RESEARCH REPORT
SWINE HEALTH

Title: Identification of Genetic Mutations that Confer Escape from Innate or Adaptive Host Immune Responses During PRRSV Infection In Vivo – **NPB #12-173**

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Industry Summary:
The major goal of the proposed study was to identify specific genetic mutations in the PRRS virus genome that confer escape from host immune responses during PRRSV infection *in vivo.* The study used samples from the PRRS Host Genetics Consortium, in which approximately 30% of pigs experimentally infected with PRRSV initially cleared the virus, but had a rebound in viremia by 42 dpi. We did extensive sequence analyses of the nsp2 and ORF2-6 region of PRRSV during the acute and rebound period. The results revealed that there was less than 1% genetic variation within or between pigs during the six-week period following infection. Despite the low level of variation, we were able to detect genetically distinct subpopulations of virus genotypes in acute and rebound periods. Multiple sub-populations of virus co-existed in most pigs, and each pig had it's own distinct population of viral genotypes. In addition, we identified specific mutations in the viral genome that were under selection. The location and frequency of these mutations indicated they are important in escape from virus neutralizing antibody. The rebound pigs had detectable neutralizing antibody to the inoculum virus, while pigs that did not clear virus had no neutralizing antibody to the inoculum virus. Together, these finding indicate that recurrence of viremia in PRRSV infected pigs is due to immune escape variants. Identification of specific mutations that contribute to immune escape will aid design of more effective vaccines for control of PRRS.

Keywords: Immune escape, recrudescence, neutralizing antibody, immune selection, virus quasispecies

Scientific Abstract:
Porcine reproductive and respiratory syndrome virus (PRRSV) infection results in a devastating disease of swine characterized by reproductive failures in sows and respiratory disease in growing pigs. The virus is characterized by a high error rate and genetic diversity that have contributed to vaccine failure and virus persistence. However, the role of genetic variation in immune escape is not well understood. In order to examine genetic diversity in virus populations during the course of infection, we used samples from the PRRS Host Genetics Consortium (PHGC). The PHGC experimentally infected over 1500 pigs with PRRSV and followed the pigs through 42 days post infection (dpi). In approximately 30% of pigs, the virus was initially cleared, and then had a rebound in viral load by 42 dpi, indicating possible escape from immune control. We examined genetic diversity in seven pigs that differed in virological outcome of infection: two pigs that successfully cleared the virus, two pigs that had persistent viremia through 35 dpi, and three pigs that initially

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cleared the virus and then rebounded in viremia by 42 dpi. Viral RNA was isolated from the inoculum and from serum collected from day 7 of each pig, and a late day from persistent and rebound pigs. Approximately 3kb from nonstructural protein 2 (nsp2) and ORF2-6 (envelope proteins GP2-5, M) was amplified, and up to 30 individual clones were sequenced per pig/day and the inoculum. The amount of variation during infection was determined by calculating average pairwise-identity. The inoculum had 0.3% and 0.4% variation within sequences from ORF2-6 and nsp2, respectively. All sequences across each gene region had 0.4%, showing that there was no more variation within or between pigs during infection than was present in the starting inoculum. However, changes from the inoculum in both regions were identified across all pigs by day 7, demonstrating selection for growth *in vivo.* To determine if the small amount of variation that occurred could be the result of selection, the sequences were analyzed by discriminant analysis of principal components (DAPC) to identify population structures between groups of sequences. Temporal analyses revealed ORF2-6 continuously changed over the course of infection, whereas nsp2 had the most change between the inoculum and day 7 sequences. These results suggest that the envelope proteins and nsp2 are under different selective pressures during infection. DAPC applied to sequences grouped by virological outcome revealed that in both ORF2-6 and nsp2, persistent and rebound virus populations were distinct from each other. However, rebound sequences in ORF2-6 were significantly more separable from the inoculum and early populations than rebound virus in nsp2, suggesting variation in ORF2-6 is more likely to contribute to rebound. To determine if the separation of rebound virus in ORF2-6 was due to rebound pigs contracting the same external, sweeping infection, we applied DAPC again to sequences grouped by individual pig/day. We found that the late day of rebound pigs were the most separable from each other and from all other sequences, showing that rebound arises due to accumulated mutations within pigs. To determine if there was genetic evidence of selection, we mapped the genetic changes that occurred across the ORF2-6 envelope region within each pig. Dominant changes occurred across all envelope proteins, but changes were specific to each pig. Evaluation of sites within pigs with a minor variant frequency of at least 25% revealed distinct haplotypes in the late days of most persistent and rebound virus populations. In addition, there was significantly more genetic variation within B-cell epitopes than non-epitope regions, as determined by computing the average entropy across sites within and outside of known B-cell epitopes. Due to the high amount of variation in B-cell epitopes, we tested for immunological evidence of selection by performing neutralizing antibody assays. Day 42 sera from all pigs were tested for neutralizing activity against the inoculum virus. All rebound pig sera had high levels of neutralizing activity against the inoculum, whereas persistent pig sera had no detectable neutralizing activity. Together, these data suggest the PRRSV envelope genes are under immune selection during infection, which contributes to immune escape and virus rebound.

Introduction:

PRRS continues to cause significant economic losses among US pork producers. Although a number of PRRSV vaccines have been developed, they provide only limited protection against genetically diverse field strains. Genetic variation is thought to play an important role in immune evasion of PRRSV, and one of the major challenges in developing effective vaccines to PRRSV is overcoming the multiple and diverse strategies the virus employs to evade both innate and adaptive host immune responses. Neutralization escape is the proposed mechanism for the emergence of PRRS in vaccinated herds; however, it has been difficult to demonstrate a causative relationship between specific mutations and immune escape. Mutations in the structural proteins result in antigenic variation and altered glycosylation that can allow the virus to escape from neutralizing antibody. In addition, the high rate of genetic variation in the non-structural proteins suggests genetic variation may contribute to evasion of both innate and adaptive immunity. The major goal of our research was to identify specific genetic mutations that confer escape from host immune responses during PRRSV infection *in vivo.* Identification of specific mutations that alter immunological properties of the virus and facilitate escape from host immune response will aid in immune control strategies and improve design of more effective, broadly reactive vaccines. Such vaccines are key for control and/or elimination of PRRSV from US swine herds.

Objectives:

1. Sequence PRRSV rebound virus and identify sites and mutations subject to positive selection
2. Determine the effect of variation in PRRSV on immune escape.
3. Determine the effect of variation on virus replication phenotype

Materials & Methods:

Selection of samples for sequencing All samples were taken from pigs from PHGC trials one and three, which consisted of pigs from similar genetic backgrounds. Serum samples from seven pigs were selected; two that successfully cleared the virus (cleared), two that maintained high levels of viremia throughout 35 dpi (persistent), and three that initially cleared the virus, then rebounded by 41 dpi (rebound). Samples for sequencing included the inoculum virus NVSL97-7895 and serum samples collected from each pig at 7 dpi, as well as from high-viremia late day sera from persistent and rebound pigs.

Viral RNA Isolation and Cloning Viral RNA was isolated from the NVSL97-7895 inoculum, day 7 sera from all pigs, and from late day sera from persistent and rebound pigs using the QIAamp Viral RNA Mini Kit according manufacturer's instructions (Qiagen). Viral RNA was converted to cDNA via random hexamer primers using the Superscript III first strand synthesis kit according to manufacturer's protocol (Invitrogen). PRRSV ORF2-6 and nsp2 were amplified using PRRSV specific primers in three separate PCR reactions per sample to minimize polymerase error. ORF2-6 was amplified with forward primer ACCAGGTACCGGCCTGAATTGAAATGAAA and reverse primer GGTTGAATTCGGTCAAGCATCTCCCCAAC. Nsp2 was amplified using forward primer CGCCAACCGGATGAATTCCA and reverse primer GAATGGCAAGACCGAGACCGACG. The PCR reactions for each sample were pooled, purified with IBI's PCR cleanup kit, TA-cloned into pGEM-T easy vectors (Promega) and transformed in Stbl2 *Escherichia coli* cells. Individual colonies were screened for the correct size insert, and positive clones were selected for sequencing.

Sequencing and Assembly Multiple clones from each sample were sequenced via high throughput Sanger sequencing at the Iowa State University DNA Facility (Ames, IA). Multiple primers were used to maximize coverage across the ~3kb regions of ORF2-6 and nsp2. Both regions were sequenced with pGEM-T easy cloning site primers T7 and SP6 (Promega). In addition, ORF2-6 was sequenced with forward primers 5'CCGGGGTACCCTTCATGATTTTCAGCAATGGCTA and 5'GACGGGTACCATTGGTTTCACCTAGAATGGC and reverse primers 5'GATGGAATTCGTCTCCACTGCCC and 5'CAATTCAGAAAGATCAAAAGGTGC. In addition to T7 and SP6, nsp2 was sequenced with forward primers 5'CCTAGCAGTGTCATCCCAGC, 5'TGATTGGGGTTTTGCAGCTT, and 5'AGGGGGTTGTTCGTGAGGAATA and reverse primers 5'AACCTGACGGCTCAATTGGT and 5'CCAAGTCAGCATGTCAACCCTA. The sequences were assembled using Phred and Phrap algorithms in MacVector. ORF2-6 was then separated into the individual genes (E, GP2, GP3, GP4, GP5, ORF5a, and M), and the individual genes and nsp2 were translated to the amino acid sequence. The sequences were compared via multiple sequence alignment (ClustalW) to determine average pairwise identity and to generate consensus sequences.

Identification of Population Structures We applied discriminant analysis of principal components (DAPC) for finding population structure in samples of genetic sequences. Briefly, an n×l sequence alignment of n sequences at l positions is converted into a n × 4l matrix X of allele frequencies, where the possible alleles at each site are the four nucleotides. Deletions in the alignment are replaced with the mean allele proportions from all gap-free sequences at the position. Once the data are reformulated as matrix X, principal component analysis (PCA) is used to reduce the data to a lower-dimensional n×q representation, with q < 4l. We choose q large enough so the reduced data retain at least 90% of the variance in the original data. Finally, discriminant analysis (DA) finds linear combinations of the q coordinates that optimally separate user-defined groupings of the data. In particular, we used DA to explore how well the sequences from disease stages (inoculum, early, late), disease courses (rebound, persistent) could be separated. To determine if there is statistical support for the observed

separation between groups, we randomly permuted the labels and repeated the DAPC analysis 1000 times. To measure separation, we use the manova F statistic computed from the observations as projected in discriminant space. The fraction of permutations where the F statistic exceeds that of the original data is reported as the p-value for rejecting the null hypothesis of no genetic differences between groups.

Mapping sites of antigenic variation. To test whether known B-cell epitopes in ORF2-6 and nsp2 were unusually divergent compared to non-epitope regions, we computed the average entropy across sites in epitope and non-epitope regions for each pig and day and then fit a linear model of entropy. Because we utilized single clone sequencing we were able to identify linked sites of variation. Due to the large region sequenced (~3 kb) and the high mutation rate of the virus, we expect random variation within each haplotype. To decrease the amount of background variation, only sites with a minor variant frequency greater than 25% were used to determine haplotypes within pigs.

Virus Neutralization Assays Neutralizing antibody assays were done using a focus-reduction assay. Briefly, 42 dpi sera from the seven pigs was heat-inactivated and serially diluted 2-fold in media, incubated 1 hour at 37°C with 200 focus-forming units of PRRSV strain NVSL97-7895, and inoculated in triplicate onto MARC-145 cells seeded the previous day in 12-well plates. Cells and virus were incubated an additional 24 hours, then the cells were fixed in ice-cold acetone:methanol and stained for PRRSV by immunocytochemistry using the monoclonal antibody SDOW17 as the primary antibody and sheep anti-mouse IgG conjugated HRP as the secondary antibody. Following addition of the HRP substrate, cells were rinsed with distilled water, air-dried, and foci of infected cells enumerated by light microscopy. The percent neutralization compared to a virus-only control was calculated, and the serum dilution that neutralized 50% of virus (ND50) was determined.

Generation of PRRSV shuttle plasmid. The pFL12 infectious molecular clone was selected as the backbone virus because it contains the full-length genome from the NVSL97-7895 strain, which is the same strain as the inoculum The full-length infectious molecular clone is over 19kb, so it contains very few unique restriction enzyme sites. The envelope gene region we amplified is only about 3kb, so we are limited in the number of restriction sites available for use in introducing envelope mutations into the infectious clone. To overcome these challenges, two shuttle plasmid systems were developed to “shuttle” the envelope mutations from our virus clones and introduce them into pFL12. The first shuttle plasmid utilized two unique restriction sites in pFL12, an EcoRV site in the envelope ORF2, and a PacI site downstream of the poly(A) sequence. An approximately 4kb region of pFL12, from 500 bp upstream of EcoRV and 200 bp downstream of PacI, was amplified and ligated into a pGEM-T easy vector and cloned. This shuttle plasmid contains a restriction site at the end of ORF5 that is unique to the envelope region, BstEII, that is used to introduce envelope variants into the shuttle plasmid. Because some haplotypes contained mutations in GP2 upstream of the EcoRV restriction site, a second shuttle was created to introduce these mutations. This shuttle plasmid utilized the pFL12 unique restriction site AsiSI (in ORF1b), and EcoRV. As with the first shuttle plasmid, primers were designed to amplify upstream of AsiSI and downstream of EcoRV, creating an approximately 3500 bp fragment, which was then ligated into pGEM-T easy vector and cloned. This AsiSI-EcoRV shuttle fragment contained the unique restriction site BssHII upstream of the start of ORF2. Envelope fragments could then be digested with BssHII and EcoRV for introduction into the shuttle plasmid, and digested with AsiSI and EcoRV for introduction into the full-length pFL12 infectious clone.

Generation of chimeric full-length molecular clones. To generate infectious clones, the shuttle plasmid was digested with EcoRV and PacI and the insert fragment was gel purified for reintroduction into pFL12, creating a chimeric infectious clone with FL12 ORF 1, 2, 6, and 7 and ORF2-ORF5 from a selected viral variant. In some cases, the ORF2-6 regions was subcloned from our amplified clones, in other cases, the variation sequence was synthesized and then inserted into the full-length clone. The entire envelope region of the dominant and subdominant haplotypes of rebound pig were used to generate infectious molecular clones

In vitro transcription of viral genomic RNA Full length viral RNA was produced by *in vitro* transcription using the mMESSAGE mMACHINE T7 Ultra *in vitro* transcription kit (Ambion) following manufacturer's instructions. Briefly, 10 μg of pFL12 and associated chimeras were linearized by restriction digest with AclI overnight. Linearized plasmids were purified by phenol-chloroform extraction and ethanol precipitation, and 3 μg of purified plasmids were used as templates for *in vitro* transcription reaction. The transcribed RNA was purified by phenol-chloroform extraction and isopropanol precipitation and the size and concentration were determined by gel electrophoresis using the glyoxal RNA gel system (Ambion).

Generation and characterization of infectious chimeric virus MARC-145 cells were maintained in DMEM supplemented with 10% FBS and antibiotics (complete media) and seeded in a T75 flask 48 hours prior to electroporation. The cells were harvested by trypsinization and collected in complete media and centrifuged at 4^{o}C at 1000xg for 7 minutes. The supernatant was aspirated and the cell pellet washed once with serum-free DMEM and spun, and washed twice with electroporation buffer (serum free DMEM + 1.25% DMSO). Cells were counted and resuspended at $2x10^6$ cells/400ul in electroporation buffer. Electroporations were carried out by mixing 5ug *in vitro* transcribed viral RNA and 5ug total MARC cellular RNA with 400ul cell suspension ($2x10^6$ cells) in 4 mm cuvettes. Reactions were immediately pulsed at 250V and 925uF capacitance on BioRad's Gene Pulser Xcell. Following electroporation, cells were seeded in DMEM supplemented with 10% FBS and antibiotics plus 1.25% DMSO. At 16-18 hours post-transfection, cells were washed and media changed to DMEM with 5% FBS and antibiotics. Cells and virus were incubated for an additional 3 days. At 48 hours and 96 hours post-electroporation, supernatants were collected and cells were washed with TN buffer+1% FCS and fixed with methanol:acetone (1:1) on ice for 10 minutes and virus replication was assessed by immunocytochemistry using anti-N monoclonal antibody SDOW17 (Rural Technologies) as the primary antibody. Cells were evaluated by microscopy for red staining of cells indicating expression of viral N protein expression. For amplification of virus, supernatants collected at 96h were passaged on new MARC-145 cells for 48 hours, and the p2 supernatants were titrated on MARC-145 cells. In addition, viral RNA was isolated from 96h and p2 supernatant, and ORF2-6 was amplified and sequenced to verify sequence integrity.

Results:

Aim 1. Sequence PRRSV rebound virus and identify sites and mutations subject to positive selection The goal of this aim was to identify PRRSV genotypes present in the inoculum, day 7 and rebound sera samples from PRRSV-infected pigs that experienced virus rebound within 42 days post-infection. A variety of statistical and phylogenetic tools were used to identify sites undergoing positive selection in vivo.

1A. Selected pigs and analysis of variation in ORF2-6 and nsp2. Seven pigs with differing virological outcomes were selected from PHGC trials one and three. Two pigs successfully cleared the virus (cleared), two pigs maintained a high level of viremia until day 41 (persistent), and three pigs initially cleared the virus, but rebounded in viral load by day 41 (rebound). Samples from the inoculum, serum from each pig at day 7, and late day sera from persistent and rebound pigs were used to isolate viral RNA, amplify ~3kb from ORF2-6 and nsp2, and sequence individual clones. In total, we sequenced 223 clones from ORF2-6 and 239 clones from nsp2. Multiple sequence alignments of all sequences were made for each gene region in order to identify the amount of variation within ORF2-6 and nsp2. In both regions, the average pairwise identity within each pig was greater than 99%, and was similar to that observed in the inoculum. In addition, the average pairwise identity across all sequences of ORF2-6 and nsp2 were 99.59% and 99.57%, respectively. These results show that very little genetic variation occurred in vivo during the six weeks following infection.

1.B. Population structures of ORF2-6 and nsp2 Despite the small amount of observed variation, selective pressures in vivo may alter virus population structures over time. To examine this, we applied discriminant analysis of principal components (DAPC) to viral sequences grouped by time after infection (inoculum, early, and late). Results showed that the inoculum, day 7 (early), and late days populations were clearly separable in both ORF2-6 and nsp2 (p-values <0.001). In addition, we observed different patterns of evolution between the two gene regions. ORF2-6 sequences continually evolved over time within the pigs, suggestive of ongoing

selective pressure on the envelope proteins. In contrast, the greatest separation among nsp2 sequences occurred between the inoculum and early sequences, suggesting selection for replication in vivo. To determine if late day virus populations of different virological outcomes can further be distinguished, we applied DAPC again to sequences grouped by time and virological outcome. In both gene regions, rebound and persistent virus were easily distinguished. The p-values for testing the null hypothesis of no group structure was less than 0.001 for groups by virological outcome, showing overwhelming genetic differentiation between inoculum, early, rebound, and persistent virus. In addition, ORF2-6 rebound genotypes were significantly more separable from inoculum and early virus populations than nsp2 rebound genotypes, suggesting that rebound in viremia was associated with variation in ORF2-6.

1.C. Rebound in viremia is not due to an external infection It is possible that persistent and rebound virus separate because the rebound virus is caused by an external sweeping infection in all rebound pigs. If so, we would expect rebound virus within the same PHGC trial to group together. To test this, we applied DAPC again to ORF2-6 sequences from individual pigs and time point. The results showed the virus populations in these two pigs were clearly distinct from each other as well as virus found in any other pig or time point. These results indicate that rebound virus is a recrudescence of viral replication due to an accumulation of distinct genetic changes occurring within pigs and not from an external sweeping infection. This further supports the hypothesis that rebound virus arose due to selective pressures on the virus from the host and is comprised of immune escape variants.

1.D. Quasispecies structure and Sites of Selection To identify and visualize where variation occurred and the pattern of variation over time, we looked at the nucleotide changes occurring in each viral population. Both gene regions contained four general patterns of single nucleotide variation: 1) nucleotide changes that occurred in multiple pigs and days that became fixed by the late day, 2) nucleotide changes that were fixed by the late day in a single pig, 3) nucleotide variants with distinct dominant and subdominant variants in multiple or single pigs demonstrating quasispecies structure, and 4) random changes observed in single clones. To identify if any of these variable nucleotide positions may be under selection over time or have significant changes in variant frequency, we used the Cochran-Mantel-Haenszel (CMH) test to identify associations between single nucleotide variants (SNV) and dpi. We tested all variable sites in the five persistent and rebound pigs (421 in ORF2-6; 504 in nsp2) for association between the variants and dpi. After controlling for multiple testing, we identified 18 SNV in ORF2-6 and 21 SNV in nsp2 showing significant association with dpi. Fifteen of the 18 SNV in ORF2-6 resulted in amino acid changes, including 3 SNVs that led to non-synonymous changes in two overlapping reading frames. In nsp2, 10 of the 21 SNV were non-synonymous. These data provide good evidence that variation in multiple envelope proteins contribute to immune evasion.

1.E. Evidence of *in vivo* selection The NVSL97-7895 inoculum virus was passaged a limited number of times in MARC-145 cells prior to inoculation in pigs and likely accumulated mutations adapted for *in vitro* growth. It is possible that SNV under selection might include changes best suited for growth *in vivo*. Indeed, several of the significant SNV identified in the CMH test started as a minor variant in the inoculum, increased in frequency in multiple pigs by day 7 and were fixed at late days post-infection. The patterns of change, and the fact that identical changes were observed across multiple pigs, argue that each change was likely selected for growth in vivo.

Specific Aim 2: Determine the effect of variation in PRRSV on immune escape. The goal of this aim was to determine if sites under selection in ORF 2-6 and/or nsp2 alter immunologic phenotype of PRRSV. The overall approach was to identify sites of antigenic variation and to generate infectious molecular clones containing sites of interest. Neutralizing antibody assays are used to determine the effect of variation on immune escape.

2.A. Evaluating B-cell epitopes for selection: To test whether known B-cell epitopes in ORF2-6 and nsp2 were unusually divergent compared to non-epitope regions, we computed the average entropy across sites in epitope and non-epitope regions for each pig and day. We then fit a linear model of entropy and found that

epitopes in ORF2-6 were significantly more variable than non-epitopes (p-value 0.0301). There was no significant association with dpi or any other measure of disease stage. No significant difference between entropy in epitopes and non-epitopes was observed in nsp2. These results are consistent with the hypothesis that variation in ORF2-6 is associated with rebound virus, and that selective pressure in ORF2-6 may result in immune escape variants by rebound virus.

2.B. Identification of Linked sites of Variation in ORF2-6 Our statistical analyses indicated that changes in multiple envelope proteins may contribute to selection in vivo. There are extensive protein-protein interactions among the PRRSV envelope proteins, and we hypothesized that there may be sites of co-variation across ORF2-6, resulting in distinct viral haplotypes. Because we utilized single clone sequencing we were able to identify linked sites of variation. In four of five pigs, late day populations were composed of at least two distinct haplotypes. In all pigs the dominant and subdominant haplotypes represented very distinct variants and, in most pigs, did not share any sites in common. The PRRSV envelope proteins form extensive protein-protein interactions and the presence of distinct haplotypes within the late day samples suggests that mutations linked within a haplotype may be biologically important. For example, the linked sites may represent conformational epitopes in oligomeric proteins. It is also plausible the changes in one envelope may effect conformational changes in another protein that modifies antigenic epitopes.

2.C. Neutralizing Antibody Assays: If rebound virus resulted from immune escape, sera from rebound pigs should have neutralizing activity against the inoculum virus. To test this, we used a focus-reduction assay to quantify neutralizing antibody titer to the NVSL97-7895 inoculum virus in day 42 serum from each pig. The neutralizing antibody titer was calculated as the serum dilution required to neutralize 50% of the virus (ND50). All three rebound pigs showed moderate to high levels of neutralizing activity against the inoculum virus, with ND50 titers ranging between 34-48. In contrast, the two persistent pigs had no detectable neutralizing activity against the inoculum. These results suggest that rebound viremia arises from replication of immune escape variants, whereas persistent viremia results in part from a lack of a neutralizing antibody and resolution of acute viremia.

Discussion:

In this study we characterized the genetic variation in nsp2 and ORF2-6 of PRRSV over the course of experimental infection. We performed population structure analyses, sequence variation analyses, and neutralizing antibody assays to characterize variation, selection, and immune escape within and between pigs of differing virological outcomes. Even though pairwise comparisons of the viral sequences revealed less than 1% variation in each of the gene regions, we identified distinct population structures and specific sites in ORF2-6 and nsp2 with significant variation. Changes in both nsp2 and ORF2-6 likely contribute to selection for replication in vivo at early times after infection. In contrast, the PRRSV envelope proteins appear to be under continual selective pressure from the host's immune response, which results in NAb escape variants and recrudescence in viremia. Of particular interest is the finding that PRRSV isolates that are greater than 99% identical at the nucleotide level can have distinct biological and/or immunological properties. This is an important finding for epidemiological studies investigating the genetic basis of new outbreaks of PRRS, and for vaccine studies that seek to develop broadly neutralizing antibody.

The escape from the neutralizing immune response by rebound virus is likely mediated by selective immune pressure on the envelope proteins from NAb. The three rebound pigs initially cleared the virus between 21-35 dpi, which is consistent with the previously determined time-frame of PRRSV clearance by NAb. Further support for rebound virus resulting from escape from NAb is provided by entropy computations between known epitope regions and non-epitope regions. Many B-cell epitopes have been reported in GP2, GP3, GP4, GP5, M, and nsp2, and we found that epitopes showed significantly more variation than non-epitope regions in ORF2-6. Rebound virus escape from NAb is further supported by NAb assays against the inoculum virus. Day 42 serum from all rebound pigs had neutralizing activity against the inoculum virus, but neither of the persistent pigs' serum had neutralizing activity. This supports the hypothesis that rebound virus is comprised of NAb escape

variants and persistent virus is due to a lack or delay of an immune response. The rebound virus was able to replicate in pigs despite the presence of NAb to the inoculum virus, whereas pigs with persistent virus did not produce productive antibodies against the inoculum virus. Taken together, these analyses provide substantial evidence for immune selection acting on the envelope proteins, which results in variants that are able to escape the host immune response. Ongoing studies to identify the effect of specific nucleotide/amino acid changes on immune escape will provide key information as to the linked sites of variation recognized by neutralizing antibody, and may identify conformational epitopes. Such information will allow us to map regions of PRRSV targeted by type-specific and broadly neutralizing antibody – which is key to developing vaccines that offer broad protection against diverse isolates of PRRSV.

In summary, PRRS continues to cost the pork industry millions of dollars every year because of the major challenges that remain in the control and elimination of PRRSV. While vaccines are available, they are not effective against heterologous strains, leading to poor protection of animals and herds. This is in large part due to the high degree of genetic variation among PRRSV strains, and our limited understanding of the role of genetic and antigenic variation in immune escape. This study identifies evidence of immune selection in B-cell epitopes on the envelope proteins of PRRSV, and identifies specific mutations and/or sets of mutations that may be responsible for the neutralizing antibody escape. Identification of these variants provides new insights into the role of genetic variation in immune escape and PRRSV persistence and recurrence that will aid efforts to control and/or prevent PRRS.