified fetal organs, which experience the most significant disruption in development during PRRSV2 infection which may lead to better management of congenitally infected piglets.

### Refereed Publications

1. Van Goor A, Pasternak A, Walker K, Hong L, Malgarin C, MacPhee DJ, Harding JCS, Lunney JK. Differential responses in placenta and fetal thymus at 12 days post infection elucidate mechanisms of viral level and fetal compromise following PRRSV2 infection. BMC Genomics. 2020 Nov 4;21(1):763. doi: 10.1186/s12864-020-07154-0. PMID: 33148169
2. Pasternak JA, MacPhee DJ, Harding JCS. Maternal and fetal thyroid dysfunction following porcine reproductive and respiratory syndrome virus2 infection. Vet Res. 2020 Mar 30;51(1):47. doi: 10.1186/s13567-020-00772-2. PMID: 32228691
3. Alex Pasternak J, MacPhee DJ, Harding JCS. Fetal cytokine response to porcine reproductive and respiratory syndrome virus-2 infection. Cytokine. 2020 Feb;126:154883. doi: 10.1016/j.cyto.2019.154883. Epub 2019 Oct 16. PMID: 31629108

### Abstracts or Proceedings

1. Van Goor A, Pasternak JA, Walker K, Hong L, Malgarin C, MacPhee DJ, Harding JCS and Lunney JK. Reproductive PRRS: Gene expression differences elucidate mechanisms of fetal viral level and demise. 101st Annual Conference of Research Workers in Animal Diseases. Dec 4-8, 2020. Chicago, IL.
2. Pasternak JA, Hamonic G, Ciobanu DC, MacPhee DJ, Plastow G, Harding JCS. Non-Thyroidal Illness Syndrome (allostatic hypothyroidism) following

PCV2 infection. 26th IPVS Conference June 2-5 2020, Rio de Janeiro. (postponed due to COVID)

3. Malgarin CM, Pasternak JA, MacPhee DJ, Harding JCS. How are PRRSV-infected fetuses dying? 26th IPVS Conference June 2-5 2020, Rio de Janeiro. (postponed due to COVID)
4. Guidoni PB, Pasternak JA, MacPhee DJ, Harding JCS. Lower intensity of tight junctions in maternal fetal interface of PRRSV2 infected fetuses. 26th IPVS Conference June 2-5 2020, Rio de Janeiro. (postponed due to COVID)
5. Van Goor A, Walker K, Pasternak JA, Malgarin C, MacPhee DJ, Harding JCS, Lunney J.K. Differentially Expressed Immune Genes Identified in the Placenta and Fetal Thymus in Fetuses of Pregnant Gilts Infected with PRRS virus. Plant and Animal Genome XXVIII Conference, Jan 11-15, 2020. San Diego CA.
6. Malgarin CM, Pasternak JA, MacPhee DJ, Harding JCS. PRRSV-infected fetuses show evidence of hypoxia and apoptosis. Banff Pork Seminar. Jan 6-9, 2020. Banff AB.
7. Ko H. Sammons J. Pasternak JA, MacPhee DJ, Plastow GS. Harding JCS. Effects of a genomic marker on developmental responses in PRRSV infected fetuses. Banff Pork Seminar. Jan 6-9, 2020. Banff AB.

### Book Chapters or Monographs

### Non-refereed Articles

No External Funding.

- Continue analysis of fetal tissues derived from the Pregnant Gilt Challenge model
- Continue assessment of the impact of PRRSV infection on intestinal development in weaned pigs.
- Our lab was shut down for an extended period and is now running at limited capacity to meet social distancing requirements. No new animal experiments were initiated to reduce the risk of infecting farm staff.

Impact and Value of Research to Stakeholder

Pertinent (Swine Virology) Publications Issued or "In Press"

### Funding Sources

### Work Planned for Next Year

### Changes/ Problems Due to COVID-19

# Annual Station Report: Project NC-229

## South Dakota State University

<b>Institution/Station</b>	South Dakota State University, Brookings, SD 57007
<b>Period Covered</b>	November 30, 2019 to November 1, 2020
<b>Personnel</b>	<p><b>NC-229 Station Representative:</b></p> <ul style="list-style-type: none"> <li>Eric A. Nelson, Distinguished Professor, eric.nelson@sdstate.edu</li> </ul> <p><b>Other Principal Leaders Associated With the Projects:</b></p> <ul style="list-style-type: none"> <li>Xiuqing Wang, Professor, xiuqing.wang@sdstate.edu</li> <li>Benjamin Hause, Assistant Professor, Benjamin.hause@sdstate.edu</li> <li>Chun-Ming Lin, Assistant Professor, chunming.lin@sdstate.edu</li> <li>Jane Hennings, Director &amp; Department Head, jane.hennings@sdstate.edu</li> <li>Steve Lawson, Research Associate III, steven.lawson@sdstate.edu</li> </ul> <p><b>Staff and Students:</b></p>
<b>Progress of Work and Principal Accomplishments</b>	<p><b>Objective 1. Control of PRRSV</b></p> <p><b>1.1. PRRS immunology/vaccinology:</b> The SDSU station contributed to the understanding of cellular innate factors such as IFITM3 and ZMPSTE24 in PRRSV replication in vitro. The antiviral activities of both cellular proteins were observed in MARC-145 cells. We are currently exploring the molecular mechanisms by which IFITM3 and ZMPSTE24 inhibit PRRSV replication.</p> <p><b>1.2. PRRS epidemiology:</b> The SDSU Station contributed PRRSV sequence data from field cases to collaborative efforts using bioinformatics tools to summarize and report routine pathogen detection to inform the U.S. swine industry on key macro-epidemiological aspects of agent detection. Over 900 PRRSV sequences were contributed by the SDSU Station.</p> <p><b>1.3. PRRS Surveillance and Diagnostics:</b> We continued to provide numerous reagents, including monoclonal antibodies, as well as diagnostic assay support to collaborating stations, other universities and industry for research and diagnostic applications.</p>

### Impact and Value of Research to Stakeholder

### Objective 2. Developing effective and efficient approaches for detection, prevention and control of pressing viral diseases of swine of recent emergence

#### 2.1. ASFV:

#### 2.2. Swine Influenza Virus:

The SDSU Station contributed swine influenza virus diagnostic and sequence data from field cases to collaborative efforts using bioinformatics tools to summarize and report routine pathogen detection to inform the U.S. swine industry on key macro-epidemiological aspects of agent detection. Additional monoclonal antibodies against selected epitopes of influenza D virus were developed and are being fully characterized for application in collaborative research projects.

#### 2.3. Porcine Circovirus:

#### 2.4. Swine Pestiviruses:

#### 2.5. Senecavirus:

We continued to develop additional reagents for detection of Senecavirus A antigen and antibody. Serum virus neutralization assays (SVN) and immunohistochemistry (IHC) methods are now readily available to the industry and provide important tools to monitor and differentiate incidences of vesicular disease outbreaks. A recently developed monoclonal antibody-based blocking ELISA to detect antibody responses to Senecavirus A (SVA) was validated and licensed for commercialization.

#### 2.6. Sapelovirus:

#### 2.7. Viruses with potential interest to Xeno-transplantation science:

1. A recently developed monoclonal antibody-based blocking ELISA to detect antibody responses to Senecavirus A (SVA) was validated and licensed for commercialization.
2. Monoclonal antibodies against Senecavirus A were further characterized and applied to serum virus neutralization assays (SVN) and immunohistochemistry (IHC) methods, providing important tools to monitor and differentiate incidences of vesicular disease outbreaks.

## Annual Station Report: Project NC-229 South Dakota State University (Continued)

### Pertinent (Swine Virology) Publications Issued or "In Press"

3. Large panels of monoclonal antibodies against the nucleocapsid and spike proteins of SARS-CoV-2 were developed. These antibodies will be of value in a wide range of research and diagnostic applications related to COVID-19.
4. Through evaluation of the role of host's natural virus restriction factors in PRRSV replication, we demonstrated that over-expression of IFITM3 and ZMPSTE24 reduces PRRSV replication. Further understanding on the mechanistic basis by which these cellular virus restriction factors inhibit PRRSV replication in vitro may lead to improved control and prevention of this disease.

### Refereed Publications

1. An evaluation of additives for mitigating the risk of virus-contaminated feed using an ice-block challenge model. Dee SA, Niederwerder MC, Edler R, Hanson D, Singrey A, Cochrane R, Spronk G, Nelson E. *Transbound Emerg Dis.* 2020 Jul 24. doi: 10.1111/tbed.13749. Online ahead of print. PMID: 32706431
2. Use of a demonstration project to evaluate viral survival in feed: Proof of concept. Dee S, Shah A, Cochrane R, Clement T, Singrey A, Edler R, Spronk G, Niederwerder M, Nelson E. *Transbound Emerg Dis.* 2020 Jun 14. doi: 10.1111/tbed.13682. Online ahead of print. PMID: 32536022
3. The risk of viral transmission in feed: What do we know, what do we do? Dee SA, Niederwerder MC, Patterson G, Cochrane R, Jones C, Diel D, Brockhoff E, Nelson E, Spronk G, Sundberg P. *Transbound Emerg Dis.* 2020 May 2. doi: 10.1111/tbed.13606. Online ahead of print. PMID: 32359207 Review.
4. The NC229 multi-station research consortium on emerging viral diseases of swine: Solving stakeholder problems through innovative science and research. Benfield D, Lunney JK, Murtaugh M, Nelson E, Osorio F, Pogranichniy R, Ramamoorthy S, Rowland RRR, Zimmerman JJ, Zuckermann FA. *Virus Res.* 2020 Apr 15;280:197898. doi: 10.1016/j.virusres.2020.197898. Epub 2020 Feb 28. PMID: 32061619
5. A Minimally Replicative Vaccine Protects Vaccinated Piglets Against Challenge With the Porcine Epidemic Diarrhea Virus. Singh G, Singh P, Pillatzki A, Nelson E, Webb B, Dillberger-Lawson S, Ramamoorthy S. *Front Vet Sci.* 2019 Oct 22;6:347. doi: 10.3389/fvets.2019.00347. eCollection 2019. PMID: 31696121 Free PMC article.

6. GTPase-activating protein-binding protein 1 (G3BP1) plays an antiviral role against porcine epidemic diarrhea virus. Pandey K, Zhong S, Diel DG, Hou Y, Wang Q, Nelson E, Wang X. *Vet Microbiol.* 2019 Sep;236:108392. doi: 10.1016/j.vetmic.2019.108392. Epub 2019 Aug 19. PMID: 31500725 Free PMC article.
7. Inactivation of porcine epidemic diarrhea virus in contaminated swine feed through inclusion of a dry lactic acid-based product. Dee, S., J. DeJong, C. Neill, B. Ratliff, A. Singrey, E. Hansen, E. Nelson, J. Keegan, A. Gaines. 2020. *JSHAP* 28:213-216.
8. Delivery of a thermo-enzymatically treated influenza vaccine using pulmonary surfactant in pigs. Vinson, H., G. Singh, A. Pillatzki, B. Webb, E. Nelson, S. Ramamoorthy. 2019. *Vet. Micro.* 239:108492. DOI: 10.1016/j.vetmic.2019.108492
9. An amphiphilic invertible polymer as a delivery vehicle for a M2e-HA2-HA1 peptide vaccine against an Influenza A virus in pigs. Singh, G., O. Zholobko, A. Pillatzki, B. Webb, E. Nelson, A. Voronov, S. Ramamoorthy. 2019. *Vaccine.* 37(31):4291-4301. doi.org/10.1016/j.vaccine.2019.06.030.

### Abstracts or Proceedings

1. Development of a novel multiplex real-time PCR for the detection and differentiation of SADS-CoV, an emerging swine coronavirus. Rauh, R., T. Clement; J. Christopher Hennings; F. Li; S. Lawson; S-L Zhai; J-Y Ma; J.D. Callahan; Z. Sankoh; W. M. Nelson; E.A. Nelson; Diego G. Diel. American Association of Swine Veterinarians. Orlando, FL, Mar 8-12, 2019.
2. Detection and control of PRRS virus and emerging viral diseases of swine (NC-229 South Dakota) D. Diel, S. Lawson, F. Li, E. Nelson, D. Wang, X. Wang, J. Christopher-Hennings. Abstract #65. North American PRRS Symposium, Chicago, Il. Dec. 2-3, 2019.
3. Development of a novel multiplex real-time PCR for the detection and differentiation of SADS-CoV, an emerging swine coronavirus. R. Rauh; T. Clement; J. Christopher Hennings; Steve Lawson; S-L Zhai; J-Y Ma; J.D. Callahan; E.A. Nelson; Diego G. Diel. Abstract #60. North American PRRS Symposium, Chicago, Il. Dec. 2-3, 2019.

### Book Chapters or Monographs

### Non-refereed Articles

## Annual Station Report: Project NC-229 South Dakota State University (Continued)

### Funding Sources

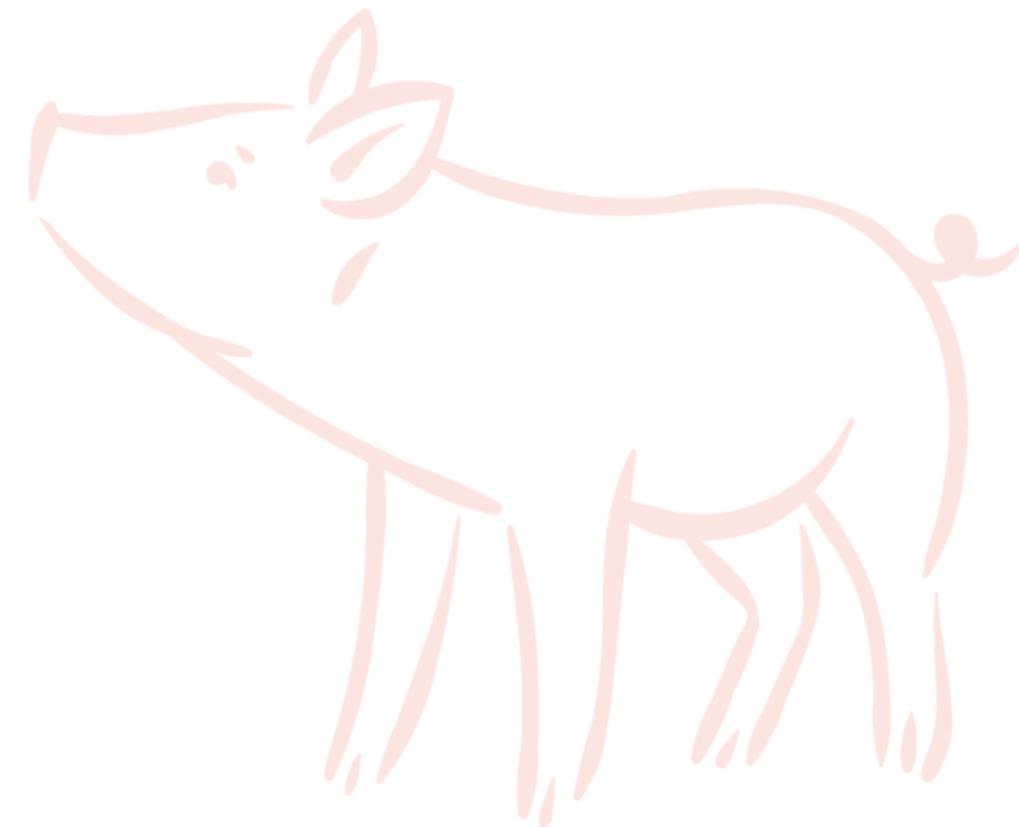
1. USDA Hatch/Multi State
2. USDA/NIFA
3. USDA/ARS
4. Swine Health Information Center
5. South Dakota Governor's Office of Economic Development
6. South Dakota Animal Disease Research & Diagnostic Laboratory

### Work Planned for Next Year

- Dr. Wang will continue to evaluate the role of cellular virus restriction factors in PRRSV and Senecavirus A replication in vitro. The mechanistic basis of virus replication inhibition will be examined.
- Dr. Hause is developing a universal swine influenza virus vaccine and efficacy will be evaluated in pigs with heterologous viruses in early 2021. Dr. Hause and Dr. Lin will evaluate the etiologic significance of the emerging porcine parvovirus 2 in swine respiratory disease using real time PCR and in situ hybridization of archived respiratory disease diagnostic submissions. Metagenomic sequencing is also planned for over 100 swine respiratory disease diagnostic submissions to evaluate pathogens associated with respiratory disease.

### Changes/ Problems Due to COVID-19

Starting in early March 2020, substantial time and resources were shifted to research efforts focused on SARS-CoV-2, resulting in some delays in NC-229 related research. By utilizing experience and technologies previously applied to NC-229 research projects, we developed large panels of monoclonal antibodies against the nucleocapsid and spike proteins of SARS-CoV-2, developed and are evaluating a fluorescent microsphere immunoassays (FMIA) for detection of antibody responses to SARS-CoV-2 infection, and developed fluorescence-based virus neutralization assays.



# Annual Station Report: Project NC-229

## University of Florida

<b>Institution/Station</b>	University of Florida, Gainesville, FL 32611	
<b>Period Covered</b>	November 30, 2019 to November 1, 2020	
<b>Personnel</b>	<p><b>NC-229 Station Representative:</b></p> <ul style="list-style-type: none"> <li>John Driver, Associate Professor, jdriver@ufl.edu</li> </ul> <p><b>Other Principal Leaders Associated With the Projects:</b></p> <ul style="list-style-type: none"> <li>Jürgen Richt, Distinguished Professor, jricht@k-state.edu</li> </ul> <p><b>Staff and Students:</b></p> <ul style="list-style-type: none"> <li>Weihong Gu, PhD student, guweihong@ufl.edu</li> <li>Melany Madrid, PhD student, madrid.m@ufl.edu</li> <li>Sadie Auer, Biological Scientist, dnsilly@ufl.edu</li> <li>Bianca Artiaga, Postdoctoral fellow, blartiaga@vet.k-state.edu</li> </ul>	
<b>Progress of Work and Principal Accomplishments</b>	<p><b>Objective 1. Control of PRRSV</b></p> <p>1.1. PRRS immunology/vaccinology:</p> <p>1.2. PRRS epidemiology:</p> <p>1.3. PRRS Surveillance and Diagnostics:</p> <p><b>Objective 2. Developing effective and efficient approaches for detection, prevention and control of pressing viral diseases of swine of recent emergence</b></p> <p>2.1. ASFV:</p> <p>2.2. Swine Influenza Virus:</p> <ul style="list-style-type: none"> <li>We compared intranasal natural killer T (NKT) cell agonist therapy to the neuraminidase inhibitor oseltamivir as a treatment for reducing influenza virus infections in pigs. NKT cells are a minor innate-like T cell population that can be induced to secrete large quantities of cytokines by synthetic glycolipid agonists.</li> <li>We determined whether prophylactically treating pigs with NKT cell agonists would protect them from a subsequent influenza virus infection.</li> <li>We characterized the pathology and immunology of influenza infection</li> </ul>	<p style="text-align: center;"><b>Impact and Value of Research to Stakeholder</b></p> <p>during the acute stage of disease in NKT cell-deficient pigs to determine how NKT cells exacerbate influenza virus shedding.</p> <ul style="list-style-type: none"> <li>We evaluated whether NKT cell agonists could be used to adjuvant a modified live influenza vaccine to enhance cross protection against a heterosubtypic virus infection.</li> <li>We evaluated whether NKT cell agonists could be used to adjuvant an inactivated influenza vaccine to enhance cross protection against a heterologous virus infection.</li> </ul> <p>2.3. Porcine Circovirus:</p> <p>2.4. Swine Pestiviruses:</p> <p>2.5. Senecavirus:</p> <p>2.6. Sapelovirus:</p> <p>2.7. Viruses with potential interest to Xeno-transplantation science:</p> <p>Natural killer T cells have long been studied for human therapeutic applications. We are attempting to transfer some of these applications to improve animal health. Although our project is focused on harnessing NKT cells to improve swine immunity against influenza, we expect our results to be broadly applicable to other infectious diseases. Some of our achievements and findings this year include:</p> <ol style="list-style-type: none"> <li>We have tested the feasibility of therapeutically harnessing NKT cells to suppress disease and inhibit virus transmission during a swine influenza outbreak.</li> <li>We have determined that therapeutically increasing pig NKT cells to high levels does not protect them from a subsequent influenza infection.</li> <li>We have determined the feasibility of harnessing the adjuvant effects of therapeutically activated NKT cells to improve the cross protection of modified live influenza virus vaccines.</li> <li>We have determined the feasibility of harnessing the adjuvant effects of therapeutically activated NKT cells to improve the cross protection of inactivated influenza virus vaccines.</li> <li>We are determining the contribution of NKT cells to natural influenza immunity as the concentration of these cells varies widely among pigs.</li> </ol>

## Annual Station Report: Project NC-229 University of Florida (Continued)

### Pertinent (Swine Virology) Publications Issued or "In Press"

#### Refereed Publications

1. Driver JP, de Carvalho Madrid DM, Gu W, Artiaga BL, Richt JA. Modulation of Immune Responses to Influenza A Virus Vaccines by Natural Killer T Cells. *Front Immunol.* 2020 Oct 20;11:2172. doi:10.3389/fimmu.2020.02172. PMID: 33193296; PMCID: PMC7606973.
2. Gu W, Madrid DMD, Yang G, Artiaga BL, Loeb JC, Castleman WL, Richt JA, Lednicky JA, Driver JP. Unaltered influenza disease outcomes in swine prophylactically treated with alpha-galactosylceramide. *Dev Comp Immunol.* 2021 Jan;114:103843. doi: 10.1016/j.dci.2020.103843. Epub 2020 Aug 29. PMID: 32871161.

#### Abstracts or Proceedings

1. Madrid DM, Driver JP, Gu W, Yang G, Artiaga BL. Oseltamivir for influenza infection in pigs. Conference or Research Workers in Animal Diseases (CRWAD). Chicago IL, November 2-5 2019
2. Gu W, Madrid DM, Artiaga BL, Yang G, Driver JP. Prophylactically activating iNKT cells in swine does not alter the course of an influenza infection. Conference or Research Workers in Animal Diseases (CRWAD). Chicago IL, November 2-5 2019

#### Book Chapters or Monographs

#### Non-refereed Articles

1. U.S. Department of Agriculture Grant 2016-09448
2. National Institutes of Health Grant HD092286

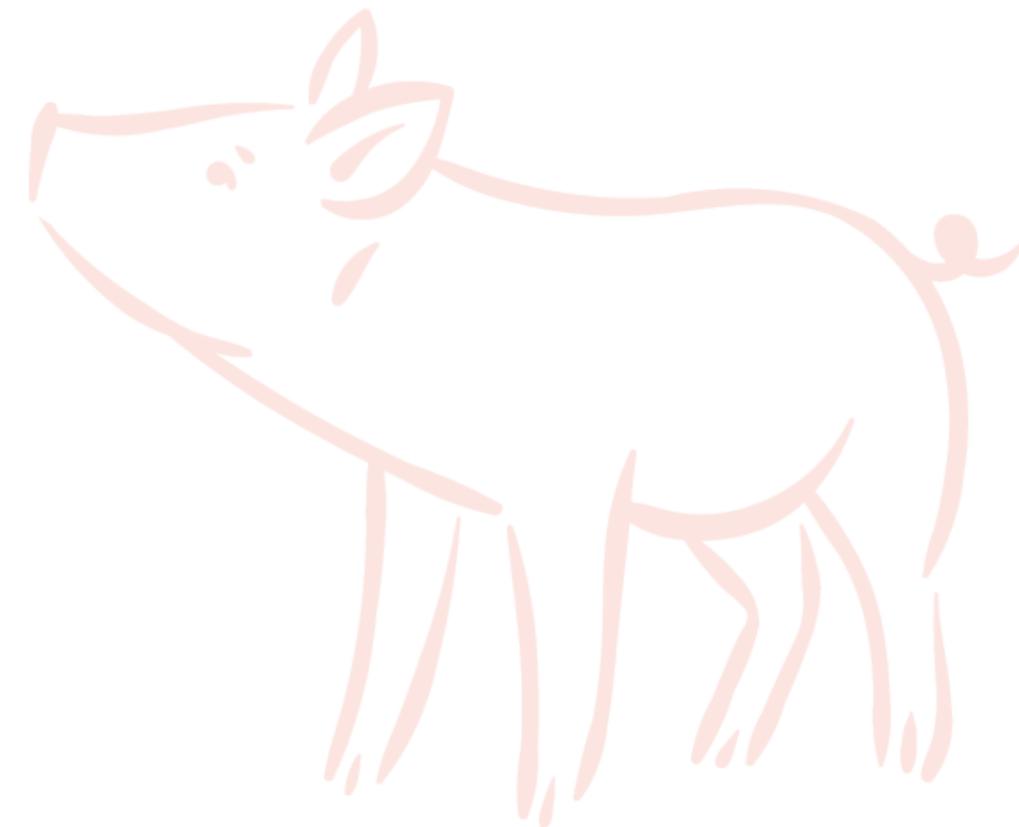
- We intend to test whether NKT cells are necessary for generating immune memory against subsequent influenza infections.
- We intend to test the safety of adjuvanting influenza vaccines that have the potential to induce vaccine associated enhanced respiratory disease.
- We will perform studies to optimize NKT cell therapy for treating ongoing influenza virus infections.

- We have had challenges performing influenza infection studies due to a lack of personal protective equipment, nasal swabs and other reagents, which has been redirected for COVID-19.
- We have had difficulty breeding our genetically modified sows.
- These difficulties have delayed the studies we planned in 2020.

### Funding Sources

### Work Planned for Next Year

### Changes/ Problems Due to COVID-19



# Annual Station Report: Project NC-229

## University of Illinois

<b>Institution/Station</b>	University of Illinois at Urbana-Champaign, Urbana, IL 61802
<b>Period Covered</b>	November 30, 2019 to November 1, 2020
<b>Personnel</b>	<p><b>NC-229 Station Representative:</b></p> <ul style="list-style-type: none"><li>• Frederico A. Zuckermann, Professor, fazaaa@illinois.edu</li></ul> <p><b>Other Principal Leaders Associated With the Projects:</b></p> <ul style="list-style-type: none"><li>• James Lowe, Associate Professor, jlowe@illinois.edu</li><li>• Dongwan Yoo, Professor, dyoo@illinois.edu</li><li>• Daniel L. Rock, Professor, dlrock@illinois.edu</li><li>• Ying Fang, Professor, yingf@illinois.edu</li><li>• Raymond Rowland, Professor, rowland7@illinois.edu</li></ul> <p><b>Staff and Students:</b></p> <p><u>Fang Laboratory</u></p> <ul style="list-style-type: none"><li>• Jin Cui, Postdoc, jincui@illinois.edu</li><li>• Fangfeng Yuan, PhD student, fy8@illinois.edu</li><li>• Xingyu Yan, PhD student, xingyuy4@illinois.edu</li><li>• Han Gao, MS student, hang7@illinois.edu</li></ul> <p><u>Zuckermann Laboratory</u></p> <ul style="list-style-type: none"><li>• Robert Husmann, Research Associate, rhusmann@illinois.edu</li></ul>
<b>Progress of Work and Principal Accomplishments</b>	<p><b>Objective 1. Control of PRRSV</b></p> <p><b>1.1. PRRS immunology/vaccinology:</b></p> <p><u>Fang Laboratory</u></p> <p>Mechanisms of hyper-phosphorylation of PRRSV nsp2-related proteins: We have studied the mechanism of phosphorylation of PRRSV nsp2-related proteins and its potential role in host immunity and viral pathogenesis. Our results showed that two nsp2-related -2/-1 frameshifting products, nsp2TF and nsp2N, are hyper-phosphorylated. Mass-spectrometric analysis of PRRSV identified that most of the phosphorylation sites are located in the hypervariable regions (HVRs) and inter-HVR regions (IHCD region). More importantly, some of these specific phosphorylation sites are associated with the B-cell or predicted T-cell epitopes. Abolishing phosphorylation of the inter-species conserved residue serine918 abrogates accumulation of viral genomic and subgenomic RNAs and recombinant virus production. These results indicate that HVR and IHCD regions of nsp2-related proteins</p>

potentially interact with the host immune system and these regions are associated with viral fitness and replication ability, which presents potential links to viral pathogenesis.

### 1.2. PRRS epidemiology:

### 1.3. PRRS Surveillance and Diagnostics:

## Objective 2. Developing effective and efficient approaches for detection, prevention and control of pressing viral diseases of swine of recent emergence

### 2.1. ASFV:

#### Fang Laboratory

ASFV-specific monoclonal antibody (mAb) development and application in diagnostics: We have generated a panel of specific monoclonal antibodies (mAbs) against selected immunogenic ASFV proteins, including p10, p14.5, p22, p30, p49, p54, p72, and CD2v. These mAbs were initially screened by immunofluorescent assay using in vitro expression system. The antibody reactivity was confirmed in virus-infected cells. Their application in the detection of ASFV infection was further tested using the methods of Western blotting, immunoprecipitation and ELISA. The anti-p30 mAb was further utilized to develop a mAb-based blocking ELISA (bELISA). The bELISA demonstrates a diagnostic sensitivity of 98.0% and diagnostic specificity of 99.78%. The assay is highly repeatable with repeatability determined to be less than 10%.

#### Zuckermann Laboratory

A manuscript was published demonstrating that the porcine alveolar macrophage cell line ZMAC is permissive to ASF virus replication. This was demonstrated using field strains of ASF virus representing genotypes I, II and IV, including the Georgia 2007 strain, and the attenuated strain OURT 88/3.

### 2.2. Swine Influenza Virus:

#### Fang Laboratory

Development of broadly protective influenza vaccines: We used parainfluenza virus 5 (PIV-5) as a vector to express a chimeric HA antigen, HA-113. Recombinant PIV-5 expressing HA-113 (PIV5-113) were rescued, and immunogenicity and protective efficacy were tested in the pig model. The results showed that PIV5-113 can protect pigs against challenge with viruses

## Annual Station Report: Project NC-229 University of Illinois (Continued)

expressing parental HAs. The protective immunity was extended against other genetically diversified influenza H1-expressing viruses.

### 2.3. Porcine Circovirus:

### 2.4. Swine Pestiviruses:

### 2.5. Senecavirus:

### 2.6. Sapelovirus:

#### Fang Laboratory

Genetic characterization and diagnostic tool development of porcine sapelovirus: An emerging porcine sapelovirus was isolated in a diagnostic specimen from a US swine farm, designated as PSV KS18-01. Full-length genome sequence was obtained through next-generation sequencing. Phylogenetic analysis showed that the virus is more closely related to two Japanese strains but is distantly related to two known US strains. PSV specific diagnostic tools were developed, including the monoclonal antibodies against VP1 and VP2, and a VP1-VP2 antigen-based indirect ELISA. Using this assay, the dynamic response of PSV antibody was investigated in a group of post-weaned pigs that naturally exposed with PSV.

### 2.7. Viruses with potential interest to Xeno-transplantation science:

1. Basic mechanism study of PRRSV protein phosphorylation expands the nidovirus phospho-proteome and reveal important roles for the phosphorylation of nsp2-related proteins in the regulation of virus replication, as well as potential effects on viral pathogenesis.
2. The panel of mAbs and mAb-based diagnostic assays represent valuable tools for ASFV detection and surveillance. They are also important tools for basic mechanism studies toward developing vaccines and antiviral agents.
3. The PIV5 vaccine vector and chimeric HA antigen established a platform for development of universal influenza vaccines.
4. The availability of the PSV isolate (KS18-01) and the specific diagnostic reagents and assays provide important tools for PSV control and prevention.
5. The availability of a USDA approved Master cell stock of the ZMAC cell line, combined with the demonstrated scalability of its culture for PRRS virus autogenous vaccine production, and its ability to replicate ASF

### Pertinent (Swine Virology) Publications Issued or "In Press"

virus, has set the stage for the use of this cell line for the commercial production of ASF virus vaccines.

### Refereed Publications

#### Fang Laboratory

1. Shang P, Yuan F, Misra S, Li Y, Fang Y. Hyper-phosphorylation of nsp2-related proteins of porcine reproductive and respiratory syndrome virus. *Virology*. 2020 Apr;543:63-75. doi: 10.1016/j.virol.2020.01.018. Epub 2020 Feb 4. PMID: 32174300; PMCID: PMC7112050.  
Viruses exploit phosphorylation of both viral and host proteins to support viral replication. In this study, we demonstrate that porcine reproductive and respiratory syndrome virus replicase nsp2, and two nsp2-related -2/-1 frameshifting products, nsp2TF and nsp2N, are hyper-phosphorylated. By mapping phosphorylation sites, we subdivide an extended, previously uncharacterized region, located between the papain-like protease-2 (PLP2) domain and frameshifting site, into three distinct domains. These domains include two large hypervariable regions (HVR) with putative intrinsically disordered structures, separated by a conserved and partly structured interval domain that we defined as the inter-HVR conserved domain (IHCD). Abolishing phosphorylation of the inter-species conserved residue serine918, which is located within the IHCD region, abrogates accumulation of viral genomic and subgenomic RNAs and recombinant virus production. Our study reveals the biological significance of phosphorylation events in nsp2-related proteins, emphasizes pleiotropic functions of nsp2-related proteins in the viral life cycle, and presents potential links to pathogenesis.
2. Li Z, Zaiser SA, Shang P, Heiden DL, Hajovsky H, Katwal P, DeVries B, Baker J, Richt JA, Li Y, He B, Fang Y\*, Huber VC\*. A chimeric influenza hemagglutinin delivered by parainfluenza virus 5 vector induces broadly protective immunity against genetically divergent influenza A H1 viruses in swine. *Vet Microbiol*. 2020. (\*co-corresponding authors).  
Pigs are an important reservoir for human influenza viruses, and influenza causes significant economic loss to the swine industry. As demonstrated during the 2009 H1N1 pandemic, control of swine influenza virus infection is a critical step toward blocking emergence of human influenza virus. An effective vaccine that can induce broadly protective immunity against heterologous influenza virus strains is critically needed. In our previous studies [McCormick et al., 2015; PLoS One, 10(6):e0127649], we used molecular breeding (DNA shuffling) strategies to increase the breadth of the variable and conserved epitopes expressed within a single influenza A virus chimeric hemagglutinin (HA) protein. Chimeric HAs were constructed using parental HAs from the 2009 pandemic virus and swine influenza viruses that had a history of zoonotic transmission to humans. In the

## Annual Station Report: Project NC-229 University of Illinois (Continued)

current study, we used parainfluenza virus 5 (PIV-5) as a vector to express one of these chimeric HA antigens, HA-113. Recombinant PIV-5 expressing HA-113 (PIV5-113) were rescued, and immunogenicity and protective efficacy were tested in both mouse and pig models. The results showed that PIV5-113 can protect mice and pigs against challenge with viruses expressing parental HAs. The protective immunity was extended against other genetically diversified influenza H1-expressing viruses. Our work demonstrates that PIV5-based influenza vaccines are efficacious as vaccines for pigs. The PIV5 vaccine vector and chimeric HA-113 antigen are discussed in the context of the development of universal influenza vaccines and the potential contribution of PIV5-113 as a candidate universal vaccine.

3. Wang Y, Yim-Im W, Porter E, Lu N, Anderson J, Noll L, Fang Y, Zhang J, Bai J. Development of a bead-based assay for detection and differentiation of field strains and four vaccine strains of type 2 porcine reproductive and respiratory syndrome virus (PRRSV-2) in the USA. *Transbound Emerg Dis.* 2020 Aug 20. doi: 10.1111/tbed.13808. Epub ahead of print. PMID: 32816334.

Porcine reproductive and respiratory syndrome (PRRS) remains one of the most economically devastating diseases in swine population in the United States of America. Due to high mutation rate of the PRRS virus (PRRSV) genome, it is difficult to develop an accurate diagnostic assay with high strain coverage. Differentiation of field strains from the four vaccines that have been used in the USA, namely Ingelvac PRRS MLV, Ingelvac ATP, Fosterera PRRS and Prime Pac PRRS, adds an additional challenge. It is difficult to use current real-time PCR systems to detect and differentiate the field strains from the vaccine strains. Luminex xTAG technology allows us to detect more molecular targets in a single reaction with a cost similar to a single real-time PCR reaction. By analysing all available 678 type 2 PRRSV (PRRSV-2) complete genome sequences, including the 4 vaccine strains, two pairs of detection primers were designed targeting the conserved regions of ORF4-ORF7, with strain coverage of 98.8% (670/678) based on in silico analysis. The virus strains sharing  $\geq 98\%$  identity of the complete genomes with the vaccine strains were considered vaccine or vaccine-like strains. One pair of primers for each vaccine strain were designed targeting the nsp2 region. In silico analysis showed the assay matched 94.7% (54/57) of Ingelvac PRRS® MLV (MLV) strain and the MLV-like strains, and 100% of the other three vaccine strains. Analytical sensitivity of the Luminex assay was one to two logs lower than that of the reverse transcription real-time PCR assay. Evaluated with 417 PRRSV-2 positive clinical samples, 95% were detected by the Luminex assay. Compared to ORF5 sequencing results, the Luminex assay detected 92.4% (73/79) of MLV strains, 78.3% (18/23) of Fosterera strains and 50% (2/4) of ATP strains. None of the 472 samples were the Prime Pac strain tested by either ORF5 sequencing or the Luminex assay.

### Yoo Laboratory

1. Hicks JA, Yoo D, Liu HC. Transcriptional Immune Signatures of Alveolar Macrophages and the Impact of the NLRP3 Inflammasome on Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) Replication. *Viruses.* 2020 Nov 12;12(11):E1299.  
Porcine Reproductive and Respiratory Syndrome (PRRS) is a contagious viral (PRRSV) disease in pigs characterized by poor reproductive health, increased mortality, and reductions in growth rates. PRRSV is known to implement immuno-antagonistic mechanisms to evade detection and mute host responses to infection. To better understand the cellular immunosignature of PRRSV we have undertaken transcriptome and immunomodulatory studies in PRRSV-infected porcine alveolar macrophages (PAMs). We first used genome-wide transcriptome profiling (RNA-seq) to elucidate PRRSV-induced changes in the PAM transcriptome in response to infection. We found a number of cellular networks were altered by PRRSV infection, including many associated with innate immunity, such as, the NLRP3 inflammasome. To further explore the role(s) of innate immune networks in PRRSV-infected PAMs, we used an NLRP3-specific inhibitor, MCC950, to identify the potential functionality of the inflammasome during PRRSV replication. We found that PRRSV does quickly induce expression of inflammasome-associated genes in PAMs. Treatment of PAMs with MCC950 suggests NLRP3 inflammasome activation negatively impacts viral replication. Treatment of PAMs with cell culture supernatants from macrophages subjected to NLRP3 inflammasome activation (via polyinosinic-polycytidylic acid (poly I:C) transfection), prior to PRRSV infection resulted in significantly reduced viral RNA levels compared to PAMs treated with cell culture supernatants from macrophages subjected to NLRP3 inflammasome inhibition (MCC950 treatment/poly I:C transfection). This further supports a role for NLRP3 inflammasome activation in the innate macrophagic anti-PRRSV immune response and suggests that PRRSV is sensitive to the effects of NLRP3 inflammasome activity. Taken together, these transcriptome and immunoregulatory data highlight the complex changes PRRSV infection induces in the molecular immune networks of its cellular host.

2. Zhang G, Li B, Yoo D, Qin T, Zhang X, Jia Y, Cui S. Animal coronaviruses and SARS-CoV-2. *Transbound Emerg Dis.* 2020 Aug 16;10.1111/tbed.13791.

COVID-19 is a highly contagious disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). It has rapidly spread to 216 countries and territories since first outbreak in December of 2019, posing a substantial economic losses and extraordinary threats to the public health worldwide. Although bats have been suggested as the natural host of SARS-CoV-2, transmission chains of this virus, role of animals during cross-species transmission, and future concerns remain unclear. Diverse animal coronaviruses have extensively been studied since the discovery of avian coronavirus in

## Annual Station Report: Project NC-229 University of Illinois (Continued)

1930s. The current article comprehensively reviews and discusses the current understanding about animal coronaviruses and SARS-CoV-2 for their emergence, transmission, zoonotic potential, alteration of tissue/host tropism, evolution, status of vaccines and surveillance. This study aims at providing guidance for control of COVID-19 and preventative strategies for possible future outbreaks of zoonotic coronavirus via cross-species transmission.

3. An TQ, Li JN, Su CM, Yoo D. Molecular and Cellular Mechanisms for PRRSV Pathogenesis and Host Response to Infection. *Virus Res.* 2020 Sep;286:197980.

Porcine reproductive and respiratory syndrome virus (PRRSV) has caused tremendous amounts of economic losses to the swine industry for more than three decades, but its control is still unsatisfactory. A significant amount of information is available for host cell-virus interactions during infection, and it is evident that PRRSV has evolved to equip various strategies to disrupt the host antiviral system and provide favorable conditions for survival. The current study reviews viral strategies for modulations of cellular processes including innate immunity, apoptosis, microRNAs, inflammatory cytokines, and other cellular pathways.

4. Yoo HS, Yoo D. COVID-19 and veterinarians for one health, zoonotic- and reverse-zoonotic transmissions. *J Vet Sci.* 2020 May;21(3):e51.  
A novel coronavirus emerged in human populations and spread rapidly to cause the global coronavirus disease 2019 pandemic. Although the origin of the associated virus (severe acute respiratory syndrome coronavirus 2 [SARS-CoV-2]) remains unclear, genetic evidence suggests that bats are a reservoir host of the virus, and pangolins are a probable intermediate. SARS-CoV-2 has crossed the species barrier to infect humans and other animal species, and infected humans can facilitate reverse-zoonotic transmission to animals. Considering the rapidly changing interconnections among people, animals, and ecosystems, traditional roles of veterinarians should evolve to include transdisciplinary roles.
5. Yoo D, Kim H, Lee JY, Yoo HS. African swine fever: Etiology, epidemiological status in Korea, and perspective on control. *J Vet Sci.* 2020 Mar;21(2):e38.

African swine fever (ASF), caused by the ASF virus, a member of the Asfarviridae family, is one of the most important diseases in the swine industry due to its clinical and economic impacts. Since the first report of ASF a century ago, ample information has become available, but prevention and treatment measures are still inadequate. Two waves of epizootic outbreaks have occurred worldwide. While the first wave of the epizootic outbreak was controlled in most of the infected areas, the second wave is currently active in the European and Asian continents, causing severe economic losses to the pig industry. There are different patterns of spreading in the outbreaks between those in European and Asian countries. Prevention and control of ASF are very difficult due to the lack of available vaccines and effective therapeutic measures. However,

recent outbreaks in South Korea have been successfully controlled on swine farms, although feral pigs are periodically being found to be positive for the ASF virus. Therefore, we would like to share our story regarding the preparation and application of control measures. The success in controlling ASF on farms in South Korea is largely due to the awareness and education of swine farmers and practitioners, the early detection of infected animals, the implementation of strict control policies by the government, and widespread sharing of information among stakeholders. Based on the experience gained from the outbreaks in South Korea, this review describes the current understanding of the ASF virus and its pathogenic mechanisms, epidemiology, and control.

### Zuckermann Laboratory

1. Portugal R, Goatley LC, Husmann R, Zuckermann FA, Dixon LK. A porcine macrophage cell line that supports high levels of replication of OURT88/3, an attenuated strain of African swine fever virus. *Emerg Microbes Infect.* 2020 Dec;9(1):1245-1253. doi: 10.1080/22221751.2020.1772675. PMID: 32515659; PMCID: PMC7448849.

The main target cells for African swine fever virus (ASFV) replication in pigs are of monocyte macrophage lineage and express markers typical of the intermediate to late stages of differentiation. The lack of a porcine cell line, which accurately represents these target cells, limits research on virus host interactions and the development of live-attenuated vaccine strains. We show here that the continuously growing, growth factor dependent ZMAC-4 porcine macrophage cell line is susceptible to infection with eight different field isolates of ASFV. Replication in ZMAC-4 cells occurred with similar kinetics and to similar high titres as in primary porcine bone marrow cells. In addition, we showed that twelve passages of an attenuated strain of ASFV, OURT88/3, in ZMAC-4 cells did not reduce the ability of this virus to induce protection against challenge with virulent virus. Thus, the ZMAC-4 cells provide an alternative to primary cells for ASFV replication.

2. Benfield D, Lunney JK, Murtaugh M, Nelson E, Osorio F, Pogranichniy R, Ramamoorthy S, Rowland RRR, Zimmerman JJ, Zuckermann FA. The NC229 multi-station research consortium on emerging viral diseases of swine: Solving stakeholder problems through innovative science and research. *Virus Res.* 2020 Apr 15;280:197898. doi: 10.1016/j.virusres.2020.197898. Epub 2020 Feb 28. PMID: 32061619.  
The NC229 research consortium was created in 1999 in response to the emergence of porcine reproductive and respiratory syndrome virus (PRRSV), a viral agent responsible for devastating economic losses to the swine industry. The project follows the traditional "consortium" approach for Multistate Agricultural Research driven through the US State Agricultural Experiment Stations (SAES), wherein stakeholder-driven needs to combat swine infectious

## Annual Station Report: Project NC-229 University of Illinois (Continued)

diseases are identified and scientific solutions pursued by combining funds from federal, state, commodity groups, and the animal health industry. The NC229 consortium was the main driving force in successfully competing for a USDA multi-station Coordinated Agricultural Project (PRRS CAP-I) in 2004-2008, immediately followed by a renewal for 2010-2014 (PRRS CAP-II), resulting in an overall record achievement of almost \$10 million dollars. The CAP funding was not only useful for quality research, extension, and education in PRRS and related diseases, but also instrumental in enabling the group to leverage swine industry funding of more than \$34 million dollars, distributed between creative research and extension on PRRS during the last 20 years. The North American/International PRRS Symposium, now recognized by the community as a highly effective platform for the exchange of basic research findings and fundamental translational technology, is directly derived from the NC229 consortium. Other significant offshoots from NC229 include the PHGC (PRRS Host Genomic Consortium), a platform for discoveries on the role of host genetics during PRRSV infection, since 2007. Since 2009, the NC229 consortium has expanded its collective research interests beyond PRRSV to include nine other emerging viral diseases of swine. In the current project (2019-2024), African Swine Fever Virus (ASFV) retains a central focus, with the goal of harnessing the group's expertise in promoting preparedness for the global control of ASFV.

### Abstracts or Proceedings

1. Fangfeng Yuan, Xingyu Yan, Ana Stoian, Han Gao, Vlad Petrovan, Lihua Wang, Jishu Shi, Raymond R. R. Rowland, Ying Fang. Development and characterization of monoclonal antibodies against African swine fever virus. 2020 NAPRRS/NC229 Compendium.
2. Fangfeng Yuan, Xingyu Yan, Brandi Feehan, Rui Guo, Yanhua Li, Giselle Cino, Ying Fang, Douglas Marthaler. An emerging porcine sapelovirus in the United States: Genetic characterization and diagnostic tool development. 2020 NAPRRS/NC229 Compendium.
3. Fangfeng Yuan, Vlad Petrovan, Jishu Shi, Luis Gimenez-Lirola, Jeff Zimmerman, Raymond R. R. Rowland, Ying Fang. Development of a Blocking Enzyme-Linked Immunosorbent Assay for Detection of Antibodies Against African Swine Fever Virus. 2020 Conference of Research Workers in Animal Disease.
4. Federico A. Zuckermann, Robert Husmanmn, WeiYu Chen, Kyle Leistikow, Megan Duersteler, Sona Son, Mike R. King, Nathan R. Augspurger, Diego M. D. L. Navarro. *Bacillus*-based microbials lower the pathogenic synergy of a *Salmonella* Choleraesuis and PRRS virus co-infection. 2020 Conference of Research Workers in Animal Disease.

### Funding Sources

### Work Planned for Next Year

### Book Chapters or Monographs

### Non-refereed Articles

1. Yoo, D. USDA NIFA AFRI grants # 2013-67015-21243 and # 2018-67015-28287
2. Fang, Y., R.R.R. Rowland, D. Rock, Y. Li., M. Niederwerder, B. Faburay, J. Zimmerman, L. Gimenez-Lirola, B. He. Development of ASFV-specific monoclonal antibodies and mAb-based blocking ELISA (National Pork Board, 04/01/2019-12/31/2020).
3. Fang, Y., P. Roady, R.R.R. Rowland, V. Huber. Establish a pregnant sow model for assessment of influenza vaccine efficacy during pregnancy (NIH, 01/07/2018 – 12/31/2021).
4. Fang, Y., R.R.R. Rowland, J. Shi, J. Zimmerman, L. Gimenez-Lirola. Develop oral fluid-based blocking ELISA for detection of ASFV infection. (USDA Hatch Funds, 10/01/20-09/30/2021).
5. Fang, Y. A novel arterivirus protein and expression mechanism: implication in vaccine and companion diagnostic assay development (USDA-NIFA, 08/14/2020 – 02/14/2022).
6. Biao, H., Y. Fang. Developing a Parainfluenza Virus 5 (PIV5)-based PRRS Vaccine (USDA-NIFA, 4/1/2016 – 3/31/2021).
7. Zuckermann, FA. Pathogenesis of exacerbated pneumonia in PRRSV-bacterial co-infections. (USDA NIFA. FY 2017. 06/15/2017 - 06/14/2020).
8. Zuckermann, FA, Sligar, S. Increasing the breadth of protection afforded by influenza A virus vaccines for swine by targeting the matrix protein 2. (USDA NIFA. FY 2020. 07/15/2020 - 06/14/2023).

### Ying Fang

- Continue to study the role of PRRSV nsp2-related proteins in viral pathogenesis and host immunity;
- Test candidate PIV5-based PRRSV vector vaccine in nursery pig model;
- Assess the efficacy of influenza candidate vaccine in pregnant sow model;
- Develop oral-fluid based ASFV bELISA.

### Federico Zuckermann

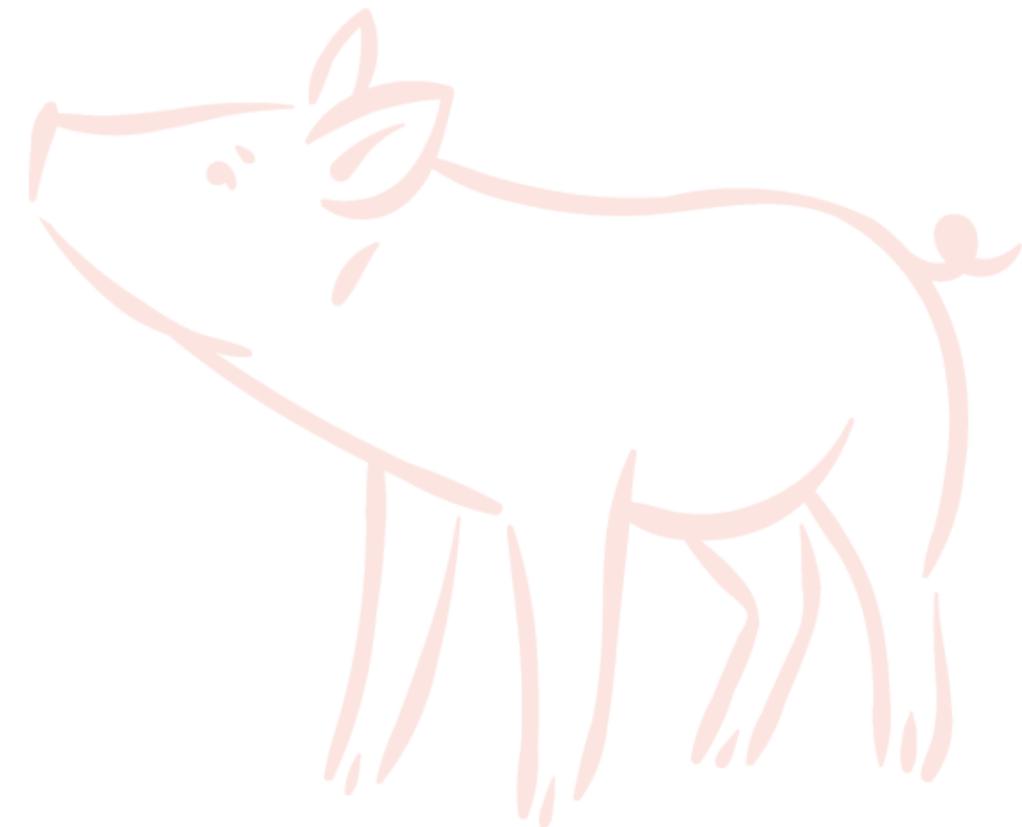
- Conduct studies to assess the ability of influenza M2 protein to increase the breath of protection afforded by conventional swine influenza A

## Annual Station Report: Project NC-229 University of Illinois (Continued)

### Changes/ Problems Due to COVID-19

vaccine to protect swine against this virus.

- Delays in the performance of animal experiments combined with a reduced access to the laboratories to satisfy COVID-19-associated safety procedures, has resulted on a reduced rate of expenditures.
- The BSL2 large animal containment facility at the University of Illinois at Urbana-Champaign was closed for several months. This closure created a backlog of animal studies. Scheduling of these studies is now moving forward, but it has been made difficult due to the high demand of the facility.



# Annual Station Report: Project NC-229

## University of Maryland

<b>Institution/Station</b>	University of Maryland, College Park, MD 20742
<b>Period Covered</b>	November 30, 2019 to November 1, 2020
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<b>Progress of Work and Principal Accomplishments</b>	<p><b>Objective 1. Control of PRRSV</b></p> <p><b>1.1. PRRS immunology/vaccinology:</b> We continued studying the atypical PRRSV strain, A2MC2, which is able to induce type I interferons in cultured cells. A2MC2 was found to induce higher level of neutralizing antibodies <i>in vivo</i> compared with the Ingelvac PRRS MLV and VR-2385. We discovered that the middle half of the A2MC2 genome is needed for triggering the interferon synthesis. Further study is undertaken to determine the critical nucleotides in the activation of interferon production. This project aims to the development of an improved vaccine against PRRS.</p> <p><b>1.2. PRRS epidemiology:</b></p> <p><b>1.3. PRRS Surveillance and Diagnostics:</b></p> <p><b>Objective 2. Developing effective and efficient approaches for detection, prevention and control of pressing viral diseases of swine of recent emergence</b></p> <p><b>2.1. ASFV:</b></p> <p><b>2.2. Swine Influenza Virus:</b></p> <p><b>2.3. Porcine Circovirus:</b></p> <p><b>2.4. Swine Pestiviruses:</b></p>

**Impact and Value of Research to Stakeholder**

**Pertinent (Swine Virology) Publications Issued or "In Press"**

**Funding Sources**

**Work Planned for Next Year**

**Changes/ Problems Due to COVID-19**

**2.5. Senecavirus:**

**2.6. Sapelovirus:**

**2.7. Viruses with potential interest to Xeno-transplantation science:**

Our studies on the interferon-inducing PRRSV A2MC2 is beneficial for vaccine development. Better protective immunity against PRRS is expected from an optimized A2MC2.

**Refereed Publications**

**Abstracts or Proceedings**

**Book Chapters or Monographs**

**Non-refereed Articles**

Maryland Agricultural Experiment Station

We will continue to characterize the mechanism of PRRSV A2MC2 in inducing production of type I interferons and explore it for vaccine development. We will also continue to examine PRRSV-cell interactions.

COVID-19 has an impact on our daily life and research progress. UMD is still at phase 2 opening for research, that is one person per 150 sqf. Delays are expected in research progress.

## Annual Station Report: Project NC-229 University of Minnesota

### Institution/Station

University of Minnesota, St. Paul, MN 55124

### Period Covered

November 30, 2019 to November 1, 2020

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### Progress of Work and Principal Accomplishments

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#### Objective 1. Control of PRRSV

##### 1.1. PRRS immunology/vaccinology:

- UMN continued work on mechanisms of immune protection and correlates of immunity, particularly in the area of neutralizing antibodies.
- UMN, in collaboration with cooperating veterinarians and producers, characterized individual variation in anti-PRRSV antibody responses that may have a genetic basis.
- UMN evaluated how partial immunity influences quasispecies evolution within and between hosts.
- UMN assessed the impact of cross-immunity on within-herd viral evolution and frequency and invasion success of new introductions into farms.
- UMN sought to quantify how landscapes of cross-immunity and population connectivity influence co-circulation and interactions among viral lineages.

##### 1.2. PRRS epidemiology:

- UMN investigated the genome variation in highly pathogenic PRRSV from U.S. outbreaks.
- UMN investigated the association between PRRS elimination in sow herds and epidemiological factors.
- UMN sought to parameterize the role of animal movement networks in PRRS epidemiology.
- UMN developed methods to detect PRRSV in used air filters from pig farms to better quantify risk of airborne exposure.
- UMN tested ultraviolet type C light farm protocols to assess inactivation of pathogens by on-farm biosecurity methods.
- UMN tested biosecurity methods to inactivate airborne PRRSV.
- UMN investigated the effect of pooling processing fluids and aggregating litters on the detection of PRRSV in breeding herds.
- UMN assessed the usage of processing fluids to detect PRRSV at the litter level.

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- UMN compared different sampling methodologies for the detection of PRRSV in the farrowing barn.
- UMN investigated spatio-temporal clusters of PRRSV ORF5 sequences in the United States.
- UMN investigated the incidence of wild type PRRSV introductions into wean to finish herds located in the Midwest and association with growing pig mortality.
- UMN in collaboration with other researchers published a critical review of the evidence and knowledge gaps of the aerosol detection and transmission of PRRSV.
- UMN assessed performance of models for the airborne spread of PRRSV.
- UMN estimated the prevalence of PRRSV in near-to-market pigs.
- UMN investigated the presence of PRRSV in pig barn manure pits.
- UMN investigated PRRSV diversity within litters and pigs in a sow farm undergoing an outbreak.

### 1.3. PRRS Surveillance and Diagnostics:

- UMN developed a rapid custom genomic toolkit to describe PRRSV diversity within animals, parities, barns, herds and farms.
- UMN described the virulent PRRSV quasispecies in re-emergent populations within piglets, parities and herds.
- UMN assessed within-herd PRRSV variability and its impact on production parameters.
- UMN related PRRSV quasispecies to re-emergence and disease outcomes.
- UMN, in collaboration with colleagues in Ohio State University, described PRRSV microevolution and diversity over time in growing pigs weaned from PRRSV positive farms.
- UMN developed an unbiased method to identify the strains of PRRSV present in pigs using RNA sequencing and bioinformatics.

### Objective 2. Developing effective and efficient approaches for detection, prevention and control of pressing viral diseases of swine of recent emergence

#### 2.1. ASFV:

- UMN developed a risk-free in situ non-animal (RISNA) surrogate assay to validate ASFV mitigation protocols.
- UMN applied the RISNA assay to confirm whether ASFV can survive in feed ingredients and complete feeds.

- UMN evaluated characteristics of supply chains (vitamins and soybean products) for the transmission of foreign viral animal diseases and application of block-chain technology to trace imported ingredients.
- UMN evaluated characteristics of supply chains (vitamins and soybean products) for the transmission of foreign viral animal diseases and application of block-chain technology to trace imported ingredients.
- UMN evaluated how Food Safety Modernization Act Preventive Controls, Foreign Supplier Verification, and Sanitary Transport rules may be used by feed industry to mitigate risk of virus spread through feed.
- UMN evaluated the potential for blockchain as a traceability solution to mitigate risk of virus spread through feed.
- UMN evaluated the risk for introduction of ASF into the US through smuggling of products through air passenger's luggage.
- UMN monitored, in collaboration with the swine industry, the evolution of the global spread of ASF through the Swine Disease Global Surveillance project.
- UMN worked with the Vietnamese Department of Animal Health to create capacity for ASF control.
- UMN worked with the Vietnamese National University of Agriculture to begin performing risk assessments and identify pathways of ASF into USA boar studs informed by Vietnamese ASF outbreak data, epidemiology reports, and experience.

#### 2.2: Swine Influenza Virus:

- UMN assessed influenza surveillance methods taking into consideration individual and group sampling approaches, and the environment.
- UMN developed a new sampling technique, udder wipes, to detect influenza in breeding herds in animals prior to weaning.
- UMN established a nurse sow model to study the experimental transmission of influenza virus.
- UMN investigated farm management factors that contribute to the transmission and recirculation of influenza in breeding herds.
- UMN researchers described factors associated with influenza A virus infection in piglets at weaning.
- UMN identified the use of nurse sows as a source of virus transmission within herds with piglets adopted by nurse sows resulting in higher likelihood of influenza infections shortly after adoption.
- UMN, investigated the persistence between seasons of influenza A virus in live animal markets.
- UMN investigated the transmission of a newly commercialized live

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attenuated influenza vaccine to non-vaccinated in contact pigs and the environment.

- UMN is in the process of investigating the role of farm workers at introducing seasonal influenza viruses into swine farms.
- UMN in collaboration with researchers in India evaluated the potential risk of transmission of avian influenza A viruses at the human-animal interface in response to unusual crow die-offs in Bangladesh.
- UMN in collaboration with researchers in India developed a protocol for a cross-sectional observational study with a One Health approach to study the transmission of avian influenza A viruses from animals to humans at the human-animal interface in Bangladesh.
- UMN investigated different vaccination approaches exploring heterologous prime-boost using whole inactivated vaccines to enhance influenza control in pigs.
- UMN evaluated the effect of influenza A virus sow vaccination on infection in pigs at weaning.
- UMN investigated patterns and parameters of influenza A virus transmission in nursery pigs.
- UMN investigators worked in collaboration with colleagues in Chile to identify novel human derived influenza viruses in pigs with zoonotic potential.
- UMN established and validated novel sampling methods to conduct surveillance of influenza virus.
- UMN developed a magnetic particle spectroscopy (MPS) based hand-held device to detect Influenza A virus in a single step reaction from clinical samples.

### 2.3. Porcine Circovirus:

### 2.4. Swine Pestiviruses:

### 2.5. Senecavirus:

- UMN estimated the seroprevalence of Senecavirus A and assessed risk factors in the US swine industry.
- UMN developed a method to identify and characterize emerging viral diseases using a hand-held sequencer and bioinformatics tools using SVA as a model.
- UMN is investigating the time-to-negative processing fluids in farms undergoing an outbreak.
- UMN investigated the duration of IgG in sows after an outbreak of SVA.

### Impact and Value of Research to Stakeholder

### 2.6. Sapelovirus:

### 2.7. Viruses with potential interest to Xeno-transplantation science:

#### UMN Outreach to Veterinarians & Scientific community:

The Allen D. Leman Swine Conference is an annual educational event for the global swine industry organized by faculty at the University of Minnesota College of Veterinary Medicine. It is internationally acclaimed for bringing science-driven solutions to the complex challenges facing the industry. This year the conference was attended by over nine hundred participants from over 20 countries, including swine veterinarians and other professionals working in swine production and animal health management. The conference had a good mix of applied research in areas of swine health and productivity and highlighted topics such as African swine fever, PRRS, *Mycoplasma hyopneumoniae*, influenza, pre-weaning mortality, antibiotic resistance, models of pig production, *Streptococcus suis*, risk assessment and biosecurity.

#### Specific outcomes & accomplishments by UMN:

##### 1. PRRSV

- UMN was able to quantify the co-circulation, emergence and sequential turnover of multiple PRRSV lineages in a single swine-producing region in the United States over a span of 9 years (2009–2017). We reported on the rapid emergence of novel sub-lineages (within 1-7-4 RFLP clade) that appeared to be absent globally pre-2008. The sequential dominance of different lineages, as well as three different sub-lineages within lineage 1, is consistent with the immune-mediated selection hypothesis for the sequential turnover in the dominant lineage.
- UMN investigated time-to-stability (TTS) after a PRRSV outbreak in six production systems located in the Midwestern United States. We found the median TTS was 41.0 weeks. Farms that broke in the winter achieved stability sooner than farms that experienced PRRS outbreaks during summer. Farms that had a PRRS outbreak associated with a 1-7-4 PRRSV lineage took significantly longer to achieve stability.
- UMN explored the role of area spread on the occurrence of new PRRSV cases by combining information on genetic similarity among recovered PRRSV isolates and publicly available weather data. Our data did not support the area spread theory as the main cause for

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PRRSV outbreaks. We suggest that for future studies, analysis of animal movement and other links between farms such as personnel, equipment and sharing of service providers should be incorporated for better insights on source of the virus.

- UMN validated a sampling technique known as processing fluids (PFs) that accumulate from tissues obtained during tail docking and castration as an alternate sample type to quantify PRRSV infection. We estimated that aggregation of at least 50 litters was possible when a pig with a Ct value of ~22 was present in the sample, and aggregation of up to 40 litters was possible when there was a sample with a Ct value of ~33.
- UMN modeled the air filtration conditions used on sow farms and found that higher barn PRRSV concentrations were obtained with lower mechanical ventilating rates and higher barn infiltration rates. Overall, the results indicated that certain filter combinations reduced overall virus penetration and barn virus concentrations by 57% to 80% for the conditions modeled.
- UMN assessed the presence of PRRSV in the environment (surfaces and air) of farrowing rooms, and udder skin of lactating sows as an indirect measure of piglet PRRSV status. PRRSV was detected at processing in udder skin wipes, environmental wipes and airborne deposited particle samples up to 14 weeks post outbreak and at weaning in udder skin wipes up to 17 weeks post outbreak. PRRSV was detected in the environment and the udder skin of lactating sows, which indicates that aggregate samples of the environment or lactating sows may be used to evaluate the PRRSV status of the herd in pigs prior to weaning. Our findings also highlight potential sources of PRRSV infection for piglets in breeding herds.
- UMN provided evidence of a national program, MSHMP, for voluntary sharing of disease status data that has helped the implementation of surveillance activities that, ultimately, allowed the generation of critically important scientific information to better support disease control activities.
- UMN estimated the prevalence of PRRSV in near-to-market pigs. A quarter of the farms (n=85) involved in the study had at least one PCR+ positive pool. The majority of the positive results were due to Type II PRRSV. Only 5 sequences were obtained, and all belong to Type II viruses. No vaccine viruses were sequenced from PCR+ samples. Overall, this study indicated that pigs that are being sent to market may still be shedding virus which can potentially contaminate trucks.

- UMN assessed the presence of PRRSV in manure pits and 9% of the pit barns tested yielded a PCR positive results. No viable virus was found. Most of the positive pits were from growing pig farms.

### 2. IAV:

- UMN evaluated the association between IAV infection in piglets at weaning and farm factors including farm features, herd management practices and gilt- and piglet-specific management procedures performed at the farm. Among all the factors evaluated (n = 24), and considering the season-adjusted multivariable analysis, only sow IAV vaccination and gilt IAV status at entry significantly reduced (p-value<0.05) IAV infections in piglets at weaning.
- UMN evaluated the effect of maternally-derived antibodies at weaning on IAV prevalence at weaning, time of influenza infection, number of weeks that pigs tested IAV positive, and estimated quantity of IAV in nursery pigs. We observed that sow vaccination or infection status that results in high levels of IAV strain-specific maternally-derived antibodies may help to reduce IAV circulation in both suckling and nursery pigs.
- UMN provided new information on sampling approaches to conduct effective influenza surveillance in pigs and identifies udder wipes from lactating sows as a novel sample type that offers a convenient, cheap and sensitive manner to monitor IAV in litters prior to weaning.
- UMN evaluated the effect of IAV vaccination on aerosol shedding in pigs housed in warm environmental conditions. A significantly higher proportion of infected pigs was detected in the non-vaccinated than in the vaccinated group. We showed that both the decrease in shedding and the increase in environmental temperature may have contributed to the inability to detect airborne IAV in vaccinated pigs.
- Using functionalized magnetic nanoparticles, the University of Minnesota developed a highly sensitive Influenza A antigen detection assay that detects H1N1 nucleoprotein at concentrations as low as 4.4 pico moles.

### 3. PEDV:

- UMN evaluated the survival of PEDV on inanimate objects routinely used on swine farms such as styrofoam, rubber, plastic, coveralls, and other equipment. Our findings indicate that the type of fomite material and temperatures impact PEDV stability, which is important in understanding the nuances of indirect transmission and epidemiology of PEDV
- UMN integrated data on animal movements with environmental

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risk factors to identify the occurrence of PED) outbreaks. Our best algorithm was able to correctly predict whether an outbreak occurred during one-week periods with >80% accuracy. Our model forms the foundation for near real-time disease mapping and will advance disease surveillance and control for endemic swine pathogens in the United States.

#### 4. Rotavirus C

- UMN sequenced 45 Rotavirus C (RVC) complete genomes from swine samples collected in the United States and Mexico. A phylogenetic analysis of each genome segment indicates that RVC populations have been evolving independently in human, swine, canine, and bovine hosts for at least the last century, with inter-species transmission events occurring deep in the phylogenetic tree, and none in the last 100 years. Pigs may act as a reservoir host for RVC, and a source of the lineages identified in other species, including humans, but additional sequencing is needed to understand the full diversity of this understudied pathogen across multiple host species.

#### Refereed Publications

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34. Schroeder DC. (2019) The ABC's of ASF (African Swine Fever) spread and testing -80th Minnesota Nutrition Conference, September 18-19, 2019, Mankato Civic Center, Mankato, MN
35. St. Charles K, Walz E, Culhane M (2020). Workload schedules, biosecurity practices, and communication preferences of truck drivers transporting pigs and their potential implications for disease spread in the United States. . EuFMD Open Session, December 8, 10, 15, 17 2020 eufmdvirtual.com
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37. Tan, S., Dvorak, C. Viral strain identification in field samples using nanopore MinION direct RNA sequencing. American Society for Virology Conference July 20-24, 2019. Poster P27-2.
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39. Torremorell M (2018). Transmission and control of influenza: the role of the piglet. Proc Conf Research Workers Anim Diseases, Chicago, IL, # 6.
40. Torremorell M (2018). Transmission of influenza A virus in pigs: the role of the piglet. Proc Int Pig Vet Soc, Chongqing, China, p:71-73.
41. Torremorell M (2019). Influenza: Herd immunity and transmission. Proc Am Assoc Swine Veterinarians, Orlando, FL, p: 408-410.
42. Torremorell M, Janni K (2018). To filter or not to filter, that's not the question anymore. National Hog Farmer, November 2018. <https://www.nationalhogfarmer.com/buildings/filter-or-not-filter-s-not-question-anymore>.
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45. van de Ligt, J, Urriola P (2019) Geeks to Geeks Case study #1: Introduction of ASF to via contaminated feed. Allen D. Leman Swine Conference. St. Paul, MN.
46. van de Ligt, J, Urriola P, Shurson G, Schroeder D, Sampedro F (2019) Risk of ASF virus introduction in vitamins, soybean products, and other feed additives. Allen D. Leman Swine Conference. St. Paul, MN.
47. van de Ligt, J. (2018) Blockchain: What Is It and Why Should I Care? 122nd Annual Meeting of the United States Animal Health Association. Kansas City, MO.
48. VanderWaal, K. (2018) Forecasting outbreaks of PRRS and PED in swine movement networks? North American PRRS Symposium, Chicago, IL. December 1-3, 2018.
49. VanderWaal, K., I. Paploski, R. Bhojwani, C. Corzo. Forecasting outbreaks of PEDV. Allen D. Leman Swine Conference. St. Paul, MN. September 14-17, 2019.
50. VanderWaal, K., I. Paposki, R. Bhojwani, A. Perez, G. Machado, C. Vilalta, C. Corzo. Can we predict outbreaks of PRRS and PED viruses? (2018) Allen D. Leman Swine Conference, St. Paul, MN Sept 15-17, 2018. Swine Health Information Center sponsored speaker.
51. Vilalta C, Janni K, Alonso C, Torremorell M (2018). Model to estimate PRRSV introduction in filtered farms with negative pressure. Proc Am Assoc Swine Veterinarians, San Diego, CA, p: 386.

## Annual Station Report: Project NC-229 University of Minnesota (Continued)

52. Xia T, Yang M, Marabella I, Zarleng D, Olson B, Torremorell M, Clack HL (2018). Inactivation of airborne porcine reproductive and respiratory syndrome virus (PRRSV) by non-thermal plasma. International Conference on Plasma Medicine.
53. VanderWaal, K. & I. Paploski. Machine learning, disease forecasting, and big data in epidemiology. "Emerging technologies" session. American Association of Swine Veterinarians. Atlanta, GA.
54. VanderWaal, K. Modeling the population-level effects of vaccination & control of infectious diseases. Leman China Swine Conference. October 14-16, 2020.
55. VanderWaal, K., L. Black, J. Hodge, A. Bedada, S. Dee. Modeling transmission of SARS-CoV-2 in pork processing plants. Allen D. Leman Swine Conference. St. Paul, MN. September 28-20, 2020.
56. Pamornchainavakul, N., I. Paploski, D. Makau, C. Corzo, VanderWaal, K. Estimating farm-level R's for PRRSV using sequence-based transmission trees. Allen D. Leman Swine Conference. St. Paul, MN. September 28-20, 2020.
57. VanderWaal, K. Machine learning as a tool for animal health and productivity. Allen D. Leman Swine Conference. St. Paul, MN. September 28-20, 2020.
58. Kikuti, M. K. VanderWaal, D. Schroeder, C. Vilalta, J. Sanhueza, C. Corzo. Understanding PRRSV diversity at the pig and litter levels using whole-genome sequencing. Allen D. Leman Swine Conference. St. Paul, MN. September 28-20, 2020.
59. Young, J.E., Goldeck, D., Dvorak, C., Graham, S., Murtaugh, M. Novel Technique for the Isolation of Neutralizing Antibodies against PRRSV. American Society for Virology Conference July 20-24, 2019. Poster P27-3.
60. Young, J.E, C. Dvorak, S. Graham, M. Murtaugh. Isolation and immortalization of PRRSV GP5 specific porcine B-cells. CRWAD proceedings 2019. Oral presentation #8.
61. VD Krishna, M Pieters, M Surendran Nair, F Munhoz dos Anjos Leal, JP Wang, and M C-J Cheeran. Development of monoclonal antibodies specific to Mycoplasma hyopneumoniae. Leman Swine Health Conference 2019.

### Book Chapters or Monographs

1. Culhane M, Garrido J, Torremorell M. Chapter 21 - Specimen Types, Collection, and Transport for Influenza A Viruses of Swine. In Animal Influenza Virus: Methods and Protocols, Third Edition, Springer, Methods

- in Molecular Biology. Erica Spackman, PhD, Editor (2019, in press)
2. Martelli P, Segalés J, Torremorell M et al. Swine Respiratory Disease. Chapter titled "Porcine respiratory disease complex: Influenza". Editorial Servet. 2019. n° ISBN del libro es 978-84-17225-86-5
3. Shepard F, Culhane M, Marthaler D(2019). Reoviruses (Rotaviruses and Reoviruses). Diseases of Swine. Eds: Zimmerman JJ, Karriker LA, Ramirez A, Schwartz KJ, Stevenson GW, Zhang J. 11th Edition, Wiley-Blackwell Publisher, ISBN: 978-1-119-35085-9
4. Zimmerman J, Benfield DA, Dee SA, Murtaugh MP, Osorio F, Stevenson GW, Torremorell M (2019). Porcine reproductive and respiratory syndrome virus (porcine arterivirus). Diseases of Swine. Eds: Zimmerman JJ, Karriker LA, Ramirez A, Schwartz KJ, Stevenson GW, Zhang J. 11th Edition, Wiley-Blackwell Publisher, ISBN: 978-1-119-35085-9

### Non-refereed Articles

1. 2016-2019 Broadly neutralizing antibodies to PRRSV. Principal Investigator, Murtaugh. USDA NIFA.
2. 2016-2021 Optimizing assessment of virus containing particles in animal agriculture. Principal Investigator, Raynor. NIOSH/NIH.
3. 2016-2021 Longitudinal study of infectious disease risks at the human-swine interface. Principal Investigator, Davies. NIOSH/NIH
4. 2017-2018 Dynamic mapping of PRRS and PED infection risk across space and time. Principal Investigator, VanderWaal. Swine Health Information Center.
5. 2017-2018 A near-real time global surveillance system for swine diseases. Principal Investigator, Perez. Swine Health Information Center.
6. 2017-2018 Development and implementation of a domestic swine bio-surveillance monitoring and surveillance system. Part 2: Multiple data streams integration and reporting. Principal Investigator, Torrison. Swine Health Information Center.
7. 2017-2018 Using Swine Health Monitoring Project to Facilitate Business Continuity. Principal Investigator, Perez. MN Board of Animal Health.
8. 2017-2019 Developing multiplex Giant magnetoresistance (GMR) biosensors for the detection of swine respiratory pathogens. Principal Investigator, Cherran. CVM Emerging and Zoonotic Diseases.
9. 2017-2019 A comprehensive surveillance system to control influenza in pigs. Principal Investigator, Torremorell. Rapid Agricultural Response Fund (renewal).
10. 2018-2019 Comparison of different sampling techniques for PRRSV

### Funding Sources

## Annual Station Report: Project NC-229 University of Minnesota (Continued)

- detection during stabilization. Principal Investigator, Corzo. Swine Diseases Eradication Center.
11. 2018-2019 Estimating the seroprevalence of Senecavirus A in the United States Swine Industry. Principal Investigator, Corzo. National Pork Board.
  12. 2018-2019 Using genomic tools to link PRRSV quasispecies to disease severity in pigs. Principal Investigator, Torremorell. National Pork Board.
  13. 2018-2019 Building a HARPC framework and use of blockchain technology to evaluate and minimize risk of transmission of foreign viruses in imported animal feed ingredients. Principal Investigator, Urriola. SHIC.
  14. 2018-2020 Assessing within-herd PRRS variability and its impact on production parameters. Principal Investigator, Arruda and Schroeder. National Pork Board.
  15. 2018-2019 Developing the Morrison Swine Health Monitoring Project (SHMP) to build capacity and enable the Swine Health Information Center: Year 4. Principal Investigator, Corzo. Swine Health Information Center.
  16. 2018-2019 Using genomic tools to link PRRSV quasispecies to disease severity in pigs infected with contemporary virulent isolates. Principal Investigator, Torremorell and Schroeder. National Pork Board.
  17. 2018-2019 Efficacy of prime boost vaccination protocols in pigs challenged with influenza A viruses (renewal). Principal Investigator, Torremorell. Zoetis.
  18. 2018-2019 Shedding of live attenuated influenza vaccine (LAIV) Ingelvac Provenza under field conditions. Principal Investigator, Torremorell. Boehringer Ingelheim.
  19. 2018-2019 Are nurse sows a risk for influenza transmission in piglets? Principal Investigator, Torremorell. Minnesota Pork Board.
  20. 2018-2020 Redefining the PRRSV paradigm: Persistence, Re-infection, Re-emergence and Spread. Principal Investigator, Schroeder, Perez, VandeWaal, and Torremorell. UMN AES
  21. 2018-2020 Exploring the viral composition of influenza in swine to determine if swine have a role in the transmission of avian influenza in Minnesota. Principal Investigator, Torremorell. MAES.
  22. 2018-2020 Studying the bidirectional transmission of influenza between pigs and people in swine herds. Principal Investigator, Torremorell. National Pork Board.
  23. 2018-2020 Development of a multi-strain modeling framework for endemic swine pathogens. Principal Investigator, Craft. Animal Health Formula.
  24. 2018-2020 Rapid, Pen-side Molecular Diagnostic Tool for Foreign Animal Disease. Principal Investigator, Dvorak. National Pork Board.

### Work Planned for Next Year

25. 2019-2020 Prevalence of PRRSV and PEDv in barn pits during manure pumping and land application in Minnesota. Principal Investigator, Corzo. UMN Signature Program.
  26. 2019-2020 Risk-free in situ non-animal (RISNA) surrogate assay for evaluating mitigation and inactivation strategies of African Swine Fever virus in feed ingredients. Principal Investigator, van de Ligt and Shurson. USB.
  27. 2019-2020 Genetic diversity of PRRSV in piglets during an outbreak. Principal Investigator, Kikuti. Boehringer Ingelheim.
  28. 2019-2020 Towards the best strategy for PRRSV stability: time-to-negative processing fluids in breeding herds. Principal Investigator, Sanhueza. Boehringer Ingelheim.
  29. 2019-2020 Porcine reproductive and respiratory syndrome virus (PRRSV) microevolution and diversity over time in growing pigs weaned from PRRSV positive farms. Principal Investigator, Kikuti. Swine Diseases Eradication Center.
  30. 2019-2020 Evaluation of the role of parity, personnel and cross-fostering in influenza virus infection during the pre-weaning period. Principal Investigator, Torremorell. Boehringer Ingelheim.
  31. 2019-2021 Epidemiological workforce development in support of the national Agro and Bio-defense facility. Principal Investigator, Perez. USDA: ARS.
  32. 2019-2023 Drivers of diversity and transmission of co-circulating viral lineages in host meta-populations. Principal Investigator, VandeWaal, Cheeran, Schroeder, and Corzo. NSF-USDA.
  33. 2020-2021 Determining the pathways for ASF introduction into boar studs and risk of ASF transmission via semen movements during an ASF outbreak. Swine Health Information Center Culhane
  34. 2020-2021 Farm level factors associated with PRRSV shedding status in near-to-market pigs. Principal Investigator, Corzo. Boehringer Ingelheim.
  35. 2020-2021 Assessing time to negative processing fluids in breeding herds after a Senecavirus A outbreak. Principal Investigator, Corzo, American Association of Swine Veterinarians Foundation.
  36. 2020-2021 Portable magnetic particle spectroscopy (MPS) platform for on-field detection of Influenza A virus and Mycoplasma hyopneumoniae. Principal Investigator, Wang, Cheeran, and Pieters. USDA/AFRI.
- To expand investigations into the bidirectional transmission of influenza between pigs and people.
  - To explore the use of use air filters as a novel surveillance method for airborne pathogens.

## Annual Station Report: Project NC-229 University of Minnesota (Continued)

- To investigate risk factors that contribute to wild type PRRS virus introductions into grow-finish pigs.
- To develop the RISNA surrogate assay for ASFV mitigation and inactivation.
- To investigate the role of neutralizing antibodies in PRRSV cross-protection.
- To investigate host factors associated with PRRSV susceptibility and resistance.
- To investigate host factors associated with PRRSV susceptibility and resistance.
- To determine infection incidence in growing pigs, specifically to identify when and how often new PRRSV infections happen in wean-to-finish pigs.
- To evaluate risk factors associated to PRRSV infection in growing pigs.
- To associate production and economic impact of PRRSV infections in growing pigs.
- To evaluate how partial immunity influences quasispecies evolution within and between hosts.
- To assess the impact of cross-immunity on within-herd viral evolution and frequency and invasion success of new introductions into farms.
- To quantify how landscapes of cross-immunity and population connectivity influence co-circulation and interactions among viral lineages.
- To formulate models for forecasting risk for PRRSV spread.
- To investigate host factors associated with PCV2 susceptibility and resistance.
- To build a risk based model of porcine virus transmission in feed ingredients.
- To formulate models for between-farm transmission of exotic viruses.
- To evaluate mechanisms of influenza virus transmission and persistence in piglets.
- To evaluate the effect of maternally derived antibodies against influenza A virus on infection dynamics in growing pigs.
- To investigate patterns and dynamics of influenza A virus transmission in growing pigs.
- To investigate farm factors associated with influenza A virus detection in piglets at weaning.
- To investigate the bi-directional transmission of influenza A virus between pigs and people.
- To evaluate the impact of vaccination on influenza A virus genetic and antigenic diversity in piglets.

### Changes/ Problems Due to COVID-19

- To evaluate strategies of vaccination to control influenza in piglets at weaning.
- To investigate methodologies and approaches to inactivate airborne viruses.
- To develop and optimize methods to assess virus containing particles in animal agriculture.
- To develop a standard ELISA test that can distinguish *Mycoplasma hyopneumonia* from other pathogenic and commercial mycoplasma in clinic samples.
- To develop a diagnostic GMR biosensor array and hand-held MPS system that can detect influenza, PRRSV and *Mycoplasma hyopneumoniae* in clinical samples.
- To determine the risk of introducing African Swine Fever (ASF) to a sow farm as a result of semen movement from apparently healthy boar studs located in an ASF disease control area.

## Annual Station Report: Project NC-229

### University of Nebraska-Lincoln

<b>Institution/Station</b>	University of Nebraska-Lincoln, Lincoln, NE 68583
<b>Period Covered</b>	November 30, 2019 to November 1, 2020
<b>Personnel</b>	<p><b>NC-229 Station Representative:</b></p> <ul style="list-style-type: none"> <li>Hiep Vu, Assistant Professor, hiepvu@unl.edu</li> </ul> <p><b>Other Principal Leaders Associated With the Projects:</b></p> <ul style="list-style-type: none"> <li>Daniel Ciobanu, Professor, dciobanu2@unl.edu</li> <li>Fernando Osorio, Professor, fosorio@unl.edu</li> </ul> <p><b>Staff and Students:</b></p> <ul style="list-style-type: none"> <li>Lianna Walker, PhD Student</li> <li>Hiruni Wijesena, PhD Student</li> <li>Kylee Sutton, MS Student</li> <li>Jayeshbhai Chaudhari, PhD Student, jayeshvet03@gmail.com</li> <li>Sushmita Kumari, MS Student, sushmitak.726@gmail.com</li> <li>Hung Luong, Visisting Scholar, lhungpt@gmail.com</li> <li>Raquel Arruda Leme, Visiting Scholar, raquelarrudaleme@gmail.com</li> <li>Kassandra Durazo, Visiting Scholar, yuruvithdurazo@gmail.com</li> </ul>
<b>Progress of Work and Principal Accomplishments</b>	<p>We had previously shown that PRRSV replicates in testicular germ cells of sexually mature boars, altering spermatogenesis, and inducing germ cell death by apoptosis. Continuing along such research line, we have now established models of in vitro infection by PRRSV on purified single-cell suspensions of spermatogonial stem cells (SCCs) obtained from neonate pigs, and use such model to study the kinetics of PRRSV replication in this testicular cell population.</p> <p>We performed a transcriptomic study to identify host factors that facilitate PRRSV live attenuated vaccine</p> <p>We established a high throughput assay for simultaneous measurement of swine antibody response against different viral proteins</p> <p>During this year our research focused on 1) dissection of the role of Swine Leukocyte Antigen II (SLAII) locus on PCV2 and PRRSV susceptibility, and 2) development of a robust assay for detection of APPV</p>

#### Objective 1. Control of PRRSV

1.1. PRRS immunology/vaccinology:

1.2. PRRS epidemiology:

1.3. PRRS Surveillance and Diagnostics:

#### Objective 2. Developing effective and efficient approaches for detection, prevention and control of pressing viral diseases of swine of recent emergence

2.1. ASFV:

2.2. Swine Influenza Virus:

2.3. Porcine Circovirus:

- Development of custom genotyping array to assist in the dissection of the QTL located on chr. 7, near SLA/MHC II locus.  
Genetic dissection of the QTL located on chromosome 7 near Swine Leukocyte Antigen Complex II (SLAII) was affected by the scarcity of the genetic markers in the commercially available genotyping arrays. The SLAII region was found to be associated with PCV2 replication and PRRSV immune response. In order to correct for this shortfall, we designed a new custom genotyping arrays, called SowPro91. This array is enriched in Single Nucleotide Polymorphisms (SNP) in the SLAII genomic region, and especially in the region that separates SLAI from SLAII. The number of SNPs in the SLA region in the commercially available genotyping arrays (e.g., Illumina Porcine SNP60 BeadArray) is limited (45 SNPs) compared to our SowPro91 array (3,432 SNPs).
- Infer SowPro91 Haplotypes and Genome-wide Association Analyses in the UNL Population Using BayesIM. Since the majority of the PCV2 dataset (total n=974, ~80%) was only genotyped with the more limited Porcine SNP60 BeadArray, a Bayesian method (BayesIM) that utilizes haplotypes instead of individual SNP in association analysis was implemented to infer SowPro91 haplotypes to the entire PCV2 dataset. This approach, which is alternative to imputation, extended the number of SNPs from 53,529 (Illumina SNP60 Bead Array) to 105,601 SNPs (SowPro91). This method increased substantially the number of genetic markers, especially in the SLAII region, improving our ability to dissect the role of host genetics in viral disease susceptibility.

## Annual Station Report: Project NC-229 University of Nebraska-Lincoln (Continued)

### 2.4. Swine Pestiviruses:

### 2.5. Senecavirus:

### 2.6. Sapelovirus:

### 2.7. Viruses with potential interest to Xeno-transplantation science:

### 2.8. APPV:

In the last year we continue to sample new cases of congenital tremor leading to sequencing novel strains of APPV from multiple sites across Midwest. Substantial variability across the APPV genome impacts the ability to detect with high-precision APPV presence. Preliminary analysis uncovered a conserved genomic region that could be exploited as a site for a diagnostic test able to uncover any APPV strain.

1. Development of a novel genotyping tool for dissection the SLAI region for its effect in viral disease susceptibility
2. Evaluation of a new statistical approach to detect DNA markers associated with viral disease susceptibility
3. Development of a novel diagnostic assay for detection of APPV
4. Development of a new assay for studying antibody responses to different viral proteins
5. Identification of host factors that might support PRRSV persistence in pigs
6. Identification of Swine testicular cell that is susceptible to PRRSV infection

### Refereed Publications

1. Chaudhari J, Vu HLX. Porcine Reproductive and Respiratory Syndrome Virus Reverse Genetics and the Major Applications. *Viruses*. 2020 Oct 31;12(11). doi: 10.3390/v12111245. Review. PubMed PMID: 33142752; PubMed Central PMCID: PMC7692847.
2. Luong HQ, Lai HTL, Vu HLX. Evaluation of Antibody Response Directed against Porcine Reproductive and Respiratory Syndrome Virus Structural Proteins. *Vaccines (Basel)*. 2020 Sep 16;8(3). doi: 10.3390/vaccines8030533. PubMed PMID: 32947931; PubMed Central PMCID: PMC7564207.
3. Chaudhari J, Liew CS, Workman AM, Riethoven JM, Steffen D, Sillman S, Vu HLX. Host Transcriptional Response to Persistent Infection with a Live-Attenuated Porcine Reproductive and Respiratory Syndrome Virus Strain.

*Viruses*. 2020 Jul 28;12(8). doi: 10.3390/v12080817. PubMed PMID: 32731586; PubMed Central PMCID: PMC7474429.

4. Benfield D, Lunney JK, Murtaugh M, Nelson E, Osorio F, Pogranichniy R, Ramamoorthy S, Rowland RRR, Zimmerman JJ, Zuckermann FA. The NC229 multi-station research consortium on emerging viral diseases of swine: Solving stakeholder problems through innovative science and research. *Virus Res*. 2020 Apr 15;280:197898. doi: 10.1016/j.virusres.2020.197898. Epub 2020 Feb 28. PubMed PMID: 32061619

### Abstracts or Proceedings

### Book Chapters or Monographs

### Non-refereed Articles

1. Vu H., Evaluate the diagnostic performance of pen-side tests for ASF detection, Swine Health Information Center grant No. 20-078
2. Vu H., Time and temperature required for complete inactivation of African swine fever virus, Swine Health Information Center grant No. 20-071
3. Vu H., Gauger P. and Ly H., Development of a broadly protective vaccine against swine influenza virus. USDA-NIFA Gant No 2020-67015-31414
4. Ciobanu D., Vu H., and Kachman SD., Deconstructing the role of SYNGR2 in viral disease susceptibility in livestock. USDA-NIFA
5. Vu H., and Osorio F., Development of a broadly protective DIVA marker vaccine against porcine reproductive and respiratory syndrome virus. USDA NIFA Grant No. 2018-67015-28294

We will continue working on both objective 1 and 2 of this project.

Specifically, we will work on the following topics

1. Evaluate the diagnostic performance of pen-side tests for ASF detection
2. Development of a broadly protective DIVA marker vaccine against porcine reproductive and respiratory syndrome virus
3. Development of a broadly protective vaccine against swine influenza virus
4. Deconstructing the role of SYNGR2 in viral disease susceptibility in livestock
5. Establish models of in vitro infection by PRRSV on purified single-cell suspensions of spermatogonial stem cells (SCCs) obtained from neonate pigs, and use such model to study the kinetics of PRRSV replication in this testicular cell population

### Impact and Value of Research to Stakeholder

### Pertinent (Swine Virology) Publications Issued or "In Press"

### Funding Sources

### Work Planned for Next Year

### Changes/Problems Due to COVID-19

# Annual Station Report: Project NC-229

## USDA-ARS-National Animal Disease Center

<b>Institution/Station</b>	USDA-ARS-National Animal Disease Center, Ames, IA, 50010
<b>Period Covered</b>	November 30, 2019 to November 1, 2020
<b>Personnel</b>	<p><b>NC-229 Station Representative:</b></p> <ul style="list-style-type: none"> <li>Laura C. Miller, Research Microbiologist, <a href="mailto:laura.miller@usda.gov">laura.miller@usda.gov</a></li> </ul> <p><b>Other Principal Leaders Associated With the Projects:</b></p> <ul style="list-style-type: none"> <li>Kay S. Faaberg, Research Microbiologist, <a href="mailto:kay.faaberg@usda.gov">kay.faaberg@usda.gov</a></li> <li>Kelly M. Lager, Supervisory Research VMO, <a href="mailto:kelly.lager@usda.gov">kelly.lager@usda.gov</a></li> <li>Alexandra C. Buckley, Research VMO, <a href="mailto:alexandra.buckley@usda.gov">alexandra.buckley@usda.gov</a></li> </ul> <p><b>Staff and Students:</b></p> <ul style="list-style-type: none"> <li>Damarius S. Fleming, ORISE, <a href="mailto:damarius.fleming@usda.gov">damarius.fleming@usda.gov</a></li> <li>Deborah S. Adolphson, Biological Science Lab Technician, <a href="mailto:adolphson@usda.gov">adolphson@usda.gov</a></li> <li>David D. Michael, Microbiologist, <a href="mailto:david.michael@usda.gov">david.michael@usda.gov</a></li> <li>Sarah J. Anderson, Biological Science Lab Technician, <a href="mailto:sarah.j.anderson@usda.gov">sarah.j.anderson@usda.gov</a></li> <li>Miranda Dietze, Biological Science Lab Technician, <a href="mailto:miranda.dietze@usda.gov">miranda.dietze@usda.gov</a></li> <li>Colin Stoy, Biological Science Lab Technician, <a href="mailto:colin.stoy@usda.gov">colin.stoy@usda.gov</a></li> </ul>
<b>Progress of Work and Principal Accomplishments</b>	<ol style="list-style-type: none"> <li>Developed a modified-live attenuated porcine epidemic diarrhea virus (PEDV) vaccine. Using an infectious clone of CoV porcine epidemic diarrhea virus (icPEDV), we generated viruses with inactive versions of three interferon antagonist regions individually or combined in one virus designated icPEDV-mut4.</li> <li>Examined the expression profile of miRNA and tRNA expressed in whole blood between healthy and highly pathogenic PRRSV-infected young pigs.</li> <li>Completed comparison of the host RNA expression to acquire a better understanding of PRRS disease by comparing gene expression changes that occur in tracheobronchial lymph nodes of pigs infected with either PRRSV, porcine circovirus type 2 (PCV2), or swine influenza A virus (IAV-S) infections. The results showed that PRRSV, IAV-S and PCV-2 viral infections followed a clinical course typical of experimental infection of young pigs. Gene expression results uncovered genes related to shared and unique host immune responses to the 3 viruses.</li> <li>Performed gene ontology enrichment to facilitate pathway and network analyses of candidate gene lists from high-throughput studies. A</li> </ol>

workflow was constructed through the use of open-sourced freely available software and genomic databases (termed the (w)HOL(e)ISTIC Gene Ontology enrichment) to provide a cost-free method to group similar genes together to examine the different processes effecting the results of an experiment.

5. Evaluated cross-species ACE2 genetic (and especially epigenetic) diversity in regulation of ACE2 expression and functionality to determine the cell tropism and animal susceptibility to SARS-CoV2.
6. Evaluated a whole-virus ELISA assay with swine oral fluids for detection of pseudorabies virus (PRV) antibodies. Preparedness for PRV or another emerging virus outbreak may involve rapid testing of large groups of swine which can be done testing oral fluid samples for specific antibody.
7. Developed an animal model for Senecavirus A challenge that can be utilized to study isolate pathogenicity and test vaccine efficacy.

### Objective 1. Control of PRRSV

#### 1.1. PRRS immunology/vaccinology:

Initiated a study to identify the presence of the tRFs in HP-PRRSV infected pigs. Discovery and mapping of possible porcine tRFs from the S.scrofa 11.1 reference genome were conducted using the whole blood of both healthy and HP-PRRSV infected pigs confirming the presence of circulating tRFs during healthy and infected states and paves a way for the identification of these rarely studied sncRNAs and how their expression changes during HP-PRRSV infection.

Investigated the impact of PRRSV and Influenza B (IBV) coinfection on antiviral response in the porcine lung during infection through transcriptomic analysis. At early times after infection, the IBV and IBV/PRRSV coinfection groups showed greater expression of antiviral genes than the PRRSV group. All groups showed a reduction in upregulation for antiviral genes at later times post-infection. The downregulated genes for these comparisons indicated changes in pathways effecting lung development, wound healing, and cellular integrity. For all three treatment groups, the neutrophil degranulation pathway activity increased which may be related to lung lesions these respiratory infections. The IBV infection increased expression for interferon and antiviral genes that lead to viral mRNA degradation and assembly and transcription inhibition.

The replication-competent expression of IFNs using a PRRSV infectious clone

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has been characterized for the viral replication kinetics, IFN expression and stability, and proof-of-concept studies in pigs. Compared with a commercial vaccine, it was shown that some vaccine candidates were more effective in protecting pigs from a field-isolate challenge.

### 1.2. PRRS epidemiology:

### 1.3. PRRS Surveillance and Diagnostics:

### Objective 2. Developing effective and efficient approaches for detection, prevention and control of pressing viral diseases of swine of recent emergence

#### 2.1. ASFV:

#### 2.2. Swine Influenza Virus:

#### 2.3. Porcine Circovirus:

#### 2.4. Swine Pestiviruses:

Atypical porcine pestivirus was recently shown to be a causative agent for congenital tremors in piglets. Field studies showed that some animals become persistently infected with the virus. Nine persistently infected animals born in litters with piglets demonstrating congenital tremors were brought to the National Animal Disease Center to study transmission potential and five gilts were bred to determine if persistently APPV infected animals give birth to piglets with congenital tremors. Animals were positive by PCR for APPV in oral fluids/swabs throughout the study and were able to infect 3 groups of naïve contact pigs placed at timepoints during breeding and gestation. Piglets born to these gilts did not display clinical signs of congenital tremors.

#### 2.5. Senecavirus:

Pathogenesis studies have been performed in both nursery aged swine and market weight gilts. These studies have helped characterize timing of viral replication and shedding of SVA in serum and rectal swabs and the antibody response. Subsequent animal studies have focused on comparison of isolate pathogenicity, minimum infectious dose, and efficacy of an inactivated SVA vaccine.

### Impact and Value of Research to Stakeholder

#### 2.6. Sapelovirus:

#### 2.7. Viruses with potential interest to Xeno-transplantation science:

1. Inactivating these three coronavirus interferon antagonists is an approach for generating candidate vaccines to limit the replication and disease caused by enteric coronaviruses.
2. Elucidating how gene function in the pig can become dysregulated due to PRRSV through changes in miRNA and tRNA expression will serve to bring researchers closer to understanding the many complications to swine health caused by porcine reproductive and respiratory virus (PRRSV) and find ways to reduce losses in commercial pig populations.
3. The intracellular changes that occur in pigs following viral respiratory infections are still scantily understood for PRRSV, as well as, other viral respiratory infections. By testing and observing the host response to other respiratory viruses, our study has elucidated similarities and differences that can assist in development of vaccines and therapeutics that shorten or prevent a chronic PRRSV infection.
4. The (w)HOL(e)ISTIC Gene Ontology enrichment allows for similar genes to be grouped together by a common theme, such as disease resistance, which aids researchers to gain insight into the problems they study, and how the grouped genes interact with each other.
5. For cross-species animal tropism, the potential infectivity of SARS-CoV2 in both wild and domestic animals, and potential for zoonotic transmission is a big public health concern that concern involves two aspects: (1) screening to identify the animal species that serve as a virus reservoir originally passing SARS-CoV2 to humans; and (2) the existing risk of infected people to pass the virus to animals, particularly the domestic species, thus potentially forming into an amplifying zoonotic cycle to worsen SARS-CoV2 evolution and prevalence. Our findings may relieve relevant public concerns regarding COVID-19-like risk in domestic animals, highlight virus-host coevolution, and evoke disease intervention through targeting ACE2 molecular diversity and interferon optimization.
6. Emerging diseases in swine may involve rapid testing of large groups of swine, which can be done by testing oral fluid samples for specific antigens or antibody. Pseudorabies virus (PRV) has been eradicated from US domestic swine but continues to circulate in feral swine. Our study indicated good diagnostic performance and excellent repeatability of a whole-virus indirect ELISA to detect PRV antibodies in swine oral fluids; therefore, oral fluids could be a useful tool for PRV surveillance and detection.

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### Pertinent (Swine Virology) Publications Issued or "In Press"

7. Developing timely animal models for emerging diseases in swine is critical to understanding disease pathogenesis as well as aiding the development of diagnostic assays and measures to control the spread of the agent. Animal models were established for Senecavirus A (SVA) in various aged swine. Samples were used for ELISA assay validation and the model can be utilized to test efficacy of vaccine candidates for SVA.

### Refereed Publications

1. Fleming DS, Miller LC. Differentially Expressed MiRNAs and tRNA Genes Affect Host Homeostasis During Highly Pathogenic Porcine Reproductive and Respiratory Syndrome Virus Infections in Young Pigs. *Front Genet.* 2019 Aug 2;10:691. doi: 10.3389/fgene.2019.00691. PMID: 31428130; PMCID: PMC6687759.
2. Buckley A, Kulshreshtha V, van Geelen A, Montiel N, Guo B, Yoon KJ, Lager K. Experimental Seneca Valley virus infection in market-weight gilts. *Vet Microbiol.* 2019 Apr;231:7-10. doi: 10.1016/j.vetmic.2019.02.019. Epub 2019 Feb 13. PMID: 30955826.
3. Lager KM, Buckley AC. Porcine Anti-viral Immunity: How Important Is It? *Front Immunol.* 2019 Sep 27;10:2258. doi: 10.3389/fimmu.2019.02258. PMID: 31611874; PMCID: PMC6776623.
4. Miller LC, Fleming DS, Lager KM. Comparison of the Transcriptome Response within the Swine Tracheobronchial Lymphnode Following Infection with PRRSV, PCV-2 or IAV-S. *Pathogens.* 2020 Feb 5;9(2):99. doi: 10.3390/pathogens9020099. PMID: 32033425; PMCID: PMC7168592.
5. Cheng TY, Buckley A, Van Geelen A, Lager K, Henao-Díaz A, Poonsuk K, Piñeyro P, Baum D, Ji J, Wang C, Main R, Zimmerman J, Giménez-Lirola L. Detection of pseudorabies virus antibody in swine oral fluid using a serum whole-virus indirect ELISA. *J Vet Diagn Invest.* 2020 Jul;32(4):535-541. doi: 10.1177/1040638720924386. Epub 2020 May 25. PMID: 32450768; PMCID: PMC7438652.
6. Deng X, Buckley AC, Pillatzki A, Lager KM, Faaberg KS, Baker SC. Inactivating Three Interferon Antagonists Attenuates Pathogenesis of an Enteric Coronavirus. *J Virol.* 2020 Aug 17;94(17):e00565-20. doi: 10.1128/JVI.00565-20. PMID: 32554697; PMCID: PMC7431798.

### Abstracts or Proceedings

1. Miller, L.C. and Fleming, D.S. Porcine messenger RNA and microRNA pathway analysis of Porcine Reproductive and Respiratory Syndrome

Virus infection. *J Immunol* May 1, 2020, 204 (1 Supplement) 92.14; [https://www.jimmunol.org/content/204/1\\_Supplement/92.14](https://www.jimmunol.org/content/204/1_Supplement/92.14) 2020 (proceedings).

2. Miller L.C., Anderson, S.J., Lager, K.M., Fleming, D.S., Jennings, J., Van Geelan, A., and Sang, Y. 2020. Validation of a PRRSV live-virus potentiated by replication-competent expression of porcine interferons. (proceedings).
3. Baker, S.C., Deng, X., Hackbart, M., Mettelman, R.C., O'Brien, A., van Geelen, A., Buckley, A.C., Lager, K., Faaberg, K.S. (2020) Targeting interferon antagonists to generate live-attenuated vaccines for existing and emerging coronaviruses. *Transforming Vaccinology.* March 15-19. Florence, Italy.
4. Buckley, A., Lager, K. (2020) Efficacy of an inactivated Seneca Valley virus vaccine in sows. *AASV.* March 9-12. Atlanta, GA.
5. Buckley, A., Arruda, B., Falkenberg, S., Miller, L., Lager, K. (2020) Persistent APPV infection in gilts. *AASV.* March 9-12. Atlanta, GA.
6. Cheng, T., Buckley, A., van Geelen, A., Lager, K., Henao-Díaz, A., Poonsuk, K., Piñeyro, P., Wang, C., Baum, D., Main, R., Zimmerman, J., Giménez-Lirola, L. (2020) Pseudorabies virus (PRV) antibody detection in swine serum and oral fluid specimens. *AASV.* March 9-12. Atlanta, GA.

### Book Chapters or Monographs

### Non-refereed Articles

1. 5030-32000-118-08R. Role of the Viral Ovarian Domain Protease in PRRSV Pathogenesis. National Pork Board.
2. 5030-32000-118-66I. The Ecology of Seneca Virus A in U.S. Swine Marketing Chain. Animal and Plant Health Inspection Service, U.S. Department of Agriculture.

### Funding Sources

### Work Planned for Next Year

1. Identify pathogenic mechanisms of swine Nidovirales, including identifying the pathogenic mechanisms of Porcine Respiratory and Reproductive Syndrome Virus (PRRSV), and the pathogenic mechanisms of Porcine Epidemic Diarrhea Virus (PEDV).
2. Discover and assess vaccines that can reduce or prevent economic losses from swine viral diseases, including identifying mechanisms to modulate innate and adaptive immune responses to swine viral pathogens and investigating technologies to override vaccine interference from passively acquired immunity.
3. Determine evolutionary antigenic and pathogenic properties of

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economically significant swine viral pathogen, including identifying and monitoring genetic and antigenic evolution in Nidovirales and emerging viral pathogens.

4. Identify mechanisms of pathogenesis, transmission, and immunity for emerging viral diseases of swine, starting with evaluating the onset and duration of Seneca A virus immunity in swine.

### Changes/ Problems Due to COVID-19

Maximizing Telework and initiating a COVID-19 research response has had a negative impact on The Intervention Strategies to Control Endemic and New and Emerging Viral Diseases of Swine project in the Virus Prion Research Unit at National Animal Disease Center in Ames, Iowa. The porcine reproductive and respiratory syndrome virus (PRRSV) and Senecavirus A (SVA) research projects were put on hold as the project initiated a COVID-19 research response that has included logistical support for challenge studies in livestock (developing IBC and IACUC protocols) and conducting a challenge study in calves. Preparations are underway for a pig study and potentially other species as well. To date, about 3 months of progress have been lost in the PRRSV and SVA research projects.

