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Use and interpretation of sequencing in PRRSV control programs

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Take-home messages

- PRRSV sequencing is a routine service of diagnostic labs serving the swine industry in Canada and the U.S.
- Comparison of sequences by alignment reveals the degree of similarity.
- Phylogenetic analysis (dendrograms or trees) with reference sequences shows relatedness to known virus families.
- Interpretation of similarities – are the viruses related or not – requires as much additional information as can be obtained since rates of genetic change can be highly variable.
- 97% or 98% sequence similarity is a useful guide indicating relatedness of two virus isolates, but genetic similarity is an unreliable predictor of immunological similarity indicative of cross-protective immunity.

Introduction

Diagnostic PRRSV sequencing began in the mid-1990's to aid producers and veterinarians in the control of PRRS. The best diagnostic method of PRRSV identification at the time was RFLP typing. This method was based on restriction enzyme cut sites in ORF5 that gave various patterns for each of three enzymes. It was most useful for differentiating the Ingelvac MLV (known then as ResPRRS and ResPRRS Repro) vaccine from field viruses, since Ingelvac MLV had a unique 2-5-2 pattern, and field viruses had other 3-digit identifiers. However, within a short period of time it became apparent that genetically unrelated viruses had the same cut pattern. For example, two common cut patterns were 1-4-2 and 1-4-4, but genetically different viruses appeared with the same cut patterns. Figure 1 shows a dendrogram from the 1990's in which genetically diverse viruses shared the same cut patterns. Also, viruses were being isolated from the field that were not 2-5-2 but by other criteria seemed to be related to Ingelvac MLV vaccine. Clearly, a better classification method was needed to understand what was happening in the field.

Diagnostic sequencing was introduced as an alternative to RFLP typing to improve the ability to differentiate PRRSV field isolates, identify vaccine reisolates, and accommodate the proliferation of vaccines (e.g. Suvaxyn, PRIMEPAC PRRS, Ingelvac ATP, Fostera PRRS) and live virus inoculums that could not be differentiated by RFLP typing from common field isolates.

ORF5, the gene encoding the major envelope glycoprotein, is the standard sequencing target because it is used for RFLP typing, and it shows extensive genetic diversity. It also was assumed to be an important target for immune protection, though its immunological significance is now uncertain.

The basic idea behind sequencing

Diagnostic PRRSV sequencing in the year 2012 is straightforward in the US and Canada. Serum or oral fluid samples with moderate to high amounts of PRRSV (Ct values of about 27 or less, depending on the diagnostic lab) are treated to isolate RNA, and submitted to an academic or commercial facility to sequence ORF5. This process generally takes one to three days. The raw data results are sent to the diagnostic lab, checked for quality, then processed and trimmed to identify the specific ORF5 region. The report typically includes sequence similarity to standard vaccine strains (e.g. Ingelvac MLV, Ingelvac ATP, Fostera PRRS, Suvaxyn), the deduced amino acid sequence, and the RFLP pattern. Some reports include a comparison to a standard reference panel of wild type PRRSV isolates, or comparison to a company or clinic PRRSV sequence database.

Analysis of PRRSV sequences

Sequence similarity, or identity, is determined by aligning two or more sequences using a computer program. An example alignment of four ORF5 sequences is shown in Figure 2. Three field virus sequences are quite similar to each other, varying only by 1.2 to 2.1 percent in pairwise comparisons (Table 1). Alternatively, they are 97.9% to 98.8% similar. A key question for producers and veterinarians is whether these differences represent normal variation in a population of viruses in a barn, or are consistent with independent viruses that are not closely related. In

Figure 1: RFLP patterns of genetically diverse PRRSV isolates. The dendrogram (black lines) contains 70 ORF5 sequences comprising 5 major PRRSV families. Forty-three (61%) of the sequences are in 6 RFLP types as indicated, including "untypeable." All types except for 252 are present in multiple families. Dashed lines are included to assist alignment of boxes with the dendrogram.

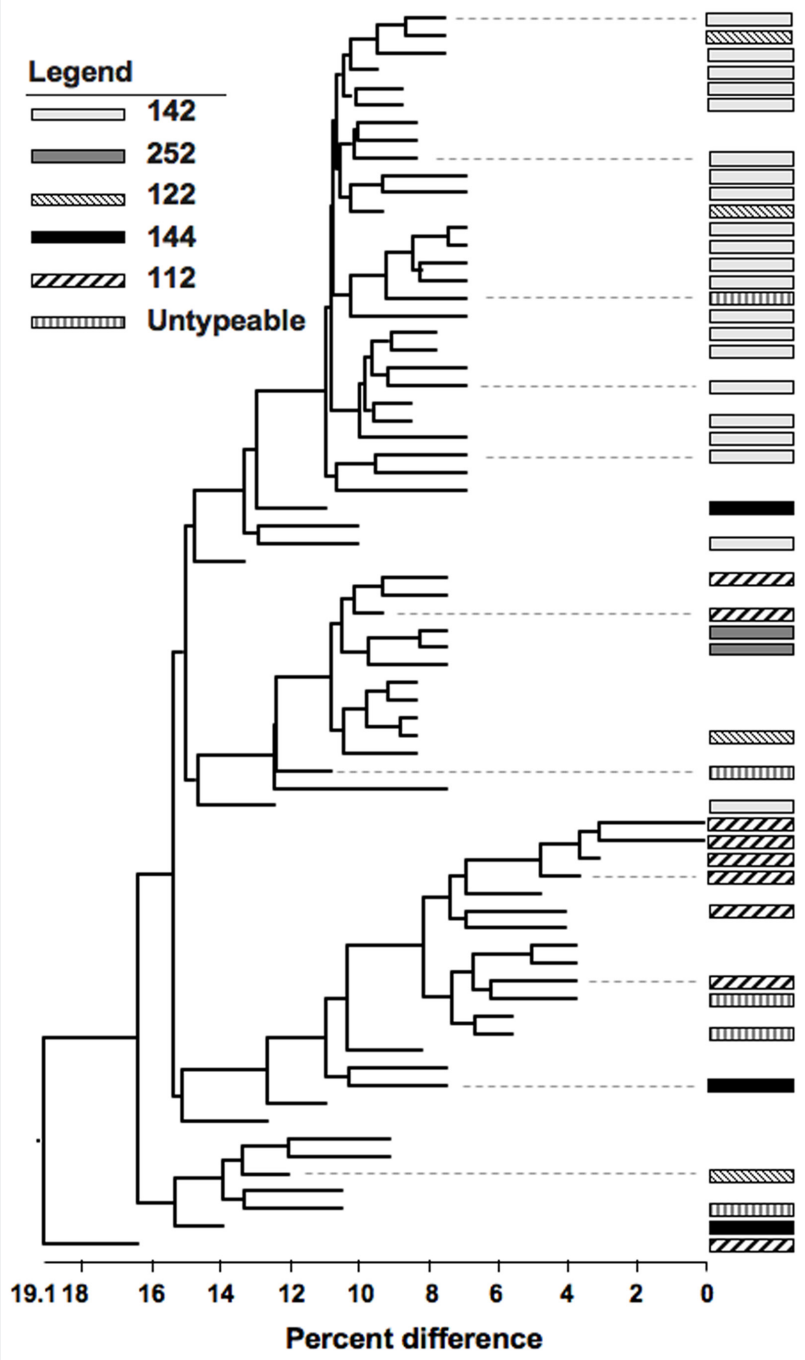


Figure 2: Base sequence alignment of 3 field isolates and Ingelvac MLV vaccine.

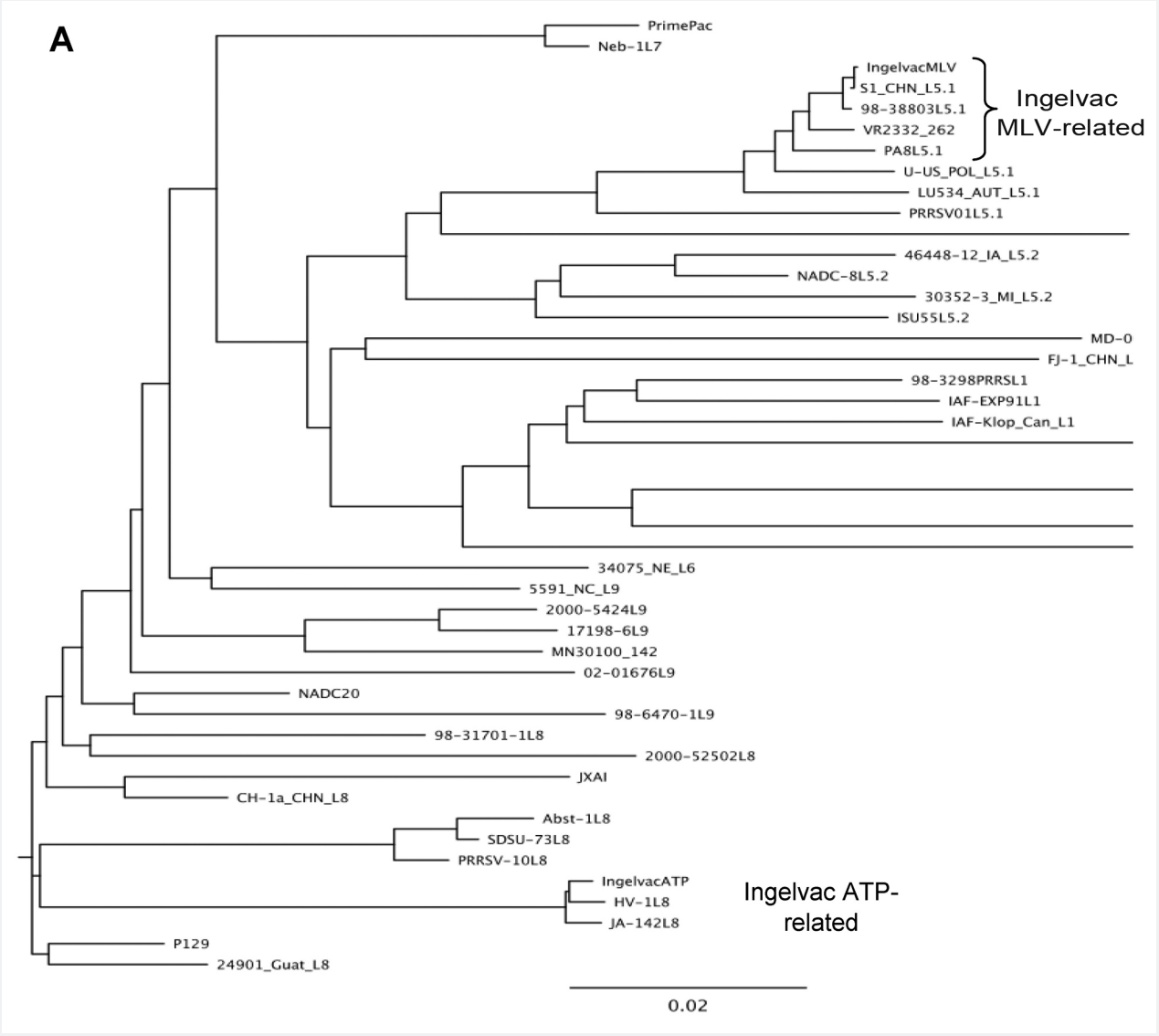
Table 1: Pairwise comparison table of PRRSV ORF5 sequences in Figure 2. Top diagonal is the pairwise percent sequence similarity. Bottom diagonal is the percent sequence difference.

Isolate	11-048146	12-018127	12-018776	Ingelvac MLV
11-048146	100	98.7	98.8	85.1
12-018127	1.3	100	97.9	85.3
12-018776	1.2	2.1	100	84.7
Ingelvac MLV	14.9	14.7	15.3	100

other words, are two viruses related or unrelated. This question is addressed below. The fourth isolate, Ingelvac MLV vaccine, is obviously different, specifically being 14.7 to 15.3% different, and showing base mutations from beginning to end (Figure 2).

Pairwise comparisons are widely used to make decisions about relatedness of PRRSV isolates, but they do not provide any genetic information about the virus type. Some PRRSV families, such as 1-8-4, 1-18-2, and 1-4-4 viruses, commonly show high virulence, airborne transmission, and a propensity to overcome pre-existing immunity. Genetic relatedness of isolates is determined from sequence alignments that contain known reference strains, and construction of dendrograms. Linear or radial dendrograms (Figure 3) show the same relationships in different ways that may be more easily digested by viewers. In our example, the three field isolates are revealed to be a closely related group that is an offshoot of the 1-18-2 family of viruses that appeared in the U.S. around 2008. Importantly, the quality of information in and resolving power of a dendrogram is only as good as the reference strains that are used. The dendrograms shown here include representatives of major PRRSV families, some of which have extensive genetic variation. The 1-8-4 family has tremendous genetic diversity. Thus, if only a few representative sequences are used, it is easy to mistakenly conclude that a new isolate is only distantly related and could thus be a new genotype. A second limitation in the example shown is the absence of European-type virus representatives. Their presence distorts the shape of a dendrogram because they are so difference, making it hard to discriminate variation among type 2 North American isolates. Identifying the presence of a type 1 European isolate is easily done by inspection of an alignment since there are obvious gaps with missing bases, and 50 to 60% of the expected bases are different.

Figure 3: Phylogenetic analysis of three PRRSV field strains. Dendrogram (A) and radial dendrogram (B) of field strains (gray outlines) in a set of 43 type 2 North American reference sequences. The Ingelvac MLV-related and Ingelvac ATP-related families are identified, in addition to the 1-8-4 family. Note that the two dendrograms are identical representations of the same phylogenetic analysis.



Sequence analysis computer programs also can translate the base sequence of ORF5 into the amino acid sequence of the envelope glycoprotein, GP5. In PRRSV, the amino acid sequence differences in proteins are often higher than the base differences in the genes. Analysis of protein sequence variation can provide insights into mechanisms that drive PRRSV evolution, and contribute to the biological properties of the virus. However, an enormous gap exists in PRRSV knowledge between gene sequence variation and biological differences in virulence, transmissibility, and cross-protective immunity. Since there is no solid evidence linking amino acid sequence of GP5 with specific biological traits, PRRSV sequence analysis should be based on genetic variation in the bases.

Interpretation of PRRSV sequences

The sequence of ORF5 is about 600 bases. While this amount is only 4% of the full-length PRRSV genome, it still contains about 40 time more resolving power to differentiate viral isolates than does RFLP, which is based on specific base patterns for three restriction enzyme cut sites in ORF5. With the hindsight of 18 years analyzing PRRSV ORF5 sequence variation, it is clear that the 600 base region of ORF5 is an exquisitely sensitive indicator of genetic variation and change in PRRSV. In one example in a commercial swine operation, Ingelvac MLV was administered to pigs, and reisolated over a period of 6 months. During this time, 20 ORF5 sequences were

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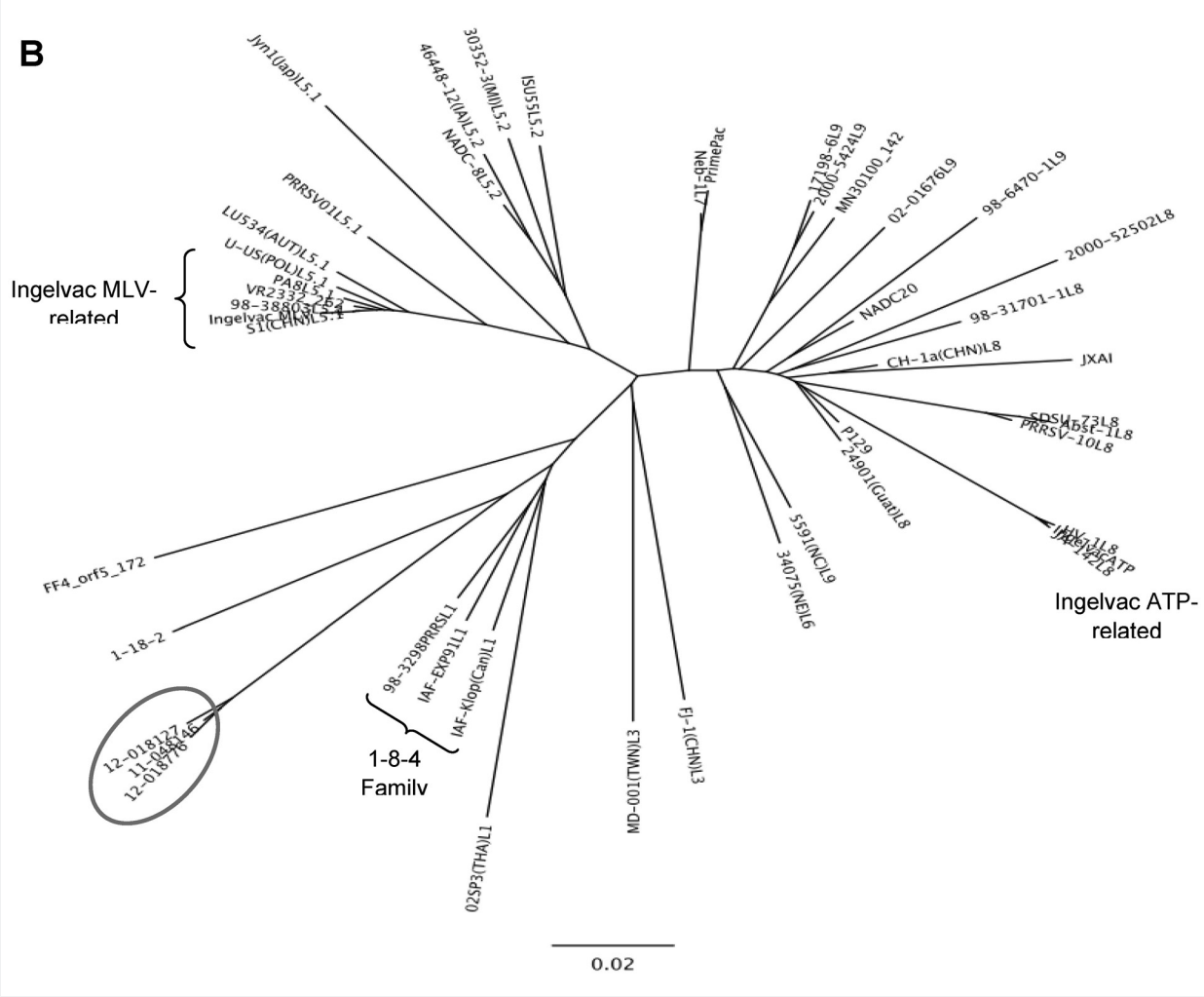

obtained, which varied between zero and 0.8% (i.e., zero to 5 base differences) from the initial vaccine sequence (Murtaugh and Harding, 2003). Additional isolates were obtained over a period of 7 years that showed increasing variation up to 3.8% difference from Ingelvac MLV. The overall rate of base change was 0.48% to 1.32% per year for all isolates in this study.

In an experimental study of PRRSV transmission carried out by sequentially infecting pigs over a one year period of time, base sequence differences varied by 0.7% from the initial inoculum (Chang et al., 2002). Taken together, the field experience and experimental study suggest that PRRSV changes at a rate of about 0.5% to 1% per year in the field. Other anecdotal observations are consistent with this estimated range. For example, ORF5 genetic diversity among type 2 PRRSV isolates collected from 1989 to 1993 was about 9%. At present it is about 25%, an increase of 16% in 19 years. It is important to remember that these figures are averages based on a limited set of observations, whereas many factors affect the rate of genetic change.

Genetic change is determined largely by mutations. The genome mutation rate is primarily a function of the fidelity of the polymerase enzyme that copies the genome. This protein is highly conserved and shows little genetic variation (Chang et al., 2002). Because it shows little variation, it will introduce mutations in all PRRSV isolates at about the same rate each time a genome is copied. Therefore, genetic variation will be influenced primarily by the number of times a virus replicates in a given period of time. PRRSV rate of growth and frequency of transmission are the primary determinants of genetic variation. As a result, variation in the rate of genetic change is determined

primarily by non-viral factors such as different levels of immunity. The level of specific anti-PRRSV immunity in pigs has a huge impact on viral growth and transmission, exerting a strong inhibitory pressure that diminished the number of genome copies. Lower viral loads result in reduced transmission rates, further reducing overall viral replication and decreasing the rate of change. Nonspecific innate immunity also exerts a substantial anti-viral effect that varies greatly among individual pigs. Age-dependent variation in PRRSV resistance is well documented, but even among pigs of the same age extensive variation in viral load occurs (Klinge et al., 2009). Likewise, transmission rate is highly dependent on viral load (Cho et al., 2007). The same virus under different host conditions, or viruses that grow at different rates in equivalent pigs, are expected to show different rates of genetic change. Thus, all of the biological factors that affect viral growth within pigs and transmission among pigs can result in higher or lower rates of genetic change outside of the suggested range of 0.5% to 1% per year.

Diagnostic issues further complicate the interpretation of PRRSV sequence information, especially the central question of whether two sequences are closely related or independent. Sporadic sampling within a barn or system may result in an incomplete picture of overall diversity in the population of indigenous viruses, and sporadic sampling over time may result in missing links that connect viruses that otherwise might appear to be unrelated. Given all of these uncertainties, it remains that PRRSV isolates are routinely determined as related or not based on a percent similarity cut-off of 97% or 98%. Unpublished data from several extensive monitoring projects suggest that these two cut-offs give equivalent results.

It is obvious that blind acceptance of a 2% or 3% genetic difference between isolates, without incorporation of additional knowledge, can lead to erroneous conclusions. The alignment example from Figure 2 shows three closely related viruses, but strict application of a 98% similarity cut-off would lead to an illogical conclusion. Likewise, in the case cited by Murtaugh and Harding, 2003, the isolate that is 3.8% different from Ingelvac MLV after 7 years is not a different virus. Intensive monitoring over time, knowledge of other PRRSV genotypes in the region, and heard health status information excludes the possibility of a field virus introduction.

Is 97% or 98% too stringent? The implementation of a 98% cut-off dates to 1998 and was based on some of the same data presented here (Collins, 1998; Murtaugh and Faaberg, 2001). A primary purpose at that time was to determine if field isolates were related to vaccines and it was generally assumed that the time frame was relatively short; i.e., one or two years. Hence, 98% was a useful cut-off. Later, when it became apparent that both vaccine-derived and field virus inoculation-based immune protection was variable, genetic relatedness was examined as a predictor of immune protection. Simply put, if homologous immune protection was good and heterologous immunity was not good, then what level of genetic relatedness defined homologous and heterologous? One hundred percent was too stringent since PRRSV sequence changes almost every time it grows in a pig (Chang et al., 2002). The 98% cut-off was generally used, or modified by ±1%, since it was already established and since no better alternative for prediction of immune protection, such as glycotype or MJ type, could be established (Murtaugh et al., 2010). Thus, 97% or 98% sequence similarity is a useful guide indicating relatedness of two virus isolates, but genetic similarity is an unreliable predictor of immunological similarity indicative of cross-protective immunity.

At this time, we have a limited understanding of PRRSV genetic variation within swine-growing regions. A comprehensive picture of viral variation at the beginning of control and elimination projects is critical for effective monitoring progress and effectiveness of implementation procedures, and for identification of new introductions to farms and to the region. Intensive sampling and sequencing is within reach due to technical improvements in viral RNA isolation, PCR amplification, and sequencing methods. Thorough sampling will eliminate substantial diagnostic uncertainty and provide better insights to biological factors that influence genetic variation.

Lastly, it is possible that ORF5 might not be the best portion of the PRRSV genome to evaluate genetic variation. The question is primarily academic at this point since there is so much genetic information for ORF5, and so little for every other region. Also, there is no evidence that any other fragment would be superior for assessing isolate relatedness. However, it is clear now that genetic variation in ORF5 neither predicts nor explains immunological protection or outbreaks in immune herds. The ongoing difficulty in obtaining reliable, durable immunological protection, using either attenuated vaccines or live virus inoculation, combined with the inability to identify predictors of success, is a major stumbling block in control of PRRSV. Assuming that some sequence feature in the PRRSV genome is associated with immune protection, whole genome sequencing has been initiated in various laboratories on a research basis to search for genetic markers. This work is in a developmental stage at this time.

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