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A summary of PRRS virus diagnostic and research data

Michael P. Murtaugh[A], Colleen Finnegan[A], Martha Fuentes[A], Craig Johnson[A], Wanqin Yu[A], Juan Abrahante[A], Barry Kerkaert[B], Gordon Spronk[B], Mark Wagner[C], Mark Fitzsimmons[D]
[A]Department of Veterinary & Biomedical Sciences, University of Minnesota, St. Paul; [B]Pipestone Veterinary Clinic, Pipestone, MN; [C]Fairmont Veterinary Clinic, Fairmont, MN; [D]Swine Graphics Enterprises, Webster City, IA

Background

Porcine reproductive and respiratory syndrome (PRRS) is a continually changing disease since its emergence in swine herds in the 1980's. Genetic change in all regions of the virus has been a constant historical feature, though the effect of genetic change on management and control of disease, on diagnosis, and on immunological resistance has not been documented. Now, there is extensive genetic diversity in PRRS virus isolates throughout the world, including North America. In 1999 the European genotype of PRRS virus was first detected in the United States. European strains of PRRS virus are, like North American strains, genetically heterogeneous and changing at an equivalent or greater rate than in North America. Yet the two genotypes are distinct from each other with no evidence of genetic change or recombination. The genetic differences, although quite substantial, are not complete. Immunological cross-reactivity and variable cross-protection to virulent challenge have been noted, as well as variation in aspects of clinical disease and pathogenesis. About five years ago a new variant North American PRRS virus appeared in Minnesota, distinguished by a distinctive restriction fragment length polymorphism (RFLP) pattern in the open reading frame (ORF) 5 gene, the so-called 184 type. More importantly, the strain was exceptionally virulent, causing death of gestating sows, piglets, and growing pigs. Unlike the European-type viruses which now appear to be widely distributed in the U.S., do not seem to requiring special strategies for treatment and control, and do not appear to be displacing endogenous North American viruses, the 184 strains are displacing other strains at the regional level, even in the face of extensive efforts in the areas of biosecurity management and immunological prevention.

In approximately five years since its first isolation, the strain known as MN184 has evolved into a large and diverse family of viruses. In 2005, approximately 20 percent (192 of 913) of all PRRS virus accessions to the PRRSV Database (prrsv.ahc.umn.edu:2501/cms/published/HomePage) were members of the 184 family. Phylogenetic analysis of 16 randomly selected isolates showed eight percent nucleotide sequence variation between isolates. They appear to have retained the highly virulent characteristic of the founding member. The precise factors responsible for its highly virulent nature are not known, but may be related to a propensity for growth to high levels for extended periods of time in the pig (Johnson et al. 2004) and to be present in nasal and oropharyngeal secretions at high concentrations relative to less virulent field strains (Cho et al. 2006). The increasing prevalence of these aggressive viruses presents new challenges to swine veterinarians and producers, and suggests that a better understanding of the interaction of PRRS virus with pigs under real world conditions of commercial swine operations will be needed to achieve control of PRRS that is present endemically or has the potential to be established. The endemic interaction of PRRS virus with large populations of swine is particularly significant due to the use of serum inoculation strategies for immunological control which ensure that virulent field viruses will be maintained in a herd. Thus, we have initiated studies to characterize the immunological status of endemically infected swine herds, assess the effect of a serum inoculation boost during mid-gestation, and to determine the potential for specific genetic change facilitating re-breaks by apparently homologous viruses in herds exposed to on-farm PRRS virus isolates.

For many swine producers the critical point in control of endemic PRRS is prevention of reproductive PRRS and in utero transmission of virus from pregnant sows to piglets. However, common-sense practices involving herd exposure to on-farm isolates that is expected to provide complete, homologous immunity are not completely successful and re-breaks with significant reproductive disease and transmission of PRRSV to the nursery still occur. Viruses recovered from affected sows and piglets appear to be directly related to the ancestral immunizing virus based on ORF 5 sequence similarities in the range of 98-100%. However, ORF 5, at 600 bases in length, is only 4% of the viral genome. In order to understand why re-breaks occur in the face of apparently solid homologous immunity, and to use this information to improve prevention protocols, it is essential to obtain a complete genetic description of the virus and link it with better information about virulence and immunological cross-reactivity in pregnant sows.

The great majority of research on PRRS virus pathogenesis and immunity is performed in young pigs using the respiratory disease model (reviewed in Rossow 1998; Murtaugh et al. 2002a, 2002b; Murtaugh 2004). But preg-

nant sows are substantially different from growing pigs physiologically and developmentally, in immune system maturity, and in the site of disease pathogenesis. Our goal to better understand why PRRS outbreaks continue to occur in sow herds that are "protected", to provide new and important information on immunological protection and potential correlates of immunity in pregnant sows, and to develop diagnostic tools for total assessment of viral genetic change in field isolates. Toward this end we have developed a reagent set for amplification and full genomic sequencing of North American PRRS viruses, performed genomic sequencing and analysis of pairs of "homologous" PRRS virus isolates used in sow herds for herd protection and then re-isolated following an outbreak of reproductive PRRS, and compared the growth characteristics of re-isolated viruses and their recent ancestors on macrophages and in adult sows for evidence of change in virulence. We seek to determine the relative contributions of immunological evasion versus virulence change in the occurrence of re-breaks in previously protected herds. The findings will increase our understanding of PRRS disease in sow herds and the role of genetic change in the virus on changes in herd protection, and provide a foundation for more complete diagnostic assessment and prevention of PRRS.

Methods and materials

Field study

A 3100-sow herd was on a quarterly vaccination program for the previous 18 months. In the 3 months preceding onset of the study the sow herd experienced 3-12 abortions per week and a wild-type field virus was isolated from one or more aborted sows. Suckling and nursery piglets were serologically positive. Three hundred pregnant sows at 30-60 days of gestation were bled and one week later were inoculated with serum containing an on-farm PRRSV isolate at approximately 10^2-10^4 $TCID_{50}$ per dose. The inoculum was prepared on-farm from replacement gilts that were infected with the wild-type virus. It was sterile-filtered, tittered by real-time PCR, and diluted with sterile saline. All sows were bled again 28 days after inoculation. Serum was tested by quantitative PCR for PRRSV and anti-PRRS antibody levels were determined using recombinant PRRSV proteins. Animals were monitored and 30 with high, low and intermediate antibody levels were followed through farrowing.

Virology and serology

Serum samples were collected seven days before and 28 days after serum inoculation in mid-gestation, from a subset of sows at farrowing, and from piglets at birth. Colostrum samples also were collected. Antibody profiles for individual PRRS virus proteins (nucleocapsid, GP5 and M) using recombinant protein-specific ELISA were obtained. Real time-PCR for PRRS was performed to determine levels of viremia. Recombinant proteins were produced in the Murtaugh laboratory or by ATG Laboratories (Eden Prairie, MN).

Whole PRRS virus genome sequencing

Overlapping 1-2 kb DNA fragments were amplified by reverse transcription and PCR to cover the entire genome of field isolates. High throughput sequencing was performed at the Advanced Genetic Analysis Center, University of Minnesota. Sequences were assembled and aligned in a publicly-available program suite known as Phred-Phrap-Consed (see www.phrap.org).

Results and discussion

Growth of field isolates viruses from serum

Initially, the growth of PRRS virus from serum samples was performed in MA104 cells. However, we discovered that many of the PRRS virus field isolates grew poorly or not at all in this cell line. Therefore, a comparative study measuring the infection efficiency of different field isolates on either MA104 cells or porcine alveolar macrophages was performed, and revealed dramatic differences. As shown in **Table 1**, 73% of field isolates were able to grow in alveolar macrophages versus 0% in MA104 cells. Over time, nearly all field viruses can be cultured on primary alveolar macrophages though multiple passages may be required to achieve measurable levels of replication. Interestingly, VR2332, a widely used laboratory strain, was able to grow efficiently in MA104 whereas its growth was indeterminant in alveolar macrophages. These findings indicate that wild-type PRRS virus growth in pigs may be facilitated by conditions in vivo that are not reproduced in vitro. Otherwise, we would expect that the viruses would routinely grow on alveolar macrophages, which are the permissive cell in the lung, the site of primary infection. In addition, wild-type viruses appear not recognize MA104 cells as permissive. The ability to grow on these cells appears to require conditions of incubation or features of the virus which are not generally present.

Field study of virological and immunological response to mid-gestation serum inoculation

A field study was performed to determine if serum inoculation would boost immunity in sows and reduce the level of reproductive disease in an endemic sow herd. Accordingly, sows at 30-60 days of gestation were inoculated with an on-farm isolate. Serum samples were taken from 300 sows seven days before and 28 days after the inoculation. Quantitative real-time RT-PCR was performed to determine the viremia status of sows before and after inoculation. All samples were tested in duplicate. Criteria for a positive result were that duplicates closely match and the test value be a minimum of 36 cycles. In this test cycle number (the Ct, or cycle threshold) is inversely proportional to the amount of target virus in the sample.

Table 1: Growth of PRRS virus isolates on MA104 cells and porcine alveolar macrophages (PAM). Infection was determined by SDOW17 staining of cells and by RT-PCR amplification of cell extracts. "+" indicates scattered positive cells in the well, "++" indicates numerous foci of infection. "C" indicates the cultures were contaminated. "n.t." indicates the test was not performed. "Pre" and "post" refer to viral samples that were used in a program of serum inoculation (pre) or were recovered from PRRS-affected pigs in an inoculated herd and had essentially the same ORF 5 nucleotide sequence (post).

	Days in culture					
	2		4		6	
Sample	MA104	PAM	MA104	PAM	MA104	PAM
H1 pre	–	+	–	+	–	+
H1 post	–	+	n.t.	n.t.	n.t.	n.t.
H2 pre	–	–	–	+	–	+
H2.7 post	–	–	–	–	–	–
H2.8 post	–	–	–	–	–	–
H3 pre	–	–	–	+	–	+
H6 pre	–	–	–	–	–	–
H7 pre	–	–	–	–	–	–
F pre	–	++	–	++	–	++
F post	–	–	–	–	–	+
B pre	–	+	–	+	–	+
B post	–	+	–	C	–	+
V pre	–	+	–	+	–	+
V post	C	C	C	C	C	C
L pre	–	+	–	+	–	+
L post	–	+	–	+	–	+
VR2332	+	+/–	+	+/–	+	+/–

As shown in **Figure 1**, pools of five serum samples before serum inoculation were all negative for virus. Two putative positive pools were negative when the individual sera were retested. At 28 days after inoculation, two of 300 sows tested positive at or near the limit of detection. We concluded from these observations that acute viremic infection is an infrequent event, at best, in endemic sow herds. No positive animals could be detected before serum inoculation, and two animals were identified at the limit of detection of the assay at 28 days after inoculation.

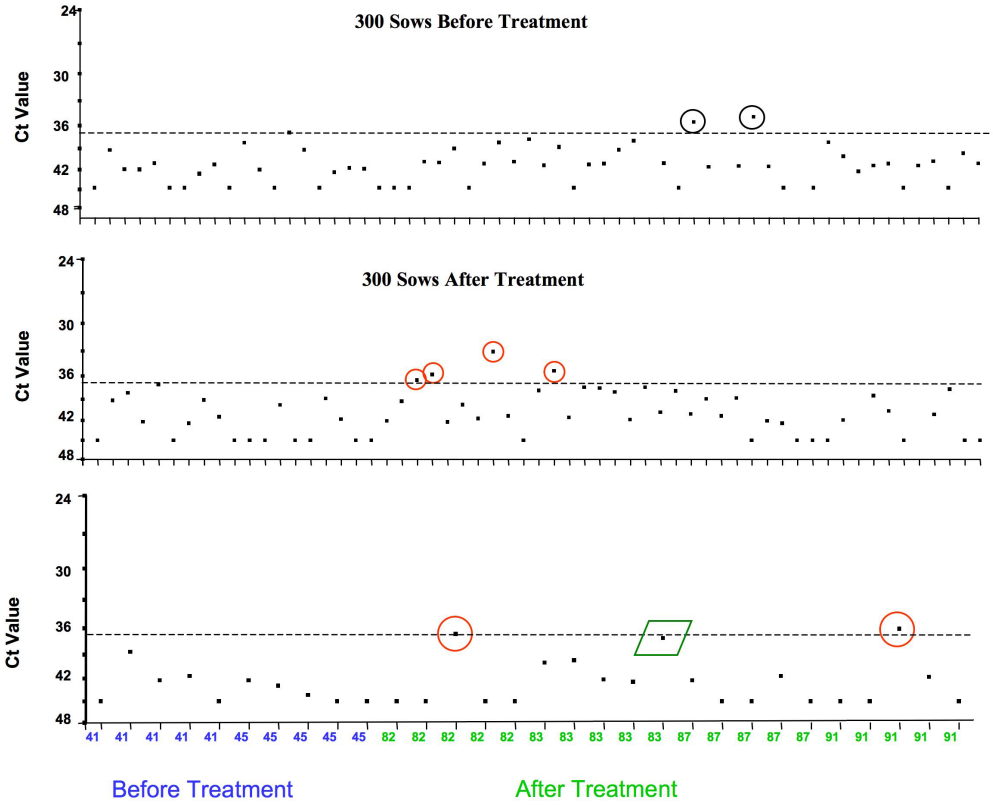
Further examination of the results in **Figure 1** shows that the quantitative PCR results require a good understanding of the assay capabilities. The assay is run for 45 cycles, and an unequivocally negative result is one in which not Ct value is obtained because the product curve never crosses the threshold. However, negative samples often cross the threshold earlier, producing false positives. Since very low levels of virus also can produce Ct values in the range of 37-44 cycles, there is an inherent range of uncertainty such that absolute conclusions of present or absent carry a risk of being incorrect.

Serological findings indicated that anti-PRRS virus antibody levels were normally distributed in the population, as determined by a combination of three conserved proteins or polypeptides (N, GP5 and M), or by a synthetic protein containing the surface components of GP5 and M that are used for viral attachment to cells (**Figure 2**). Comparison of antibody levels before and after inoculation showed that there was a decrease in titer over the 35 day period. This observation is consistent with a lack of effect of the serum inoculation, and was more pronounced for the antibodies directed against the conserved N, GP5 and M than for the ectodomains.

We also compared within each animal the serological changes. The result, in **Figure 2**, panel C, showed a correlation between the change in 3-protein and Orf 5 & 6. However, there is extensive variation, indicating that many factors affect the relative antibody response to individual viral proteins even within the same pig. The evidence suggests that antibody responses to PRRS virus proteins are largely independent of each other. One might therefore predict that conclusions about serological responses to PRRS virus might be affected by the specific proteins used in the assay.

Within the population of 247 sows, the majority showed little or no change in antibody levels before and after treatment, and others that showed substantial differences, either increasing or decreasing. Therefore, a subset of 30 sows was selected, representing the full range of observations, to determine if the differences were maintained at farrowing and if they were associated with reproductive outcomes. Interestingly, the antibody levels to 3-proteins declined substantially in almost all cases and, for anti-Orf 5 & 6, stayed the same or declined substantially (**Figure 3**). Thus, the variation observed in mid-gestation was dramatically reduced by the time of farrowing, though all

Figure 1: Quantitative real-time RT-PCR determination of viremia in serum before and after mid-gestation serum inoculation. Serum samples were pooled into groups of 5 animals for initial testing. Results are presented as Ct (cycle threshold) values, in which higher numbers correspond to lower values. The nominal negative Ct is at 36 cycles (dashed line), the limit of detection. Values shown by circles that are above the negative threshold and had good agreement between duplicates were retested individually, and are shown in the bottom panel. Circles indicate positive test. Trapezoid indicates sample without agreement between the duplicates, so is not positive.



of the animals were serologically positive. Reproductive performance was within the normal range and no association was noted between antibody levels at farrowing and reproductive outcomes.

Whole genome sequencing

We developed a standard set of reagents and protocols to sequence, assemble and compare whole genomes from two positive control strains, VR2332 and Ingelvac ATP. In addition, we sequenced and assembled whole genomes from two PRRSV field isolates, MN30100 (provided by Dr. Scott Dee, University of Minnesota) and ISU-P (provided by Dr. Jeff Zimmerman, Iowa State University). The primers utilized for amplification and sequencing were designed to completely conserved regions determined by ClustalW analysis of 11 PRRSV North American sequences available in Genbank. In total a set of 22 primer pairs was designed with an average amplification product size of 900 base pairs spanning the entire viral genome. Four whole PRRS genomes have been successfully sequenced and assembled. Five additional pairs of samples have been re-grown in alveolar macrophages and await amplification and sequencing. While this work is in progress and so we do not have results to report at this time, we have established that a conserved set of primers can be expected produce whole genome sequences. This information will be valuable for comparative studies to help identify viral genetic features that may be important in disease pathogenesis or as targets of immune intervention.

Conclusions

Routine isolation of PRRS virus from field samples does not appear to be feasible on MA104 cells, or their MARC145 derivative. Isolation on alveolar macrophages is better, but not always predictable. A better understanding of the requirements for PRRS virus growth on cells in culture is needed. The findings from our investigation of PRRS virus interaction with pigs in this field study of an endemic sow herd were somewhat inconclusive since no evidence of virus transmission was observed from sow to piglets. Nevertheless, useful descriptive information was obtained that will help in the interpretation of future experiments and for providing baseline information on

Figure 2: Effect of serum inoculation on antibody response to PRRS virus proteins. Samples before and after mid-gestation serum inoculation were matched for 247 sows and tested for antibody level against a combination of three PRRS virus protein, N, GP5 and M (3 protein), and against a synthetic protein containing the surface components that are needed for viral attachment to cells (Orf 5 & 6 (M) ectodomains). (A) Histogram showing the difference in 3-protein antibody level, after minus before. (B) Histogram showing the difference in the Orf 5 & 6 ectodomains antibody level, after minus before. (C) Correlation between 3-protein and Orf 5 & 6 antibody level differences shown in panels A and B. The no change point shows the mean at which there would be no difference in before and after antibody levels.

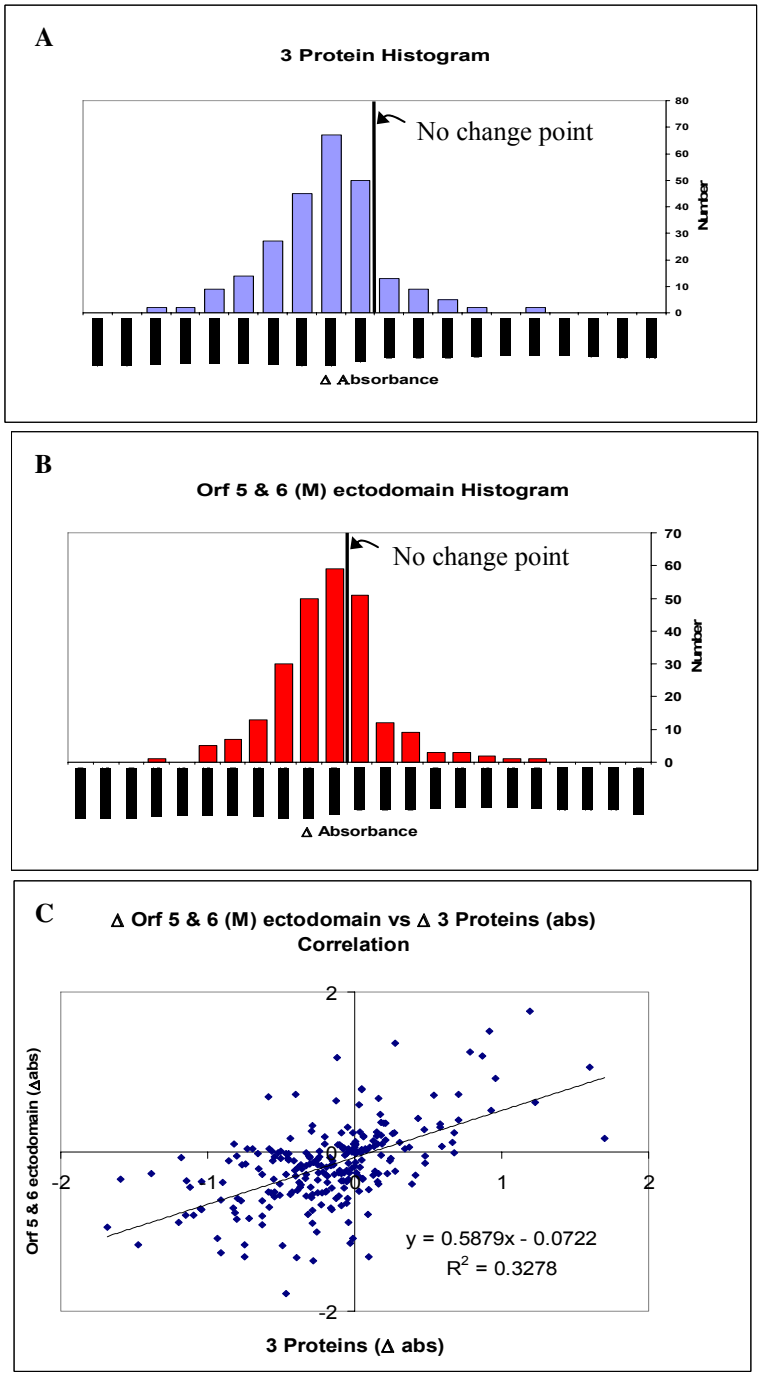
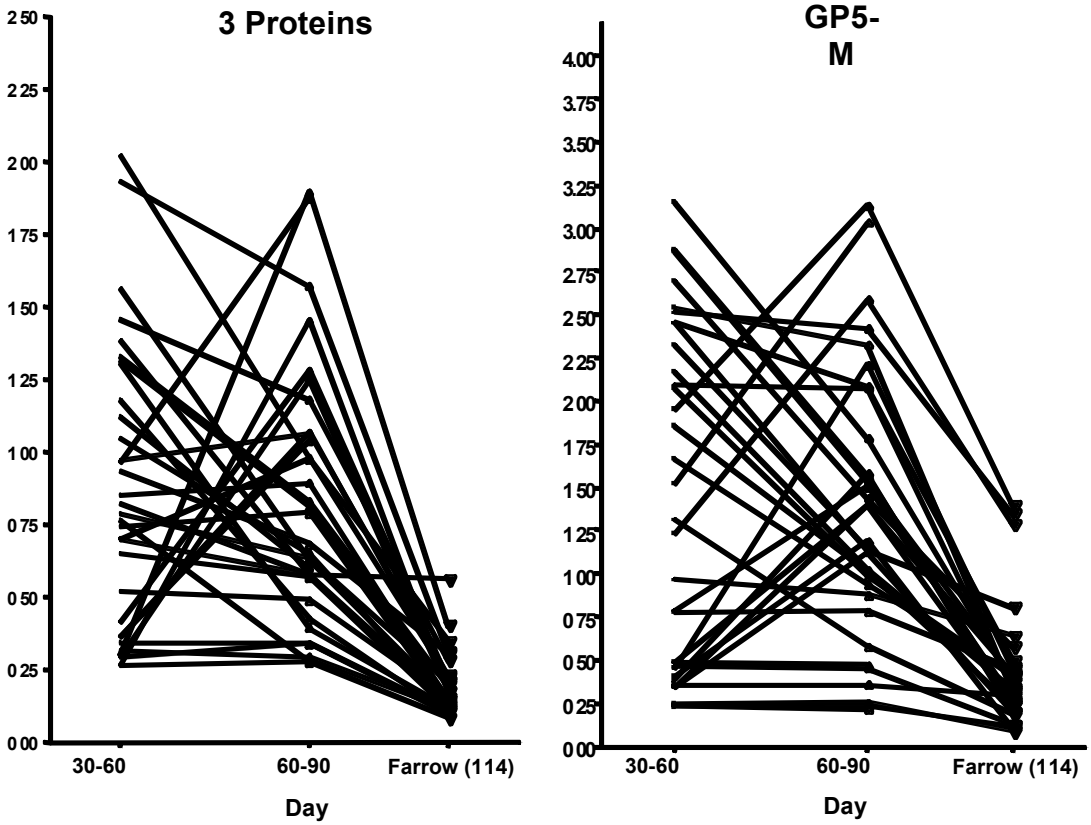


Figure 3: Three-protein and GP5-M chimera (Orf 5 & 6) antibody levels in 30 sows with various patterns of antibody response before and after treatment (day 0 and day 35). Antibody levels decline in all animals in late gestation.



sows in an endemic PRRS herd. In a herd of animals in which pigs are routinely exposed to PRRS virus as gilts, there is little or no evidence of viremic animals. Antibody levels to PRRS virus show a normal distribution with small proportions of animals showing high levels and other animals showing low levels. There was no evidence of outliers that might be considered at risk of infection and all animals were nonviremic regardless of their antibody level. Also, since there was little correlation in antibody levels obtained using different antigen compositions on the ELISA plates, the values obtained are somewhat dependent on the test used. Antibody levels decline over time in pregnant sows, even in the face of a mid-gestation boost with a virulent virus inoculum. Substantial variation was present in antibody levels in mid-gestation, but was greatly reduced at farrowing. Further studies to identify genetic factors in the virus that might explain some of the variation in the success of immunologically-based control of PRRS virus are underway, using whole genome sequencing.

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