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# Large scale parallel pyrosequencing technology: PRRSV strain VR-2332 nsp2 deletion mutant stability in swine☆

Baoqing Guo[a], Ann C. Vorwald[b], David P. Alt[c], Kelly M. Lager[b], Darrell O. Bayles[c], Kay S. Faaberg[b,*]

[a] Veterinary Diagnostic & Production Animal Medicine, Iowa State University, Ames, IA, USA
[b] Virus and Prion Research Unit, National Animal Disease Center, USDA, Agricultural Research Service, Ames, IA 50010, USA
[c] Infectious Bacterial Diseases Research Unit, National Animal Disease Center, USDA, Agricultural Research Service, Ames, IA 50010, USA

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ABSTRACT

Fifteen porcine reproductive and respiratory syndrome virus (PRRSV) isolate genomes were derived simultaneously using 454 pyrosequencing technology. The viral isolates sequenced were from a recent swine study, in which engineered Type 2 prototype PRRSV strain VR-2332 mutants, with 87, 184, 200, and 403 amino acid deletions in the second hypervariable region of nsp2, were found to be stable in the nsp2 coding region after in vivo infection (Faaberg et al., 2010). Furthermore, 3 of 4 mutants achieved replication kinetics similar to *wt* virus by study end. We hypothesized that other mutations elsewhere in the virus may have contributed to their replication fitness in swine. To further assess the stability of the engineered viruses, all sequenced genomes were compared and contrasted. No specific mutations occurred in all nsp2 deletion mutant genomes that were not also seen in the parent genome of Type 2 PRRSV strain VR-2332. Second site (non-nsp2) deletions and/or insertions were not evident after replication in swine. The number of point mutations seen increased slightly with deletion size, but even the largest deletion (403 aa) had very few consensus mutations. Thus, our findings provide further substantiation that the nsp2 deletion mutant genomes were genetically stable after in vivo passage.

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## 1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) belongs to the family *Arteriviridae* of the order *Nidovirales* (Cavanagh, 1997). The single-stranded, positive-sense RNA virus genome varies in length from 15 to 15.5 kilobases, and consists of a 5′ untranslated region (UTR) immediately followed by a long open reading frame (ORF1) that codes for the replicase polyprotein (1a and 1ab), followed by several overlapping ORFs coding for structural proteins, and a 3′ UTR. The viral replicase polyprotein is processed cotranslationally into at least 14 proteins by self-encoded papain-like cysteine proteases [PLP1α and 1β in nonstructural protein (nsp) 1, and PLP2 in nsp2] and a serine protease in nsp4. PLP2 (also known as PL2 or cysteine protease) belongs to the superfamily of proteases homologous to the *Ovarian tumor* (OTU) gene product of *Drosophila* (Frias-Staheli et al., 2007; Makarova et al., 2000; Sun et al., 2010). Consistent with other viral OTU homologs, the PLP2 protease of Type 1 PRRSV has been shown to possess deubiquitinating activity in vitro (Frias-Staheli et al., 2007; Makarova et al., 2000; Sun et al., 2010). Nsp2 harbors the PLP2 domain near the N-terminal end, flanked by two regions shown to be hypervariable among PRRSV strains (Han et al., 2006), followed by 3–4 transmembrane domains and by a relatively conserved C-terminal region. The variation in PRRSV strain genome size has been shown to be largely due to deletions or insertions in the second hypervariable (HV2) domain of nsp2 (Han et al., 2006). Several engineered genomes have used nsp2 deletions and/or insertions in this same region to investigate viral protein function and produce marker vaccines (Chen et al., 2010; Fang et al., 2008; Kim et al., 2007, 2009; Zhou et al., 2009). Our laboratory has produced several nsp2 deletion constructs in the prototype Type 2 strain VR-2332, and has inserted foreign tags to investigate the actions of the PLP2 protease in vitro (Han et al., 2007, 2009, 2010). Recently, we also investigated the stability and replication kinetics as well as selected immune parameters after infection of swine with four of these deletion mutants, coding for deletions in nsp2 varying in size from 87 to 403 amino acids (Faaberg et al., 2010). We reported that the deletion mutants initially had diminished capacities to replicate in swine, but all but the large deletion virus (403 amino acids removed from nsp2) recovered to parental viral RNA levels by study end. The prior study also showed that the ORF1 region coding for nsp2 (3688

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* Corresponding author at: USDA, Agricultural Research Service, Virus and Prion Research Unit, Mailstop 2S-209, National Animal Disease Center, 1920 Dayton Avenue, Ames, IA 50010, USA. Tel.: +1 515 337 7259; fax: +1 515 337 7428.
*E-mail address:* kay.faaberg@ars.usda.gov (K.S. Faaberg).

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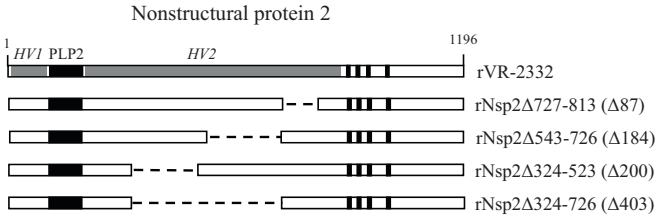


**Fig. 1.** PRRSV nsp2 deletion mutant schematic. Treatment groups used for deletion mutant sequencing study. Abbreviations: hypervariable regions of nsp2 (HV1 and HV2), defined previously, are shown in solid grey (Han et al., 2006), chymotrypsin-like cysteine protease (PLP2), predicted nsp2 transmembrane regions are indicated by short black bars (▮).

bases for strain VR-2332) was genetically stable in all four mutants (Faaberg et al., 2010). Our hypothesis was that other regions of the PRRSV deletion mutant genomes possessed alterations/adaptations that led to the recovery to *wt* virus replication levels for 3 of the 4 nsp2 deleted viruses. In order to understand the effects of the nsp2 deletions on the genetic stability of other areas of the virus genome and to probe reasons for viral fitness, a protocol was devised and implemented to acquire several PRRSV genome sequences in one sequencing effort.

We now report the successful simultaneous 454 pyrosequencing of 15 complete or nearly complete genomes of the engineered viruses after passage in swine. The complete sequences were then compared and contrasted to understand the stability of engineered mutant genomes. We found that no identical mutations occurred in all nsp2 deletion mutant genomes that were not seen in the recombinant wild-type (*wt*) VR-2332 genome. Second site (non-nsp2) deletions and/or insertions were not detected. In general, the number of mutations seen increased with deletion size, but even the largest deletion (403 aa) had very few consensus mutations. Thus, the nsp2 deletion mutant genomes were genetically stable in vivo.

## 2. Materials and methods

### 2.1. Virus

Recombinant viral strain VR-2332 (rV7; *wt*) and four VR-2332 nsp2 deletion mutants (rΔ727-813, rΔ543-726, rΔ324-523, and rΔ324-726) have been described previously (Fig. 1) (Han et al., 2007). The engineered mutants correspond to deletion sizes of 87 (Δ87), 184 (Δ184), 200 (Δ200) and 403 (Δ403) amino acids, respectively. In a previous study, all five viruses had been individually inoculated into groups of four young pigs at 4 weeks of age (Faaberg et al., 2010).

### 2.2. Swine study

In the present animal experiment, 18 conventionally raised 3-week-old weaned pigs were purchased from a PRRSV-negative herd. Upon delivery to the National Animal Disease Center (NADC), pigs were randomly allocated to one of three treatment groups ($n = 6$/group) housed separately in isolation facilities following the NADC Animal Care and Use Committee guidelines. Each group was acclimated for 1 week prior to the beginning of the study (Day 0), at which time all pigs within the group were inoculated with one of 3 virus preparations. Groups 1 and 2 received the wild type (*wt*) or large deletion (Δ403) recombinant viruses, respectively, that were used in a previous in vivo study (Faaberg et al., 2010). Pigs were inoculated with an intramuscular injection at one site with 2 ml ($2 \times 10^3$ TCID/ml) of cell culture propagated virus. In order to examine the growth kinetics and stability of the large deletion virus after an additional in vivo passage, pigs in group 3 were inoculated intramuscularly with lung lavage fluid (2 ml at each of 2 sites) collected from one pig 35 days post-challenge with the cell-culture propagated Δ403 virus (Faaberg et al., 2010). The lung lavage fluid had been negative by virus isolation, but positive by PRRSV qRT PCR for Δ403 (rΔ324-726) RNA. Serum was collected from all pigs on days 0, 2, 4, 7, 9, 11, 15, 21, 28, and 35 post-inoculation. On day 35, each pig was euthanized and necropsied to collect lung lavage fluid. The weight of the tracheobronchial lymph node located at the base of the left bronchi (LNW) was compared to the body weight (BW) to provide a clinical assessment of relative PRRSV pathogenicity. Because the purpose of the study was to examine the effect of swine passage on the replication kinetics of the large deletion virus, no negative control animals were utilized.

### 2.3. Virus isolates and cell culture

Virus isolates (Δ87, Δ184, Δ200, and Δ403, *wt*) were recovered from stored serum or lavage fluid using Marc-145 cell culture. The samples were selected from two pigs per group, the same animals as used for nsp2 sequencing as reported previously (Faaberg et al., 2010), for a total of 10 viral isolates. Additionally, viruses were isolated during the current swine study described above from serum and lavage fluid from two pigs per group for the repeat inoculation of *wt* (*wt*R) and for the pig passed large deletion virus (Δ403P), for an additional 4 viral isolates. The animals reinfected with large deletion virus cell supernatant fluid had no discernable viral titer, so the remaining sample for genome sequencing was selected based on the fact that nested RT-PCR confirmed the presence of viral RNA (Δ403R, Fig. 3). The 15 isolates were designated as the virus name (e.g., Δ87) followed by animal 1 or 2 (e.g., Δ87-1 and Δ87-2). Most isolates were generated from the last virus isolation positive sample, which was taken at 35 days post-infection, except for the swine passaged virus (Δ403P) where the two isolation positive samples were harvested at 15 dpi. The PRRSV nested RT-PCR positive serum sample (Δ403R) virus was blindly passed on MARC-145 cells. The original isolates were amplified by passage in two T75 flasks of Marc-145 cells. Virions were harvested by freeze-and-thaw of cultured cells exhibiting cytopathic effect (CPE), and then purified twice on a sucrose cushion (0.5 M sucrose, 10 mM NaCl, 10 mM Tris of pH 7.5 and 1 mM EDTA) by ultracentrifugation at 24,000 rpm at 4 °C for 3–4 h using SW28 rotor. Virus pellets were suspended in 0.5 ml STE buffer (10 mM NaCl/10 mM Tris, pH 8.0/1 mM EDTA, pH 8.0). To summarize, virus isolates Δ87-1, Δ87-2, Δ184-1, Δ184-2, Δ200-1, Δ200-2, Δ403-1, Δ403-2, *wt*-1, and *wt*-2 were recovered from stored samples obtained from a prior swine study (Faaberg et al., 2010). The present study yielded five additional virus isolates that were identified as *wt*R-1, *wt*R-2, Δ403R, Δ403P-1, and Δ403P-2.

### 2.4. Viral RNA extraction

Residual DNA contamination in the sucrose cushion purified virus suspension was eliminated by addition of 10 μl RQ1 DNase I (Promega) and incubated at room temperature for 10 min. RNase-In (25 μl, Ambion) and 50 μl of 0.1 M dithiothreitol (DTT) were added. Virus particles in suspension were then lysed by adding 0.5 ml of 2× LES buffer (0.2 M LiCl/10 mM EDTA/2.0% SDS) and 10 μl of proteinase K (20 mg/ml) and incubated at 56 °C for 30 min. The released viral RNA was extracted by adding equal volume of 0.1 M citrate buffer saturated phenol (pH 4.3) and half a volume of 24:1 chloroform:isoamyl alcohol followed by vortexing and incubation at 65 °C for 2 min, then cooled on ice for 1 min. The aqueous phase was separated by centrifugation for 3–5 min and then transferred to a new tube. Phenol extraction was repeated twice with heating, and then without heating. The aqueous phase containing viral RNA was extracted three times by an equal volume of 24:1 chlo-