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W. Christopher Scruton
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## Layout
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## Logo Design
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# Identification of genetically diverse sequences (ORF 5) of porcine reproductive and respiratory syndrome virus in a swine herd

SA Dee, M Torremorell, KD Rossow, S Otake, C Mahlum, and KS Faaberg

## Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is an economically significant pathogen of the global swine industry (1). PRRSV is a member of the genus Arterivirus of the family *Arterivirus* with a single stranded positive sense RNA nucleic acid capable of undergoing genetic change through the processes of mutation and recombination (2–4). A large number of genetically distinct PRRSV isolates have been identified across the United States, and differences in virulence among isolates have been demonstrated (3, 5–6). Traditionally, isolation of virus (VI) has been done in cell culture although this method requires the presence of viable virus and is labor-intensive (7). Recent advances in molecular diagnostics, such as polymerase chain reaction (PCR), allow for the detection of PRRSV nucleic acid, and samples of PRRSV nucleic acid can be characterized by molecular sequencing of the open reading frame (ORF) 5 region (8,9). Open reading frame 5, encoding the virus envelope protein, is a variable portion of the virus genome prone to mutation and is therefore useful for characterizing genetically distinct isolates of PRRSV (3,5).

Another method for the detection of PRRSV that is frequently encountered in research laboratories is swine bioassay (10–12). Swine bioassay is a very sensitive test, and was first used to determine whether infectious PRRSV was present in porcine tissue homogenates or samples of samples (10–12). The protocol for swine bioassay consists of inoculating PRRSV-negative pigs with the sample in question by the intramuscular, intraperitoneal or intranasal routes, followed by serial bleeding to assess whether antibodies to the PRRSV are detected.

Molecular diagnostic techniques such as PCR and sequencing have also enabled veterinarians to conduct epidemiological investigations of acute epizootics of PRRSV. A recent report characterized the molecular sequences of several PRRSV isolates collected during a series of acute cases of PRRS across 7 neighboring farms (13). Results indicated that the isolates recovered from each farm possessed a high degree of homology (96.75–100.00%) based on the sequence results of the ORF 5 structural protein. It was therefore hypothesized that this cluster of acute outbreaks represented area spread of PRRSV, despite an unidentifiable route of transmission.

Upon review of this report, a study was conducted to determine whether these techniques could be used to better understand the epidemiology PRRSV within a chronically infected farm with a history of recurrent reproductive failure secondary to PRRS. Dual infections of PRRSV have been demonstrated in experimentally infected newborn and 3-week-old pigs (14), therefore, the central question posed during this study was whether genetically diverse PRRSV isolates could co-exist in an infected farm at the population or the individual animal level. This paper consists of a case study that describes the detection and differentiation of the ORF 5 region of PRRSV isolates recovered over an 18-month period from a chronically infected farm.

## Materials and methods

### Case study

The case study will be presented in 3 sections: *farm history*, *period 1* (January–December 1999), and *period 2* (January–December 2000).

### Farm history

The case farm consisted of 1750 sows. It internally multiplied its own replacement females and received semen from an artificial insemination (AI) center. The case farm utilized 2-site production, and the nursery-finishing site was located approximately 1 km from the breeding herd, and 3.2 km from other farms in the system. The case farm was initially infected with PRRSV in October 1997. PRRSV infection was verified by specific clinical signs and the detection of PRRSV-antibodies (15,16). Attempts to isolate the virus were not successful. During the period of October 1997 to March 1998, the farm experienced an elevation in pre-weaning mortality (from 10% to 20%), sow mortality (from 10% to 16.5%), stillborn rate (from 8% to 12%), abortion rate (from 1.3% to 3.6%), and a reduction in the number of pigs weaned/week (from 800–1,000/week to 300–500/week). The use of killed autogenous PRRSV vaccines and attempts to expose replacement breeding stock to farm-specific PRRSV prior

to entry to the herd did not control the disease. Modified-live PRRSV vaccines were never employed.

**Period 1: January–December 1999**

Due to the inability to control PRRS, the decision was made to attempt PRRSV elimination from the case farm by controlled gilt entry and partial depopulation. Controlled gilt entry has successfully reduced the spread of PRRSV in 3 breeding herds (17). Therefore, the case farm was closed to any breeding stock introductions for a 9-month period from January–September 1999. Beginning in January 1999, 10 seronegative vasectomized boars were introduced to the breeding herd as sentinels, housed with pens of replacement gilts or weaned sows, and allowed to roam freely throughout the facility in an attempt to detect shedding of PRRSV. Monthly testing of sentinels for 5 consecutive months using the IDEXX ELISA test (IDEXX Laboratories, Westbrook, ME), indicated a seronegative status (sample-to-positive ratios <0.4). In contrast, seroconversion to PRRSV was detected post-weaning indicating active infection in the nusery and finisher; therefore, these facilities were depopulated to eliminate PRRSV in the weaned pig population in May 1999 (18).

From June–November 1999, an independent diagnostic investigation was conducted using animals from the case farm to determine whether PRRSV-infected animals were still present in the breeding herd (19). Sixty randomly selected adult swine (45 sows and 15 boars) were purchased (10 animals per month), necropsied, and approximately 15–17 tissue sites per animal were tested for the presence of PRRSV by Taqman™ PCR (Perkin-Elmer Applied Biosystems Foster City, CA), VI on MARC-145 cells and porcine alveolar macrophages, and immunohistochemistry (IHC) (7,20,21). Samples were collected from lymphoid tissues, male and female reproductive tracts, the central nervous system, the respiratory tract, as well as a wide range of other thoracic and abdominal viscera (19). In September 1999, infectious PRRSV was isolated from a sample of lateral retropharyngeal lymph node collected from an individual sow with an ELISA s/p ratio of 1.20 (19). The ORF 5 region of the virus, consisting of 610 nucleotides was sequenced, and determined to be dissimilar to 3 commercially available modified-live PRRSV vaccine standards, and therefore considered to be of field origin. For reasons of clarity throughout this report, this was designated as primary sequence *PRRSV-A*.

From January–June 1999, closure of the herd and the lack of replacement gilt introduction had reduced the breeding herd to 1,200 sows. To compensate for this change in inventory, a gilt-breeding project was initiated in the site 2 finishing facility following depopulation. Sources of animals for the breeding project consisted of 175 gilts from a PRRSV negative source, that had verified its status by a lack of clinical signs and 5 years of negative ELISA serology, and 400 gilts raised in the depopulated site 2 nursery-finisher facilities. During October and November 1999, the initial transfer of the bred and open gilts was initiated from site 2 to the site 1 isolation facility. All animals were tested by ELISA and determined to be negative (s/p ratio <0.4) prior to shipping.

During December 1999, ELISA antibodies were detected in site 2 replacement gilts, and the seroprevalence across groups ranging from 7–100%. Observable clinical signs were not detected. However, prior to testing, these gilts had been moved from site 2 to site 1, and testing of these animals at the site 1 facility indicated that all gilts were ELISA positive.

**Period 2: January–December 2000**

During January, clinical signs of third-trimester abortions, premature farrowings, and levels of stillborns (13.2%), mummies (4.4%), and pre-weaning mortality (14.5%) were observed in the case farm site 1 breeding herd. In February, PRRSV nucleic acid was detected by PCR in tissues from 3 clinically affected suckling piglets and 5 samples of 8- to 10-week-old nursery pig sera housed on the second site. Nucleic acid sequencing of ORF 5 revealed that the PRRSV RNA detected in the nursery pig sera was 99.7% homologous to the primary sequence of PRRSV-A. In contrast, analysis of samples from clinically affected suckling piglets revealed the presence of a distinctly different nucleic acid sequence that was only 94.2% homologous to PRRSV-A. Throughout this report, this nucleic acid sequence will be termed primary sequence *PRRSV-B*. During the months of April–December of period 2, follow-up diagnostic efforts were conducted to try to determine if sequences related to primary sequences PRRSV-A and -B would be detected in other stages of production, if a specific strain would predominate over time, or if other genetically diverse strains would be detected.

## Sampling and diagnostic methods

A series of visits to the case farm were made every other month during the period of April–December 2000. The following samples were collected during each visit:

**Set 1**

30 serum samples from 15 litters of suckling piglets (2 pigs/litter) randomly selected throughout the lactation period of 20 days. If clinically affected offspring were observed, they were humanely euthanized and tissues submitted for PCR and VI. These animals were defined as piglets originating from premature farrowings, those that were weak-born, or those displaying signs of dyspnea (rapid abdominal breathing) (15, 18).

**Set 2**

10 serum samples from clinical 8- to 10-week-old nursery pigs and 10 samples of clinical 11–14 week grow-finish pigs. In order for an animal to be classified as "clini-

cal" it had to exhibit all three of the following clinical signs: dyspnea, rough hair coat, and deteriorating condition (15,18). These animals were selected from the two rooms (five samples per room) that consisted of pens designated to house poor-doing animals collected from the eight nursery rooms and the designated two rooms that housed clinically affected pigs from the 16 grow-finish rooms. Grow-finish sampling was initiated in October.

### Set 3

10 serum samples from aclinical 8- to 10-week-old nursery pigs and 10 samples from 11–14 aclinical grow-finish pigs. These animals did not demonstrate the aforementioned clinical signs used to define clinical pigs. Samples from aclinical pigs were collected across the eight nursery rooms and 10 of the 16 grow-finish rooms. A minimum of 1 pig was sampled per nursery room, and 1 pig was selected from 10 randomly selected grow-finish rooms.

Suckling piglet sera were pooled (2:1) by litter, while the 10 samples of nursery pig sera were organized into 3 pools as follows: 3:1, 3:1, and 4:1. Samples were tested by Taqman PCR and VI on MARC-145 cells and porcine alveolar macrophages, and a subset of positive samples were submitted for nucleic acid sequencing (5,7,20). Automated sequencing reactions of the ORF 5 of the PRRSV were completed with Taq DyeDeoxy Terminator Cycle Sequencing Kit using a PE 2400 Thermocycler (Perkin Elmer Applied Biosystems Foster City, CA) at the University of Minnesota Advanced Genetic Analysis Center. Comparison of the PRRSV genomes and deduced ORF amino acid comparison was completed using computer software included in the LASERGENE package (DNASTAR Inc., Madison, WI) and Wisconsin Package Version 10.0 and (Genetics Computer Group (GCG), Madison, WI).

## Results: Bimonthly sampling

### April 2000

April PCR results indicated the presence of PRRSV nucleic acid in 1 of 15 serum pools collected from suckling piglets. This pool originated from 19-day-old piglets. Both piglets were determined to be aclinical, as were the remaining members of the litter and the dam. Nucleic acid sequencing indicated a 98.7% homology with the primary sequence PRRSV-A. One clinical piglet from another litter (dyspnea, rough hair coat, and deteriorating condition) was necropsied. PRRSV antigen was detected by IHC in lung samples; however, all samples collected from this animal were PCR and VI negative. PRRSV nucleic acid was also identified in 4 nursery pig serum pools, 2 pools from pigs classified as aclinical and the others from those previously classified as clinical (15,18). Sequencing of all 4 pools indicated that these samples possessed a high degree of homology (99.0–99.8%) to the primary sequence PRRSV-B.

### June 2000

June PCR results indicated the presence of another distinct PRRSV sequence that differed from both primary sequence PRRSV-A (91.3% homology), and primary sequence PRRSV-B (89.0% homology). This sequence was termed primary sequence *PRRSV-C*. This nucleic acid sequence was recovered from 2 separate serum pools collected from 10-week-old aclinical nursery pigs. All other serum pools were negative for PRRSV-A, -B, or -C by both PCR and VI.

### August 2000

All samples collected in August were negative by all tests described.

### October 2000

Three serum pools (2 from clinical pools and 1 from aclinical pools) from 10-week-old nursery pigs in October were positive by PCR. PRRSV nucleic acid from all 3 pools was sequenced, and results indicated a high degree of homology (99.3–99.5%) with primary sequence PRRSV-C. These sequences differed from primary sequence PRRSV-A (90.7–91.3% homology), and from primary sequence PRRSV-B (88.3–89.0% homology). No evidence of PRRSV-A or -B was detected in any samples. All grow-finish samples were PCR and VI negative.

### December 2000

Two pools of sera from aclinical 9-week-old nursery pigs, and 2 pools from clinical 9-week-old animals pigs were positive by PCR. PRRSV nucleic acid was also detected in 2 pools from 11-week-old clinical grow-finish pigs and 2 pools of aclinical 12-week-old pigs. Sequence analysis was conducted on one sample of pooled sera from clinical 9-week-old nursery pigs, and one sample of 11-week-old clinical grow-finish pigs. Nucleic acid possessing a 98.7% homology with primary sequence PRRSV-B was detected in the samples from the nursery, while nucleic acid from the finishing samples was found to be 98.8% homologous to primary sequence PRRSV-C.

## Discussion

The purpose of this paper was to report diagnostic findings from a case study involving a chronically infected swine farm that had experienced multiple outbreaks of PRRS, and to address the central question of whether genetically diverse strains of PRRSV co-existed within the case farm. Throughout the course of the study, the ORF 5 regions from a total of 16 of 25 positive samples for PRRSV nucleic acid were analyzed. Sequences appeared to cluster into 1 of 3 distinct groups (A, B and C). The heterology across the primary sequences from each group

ranged from 5.8% to 11.0%, and from 10% as compared to the VR-2332 prototype. Analysis of sequence data from the primary sequences of the 3 groups indicated no obvious evidence of recombination or single strain PRRSV evolution, therefore the conclusion is that the 3 primary sequences appeared to be 3 distinctly different viruses. This conclusion was based on examination of nucleotide sequence data, and supported by analysis of protein sequence data. In contrast, the percent homology of sequences within each group ranged from 98.7–99.8%. However, these assumptions are based solely on the evaluation of only one open reading frame (ORF 5), and not the entire viral genome. This region is known to be highly variable, and we may have obtained different results if a more conserved region, such as ORF 6, had been evaluated. Furthermore, owing to cost, not all PRRSV-positive samples were sequenced, therefore, it is possible that more than 3 strains were present.

It does not appear that a single strain became predominant during the time the case study lasted. However, sequences similar to primary sequence PRRSV-A have not been detected since April 2000, while sequences similar to B and C appeared to be circulating in the nursery and finisher at the time the study was completed.

At this time, no conclusions can be drawn regarding the frequency of multiple PRRSV within other farms, or the impact that the co-existence of genetically diverse isolates could have on the control of the disease. This study consisted of only 1 farm, and it is not known if this situation is representative of other swine operations in the United States. Because it was a field case, proper controls were not available. Therefore, further testing across a larger number of farms, as well as an evaluation of other regions of the virus is necessary before any final conclusions can be drawn. If proven to be a common occurrence in the industry, the ability to consistently acclimatize replacement breeding stock to multiple strains of PRRSV could be very difficult to accomplish, and may result in subpopulations of animals that are naïve to one or more strains. In the case farm, it is impossible to predict which strain(s) will be transmitted to the breeding herd following the introduction of internally raised replacement gilts. Finally, should the presence of multiple strains result in ineffective control of PRRS and significant economic losses, eradication may be the most cost-effective strategy long-term. How the presence of multiple strains may affect the outcome of the PRRS eradication programs is unknown at this time.

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