RESEARCH REPORT
SWINE HEALTH

**Title:** Sequencing, Cloning and Characterization of a 2007 Vietnam PRRSV isolate - **NPB #08-181**

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**Industry Summary:** Epidemics of severe swine disease, termed "Porcine High Fever Disease (PHFD)", continue to be reported in Southeast Asia, and a specific strain of porcine reproductive and respiratory syndrome virus (PRRSV) has been identified as the primary pathogen in these epidemics. This viral strain has not been identified in the United States, nor has PRRS disease been associated with such severe symptoms as reported in China, Vietnam, and other neighboring countries. The objective of this proposal was to study a Vietnam PRRSV isolate recovered from the epidemic of PHFD by primary genetic analysis of the virus, development of a reverse genetic system, and analysis of the recombinant virus in cell culture. Unfortunately, the transfer of the genetic material of the virus from our collaborators was severely delayed, such that only part of the proposed research has been completed. However, we have determined that the isolate, named SRV-07, aligns with other PHFD PRRSV isolates from other Asian countries, that these isolates still have not been detected in the United States. We have developed the tools to ensure correct diagnoses if/when similar isolates may appear in our country. The insights will benefit the US pork industry by having advanced detection tools for prompt diagnosis, and possible new virulence attributes of the Southeast Asian PRRSV isolate.

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Scientific Abstract: The genetic material from PRRSV Type 2 sampled in Vietnam (isolate SRV-07) during the outbreak of porcine high fever disease (PHFD) in SE Asia was isolated and forwarded to our Agricultural Research Laboratory, although it was severely delayed. As a result, the findings are incomplete. Only limited SRV-07 sequence has been obtained - consisting of the 5'-end, partial nonstructural protein 2 (nsp2) covering the deletion seen in other PHFD strains, partial nsp7-8, partial ORF3 and all of ORF5 - but the genetic analysis to date has shown high similarity to other Asian PHFD isolates. The genetic material was amplified for derivation of an infectious clone, and we have successfully generated 10.4 kb of the genome in 3 overlapping products. We prepared a subclone covering ORF4-3'end for in vitro generation of RNA to be analyzed in our laboratory and US diagnostic laboratories. Our findings reveal that SRV-07 has a RFLP cut pattern of 1-8-4, but is only 87.4% identical to MN184 isolates in the ORF5 region and has still not been detected in the US, as of February 2010. We prepared primers and probe for Real-Time analysis of SRV-07, and showed that optimal primers could successfully amplify SRV-07 genetic material. Remaining work to be accomplished will include the production of the infectious clone, full genotypic and phenotypic analysis, and providing verification that the prevalent US PRRSV diagnostic laboratories are able to detect similar strains if they appear.

**Introduction:** In 2006, new PRRSV isolates appeared in SE Asia, predominantly China, that were associated with high fever and swine mortality. In 2007, the National Veterinary Services Laboratory at Plum Island Animal Disease Center was requested to analyze swine samples from Vietnam. In a multi-personnel collaboration, the PRRSV isolate was shown to be similar in the nsp2 and ORF5 regions to North American (Type 2) strains that had been simultaneously isolated in China and for which severe symptoms were reported. In order to understand its potential disease severity in US swine herds, we first sought to determine the full-length sequence of the Vietnamese isolate, named SRV-07, and compare its genetic footprint to those of other North American and European isolates. We also proposed to develop a reverse genetic system for this strain of PRRSV. Our first goal, however, was to develop the genetic tools needed to ensure the existing molecular diagnostic tests are fully able to detect similar strains and to characterize the SRV-07 strain for in vitro phenotype.

**Objectives:**

**Objective 1. Genetic analysis of 2007 Vietnam PRRSV isolate.**
We will determine the complete nucleotide sequence of a Vietnam PHFD PRRSV isolate and compare it with other PRRSV isolates from the US as well as from around the world.

**Objective 2. Development of an infectious clone.**
We will produce a reverse genetic system to the Vietnam strain in order to evaluate PRRSV associated with PHFD in the absence of other pathogens.

**Objective 3. Phenotypic characterization of the Vietnam isolate.**
We will assess the ability of current US diagnostic tests available for PRRSV to accurately discern this foreign genotype if/when it appears.

**Materials & Methods:**

**Objective 1. Genetic analysis of 2007 Vietnam PRRSV isolate.**

A. **Viral Growth and Genomic RNA extraction.** The Vietnamese PRRSV isolate was amplified by passage on MARC-145 cells and/or porcine alveolar macrophages (PAM) by APHIS DVL. The medium containing the virus and the remaining infected cells was frozen at -80°C, thawed to lyse

all cells, and then spun at 1800 rpm for 10 minutes to pellet the cell debris. For infectious clone preparation, 10 ml clarified cell supernatant will be placed in each of 3 silica-based columns included in the QIAamp DNA Blood Maxi Kit. After following kit manufacturer's procedure, the viral RNA was then eluted from each column with 0.5 ml RNase-free water. The concentrated viral RNA elutions were combined and then distributed in 0.2 ml aliquots and stored at -80°C.

B. **Full-Genome Sequence Analysis.** The full genome of SRV-07 has not been completed. We have amplification of 10.4 kb in 3 separate overlapping RT-PCR products. The standard Sanger sequencing method was used to sequence small regions of SRV-07.

**Objective 2. Development of an infectious clone.**

A. **Cloning Strategy.** Purified RNA will be amplified using the SuperScript III One-Step RT-PCR Platinum Taq High Fidelity Kit (Invitrogen) and derived PRRSV primers. RT-PCR products will be purified with QiaQuick kit (Qiagen) and plasmids will be transformed into *E. coli* using TOPO® XL PCR Cloning Kit (pCR®-XL-TOPO® vector; Invitrogen). After amplification, plasmids will then be purified using the PureLink™ HiPure Plasmid DNA Purification Kit (Invitrogen), followed by DNA sequencing. QuikChange® Multi Site-Directed Mutagenesis Kit (Stratagene) will be used to modify all cDNA subclones to correct aberrant nucleotides. The correct nucleotides will be confirmed by complete DNA sequencing. The full-length infectious clone will be prepared by ligation of subclones. This in turn will be placed into a modified pOK12 vector and amplified, purified, and quantified.

B. **In vitro transcription, transfection and recombinant virus rescue.** The full-length cDNA clone (pSRV-07) will be linearized with PacI and capped RNA transcripts will be synthesized using the mMessage Machine Kit (Applied Biosystems). Confluent MA-104 cells will be separately transfected with 2.5 μg of the rSRV RNA transcript using DMRIE-C (Invitrogen). Cytopathic effect (CPE) may appear within 2-4 days. The presence of PRRSV N protein will be detected at day 2 postinfection (pi) by indirect immunofluorescence assay (IFA) using monoclonal antibody SDOW17 (gift of Eric A. Nelson). The transfection supernatants will be collected when CPE appears, and the rescued virus (recombinant Soviet Republic of Vietnam virus; rSRV) will be harvested and aliquotted. An aliquot of rSRV will be serially passaged three times. The total RNA will be extracted from passage 3 supernatant and analyzed by RT-PCR followed by sequencing. Titers will be determined on MA-104 cells by plaque forming units (PFU)/ml and by TCID50/ml to verify diagnostic capabilities and assess in vitro growth properties.

**Objective 3. Phenotypic characterization of the Vietnam isolate.**

A. **Sensitivity to Routine Diagnostic Tests.**

1. Specific primers and probe (results section) were developed to conduct primary diagnosis. rSRV, rVR-2332 and rMN184 will be assayed in parallel. The RNA will be extracted from the cell supernatant. After quantification, 10-fold dilutions of an equal amount of input RNA will be tested. NADC Real-Time RT-PCR, Tetracore PRRS Real-Time RT-PCR, and AgPath-ID™ NA and EU PRRSV Multiplex RT-PCR by Ambion will also be used to assess our ability to diagnose the SRV-07 strain, as these three tests include lab-specific and commercially available assays. Results will be summarized and compared to obtain the relative diagnostic sensitivity of rSRV to other routinely submitted PRRSV.
2. The ORF5 region of the SRV-07 genome was amplified with specific primers listed in the results. Also, ORF4-3'end were amplified and in vitro transcribed. The resulting RNA will be submitted for sequencing to Iowa State, South Dakota State and University of Minnesota Veterinary Diagnostic Laboratories

**B. Growth Curves.** Knowledge of the in vitro growth behavior of the Vietnamese isolates may be informative about how the isolate replicates in swine. rSRV, VR-2332 and MN184 viruses will be titered on MARC-145 cells. An aliquot, representing 0.1 multiplicity of infection (m.o.i.)/cell, will be used to infect fresh individual MARC-145 cell monolayers in 25 cm3 flasks in parallel. After 1 h attachment at room temperature, the inocula will be removed and the monolayers will be washed three times. After washing, 7 ml complete medium will be added and the flasks will then incubated for up to 5 days at 37°C, 5% CO2. Samples (500 ml) will be collected from infected media at different hours post-infection (hpi: typically 6, 12, 36, 48, 72) and titrated by viral plaque assay.

**C. Plaque Analysis.** Equal amounts of PRRSV-infected cell culture supernatants will be diluted $10^3$-$10^6$ fold in EMEM without FBS. Diluted virus (50 ml) will then be added to duplicate wells of a 12-well-plate that contains 90% confluent MARC-145 cell monolayers, followed by room temperature (RT) incubation for 1 h with occasional redistribution of the inoculum. An equal mixture (5 ml total) of 2X EMEM medium and 2% SeaPlaque LE agarose (FMC), prewarmed at 56°C, will then be added to each well and left at RT for 15 min to solidify the agarose overlay. The cells will then be transferred for incubation at 37°C in 5% CO2 for 96 h. Plaques will be stained with crystal violet for permanent record and to reveal their size and number. The results will be recorded as the number of plaque forming units per milliliter of inoculum (PFU/ml). The morphology of representative plaques will be recorded pictorially.

**D. Northern Analysis.** Total intracellular RNA from transfected/infected cells or in vitro transcribed/purified RNA will be denatured with glyoxyl and electrophoresced through a 0.8% agarose gel, transferred to nylon membranes (MagnaGraph, GE Osmonics) and crosslinked to the membrane. The membrane will be probed with ologomer complementary to PRRSV ORF7.

**Results:**

**Objective 1. Genetic analysis of 2007 Vietnam PRRSV isolate (SRV-07).**

**E. Viral Growth and Genomic RNA extraction.** Completed as anticipated, approximately 9 months prior to transfer.

**F. Full-Genome Sequence Analysis.**

1. The viral RNA of SRV-07 was not transferred to the Faaberg laboratory from APHIS laboratories until March of 2010. As a result, much of the research plan could not be completed using the SRV-07 as originally proposed. The SRV-07 RNA received was not infectious when transfected into Marc-145 cells. This suggests that the viral RNA was degraded during processing or incorrectly stored prior to transfer.
2. We opted to perform traditional sequence analysis, as 454 sequence analysis is much too expensive to perform on only one PRRSV strain. However, in another study, we optimized and sequenced 20 PRRSV strains in tandem (manuscript in preparation).
   i. RT-PCR amplification of SRV PRRSV RNA for sequencing: 8 pairs of primers were designed to amplify 4 overlapping regions based on the JXwn06 genome.
   ii. Three regions were amplified with a total of 10.4 kb. One PCR product has yielded a small segment of nsp7-8 (1054 bases; 99.6% identical to JXwn06), but the sequence was directly followed by 106 bases that aligned to nucleotides in ORF3 (99.1% identical to JXwn06). We will investigate this result by sequence analysis of other similar clones. The remaining full-length sequence has not been determined.
   iii. 5'-RACE was completed, and showed that the initial 251 bases are 98.4% identical to Chinese PHFD PRRSV isolate JXwn06.

iv. 2008 work had shown that a 474 base portion of nonstructural protein 2 (nsp2) was 98.5% identical to isolate JXwn06, and had the same ORF1 nonstructural protein 2 deletions noted in the PHFD isolates. Also, the prior work had shown that ORF5 (603 bases) was 99.2% identical to isolate JXwn06.

SRV-07, as sequenced to date, conforms to the genome structure and nucleotide identity of other PHFD isolates from China. However, there are several nucleotide polymorphisms and much of the nucleotide sequence remains to be analyzed. As a result, we cannot perform complete analysis of the genome with other North American and European isolates at this time.

**Objective 2. Development of an infectious clone.**

**A. Cloning Strategy.** We plan to prepare an infectious clone of SRV-07, as described in the original proposal as future time permits.

**B. *In vitro* Transcription, Transfection and Recombinant Virus Rescue.** The procedures are well established and will be completed when the full-length genome of SRV-07 has been generated.

**C. rJXwn06:** In light of the extended delay and to derive information on a similar PHFD strain, we acquired the infectious cDNA clone of isolate JXwn06 (rJXwn06; EF641008) from Dr. Hanchun Yang of China Agricultural University in Beijing, China, after proper permitting. The rescue of rJXwn06 from the cloned plasmid was as follows:

1. The DNA coding for rJXwon06 was sent to the laboratory on filter paper. The spot including the DNA was excised and rinsed in Tris-EDTA buffer in a microcentrifuge tube to elute the DNA. The filter was removed by centrifugation and the DNA supernant was stored at -80°C.
2. 0.2 µl of the eluted DNA was transformed into DH1 competent cells. Four colonies were selected, colony purified, and grown out in LB containing 100 µg/ml of ampicillin. The four clones were named: pHCY-1, pHCY-2, pHCY-3, pHCY-4.
3. Extraction of plasmids from the four clone by Qiagen miniprep showed that all formed a single band higher than 10KB on 0.8% agarose gel electrophoresis. pHCY-1 was chosen for large scale preparation.
4. In vitro transcription was completed using the mMESSAGE mMACHINE® SP6 Kit (Ambion). The synthetic RNA was purified for transfection into Marc-145 cells.
5. Transfection of rJXwn06 resulted in CPE after 3-4 days. The cell culture was then frozen and thawed to release cell-associoated virions and the derived supernatant was used to infect fresh Marc-145 cells at a 1:10 dilution for a total 4 passages.
6. Marc-145 cells infected with rJXwn06, 22 hrs pi, stained with SDOW-17-FITC (Figure 1).
7. rJXwn06 titration:

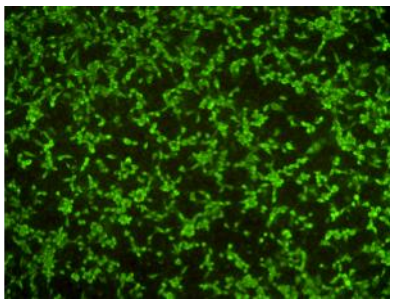


Figure 1

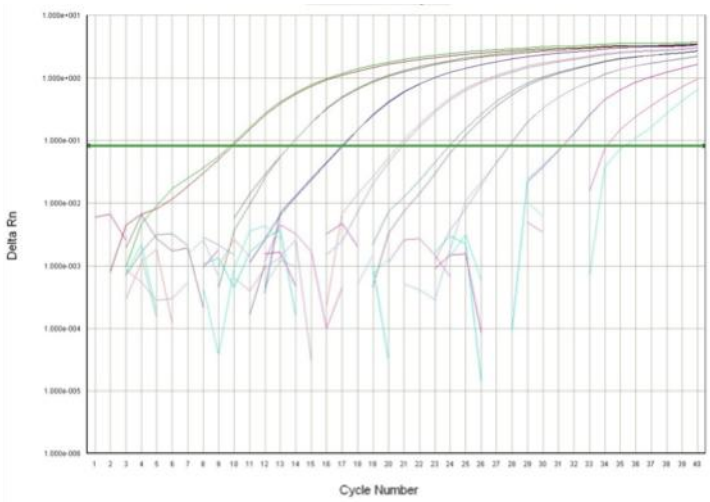
| Passage Number | Onset of CPE (Hours) | $TCID_{50}$/ml |
|---|---|---|
| 1 | 80 | $3.16 \times 10^5$ |
| 2 | 70 | $3.16 \times 10^6$ |
| 3 | 60 | $1.00 \times 10^7$ |
| 4 | 66 | $3.16 \times 10^6$ |

**Objective 3. Phenotypic characterization of the Vietnam isolate.**

**A. Sensitivity to Routine Diagnostic Tests.**

1. NADC Real-Time RT-PCR, Tetracore PRRS Real-Time RT-PCR, and AgPath-ID™ NA and EU PRRSV Multiplex RT-PCR by Ambion
   i. Synthesis of SRV-07 PRRSV ORF4-3'end transcript
      1. A pair of primers covering the ORF4, 5, 6, 7 and 3´-UTR region of JXwn06 were synthesized. The T7 promoter (red) was incorporated into the forward primer. The primer sequences are as follows:
         SRV13193F: 5'-AAATTCAAGTTAATACGACTCACTATAGGGTTCGTGGTTTCTCAGGCGTT
         SRV3'endR: 5'-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTAATTACGGCCGCATGGTTCT
      2. RT-PCR amplification (product size 2182 bp) using SRV-07 PRRSV RNA was done with a one-step RT-PCT kit (Invitrogen)
      3. In vitro transcription from the amplified RT-PCR product was successful (Promaga RiboMax).
      4. Realtime RT- PCR was performed with a pair of primers and probe designed to amplify both traditional North American Type 2 strains as well as the newly emerged PHFD isolates in Asia for evaluation of the ORF4, 5, 6, 7 and 3´-UTR transcript. They are: Forward primer: 5'- GATGCCGTTTGTGCTTGCTA; Reverse primer: 5--AGCCGGGACGCCGGACG; Probe: 5- FAM-CAAGTACATTCTGGCCCCTGCCCACCACGTTG-BHQ. Figure 2 shows the amplification output of 8 serial dilutions of the transcript from 317 ng (10-1 dilution) to 31.7 fg (10-8 dilution).

Figure 2




      5. Realtime RT-PCR also included VR2332 viral RNA serial dilutions. As shown in the table below, the SRV-07 transcript has a lower CT value than VR2332 for the same amount of RNA. This data showed the RNA transcript can be used to evaluate the US real-time protocols.

ii. NADC

RT-PCR, PRRSV Ambion

2. Ease of ORF5

i. We that Asian

| Sample Name | Avg CT |
|---|---|
| SRV transcript 317 ng (10^-1) | 9.8 |
| SRV transcript 31.7 ng (10^-2) | 13.6 |
| SRV transcript 3.17 ng (10^-3) | 17.0 |
| SRV transcript 0.317 ng (10^-4) | 20.7 |
| SRV transcript 31.7 pg (10^-5) | 24.2 |
| SRV transcript 3.17 pg (10^-6) | 27.8 |
| SRV transcript 0.317 pg (10^-7) | 31.2 |
| SRV transcript 31.7 fg (10^-8) | 34.7 |
| VR2332 25 ng (10^-1) | 23.5 |
| VR2332 2.5 ng (10^-2) | 27.4 |
| VR2332 0.25 ng (10^-3) | 30.5 |
| VR2332 0.025 pg (10^-4) | 34.3 |
| VR2332 2.5 pg (10^-5) | 37.4 |
| VR2332 0.25 pg (10^-6) | Undetermined |
| VR2332 0.025 pg (10^-7) | Undetermined |
| VR2332 2.55 fg (10^-8) | Undetermined |
| NTC | Undetermined |

Real-Time RT-PCR, Tetracore PRRS Real-Time and AgPath-ID™ NA and EU Multiplex RT-PCR by have yet to be analyzed. detection of rSRV by routine sequencing primers first established primers would easily amplify the PHFD isolates.

1. ORF5-Fwd1 (5'-CTCCTYATGCTTTCYTCTTGC)/ORF5-Rev1 (5'-ACCCGAAGGTAAAAGCACARGT) - nt. 13436-14438 = 1003 bp
2. ORF5-Fwd2 (5'-TGCTTCYGAGATGAGTGAAA)/ORF5-Rev2 (CAAAAGGTGCAGAAGCCCTA) - nt. 13465-14428 = 964 bp

ii. The RFLP cut pattern reveals the SRV-07 PHFD isolate to be 1-8-4. However, the identity with previously isolate MN184 is only 87.4% on a nucleotide basis, showing that RFLP cut-patterns are not a reliable means of categorizing PRRSV isolates. The translated protein, GP5, is predicted to have 5 N-glycosylated residues on its ectodomain.

iii. SRV-07 transcript described above will be forwarded to Iowa State, South Dakota State University and University of Minnesota Veterinary Diagnostic Laboratories to confirm that the genetic material can be sequenced by the major US laboratories.

**B. Growth Curves.** Not yet completed, waiting for SRV-07 infectious clone to be prepared.

**C. Plaque Analysis.** We performed an initial plaque analysis of Asian PHFV using rJXwn06 (Figure 3). The plaque morphology is similar to other Type 2 strains whereas MN184 has been shown to possess an unusual plaque morphology. However, the size of the SRV-07 plaques is similar to those of MN184.

**D. Northern Analysis.** Not yet completed, waiting for SRV-07 infectious clone to be prepared.

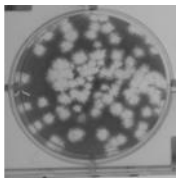


Figure 3

**Discussion:** The research to date has been primarily focused on deriving genetic tools to ensure US PRRSV surveillance methods are in place, in case Asian PHFD strains similar to SRV-07 arise in our country. To this end, we have derived an RT-PCR product that encompasses ORF4-3'end of the SRV-07 isolate. This RT-PCR product was in vitro transcribed to RNA, and will soon be submitted to various diagnostic laboratories to test the capabilities of those laboratories to identify PRRSV by RealTime RT-PCR techniques and to obtain the sequence for ORF5. Using primers developed especially for SRV-07, we revealed that the RFLP cut pattern is 1-8-4, but does NOT correspond to existing 1-8-4 strains presently

circulating in the US. Rather, sequenced US strains (MN184) show only a distant identity (87.4% on the nucleotide level) to the Asian PHFD isolates. The translated protein, GP5, is predicted to have 5 N-glycosylation sites on its ectodomain. As of February of 2010, we also confirmed that Asian PHFD strains are not yet circulating in the US. In addition, we have shown that SRV-07 appears to be very similar to the other Asian PHFD strains, but as only a small portion of the genome has been sequenced, we are hesitant to confirm SRV-07 as a PHFD strain in its entirety. Preparation of the infectious clone has proceeded to the point that we have amplified 10.4 kb in 3 overlapping RT-PCR products, we have determined the 5'end and have prepared additional primers to amplify the remaining sequence. Because the transfer of SRV-07 RNA was delayed, we acquired the infectious clone of Chinese PHFD strain JXwn06 from Dr. Hanchun Yang with the proper permit in place. We have successfully derived virus from this cDNA clone and can now expand our proposed research to test the genotypic and phenotypic characteristics of more than one PHFD strain. The results of this study will directly benefit pork producers by the assurance that PHFD strains have not been detected in the US, and that the veterinary diagnostic laboratories are nonetheless capable of correctly identifying similar strains, should they appear. Furthermore, we will better understand the cell culture phenotype, which can be used to predict its phenotype in swine. Finally, we now have the capability and permission to study the actions of rSRV-07 in swine under BSL3 conditions.