RESEARCH REPORT
SWINE HEALTH

Title: Characteristics of Unusually Virulent Contemporary PRRSV Isolates, **NPB 17-171**

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Industry Summary:

In the majority of cases involving new PRRSV isolates, outbreak severity initially is tremendous, then decreases over time as herd immunity and biosecurity is managed to reduce and prevent disease. Challenge studies have shown that commercial vaccines significantly improve outcomes compared to infection of naïve animals. PRRS viruses that break in solidly immune, vaccinated herds as if they were naïve are very difficult to manage and threaten economic swine production. An unusually aggressive family of viruses characterized by a 1-7-4 RFLP appeared in January 2014 and within 15 months was linked to severe outbreaks in well managed herds. These viruses are markedly different from other contemporary field viruses in their ability to spread rapidly and cause severe disease in immune (endemic) herds.

In this study we focused on examining the differences between historical and contemporary isolates of varying virulence levels to identify possible reasons for the unusually high virulence of contemporary viruses including the 1-7-4 RFLP viruses. The objectives of this project were to 1) examine serum neutralization titers between animals exposed to different viruses, 2) characterize the growth characteristics of contemporary virulent viruses, 3) discover unique genomic features that may be involved in viral virulence, and 4) examine new methods for classification of PRRSV isolates that may more effectively predict potential highly virulent viral strains. First, we determined that the contemporary 1-7-4 RFLP viruses were sufficiently antigenically distinct from vaccine that they evaded the vaccine-induced immune response, thus vaccine did not induce any protection against these viruses. However, once an animal was infected with a contemporary virus, they were protected against infection by other contemporary strains. Secondly, the growth characteristics of contemporary strains were compared to that of historical strains to try and explain why these contemporary strains are so virulent, but no significant growth advantages were observed. Next, genome sequences from contemporary and historic viruses of various virulence were compared to identify possible virulence genes or targets for diagnostics or vaccination. Interestingly, 6 regions of the genome were observed to contain putative proteins that were conserved across all or most of the viral genomes examined, suggesting more proteins may be involved in viral replication than previously known. Surprisingly, comparing high vs low virulence viral genomes identified 3 regions predicting different protein products depending upon virulence of the virus. In one region, a protein was predicted to be present in low virulence viruses, but was not observed in any medium or high virulence viruses, thus suggesting it is involved in maintaining low virulence and deserves further study. Finally, viral genome sequences were

examined using a variety of clustering methods and were compared with RFLP-typing to try and differentiate highly virulent strains from lower or non-virulent viruses. Unfortunately, no magic method was identified to differentiate viral virulence. However, ORF5 or whole genome clustering did give better results than that of RFLP-typing to differentiate low or non-virulent strains from virulent strains. Overall, this research suggests that contemporary viruses differ enough from vaccine strains that little or no protection against contemporary strains is obtained from these vaccines. However, contemporary strains are able to protect against infection by other contemporary viruses, suggesting that vaccine derived from contemporary strains would be protective. The identification of possible regions involved in virulence may allow for quicker vaccine development. Identification of new viral clusters using ORF5 sequencing will help identify new strains that may be able to evade vaccine-induced immunity, thus identifying the need for updated vaccines.

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Key Findings:

- Contemporary 1-7-4 RFLP viruses evade vaccine-induced immunity.
- Antibodies induced by infection of contemporary viruses protects against infection by other contemporary viruses.
- Contemporary viruses do not seem to have a growth advantage over historical strains, but rather have evolved to evade vaccine-induced protection.
- Three regions of the genome were identified that encode putative virulence factors that may enhance vaccine development.
- Clustering by ORF5 or whole genome sequence along with neutralizing antibody assays can be used to determine when new PRRSV strains are likely to evade vaccine-induced immune responses.

Keywords: Porcine reproductive and respiratory syndrome virus, PRRS, virus neutralization, viral growth, classification, characterization

Scientific Abstract:

In the majority of cases involving new PRRSV isolates, outbreak severity initially is tremendous, then decreases over time as herd immunity and biosecurity is managed to reduce and prevent disease. Challenge studies have shown that commercial vaccines significantly improve outcomes compared to infection of naïve animals. PRRS viruses that break in solidly immune, vaccinated herds as if they were naïve are very difficult to manage and threaten economic swine production. In January 2014, a new family of aggressive viruses appeared and within 15 months was linked to severe outbreaks in well managed herds. These viruses are markedly different from other contemporary field viruses in their ability to spread rapidly and cause severe disease in immune (endemic) herds, suggesting immunity due vaccine or field virus exposure is not cross-protective. This research examined the cross-protective immunity to these new viruses as well as genetic features that may be responsible for its unusual virulence and immune insensitivity, and point to a need for new directions and better ways to characterize and diagnose severe PRRS.

The results of this study identified differences in the highly virulent contemporary isolates as compared to historical isolates. Viral neutralization of serum from animals vaccinated or infected with different isolates identified a lack of neutralization of contemporary viruses by animals that had only seen vaccine or a historical isolate. Animals that had seen other different contemporary isolates were able to neutralize the 1-7-4 RFLP contemporary isolate. This suggests that the 1-7-4 isolate was sufficiently antigenically distinct from vaccine that it evaded vaccine-induced antibodies. Even though vaccine was unable to protect against 1-7-4 challenge, previous infection with other contemporary viruses was able to induce cross-protective neutralizing antibodies against 1-7-4, suggesting a new contemporary viral vaccine may be needed. Besides neutralization, another method for the 1-7-4 RFLP viruses to evade protection would be if they were able to grow more quickly or to higher titers making it more difficult to control infection. A comparison of growth characteristics in vitro were examined between the contemporary and historical isolates identifying similar growth rates, thus viral growth

does not explain the high virulence of this 1-7-4 virus. Next, genome sequences from 19 viruses were examined to determine if a unique virulence feature or putative protein could be identified to explain highly virulent strains. Interestingly, 6 conserved features were observed in both contemporary and historic isolates, including a putative ORF7a protein, and although these do not seem to be virulence targets, it is interesting to note that we continue to identify novel putative proteins for this virus. However, we did identify 3 putative virulence targets in the genome that differ between low or non-virulent strains and virulent viruses that need to be further investigated to determine if they can be exploited for vaccine development. Finally, a variety of clustering methods were examined and compared to RFLP typing in an effort to obtain a better method for identifying highly virulent strains. Examination of phylogenetic trees created from ORF5 or whole genome sequences, clustered PRRSV isolates similar to that of RFLP typing, but identified the difference between virulent 1-4-2 virus and historical non-virulent 1-4-2 virus, which are not differentiated by RFLP typing. However, these phylogenetic trees were not any more successful at identifying highly virulent strains than that of RFLP typing. Use of ORF5 clustering in conjunction with neutralizing antibody assays to determine cross-neutralization between viral strains or clusters of strains, would allow for identification of new viral clusters able to evade vaccine-induced protection. This would identify when new vaccines need to be developed and hopefully use of the 3 putative vaccine targets identified in this study will allow for development of quicker, more effective vaccines.

Introduction:

In the majority of cases involving new PRRSV isolates, outbreak severity initially is tremendous, then decreases over time as herd immunity and biosecurity is managed to reduce and prevent disease. Challenge studies have shown that commercial vaccines significantly improve outcomes compared to infection of naïve animals. PRRS viruses that break in solidly immune, vaccinated herds as if they were naïve are very difficult to manage and threaten economic swine production. In January 2014, a new family of aggressive viruses appeared and within 15 months was linked to severe outbreaks in well managed herds. These viruses are markedly different from other contemporary field viruses in their ability to spread rapidly and cause severe disease in immune (endemic) herds, suggesting immunity due vaccine or field virus exposure is not cross-protective. This research examined the cross-protective immunity to these new viruses as well as genetic features that may be responsible for its unusual virulence and immune insensitivity. Identification of new viruses, the ability of vaccine to control infection, and development of new vaccines to induce protection against these viruses is needed and this research begins the development of new ways to characterize, diagnose, and protect against severe PRRS disease.

Objectives:

1. Characterize the virus neutralization characteristics, both homologous and cross-protective, of immune serum from pigs exposed to virulent and vaccine viruses, against contemporary PRRS virus isolates (RFLP types including 1-7-4, 1-3-4, 1-4-4, 1-8-4, 1-2-4, and 1-26-2). The deliverables from this objective include evidence-based predictions of immune status of herds against 1-7-4 and other contemporary PRRS viruses, data to better understand mechanisms of protection and guide further development of immune interventions.
2. Characterize the growth characteristics of contemporary virulent isolates for insights into their exceptional ability to infect immune herds. The deliverables for this objective include improved tools for understanding and measuring growth properties of contemporary PRRS viruses, and new viral isolates to share for studies of pathogenic, epidemiologic and immunogenic mechanisms.
3. Determine unique features of contemporary virulent PRRSV genomes that are associated with atypical responses or avoidance of cross-neutralizing immune protection. The deliverables from this objective include screening for novel open reading frames that could provide an additional diagnostic tool to differentiate virus isolates according to field-relevant characteristics, and provide new ideas and targets to investigate for specific roles in virulence, immune avoidance, and other properties that make certain contemporary viruses exceptionally pathogenic.

4. Compare differentiation methods for characterization or classification of PRRS virus isolates that predict more effectively than RFLP typing the potential threat of isolates to infect immune herds with devastating consequences. The deliverables from this first broad comparative analysis of PRRS virus differentiation schemes and new whole genome analysis will be to show the relative value of these methods to cluster viruses according to virulence characteristics.

Materials & Methods:

Serum samples and viruses

A total of 78 serum samples from pigs with different virus exposures were obtained from previous studies and commercial farms and were used for examination of neutralizing antibodies against different viral strains.

Viral strains were adapted to and grown in MARC 145 cells in culture following standard protocols [1]. The viral isolates used for the neutralizing antibody assays included the following: VR2332 (C-262-1), MN184 (H-184-1, GenBank ID DQ176019.1), a contemporary highly virulent 1-7-4 strain (2016-US174, C-174-4), a second 1-7-4 contemporary strain (2018-NC174, C-174-7), and a type 1 PRRSV strain (SDEU, H-PRRS1) (Table 1). Viral growth curves were performed using these virus isolates as well as 3 others identified in Figure 3. Determination of $TCID_{50}$ infectious titers and viral copy numbers were performed as previously described [1, 2].

Whole genome sequences of viral isolates in Table 1 were either obtained from GenBank or sequenced in our lab using Illumina next generation sequencing. The genome sequence of isolates determined for this study have not yet been submitted to GenBank.

Serum neutralization assay

The PRRSV serum neutralization assay was performed as previously described [1].

Viral growth curves

Viral growth over 48h as determined by percent of virus infected MARC 145 cells using flow cytometry was performed as previously described infecting cells with the same number of viral copies instead of at a standard MOI in order to try and normalize the infectious viral load [3]. The viral load was much lower than usual due to low viral titers of some of the strains examined.

Growth curves of 5 viral isolates over 72h as determined by viral RNA copies in supernatants or cells were performed by infecting MARC 145 or PAM cells at an MOI of 0.1 as previously described [1-3]. Detection of viral RNA over time in supernatants and cells was performed by isolation of viral RNA from supernatants using a QIAamp Viral RNA minikit and from cells using the Qiagen RNeasy mini kit following manufacturer's instructions. RNA was quantified at each time point using quantitative reverse transcription PCR (RT-qPCR) as previously described [2]. Virus in supernatants was normalized to viral copies per ml of supernatant and virus in cells was normalized to viral copies per well of cells using a standard curve as described previously [2].

Confirmation of ORF7a protein by ELISA and mass spectometry

Cloning, expression and purification of the PRRSV ORF7a protein from isolate C-184-1 (83aa protein) was performed as previously described [4]. An ELISA assay and avidity assay were performed as previously described by coating ELISA plates with 500ng of ORF7a protein [4]. Serum samples were obtained from animals infected with a 1-8-4 RFLP virus containing the 83aa protein, MLV vaccine (similar to VR2332, H-262-1), or were PRRSV negative pigs. Mass spectrometry was performed on MN184 (H-184-1) or VR2332 (C-262-1) virus-infected MARC 145 cells at 48h post-infection. Cells were purified, digested into peptides, and analyzed on a Sciex 5500 triple quadrupole mass spectrometer and a Sciex 5600 quadrupole time of flight mass spectrometer and examined for peptides specific for each of the putative ORF7a proteins.

ORF identification and genome clustering

Identification of novel open reading frames (ORFs) and determination of phylogenetic trees using ClustalO were performed using CLC genomics workbench 12 in viewing mode (Qiagen).

Results:

Objective 1: Characterization of viral neutralization characteristics of immune serum from pigs exposed to virulent and vaccine viruses against contemporary PRRSV isolates.

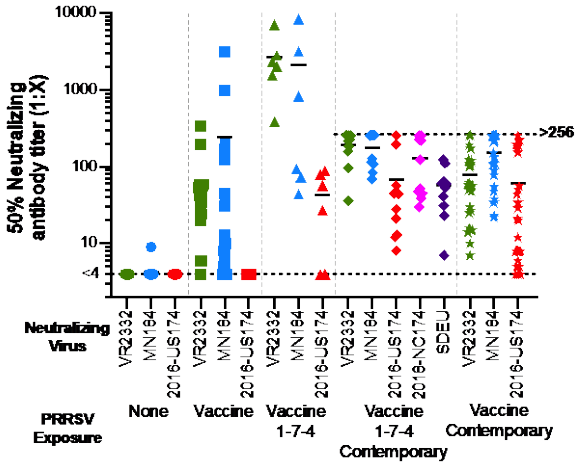
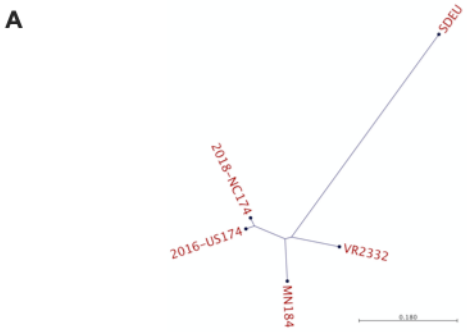


Figure 1. Neutralization titers against viral strains following varying viral exposures. Serum samples were obtained from animals with known PRRSV exposures as shown below the x-axis. Contemporary exposure designates that the animals were exposed to multiple contemporary viruses that were not 1-7-4. The virus used for neutralization is shown on the x-axis. The mean NA titer is shown as a black line for each group. The limit of detection of the assay is a titer <4 as shown by the dotted line. In both the contemporary exposure groups, the highest titer examined was 1:256, those samples with 50% NA titers >256 are shown at the dotted line at 256.

A total of 78 serum samples from pigs with different virus exposures were analyzed for serum neutralizing antibodies (NA) against 3 viruses; VR2332 (C-262-1), MN184 (H-184-1, GenBank ID DQ176019.1), and a contemporary highly virulent 1-7-4 strain (2016-US174, C-174-4) (Figure 1). Ten of these animals, those exposed to vaccine, a 1-7-4 isolate, and other contemporary isolates, were also examined for NA against a second 1-7-4 contemporary strain (2018-NC174, C-174-7) and a type 1 PRRSV strain (SDEU, H-PRRS1) (Figure 1). All vaccinated animals except 1 (vaccine only group) developed detectable NA titers against VR2332, the viral strain used to develop MLV vaccine, and all vaccinated animals except 3 (from the vaccine only group) developed detectable NA titers against MN184, a historical highly pathogenic strain isolated in 2001 (Figure 1). Only animals which were exposed to a 1-7-4 virus or another contemporary isolate were able to neutralize the 1-7-4 virus, even though they were not necessarily infected with this specific isolate (Figure 1). This suggests that vaccine was not able to induce a protective response against 1-7-4 infection, but that other contemporary isolates were able to induce a protective response (Figure 1). Interestingly, animals that were exposed to vaccine, a 1-7-4 isolate, and other contemporary isolates developed protective neutralizing antibodies against 2 different contemporary strains as well as a type 1 isolate, suggesting that either the number of exposures or the breadth of exposure to highly variable isolates was able to induce broadly neutralizing antibodies against diverse isolates (Figure 1).

The contemporary isolates examined in the neutralization assay were not the exact isolates in which the pigs were exposed, but were similar. The diversity between the isolates examined for neutralization is shown in Figure 2. VR2332 and MN184 have an 88% identity between each other, whereas they have an 87% and 89% identity against the 1-7-4 strains, respectively. The PRRSV type 1 strain, SDEU, only has a 66-67% identity between all the type 2 strains. Thus, it is expected that very broad neutralization between type 1 and type 2 strains would be necessary for animals infected with type 2 viruses to develop protection against type 1 viruses.



B

PERCENT IDENTITY

% DIVERGENCE	VR2332	MN184	2016-US174	2018-NC174	SDEU
VR2332		87.8	86.7	86.5	66.1
MN184	12.2		88.8	88.7	66.7
2016-US174	13.3	11.2		96.9	66.5
2018-NC174	13.5	11.3	3.1		66.6
SDEU	33.9	33.3	33.5	33.4	

Figure 2. Comparison of viruses used for neutralizing antibodies. Whole genome sequence of each of the viruses was obtained. A) A phylogenetic tree showing the relationship between the viruses was obtained using ClustalO in CLC genomics workbench. B) An Identity matrix was determined using NCBI's BLAST 2 sequences using the whole genome sequence of each of the viruses to compare the sequences to each other.

Objective 2: Characterize the growth characteristics of contemporary virulent isolates for insights into their exceptional ability to infect immune herds.

A total of 5 contemporary strains were successfully propagated in cell culture, although some did not grow well or to high titers. An experiment was performed to compare viral infection over time between these 5 contemporary strains, VR2332, and MN184 using flow cytometry to detect the percent of infected MARC 145 cells (Figure 3). An equal number of viral copies as determined by RT-qPCR was used to infect MARC 145 cells, which resulted in slightly different MOI's. The MOI's were quite low due to the low titer of some of the viruses (Figure 3 inset table). Unfortunately, due to the low amounts of virus used for infection, viral growth could not be effectively compared between viral isolates, so a different approach needed to be examined for comparison of viral growth between isolates.

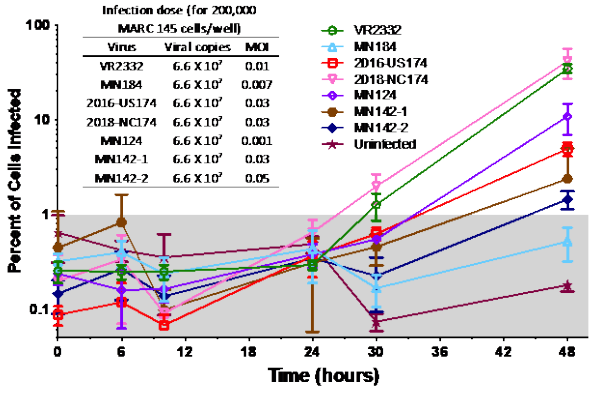


Figure 3. Infection of MARC145 cells with PRRSV isolates. Contemporary PRRSV strains from pig serum samples were grown on MARC145 cells. Viral copies and $TCID_{50}$'s were determined for each viral stock. Analysis of viral growth was performed over a 48h time course and analyzed for the percent of infected cells using flow cytometry. The inset table shows the number of viral copies/ml used for the infection and the corresponding MOI based on the $TCID_{50}$ for each virus. Values below assay background are shaded.

Two contemporary 1-7-4 PRRSV strains were able to grow to decent titers in both MARC145 cells and PAM cells and their growth as compared to 3 low virulence strains was examined over a 72h time course of infection. The low virulence strains used were VR2332 (C-262-1), MN184 (H-184-1, GenBank ID DQ176019.1), and a type 1 PRRSV SDEU (H-PRRS1). The contemporary strains examined, which were both highly virulent in pigs, were 2016-US174 (C-174-4) and 2018-NC174 (C-174-7) (see Table 1). Viral RNA levels were examined in both the supernatant and the cells at each time point and was compared between viral isolates. In MARC145 cells, all 4 PRRSV-2 isolates gave similar growth curves with virus first detected in supernatants and cells at 10 hours post-infection (hpi), but at 6 hpi virus was just barely detectable in VR2332 infected cells (Figure 4 A and C). Both VR2332 and 2018-NC174 grew to higher levels more quickly than the other virus isolates (Figure 4 A and C). The 2018-NC174, 2016-US174, and MN184 isolates were able to replicate in PAM cells, where VR2332 was detectable, but only slightly increased in viral levels at 48h in supernatants and 24h in cells (Figure 4B and D). Interestingly, in PAMs both contemporary isolates seemed to grow more quickly than that of the MN184 isolate, showing an increase in viral levels at 24hpi in supernatants, where MN184 didn't increase until 48hpi (Figure 4B and D), but this wasn't statistically significant. In the cell fraction of PAMs, 2018-NC174 had increased viral levels by 10hpi, but 2016-US174 and MN184 didn't show an increase in virus until 24hpi. The SDEU strain did not grow well in either MARC 145 or PAM cells (Figure 4). It was also observed that during propagation of 2016-US174 viral stock, a

delayed CPE was observed in MARC 145 cells as compared to the other strains, the significance of which is not known at this time since the viral copies observed in cells and supernatants do not reflect this observation.

The ability of the contemporary strains to grow more quickly in PAM cells may reflect their ability to replicate quickly in the pig leading to highly virulent viruses. However, since no significant difference was observed between the growth of the viruses, this would need to be replicated to confirm the observation. Unfortunately, the ability to more quickly replicate in cells may also be a result of how well adapted it is to grow in MARC 145 or PAM cells in culture. Thus, the viral strains would need to be grown for a few passages in PAM cells before repeating this experiment, knowing that all of the viral stocks were adapted for growth in this cell type, thus minimizing cell culture adaptation of the virus as a variable for viral growth.

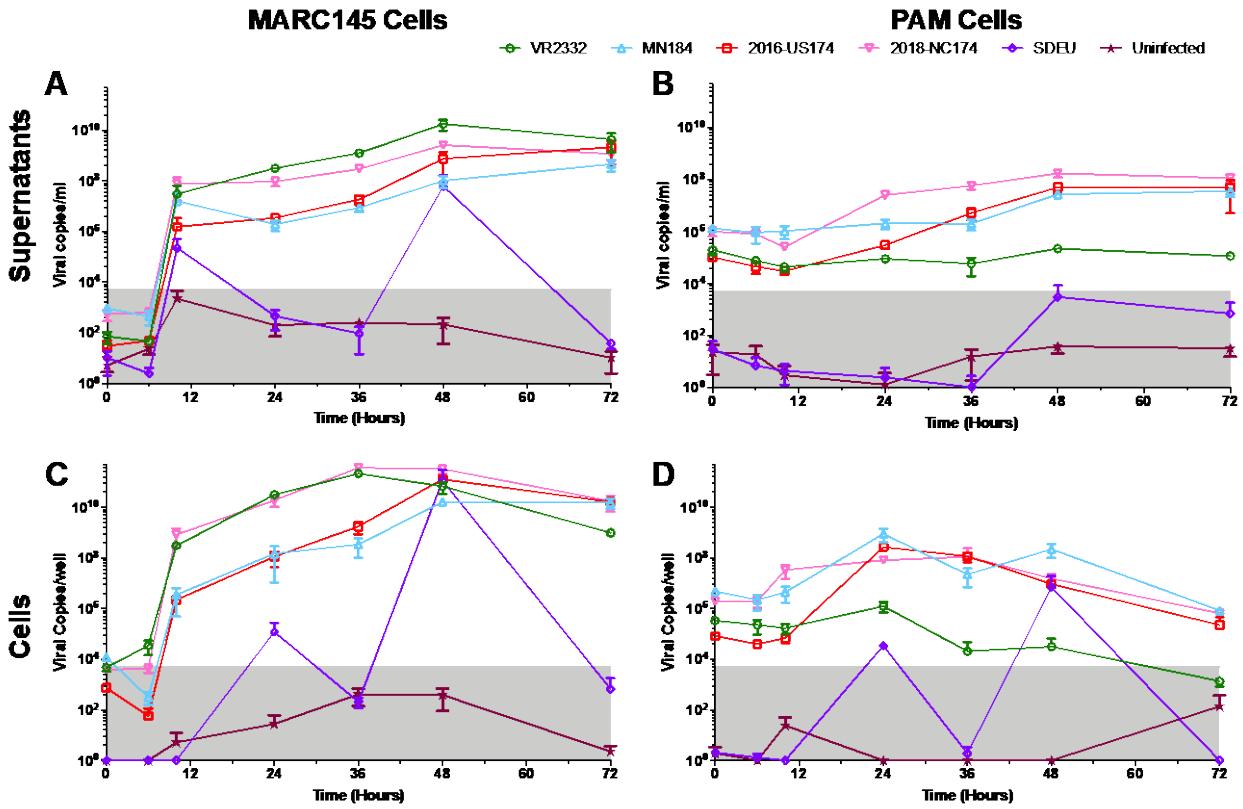


Figure 4. Viral Growth over time in various strains. A total of 2 low virulence (VR2332 and MN184) and 2 high virulence contemporary (2016-US174 and 2018-NC174) viral strains were used to infect either MARC145 or P AM cells at an MOI of 0.1 and grown over a 72h time course of infection. At each time point, supernatants and cells were seperated and the amount of virus in each was determined using a PRRSV ORF6/7 R T-qPCR assay and are presented as viral copies/ml for supernatants or viral copies/well for cells. Three replicates were performed for each virus. V alues below the detection level of the assay are shaded.

Objective 3: Determine unique features of contemporary virulent PRRSV genomes that are associated with atypical responses or avoidance of cross-neutralizing immune protection.

The whole genomes from a total of 19 historical (2006 or earlier) and contemporary (2015 or later) viral isolates were sequenced and annotated for known open reading frames (ORFs) (Table 1). All of the isolates were PRRSV type 2 except a single historical PRRSV type 1 isolate (Table 1). The RFLP type for each virus was determined

and the virulence was estimated using clinical signs from the infected pigs on the farms in which the virus was isolated (Table 1). Virulence, however, is known to vary depending upon their previous exposure history [5].

Each of the genomes was then examined for all possible ORFs greater than 35aa in length starting with any start codon. First, the predicted ORFs from all of the sequences were examined to determine those that were conserved between all or most of the genomes, identifying all of the known ORFs including the lesser identified regions of nsp2TF, GP2b, and ORF5a [6-8].

Interestingly, we identified 6 additional regions containing ORFs conserved across all or most of the sequences examined (Table 2). The ORF1a (nsp1) region contained 2 identified ORFs right next to each other that were in a different reading frame than that of ORF1a (Table 2). The first ORF was observed in all sequences, although it was slightly longer in the type 1 PRRSV sequence (H-PRRS1). The second ORF was observed in all sequences except H-262-1 and C-262-1 and it was a little different in the type 1 PRRSV sequence (H-PRRS1). The next conserved ORF overlaps with the ORF1a nsp2 and nsp2TF region, although it is translated in a different reading frame than either of those proteins (Table 2). This ORF was observed in all of the sequences including H-PRRS1, except for C-174-2. In the nsp3 region of ORF1a, a conserved ORF was observed of varying lengths, but similar sequence in all genomes except H-PRRS1. In the nsp9 and nsp10 regions of ORF2b, 2 conserved ORFs were observed in all genomes except H-PRRS1 with only slightly varying amino acid sequences and lengths. In ORF7, a long ORF was observed in most of the 1-8-4 RFLP type sequences and 2 separate ORFs, one right after the other with basically the same amino acid composition as the N- or C-terminal sequence of the long ORF, were observed in the majority of the other sequences (Figure 5). One of the 1-8-4 sequences (H-184-1) only contained the C-terminal ORF and H-PRRS1 only contained a similar, but longer C-terminal ORF which had been previously identified in the literature (Figure 5) [9]. The high conservation of all of these ORF regions throughout the different genomes suggests a protein may in fact be expressed for these regions and deserves further investigation.

The predicted ORFs observed in the ORF7 region were further examined to determine if a protein product (putatively called ORF7a) from this region or antibodies to this protein could be observed in infected cells. Interestingly, a second ORF in the ORF7 region of PRRSV-1 corresponding to the C-terminal region of the putative ORF7a protein has been previously examined demonstrating the

Table 1. Whole genome sequences obtained from PRRSV isolates

Isolate ID	Name (GenBank ID)	RFLP Type	Historical/Contemporary	Virulence
C-142-1	PRRSV2/USA/Lab1-142	1-4-2	Contemporary	High
C-144-1	PRRSV2/USA/Lab2-144	1-4-4	Contemporary	High
C-174-1	PRRSV2/USA/MN56/2015	1-7-4	Contemporary	Medium
C-174-2	PRRSV2/USA/MN58/2015	1-7-4	Contemporary	Medium
C-174-3	PRRSV2/USA/NC59/2015	1-7-4	Contemporary	Medium
C-174-4	PRRSV2/USA/Lab3-174/2016*	1-7-4	Contemporary	High
C-174-5	PRRSV2/USA/MO1/2017	1-7-4	Contemporary	Medium
C-174-6	PRRSV2/USA/MO2/2017	1-7-4	Contemporary	Medium
C-174-7	PRRSV2/USA/NC174/2018*	1-7-4	Contemporary	High
C-184-1	PRRSV2/USA/IA21/2015	1-8-4	Contemporary	High
C-184-2	PRRSV2/USA/OK5/2016	1-8-4	Contemporary	High
C-184-3	PRRSV2/USA/OK6/2016	1-8-4	Contemporary	Medium
C-184-4	PRRSV2/USA/OK7/2016	1-8-4	Contemporary	Medium
C-184-5	PRRSV2/USA/OK8/2016	1-8-4	Contemporary	Medium
C-262-1	PRRSV2/USA/Lab7-VR2332*	2-6-2	Contemporary	Low
H-142-1	JA142 (AY424271.1)	1-4-2	Historical	Low
H-184-1	MN184 (DQ176019.1)*	1-8-4	Historical	High
H-262-1	VR2332a (AY150564.1)	2-6-2	Historical	Low
H-PRRS1	PRRSV1/USA/Lab6-SDEU*	(Type 1)	Historical	Low

*Virus used for serum neutralizing assay and growth curves

Table 2. Conserved novel ORFs

GenBank ID	Region and reading frame^	Nucleotides*	# nt	# aa	Amino acid sequence
AY150564.1	ORF1a (nsp1), frame +2	195-302	108	35	LGYLIGARVPPMPGCLWRRAKYTAHDASVHGLSFP*
DQ176019.1		315-452	138	45	LSSECWACFIGPKSRSGGRCHAHSPLLSAPLLGLVGFLQFFQLHE*
AY150564.1	ORF1a (nsp2)/nsp2TF, frame +2/+1	3961-4152	192	63	LGFCSFYSIVPLFMLQLPSLWYCSPLGCVFWVFSARSNGGFWLLVGFCCWSVQACVRPSRRCL*
AY150564.1	ORF1a (nsp3), frame +1	5430-5567	138	45	LVVILMLLALSPPTTFIITPVAPAVLPPWLSHQMGPTWPLSAALR*
AY150564.1	ORF1b (nsp9), frame +1	8267-8386	120	39	LMGAPSWPRPCPPGLSYMYRPYQRLSLITLTLGLTALNS*
AY150564.1	ORF1b (nsp10), frame +1	9926-10159	234	77	LSGVELGEMKLNYQIVIMLAPPCSLPAKRSTWSLSLPMYCAAGSSSAHPVLGKHTGSFNIRSRMVMLFTHQLTRPCLT*
AY150564.1	ORF7, frame +2	14933-15061	129	42	MASQSISCARCWVRSSLSKTSPEARDRERKIRRKTRRSPIFL*
AY150564.1		15065-15184	120	39	LKMMSDITLPLVSGNCVCRQSRPPLIKALGLAPCQIQGG*
(C-184-1)		14567-14818	252	83	MASQSISCAKCWAGSSPSKTSPEVRDREEKVRREARRSPIFLSRLKMTLDITLPLVSGNCVCRQFRLPLTKALEPASCRTQGE*
(C-184-4)		14483-14764	282	93	MAGSRRKRKGMASQSISCAKCWAGSSPSKTSPEARDRGRKTRRKARRSPIFLLRLRMTLDITLPLVSGNCVCRQSRLPSTKALGLAPYQIQGE*

*Nucleotides are based on the GenBank ID (or strain) noted

^Frame is compared to ORF region shown

Figure 5. Putative ORF7a ORFs. A predicted group of ORFs (putatively called ORF7a) was observed in the ORF7 encoding region. Depending upon the viral isolate the ORF encodes for a single protein (ORF7a), a longer protein (ORF7a+), or 2 different protein fragments that encode for either the N terminus (ORF7aN) or the C terminus (ORF7aC) of the ORF7a protein. The PRRSV-1 gives a similar protein product that has been previously identified in the literature [9].

existence of an ORF7ap protein [9]. To examine the presence of an ORF7a protein in PRRSV-2 viruses, MARC145 cells were infected with different PRRSV strains that encoded various forms of the ORF7a protein and cell lysates were screened by mass spectrometry (LC-MS-MS and MRM) for the presence of these novel ORFs. Initial results suggest that the 83aa ORF7a protein is produced and can be detected using mass spectrometry.

Next, a recombinant 83aa protein was expressed and purified in *E.coli* and an ELISA assay was developed using this ORF7a protein to determine if antibodies binding this protein could be detected in infected pig serum. In fact, animals that had been exposed to a virus containing the 83aa ORF7a coding region contained antibody reactivity against this protein (Figure 6). Interestingly, animals that had been exposed to a virus containing 2 ORFs coding for the N- and C-terminal ends of the putative ORF7a protein (39aa and 42aa) also had reactivity suggesting that at least one of these proteins induced antibodies that reacted against the ORF7a protein (Figure 6). Animals that were not exposed to PRRSV showed no antibody reactivity (Figure 6). An avidity ELISA was performed to examine the strength of the antigen-antibody binding and confirmed that the reactivity was concentration dependent and specific (data not shown). This data suggests that the putative ORF7a protein is expressed, suggesting that perhaps some of the other conserved ORFs identified here may also be expressed. These conserved ORFs should be further investigated to determine if they are expressed and what function they may play in the viral life cycle.

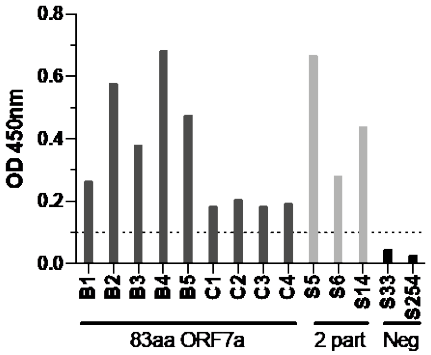


Figure 6. Serum antibody reactivity to ORF7a. Recombinant ORF7a protein (83aa) was expressed and purified from E.coli. ELISA plates were coated with 500ng of recombinant ORF7a protein and a 1:50 dilution of serum was used to examine reactivity to ORF7a. Animals were exposed to either a PRRSV containing the putative 83aa protein, the 2 part N and C terminus proteins, or were PRRSV negative. The ELISA OD values are shown with the background of the assay shown as a dotted line.

More interesting than conserved ORFs would be those that differ between low and high virulence viruses. Thus, we looked for putative ORFs that were either present in only low or high/medium virulence viruses or that differed in putative protein products between the 2 groups. Although numerous putative ORFs were present in different isolates, they usually followed RFLP type as opposed to virulence. However, 3 regions stood out as perhaps relating to virulence due to their differences between low virulence strains and the high/medium virulence strains (Table 3). In the ORF1a region (that codes for nsp7), there is another putative ORF which would be translated in a

Table 3. Novel ORFs that may be related to virulence

GenBank ID	Region and reading frame^	Nucleotides*	# nt	# aa	Amino acid sequence
AY150564.1	ORF1a (nsp7), frame +1	7311-7595	285	94	MLWAGKSTRNFGTRIPVMCFMRRSIITQMSGSVSELATLPTLTLRRELCVDMSPLKTRLTMFTPPHLVRSSWSPSTQRMEESNGKLQSFPWSRP*
(C-174-1)		7040-7213	174	57	LAFMSWMGKSTKNFGIRIPVMCSTKKSTTTQTRGNALEPATLPTWILRRGLCAGASP*
(C-174-1)		7229-7339	111	36	MFMPPLPVGSFLSLPTQRVGKPSGKLQGFPWSRPIA*
DQ176019.1	ORF1b (nsp9/10), frame +1	9038-9340	303	101	MFLSTTPPLLQYSWTVVLVWSTTLNGLKNLWLEWRYAPARTAIASPARRSSYPCGRNLSPIMRGRSQGYVGTAELRPRMPLPVVLTSVFTTLTFTSIVQS*
AY150564.1		9614-9790	177	59	MRGRSRECAGTAGPRPRTLLPVASTSAFTTPTSTSIVQSQSGVAIQRVLVLVVSANPL*
AY150564.1	ORF1b (nsp10), frame +2	9474-9599	126	42	LRGPGPVRYCLWPRRLHLPHPLPPALSSHNLVWPSSGFWFL*
AY150564.1	ORF4, frame +1	13594-13713	120	40	LAMCQASWLCVSILPATSNMSRSLPNAPWWSTMCGCSIS*

*Nucleotides are based on the GenBank ID (or strain) noted

^Frame is compared to ORF region shown

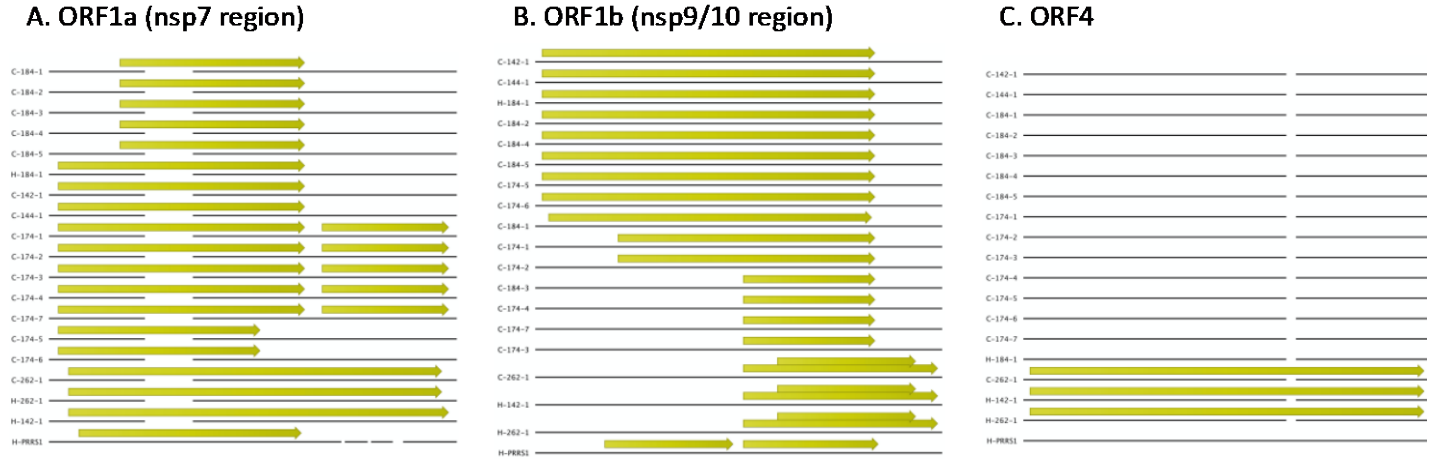


Figure 7. Putative ORFs related to viral virulence. Putative ORFs that may be involved in viral virulence were identified based on differences between ORFs due to virulence of viral isolates. Three regions were observed to contain unique ORFs that would translate different proteins depending upon the virulence of the isolate examined. The regions were found in a different frame than A) the ORF1a-nsp7 coding region, B) the ORF1b-nsp9/10 coding region, and C) the ORF4 coding region.

different frame than nsp7 (Table 3). In less virulent isolates a single longer ORF is present and in more virulent isolates either 1 smaller ORF similar to the N terminus of the larger one or 2 smaller ORFs similar to both the N- and C-terminus of the larger ORF are present (Table 3, Figure 7A). In the ORF1b region (region coding for nsp9 and 10) of less virulent strains, 2 short overlapping ORFs translated in 2 different frames are observed (nsp9/10 and nsp10, Table 3, Figure 7B). In more virulent strains a single ORF in the nsp9/10 region is observed ranging from a similar, but truncated ORF as compared to the less virulent strains, to an ORF quite a bit longer on the N-terminus (Table 3, Figure 7B). In the ORF4 region an ORF was observed only to be present in low virulence strains (Table 3, Figure 7C). Because a difference in virulence corresponds to a difference in the ORFs present, these 3 regions merit further examination to determine if the ORFs encode for expressed proteins, if these proteins are involved in virulence of the virus, and if changing, adding, or deleting these proteins can change the virulence of the virus.

Objective 4: Compare differentiation methods for characterization or classification of PRRS virus isolates that predict more effectively than RFLP typing the potential threat of isolates to infect immune herds with devastating consequences.

Using the 19 whole genome sequences from the above objectives (Table 1), different methods of clustering were performed to determine if any could predict the virulence of the isolate. As a gold standard, the methods were compared to RFLP typing and ORF5 clustering by amino acids and nucleotides (Figure 8). Whole genome sequence, ORF5 nucleotides, ORF5 amino acids, and RFLP type gave basically the same clustering, except that in sequence based clustering, 1-4-2 RFLP viruses clustered nicely based on virulence as opposed to RFLP type

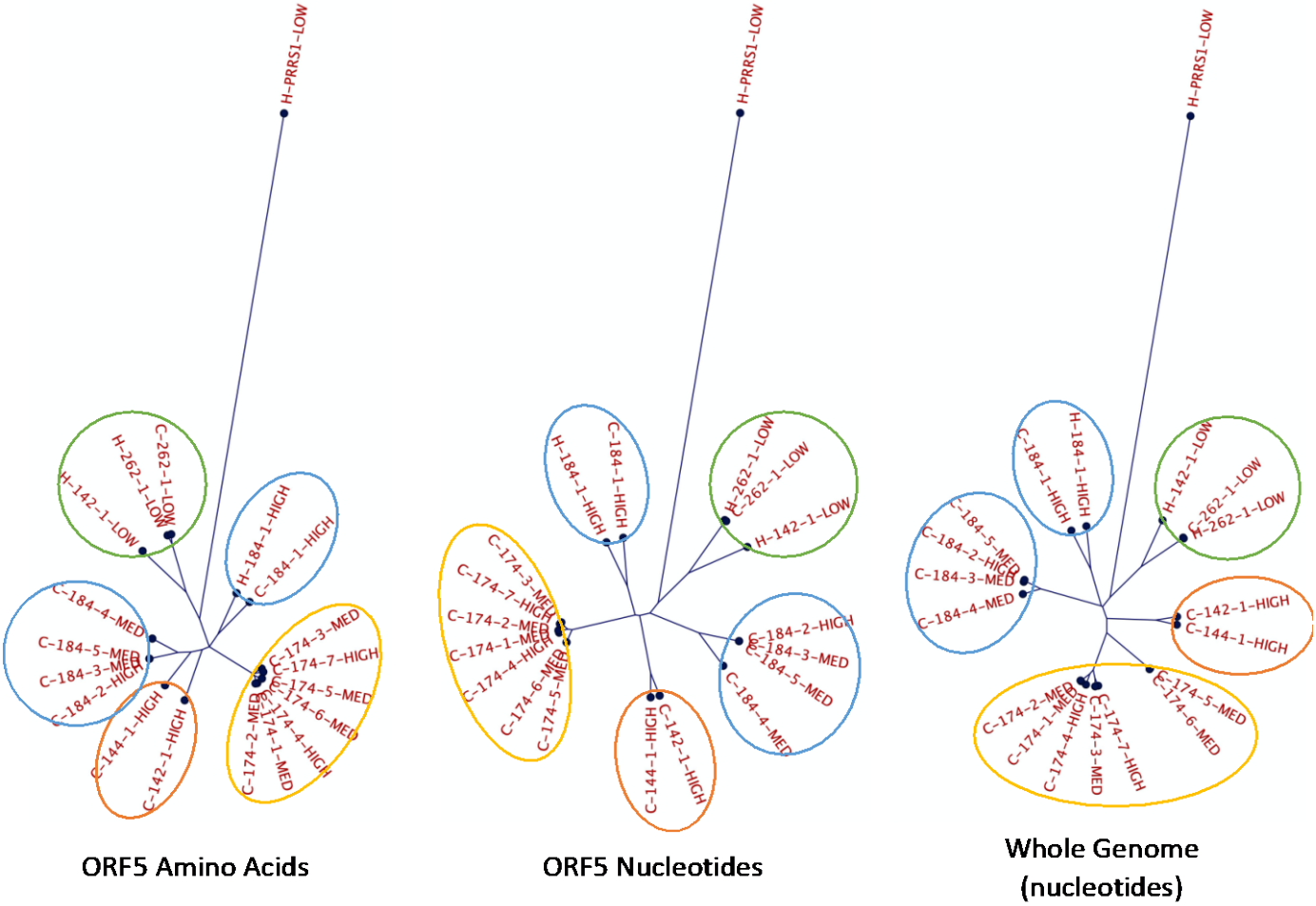


Figure 8. A comparison of categorizing PRRSV isolates using different methods. Whole genome sequences of PRRSV isolates with known virulence based on sow and piglet data. Isolates were grouped by either the ORF5 amino acid sequence, ORF5 nucleotide sequence or the whole genome nucleotide sequence. The group of low virulence viruses (RFLP 1-4-2 and 2-6-2) are shown highlighted in green. The RFLP 1-7-4 viruses are shown in yellow, RFLP 1-8-4 viruses are shown in blue, and the RFLP 1-4-4 and contemporary 1-4-2 high virulence viruses are highlighted in orange. The virulence of each isolate is noted.

(Figure 8). Thus, clustering by ORF5 or whole genome sequences was more accurate than RFLP type for virulence, however none of the methods were able to predict high vs medium virulence isolates. A variety of other clustering methods were examined to determine if highly virulent isolates could be predicted. All of the different ORFs as nucleotides and amino acids, including the less common ORFs and the ORFs predicted in objective 3, were examined, but none gave better virulence predictions than that of ORF5 or the whole genome. Next, different regions of the genome were examined based on what was observed in objective 3. Interestingly, the region spanning the end of ORF1a and beginning of ORF1b might be more predictive of virulence than ORF5 (Figure 9). This region certainly divides the isolates into more clusters, but it isn't necessarily related to the reported virulence of the viruses (Figure 9). However, the sequence in this region would need to be examined more closely and with more sequences from isolates with known virulence levels to determine if it was really more predictive of virulence. It would be interesting to further examine this region to determine if it might encode yet unidentified virulence factors. Finally, since ORF4 was identified as containing a putative ORF only observed in non-virulent isolates, this region was also thoroughly examined, but was only able to differentially cluster non-virulent isolates from the others.

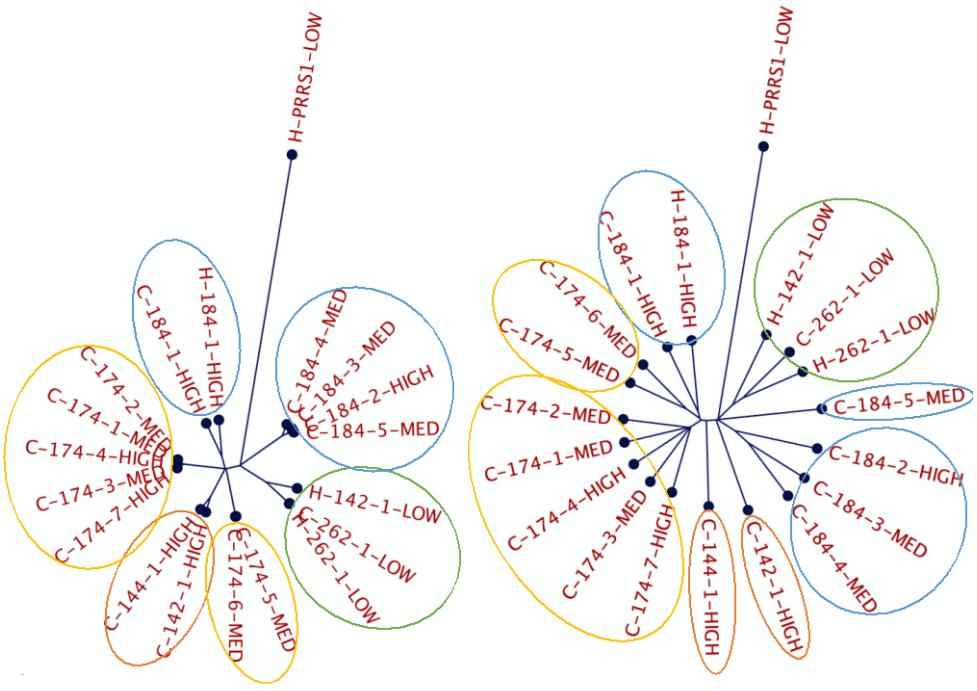


Figure 9. Clustering of isolates based on the region around the ORF1a-ORF1b junction. Approximately 3000 or 1500 bases spanning the ORF1a/1b junction were compared between 19 isolates. Isolates were clustered using the neighbor-joining method of clustering to create the phylogenetic trees. The group of low virulence viruses (RFLP 1-4-2 and 2-6-2) are shown highlighted in green. The RFLP 1-7-4 viruses are shown in yellow, RFLP 1-8-4 viruses are shown in blue, and the RFLP 1-4-4 and contemporary 1-4-2 high virulence viruses are highlighted in orange. The virulence of each isolate is noted.

Discussion:

PRRSV has an impressive ability to evade the immune response and cause disease in immune (endemic) herds due to its high mutation rate producing diverse new viral strains. Although commercial vaccination has been able to significantly improve disease outcomes in the past, new virulent isolates are now present that seem to completely evade vaccine-induced protection. The ability to identify new viral isolates in which animals would have no or little immune protection against would be an indispensable tool for managing healthy herds. Knowledge of when and how to quickly produce new vaccines based on newly identified viral clusters that are no longer protected against by vaccine-induced immunity is critical. In this study, we identified that new viral strains are highly virulent due to their ability to evade vaccine-induced immunity. We have also identified and are in the process of refining methods for determining if a new virus is likely to evade herd immunity based on their infection history. Finally, we have identified different regions of the genome that may be involved in virulence of the virus, perhaps leading towards the development of more effective vaccines.

Examination of the ability of pig serum samples to cross-neutralize diverse viruses from different clusters identifies the likelihood of protection against new viral strains. Here we determined that the more viruses the animals were exposed to, the more broadly neutralizing their antibodies. However, we do not know if broad neutralization can be induced through multiple exposures by diverse viral strains, multiple exposures of the same strain, a single inoculation with a cocktail of multiple strains, or perhaps multiple inoculations of a cocktail of strains. How to induce this broadly neutralizing activity is essential for control and protection against these new PRRSV strains.

Identification of 3 regions of the viral genome that may be involved in viral virulence is an exciting finding. These regions need to be further examined to determine if these putative proteins are expressed during viral infection, their role in viral replication, and if insertion or deletion of these proteins affects the virulence of the virus. If any of these regions are, in fact, involved in virulence, this could greatly influence our ability to create new vaccines, perhaps designing them against newly emerging highly virulent strains.

Development of a clustering method to identify viruses that may be highly virulent has proven to be difficult. One problem with determining a clustering method differentiating virus isolates by virulence is determining the actual virulence of the isolate. PRRSV virulence is greatly influenced by the animal's exposure history. If the animal had been previously exposed to a similar isolate, then the virulence of the new virus would likely be lower than if the same virus infected a naïve herd, in which case it would show up as a highly virulent virus. However, determination of the level of virulence of a virus may not be as useful as knowing which cluster the new virus belongs to and whether animals already possess protective cross-neutralizing antibodies against viruses from that cluster.

Clustering using ORF5 sequence, which is routinely obtained through diagnostic laboratories, was shown to be more effective at determining non-virulent vs. virulent strains than RFLP typing, even though viruses mainly clustered with their RFLP type. Use of clustering information should be useful for predicting if previous vaccination or exposure will effectively protect the herd against infection by a new viral strain. Our data suggests that combining the serum neutralization data with clustering will allow for the prediction of whether a farm with known viral exposures will have some immunity to a new viral isolate or whether the new virus will be highly virulent to the farm. Our identification of possible virulence regions in the genome should allow for more effective vaccine development using these regions as vaccine targets. The information gained through this study should allow producers to know when it is necessary to take immune and biosecurity measures to try to reduce and prevent disease, as well as help vaccine companies produce new, more effective vaccines against these new virulent viruses.

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