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# Use of PRRS virus sequence information within herds

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## Introduction

PRRS continues to frustrate producers, practitioners, and scientists. New technology has enabled us to learn more about the "wanderings" of the PRRS virus. We can now answer the most frequently asked questions:

Where did it come from?

Is it the same virus I had last year or a new introduction?

Is it the same virus that my neighbor has?

Is it the "bad virus" (1-8-4) I heard about?

Is it vaccine strain or a field strain?

My pigs get transported on the same truck as another herd. Did I get their virus?

Can I mix pigs from these 2 farms? Do they have the same or different viruses?

The more we learn about the PRRS virus and where/how it's spreading, the more we will be able to implement biosecurity measures and other protocols to help control it.

## Porcine reproductive and respiratory syndrome virus (PRRSV)

The porcine reproductive and respiratory syndrome virus (PRRSV) is characterized as genetically unstable in certain segments of the genome; nucleotide sequences in the genome change frequently through point mutations, deletions, insertions, or recombinations. Researchers have developed diagnostic tools that are used to determine whether various PRRSV isolates are the same or different based on detecting changes in these variable segments of the genomic sequence. Determining whether or not two or more viruses are the same is key to understanding whether clinical PRRSV infections result from the same or mutated viruses present in the herd versus new introductions of virus.

## Restriction length polymorphism—convenient, confusing categories

The restriction fragment length polymorphism (RFLP) technique has been used to assign a numeric code to a "family" of presumably related PRRSV isolates (Wesley et al., 1996). In many cases there is good correlation between the RFLP cut patterns and the degree of genetic similarity among viruses. However, there are also many cases where the RFLP patterns mischaracterize the relative similarity of PRRSV isolates, resulting in misleading conclusions regarding their similarities or differences (see Appendix A for more details). Thus, the three-digit RFLP numeric code assigned to a particular isolate may or may not indicate relatedness with other PRRSV isolates. In summary, when using RFLP cut patterns to identify relationships among PRRSV isolates, the inherent inaccuracies of the technique must be considered.

## Genomic sequencing—what it is and isn't

### Sequence of what?

Open reading frame 5 (ORF 5), the envelope coding region of the genome, is used to differentiate PRRSVs. The envelope portions of viruses, PRRSV and others, are exposed to immune and environmental pressures that are believed to direct or select for viruses that can maintain replication fitness at an advantage over another PRRSV and possibly other pathogens (Chang et al., 2002; Smith et al., 1997). The resulting changes can be demonstrated by sequencing – determining the precise base pair sequence of the portion of the viral genome analyzed. Sequencing portions of a virus for comparison has been done historically for other viruses such as the measles virus and influenza virus (Rota et al., 1998; Wright and Webster, 1996).

To definitively determine if viruses are identical the entire genome would have to be sequenced and compared—a process not financially practical or feasible at present except in research applications. Does that mean PRRSV sequencing of only one portion of the virus is meaningless? No, virus sequencing and comparison is a common, valuable, and accepted practice (Rota et al., 1998). Simi-

larities and differences in a viral region prone to change make it an excellent benchmark for comparisons.

### Different or not?

The immediate question that arises once you have more than one virus sequence is: How do I determine if these viruses are the same or different? There is no definitive answer to this question for PRRSV or other viruses, including the closely followed influenza viruses. Interpretation of virus sequences is situational and influenced by many factors. We have historically used 99.5% homology as a cut-off for interpreting viruses as the same based on several reasons:

- A virulent field strain of PRRSV (VR-2332) differs by only 0.3–0.5% from the markedly less virulent modified live vaccine virus derived from it.
- A value of 0.4% has been used in another study to determine relatedness (Rota et al., 1998).
- The fidelity/reproducibility of the sequencing reactions falls within the 0.5% cut-off (Smith et al., 1997) and has been in our experience closer to 100% (unpublished data).
- RNA viruses may mutate at a varied rate, which can be influenced by more than just the inherent nature of RNA polymerase infidelity (Rota et al., 1998).

Interpretation of PRRSV sequences is dependent on the number of sequences available and the effort to sequence routinely over a period of time—ideally more than one year depending on the herd (size, location, and pig flow). For example, viruses differing by 2% could be derived from a similar recent ancestor if they are recovered one to two years apart within a herd or downstream from a common source. Likewise, it would be difficult to interpret viruses differing by 2% obtained from one sampling as being the same or different. If the viruses within a herd or region have been characterized over time it is easier to identify new introductions, i.e., the time required for change to occur in the indigenous virus cannot be accounted for by the degree of ORF 5 difference (Rossow and Mohr, 2002).

**What do differences mean?** There are two important and related questions that we would like to be able to answer by looking at differences in PRRSV gene sequences, but we don't yet have the tools to do so. The first concerns antigenicity—at what point do differences in genomic sequence result in enough change in antigenicity such that homologous immune protection fails? This is an ambitious but important question. Unfortunately, we don't understand the protective immune response to PRRSV well enough to be able to address the question of homologous versus heterologous immunity in the first place. Additionally, sequence changes may or may not reflect antigenic differences, and no method for determining the impact of PRRSV sequence changes on antigenicity has been developed so far.

The second question has to do with virulence—what changes in the viral genome result in clinical variation in disease presentation (virulence)? While certain genomic changes have been identified which appear to occur commonly as virus isolates become adapted to continuous cell lines or pigs (Murtaugh et al., 1995; Chang et al., 2002), PRRSV ORF 5 sequencing is not meant to be a direct measure of virulence.

Thus, the interpretation of differences identified in genomic sequences at this point is quite straightforward: two or more viruses may be interpreted as different, but we don't know what the differences mean in terms of antigenicity or virulence. There is a great need to move forward with these research questions because until they are answered, we won't be able to develop effective control programs.

## Sequencing mechanics

### Sample submissions—what do you send in? i.e., what question do you want to answer?

The process of sample submission begins with selecting candidate pigs. The obvious objective is to find PRRSV in clinically affected pigs to increase the likelihood that the virus found is associated with the disease observed. In sow units we have had the most success sampling small, weak pigs at 4–5 days of age that are showing typical signs of PRRSV infection—thumping (dyspnea), gaunt/emaciated, scouring, etc. Sows have proved to yield virus in herds with active reproductive disease if the sows have high fevers at the time of sampling (>104˚F), particularly febrile sows with additional clinical signs such as inappetance, respiratory signs, and general malaise. Most sows are not viremic at the time of abortion. Pig selection in nurseries and finishers is the same—fever, inappetance, respiratory, or (rarely) nervous signs.

Samples that can be submitted for sequencing include serum, tissues, and semen. Serum has worked most consistently, in keeping with the protracted viremia associated with the clinical course of the disease. Serum samples are obtained without sacrificing the pig—another advantage. Lung and lymph node samples in pigs of any age are also high probability sources. Surprisingly, tonsils samples have yielded few positives in our experience.

Our typical sampling protocol during a disease outbreak episode includes serum samples from 10–15 piglets at 4–5 days of age and 10–15 sows showing the clinical signs described. When searching for virus in the absence of clear clinical signs we have increased the sampling to as many as 50 piglets (4–14 days of age, selecting the poorest quality pigs available).

Proper handling of samples is important. Samples should be kept cool from the time of collection until delivery to the lab. We bring coolers or boxes with freezer packs into the barns to chill the blood and tissue samples immediately upon collection. Care should be taken during the centrifugation of blood tubes not to allow the tubes to warm up.

### Diagnostic testing—what happens to it?

Sequencing can be performed on virus isolates that are cultured in the laboratory or directly from samples amplified by a PRRSV-specific polymerase chain reaction (PCR). The advantage of using samples from PCR is that it avoids potential changes in the genome that stem from adaptation of the virus to laboratory cell lines or selecting a virus predisposed to grow in cell culture such as RespPRRS. It also speeds up the process. A disadvantage is that you don't wind up with a defined isolate in "the bottle" when using PCR directly from tissue or serum because no culturing or isolation step is required.

PCR testing can be run on serum, semen, or tissue samples. The PCR process for diagnosis amplifies a conserved region of the open reading frame (ORF) 6 region of the genome, a more highly conserved region of the PRRSV. For sequencing, multiple overlapping primers are used to obtain the 600 bases in the sequence of the ORF 5 region.The entire process of running the PCR on submitted samples and developing the sequence information requires two to four weeks, depending on the yield of the PCR process and the behavior of the sequencing primers with the specific virus being analyzed. The PCR step costs $20 per sample, and each sequence determination costs $175. Separate testing for RFLP pattern determination is no longer required because the RFLP pattern is predicted from the sequence information.

### Diagnostic reports and their interpretation—what do you get back?

The diagnostic report involves three main reports: virus sequence, dendogram and homology chart.

The **sequence** is the specific ordered list of the bases determined for the ORF 5 region of the virus submitted. Charts can be constructed that compare the sequence of the submitted virus against previously sequenced viruses as a tool to identify specific sites where differences exist. We seldom make these direct comparisons, except when trying to localize changes within ORF 5 between different viruses. These efforts have not been particularly rewarding in practice.

The **dendogram** (figure 1) is a useful tool for evaluating relationships among groups of viruses. A scale line at the bottom of the chart depicts the distance or difference between the isolates on the dendogram for the ORF 5 region. Adding the distances of the solid lines from left and to right provides an estimate of the difference in the respective sequences between two viruses. The value of the dendogram is the visual grouping that provides information on "families" or clusters of viruses that are in the chart. Known standard sequences (e.g., modified-live vaccines and prototype viruses used in research) are included to provide reference information for relative comparisons. Clusters of viruses that are grouped together are interpreted as more highly related. Viruses can be included in the dendogram in any combination: by farm, system, practice, or region

The **homology chart** (figure 2) is a specific listing of the percent identity among different viruses laid out much like a road atlas mileage chart. The chart can be read by going across and up at the intersection of two viruses to find the specific percent homology between the two. Again, known standard sequences are typically included as a point of reference. The advantage of using the homology chart is that the percent identity is listed specifically, removing the need for complicated ruler and calculator work with the dendogram.

Other reporting formats are also available with advocates for each. These are the three we use on a routine basis.

## Case examples of sequencing application

### New clinical cases in PRRSV-negative herds

A new PRRSV infection in a herd previously considered free of the virus represents the simplest interpretation of sequencing information. We routinely encourage clients to request sequencing of isolates in these cases for two reasons: as baseline information for future comparisons, and as an aid to identify possible sources or routes of infection.

Figure 1. PRRSV dendogram

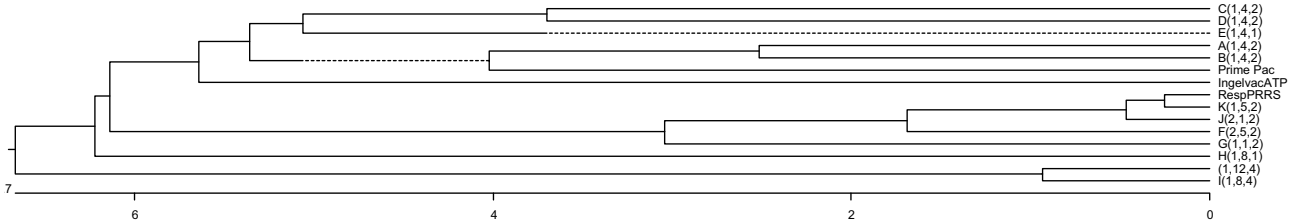

Figure 2. PRRSV homology chart

Percent Identity

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **1** | | 86.4 | 85.1 | 85.4 | 85.9 | 85.7 | 86.6 | 84.7 | 84.1 | 85.1 | 85.1 | 89.6 | 98.2 | 84.6 | 85.2 | **1** | (1,12,4) |
| **2** | 14.4 | | 94.7 | 92.0 | 92.2 | 90.2 | 92.9 | 89.7 | 87.4 | 88.6 | 85.7 | 88.2 | 85.9 | 88.1 | 88.4 | **2** | A(1,4,2) |
| **3** | 15.5 | 5.0 | | 91.4 | 92.5 | 91.4 | 93.0 | 91.5 | 87.6 | 88.2 | 87.6 | 88.6 | 85.4 | 88.4 | 88.1 | **3** | B(1,4,2) |
| **4** | 14.8 | 7.7 | 8.3 | | 92.7 | 90.5 | 93.2 | 89.1 | 88.9 | 90.4 | 88.7 | 89.2 | 85.6 | 90.2 | 90.2 | **4** | Prime Pac |
| **5** | 14.6 | 7.6 | 7.6 | 7.8 | | 92.5 | 94.9 | 90.7 | 87.6 | 88.7 | 87.7 | 88.7 | 85.9 | 88.7 | 88.4 | **5** | C(1,4,2) |
| **6** | 15.1 | 9.7 | 9.1 | 10.1 | 7.4 | | 92.5 | 88.9 | 87.9 | 88.1 | 87.6 | 87.4 | 85.6 | 88.2 | 87.9 | **6** | D(1,4,2) |
| **7** | 13.8 | 7.0 | 7.1 | 6.8 | 5.2 | 7.6 | | 92.4 | 88.7 | 90.0 | 88.4 | 89.4 | 86.6 | 89.9 | 89.7 | **7** | E(1,4,1) |
| **8** | 15.9 | 10.5 | 9.2 | 11.1 | 9.8 | 12.1 | 7.2 | | 87.6 | 89.4 | 88.7 | 88.2 | 84.9 | 89.7 | 89.6 | **8** | IngelvacATP |
| **9** | 16.7 | 12.3 | 12.1 | 11.0 | 12.3 | 12.5 | 11.0 | 12.9 | | 96.8 | 93.2 | 86.6 | 83.7 | 96.7 | 96.7 | **9** | F(2,5,2) |
| **10** | 15.2 | 10.8 | 11.1 | 9.5 | 10.9 | 11.9 | 9.3 | 10.7 | 3.2 | | 94.5 | 87.9 | 84.7 | 99.2 | 99.5 | **10** | RespPRRS |
| **11** | 15.4 | 13.7 | 12.5 | 11.3 | 11.7 | 12.9 | 10.9 | 11.2 | 6.8 | 5.4 | | 87.1 | 85.4 | 94.5 | 95.0 | **11** | G(1,1,2) |
| **12** | 11.1 | 12.3 | 11.7 | 11.4 | 11.3 | 13.3 | 10.4 | 11.9 | 13.0 | 11.7 | 12.7 | | 89.6 | 87.2 | 88.1 | **12** | H(1,8,1) |
| **13** | 1.9 | 14.8 | 15.3 | 14.6 | 14.6 | 15.5 | 13.6 | 15.5 | 17.0 | 15.5 | 15.5 | 10.8 | | 84.1 | 84.9 | **13** | I(1,8,4) |
| **14** | 15.9 | 11.4 | 11.3 | 9.7 | 10.7 | 11.7 | 9.5 | 10.3 | 3.4 | 0.8 | 5.6 | 12.3 | 16.2 | | 99.0 | **14** | J(2,1,2) |
| **15** | 15.0 | 11.0 | 11.3 | 9.7 | 11.1 | 12.1 | 9.5 | 10.5 | 3.4 | 0.5 | 4.8 | 11.4 | 15.3 | 1.0 | | **15** | K(1,5,2) |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | | |

Herds that have undertaken projects to eradicate PRRSV are of particular interest because of the concern that the PRRSV in the new outbreak may have carried over from the previous herd. In our experience with 11 such cases, virus sequences from 10 of the herds were considered different from isolates previously sequenced from those herds. In one case, the sequence was similar to a live-attenuated vaccine that had been used in the herd prior to a test-and-removal PRRSV eradication project, raising questions as to whether the isolate may have represented ongoing vaccine-like virus circulation or the possibility of introduction of a field virus extremely similar to the vaccine strain. In the other 10 herds, virus isolates were different from isolates found previously in the herd, but similar or identical to PRRSV isolates from other neighboring farms. These were encouraging from the standpoint of the success of the original eradication projects, but obviously disappointing in terms of our abilities to limit introduction of PRRSV into herds in areas of high hog density. These results do, however, provide some focus to the question of how the virus enters or re-enters negative herds.

In herds with no history of prior PRRSV infections, the concern is not whether the virus is the same as in previous cases, but centers on where the virus may have originated. As was observed in outbreaks following PRRSV eradication projects, the majority of the cases in negative herds we have observed have been found to involve viruses that were the same as viruses identified in neighboring herds.

## New or ongoing cases in PRRSV-positive herds and production systems

Interpretation of PRRSV sequence information in previously infected herds is a bigger challenge because of the ongoing mutation of the indigenous virus(es), and because of the potential for concurrent existence of multiple PRRSV sequences within a single herd and also subclinical introduction of new viruses. The potential for concurrent PRRSV infections was clearly illustrated in a revealing and somewhat alarming research paper reporting multiple virus sequences recovered from individual pigs (Chang et al., 2002). Despite the potential for concurrent infections, we typically observe a dominant strain that is recovered repeatedly, showing some degree of mutation or drift over time.

We have used several applications for sequence information in PRRS positive herds. One application is to follow the virus through a production flow, particularly where commingling of pigs from several sow herds is practiced. Recovering virus from growing pigs provides information regarding the relative activity of PRRSV in sow herds if different sequences are present in different sow herds. This can be used as a tool for determining appropriate commingling strategies. For example, if a defined group of sow herds develops clinical PRRSV outbreak (typically from a point source such as a gilt developer) involving a virus that is different from historical isolates from the downstream flow, weaned pigs from the affected sow herds are diverted from the common piglow to avoid adding novel PRRSV strains to the growing pig population.

Sequencing PRRSV though a production flow is also useful for revealing whether clinical disease associated with PRRSV in growing pigs is likely to have originated from the sow herd(s) or represents a lateral introduction at some point post-weaning. Finding a different virus in growing pigs stimulates a closer look for PRRSV activity in the sow herd to ensure that a new virus hasn't entered the herd undetected. A reasonable confirmation of a lateral introduction of PRRSV directs attention to sources of virus introduction other than the sow herd.

Another application is identifying whether or not a repeat episode of clinical PRRSV activity is associated with

previously identified isolates. This is significant in terms of understanding the effectiveness of herd PRRSV control strategies and biosecurity practices. We have used this approach extensively, and have found both old and new (mostly new) isolates associated with clinical episodes in positive herds. In some cases, unique clinical presentations have stemmed from apparent new introductions of virus into previously infected herds (Rossow et al., 1999). In other cases, typical clinical signs have resulted from the introduction of a novel strain

A final application of sequencing information in PRRSV-positive herds is tracking exposure to resident virus(es) for acclimation of breeding herd replacements when using either natural exposure or inoculation with live virus. Our reason for tracking the virus sequence in this situation stems from the belief that homologous exposure provides the best opportunity for protective immunity and the desire to avoid exposing the sow herd to a new virus. We want to ensure that replacement breeding stock are, first of all, exposed to the virus present in the herd, and that they are exposed to the specific virus that has been associated with clinical disease. In the case of natural exposure, there is concern that sequence mutations may arise through multiple passages through successive groups of pigs. These changes can be monitored through periodic sequence determination. In the case of virus inoculation, the sequencing is performed to ensure that the same virus used in the inoculum is recovered from the treated pigs.



## Summary

Using PRRSV sequence data within herds or production systems provides epidemiological information that can be used to monitor the effectiveness of control and biosecurity programs. The greatest benefit is gained from the aggregate of information that is accumulated over time through ongoing sequencing. At present, our scope is largely limited to observing and chronicling what happens in farms that experience clinical PRRSV infections. We do not have the ability to draw conclusions regarding the significance of changes in the virus genome in terms of antigenicity and virulence. Further advances in our understanding of PRRSV immunity will be needed to apply genomic sequence information for such purposes. As an industry we have to commit the resources necessary to address these questions in order to move beyond merely describing what happens in farms battling PRRSV.

## Glossary

### Trees
A mathematical structure used to model the actual evolutionary history of a group of sequences (Page and Holmes, 2000).

### Cladogram
Shows relative recency of common ancestory (Page and Holmes, 2000).

### Dendogram (ultrametric tree)
A type of additive tree or phylogram used to depict evolutionary time (Page and Holmes, 2000).

### Additive tree
Depicts the amount of evolutionary change that has occurred along different branches (Page and Holmes, 2000).

### Sequences
A list of bases comprising the section of virus analyzed. They are commonly compared to an average or consensus sequence (Page and Holmes, 2000).

### Homology charts
Tables of percent base similarities of virus sequences (Page and Holmes, 2000).

## Appendix A

### RFLP limitations
The initial need for a test capable of differentiating PRRSV isolates stemmed from the introduction of modified-live PRRSV vaccine, prompting the desire for a diagnostic test to distinguish vaccine virus from field virus. Serologic tests had been developed to distinguish North American from European strains, but no such test was or is available to determine whether pigs had been exposed to field virus versus vaccine. Restriction fragment length polymorphism (RFLP) was originally proposed in 1996 as a method to differentiate vaccine and field strains of PRRSV (Wesley et al., 1996). RFLP is defined as the fragment patterns produced by a specific restriction endonuclease on DNA from different individuals—viruses in this case. The fragment patterns from different individuals may be different owing to variations in DNA nucleotide sequences. Restriction endonuclease is defined as an enzyme that catalyzes the cleavage of a double-strand DNA at points with a specific nucleotide sequence. To characterize a sequence of genetic material with the RFLP technique, multiple enzymes reacting with different DNA sequences are used to divide the sequence into smaller pieces. If there are different nucleotide sequences in areas where the enzymes cut, different sized fragments are produced. The specific patterns of fragment sizes for

PRRSVs produced by the technique were assigned numbers, one number for each of the three lanes used for the analysis corresponding to the order in which the pattern was identified (e.g., 1-4-2, 2-5-2, etc.). The fragment sizes were then compared and used for differentiation of PRRSVs.

The section of PRRSV evaluated by RFLP was the region of the virus encoding the envelope (open reading frame 5 [ORF 5]). This portion of the genome can vary markedly among viruses, making it valuable for comparing and contrasting different isolates (Murtaugh, 1995). However, the RFLP technique ignores some differences existing within fragments that are not associated with the defined cleavage sites, leading to inappropriate conclusions on relatedness. For example, PRRSV ORF 5 sections interpreted as similar because of a cut pattern of 1-4-2 have actually varied by 10% or more when actual sequences of the virus are compared. Conversely, viruses with different cut patterns could be interpreted as different when in fact they are similar. For example, two sequences from different serum pools in one case submission had RFLP cut patterns of 2-1-2 and 1-5-2 indicating the viruses were different. Comparison of the ORF 5 sequences from these viruses revealed 99% similarity or homology. Thus, the RFLP technique can miss important genomic differences among viruses, or categorize dissimilar viruses together based on changes at a few key points.

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