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# A highly pathogenic porcine reproductive and respiratory syndrome virus generated from an infectious cDNA clone retains the in vivo virulence and transmissibility properties of the parental virus

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## Abstract

The nucleotide sequence of a highly pathogenic porcine reproductive and respiratory syndrome virus (PRRSV) was determined. Transfection of MARC-145 cells with capped in vitro transcripts derived from a full-length cDNA clone of the viral genome resulted in infectious PRRSV with growth characteristics similar to that of the parental virus. Primer extension analysis revealed that during replication, the viral polymerase corrected the two nonviral guanosine residues present at the 5' terminus of the transfected transcripts. Animal studies showed that the cloned virus induced hyperthermia, persistent viremia, and antibody response, similar to that observed with the parental virus. Contact transmission occurred rapidly within 3 days of introduction of naïve pigs into the group of clone virus-inoculated pigs. These results suggest that the cloned virus retains the in vivo virulence and contagion properties of the parental virus, thus, providing the background for reverse genetics manipulation in systematic examination of attenuation and virulence phenotypes.

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## Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is a spherical, enveloped virus, which causes highly contagious, severe disease in the natural host, the swine, with a spectrum of disease states ranging from respiratory failure in neonates to abortions in sows (Christianson, 1992; Keffaber, 1989). PRRSV is endemic to most swine producing countries and imposes a heavy economic burden due to high mortality associated with the disease (Albina, 1997; Meredith, 1995).

Along with equine arteritis virus, simian hemorrhagic fever virus, and lactate dehydrogenase-elevating virus,

PRRSV belongs to the *Arteriviridae* family (Cavanaugh, 1997; Meulenberg et al., 1993, 1994). Recently, arteriviruses have been classified within the order *Nidovirales* for shared strategies of replication and transcription with the coronaviruses (Cavanaugh, 1997) of which severe acute respiratory syndrome virus (SARS-CoV) is a member.

PRRSV possesses an approximately 15.4 kb positive polarity, single-strand RNA genome (Meulenberg et al., 1993) with a 5'-cap and a 3'-poly(A) tail. The genome is translationally active and is organized into 5'-terminal open reading frames (ORFs) encoding nonstructural proteins, ORF1a and ORF1b, which are proteolytically processed to generate replicase proteins. The 3'-terminal ORFs 2–7 encode, in succession, the glycoproteins 2–5, the matrix (M) protein, and the nucleocapsid (N) protein. Immediately following release into the cytoplasm, the genomic RNA is translated into ORF1a and ORF1ab polyproteins. The latter is translated via a–1 pseudoknot-mediated ribosomal frameshift. Both ORF1a and ORF1ab

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polyproteins are processed to generate at least 12 individual mature proteins. The viral replicase complex replicates the genomic RNA into a negative-sense intermediate that in turn serves as the template for generation of progeny genomic RNA. The replicase also generates a nested set of negative-sense subgenomic (sg) RNAs. These sgRNAs (Sawicki and Sawicki, 1990; Sethna et al., 1989) serve as templates for generation of 5'- and 3'-coterminally positive-sense sgmRNAs encoding ORF2-7 structural proteins, which participate in viral assembly and also are responsible for viral antigenicity (Conzelmann et al., 1993; Meulenberg et al., 1995, 1997). It is thought that replication and transcription occurs along double membrane compartments within the host cell (Pol et al., 1997).

One major step toward understanding PRRSV biology was achieved through the development of infectious cDNA clones. In 1998, Meulenberg et al. (1998) reported the generation of an infectious clone of the European prototype Lelystad virus (LV). Recently, the construction of an infectious clone for the prototypic North American strain, VR-2332, has been reported (Nielsen et al., 2003). In these studies, transfection of BHK-21 cells with capped *in vitro* transcripts resulted in generation of infectious PRRSV that could infect permissive CL2621 and MARC-145 cells (Kim et al., 1993). Although the rescued recombinant North American virus, VR-2332, exhibited similar growth properties in MARC-145 cells, its growth, genomic stability, and transmission by contact in swine remain largely unaddressed.

In order to study the mechanisms of replication and pathogenesis of this important swine pathogen, we used a highly virulent American PRRSV isolate (NVSL #97-7895) to construct an infectious cDNA clone. This particular isolate was obtained in southeastern Iowa from a vaccinated herd that developed PRRSV infection (Allende et al., 2000). Initial sequence analyses of limited regions of the viral genome indicated that it was not a revertant of the vaccine strains in use at that time and that the isolate represented a distinct North American virus associated with high pathogenicity, demonstrated by the abortifacient character of the isolate in the PRRS experimental model of reproductive failure (Osorio et al., 2002).

In the current study, we report the complete sequence analyses of the PRRSV genome. A full-length cDNA clone representing the consensus amino acid sequence encoded in the genome was assembled. Capped *in vitro* transcripts produced from the full-length cDNA clone, when transfected into MARC-145 cells, generated PRRSV with growth kinetics indistinguishable from the parental virus. More importantly, we demonstrate that the recombinant virus replicated in pigs causing clinical signs, persistent viremia, and high antibody titers against PRRSV. Furthermore, we show that contact transmission of the virus from the infected pigs to introduced sentinels occurred rapidly.

## Results

### *Sequence determination of the PRRSV NVSL #97-7895 genome*

In January 1997, a swine herd, with a previous history of vaccination against PRRSV, developed highly lethal, “atypical” porcine reproductive and respiratory syndrome (Halbur and Bush, 1997). As part of a survey to study the atypical form of PRRS affecting the region, PRRSV NVSL #97-7895 was isolated from a newborn pig and passaged on MARC-145 cells. The passaged virus was purified and utilized for extraction of the viral genomic RNA and subsequent sequence determination.

Sequencing of ORF5 led to the conclusion that this virus varied greatly from the vaccine strain and represented a unique isolate. To determine the genome sequence of this PRRSV, we utilized RT-PCR to generate five overlapping cDNA fragments (amplicons) encompassing the entire length of the genome (Fig. 1A). These five amplicons (A, B, 5C, 3C, and D) were individually cloned into the low-copy plasmid, pBR322. Primers (Table 1) used in amplification incorporated specific restriction enzyme sites to facilitate cloning. The top and bottom strands of 6–12 clones of each amplicon were sequenced. Thus, the consensus sequence was derived for each nucleotide position with 12- to 24-fold redundancy. Additionally, the overlapping regions between each amplicon, which contained artificial sequences introduced by primers used in the initial RT-PCR, were cloned and sequenced similarly with primer pairs representing the newly determined consensus sequences. Authentic 5'-terminal sequences of the PRRSV genome were obtained by RLM-RACE and subsequent sequence analysis of six individual clones. Amplicon A, which contained the 5' end of the genome, was repaired to reflect the authentic viral sequences.

Final assembly of the full-length consensus sequence of the PRRSV genome revealed a composition of 15413 nt with a 5'-untranslated region (UTR) containing 191 nt, two bases longer than the North American prototype VR-2332 (Genbank, accession #U87392). The genome shares 90–91% sequence identity with the North American isolates VR-2332, PA-8, and 16244B. We also observed an approximately equal ratio of serine or leucine at amino acid position 1028 and proline or serine at position 2783 of the ORF1ab polyprotein.

### *Construction of full-length cDNA clone of PRRSV*

DNA fragments A and D (Fig. 1A) were ligated using *Bsr*GI site to obtain the plasmid pAD. Bacteriophage T7 RNA polymerase promoter was introduced at the 5' terminus of the viral genome. Two additional guanosine residues were introduced between the T7 promoter and the first nucleotide of the viral genome to facilitate efficient transcription by T7 RNA polymerase. On the basis of the

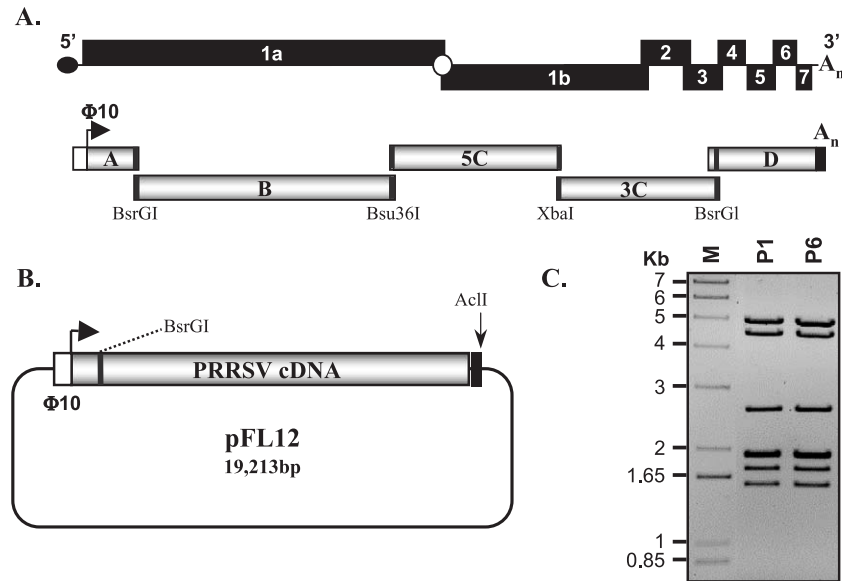


Fig. 1. Construction of PRRSV NVSL #97-7895 full-length cDNA. (A) Five overlapping regions of the viral genome were amplified to generate the cDNA fragments A, B, 5C, 3C, and D. Fragment D was amplified to introduce 41 adenosines at the 3' terminus. The T7 RNA polymerase promoter ( $\Phi$ 10) was inserted upstream of viral genomic 5'-terminal sequences in fragment A. Restriction enzyme sites used for cloning are listed below the fragments. (B) cDNA fragments with the consensus amino acid sequence were selected for assembly of the full-length clone in pBR322. An *AcII* site was incorporated immediately downstream of the poly(A) tail. The artificially introduced genetic marker, a *BsrGI* restriction enzyme site at position nt 1170, is shown. Bent arrow shows the position and direction of transcription by T7 RNA polymerase. (C) Original bacterial stock carrying pFL12 was propagated six times in *E. coli* DH5 $\alpha$ . Plasmid DNA from passage 1 (P1) and 6 (P6) bacterial cultures was extracted, digested with *MfeI*, and resolved by electrophoresis on a 0.9% agarose gel along with a size marker (M). Expected sizes of the products of digestion are 1525, 1704, 1882, 1906, 2617, 4527, and 5034 bp. Gel image is inverted.

previous report from Nielsen et al. (2003) demonstrating that 40 adenosine residues at the 3' terminus of the viral genome were sufficient to recover infectious virus, we incorporated a poly(A) tail of 41 residues at the 3' end of

fragment D. Further details of the construction of the full-length cDNA clone have been described in Materials and methods. Clones were screened to ensure insertion of fragments in the correct orientation, and three full-length cDNA

Table 1  
Primers used in the present study

Primer	Sequence <sup>a</sup>	Viral genome position <sup>b</sup>
For fragment A		
P1F	5'GCATATAAGCTTTAATACGACTCACTATAGGTGCCTCGGCATTTGTATTGTCAGG	24–47
P1146R	5'GTGTCAGTCGACGTAGATG <b>TGTAC</b> AACGATAGGCCCATC	1155–1174
T7P+1	5'ATATAAGCTTCGGACCGTAATACGACTCACTATAGGATGACGTATAGGTGTTGGC TCTATGCCATGACATTTGTATTG	1–42
For fragment B		
P1145F	5'GTGTCAGATATC <b>TGTAC</b> AGTACTTCTCTG	1169–1185
P6154R	5'GTGTCAGATATCAGCCCATATGTCTTTAATTATGTGGC	6157–6178
For fragment 5C		
P5706F	5'ATATAGATATCGCACATGTCCTTACGGG	5730–5746
P9791R	5'ATATAGATATCCAT <b>CTAG</b> AGGGCTTGTGC	9798–9815
For fragment 3C		
P9783F	5'ATATAGGATCCCTCTAGATGAGGTGTTG	9805–9823
P13625R	5'ATATAGGATCCCTTGACATGTTGGACGTAGC	13640–13660
For fragment D		
P12687F	5'GTGTCAAAGCTTGTACATTCCTCCATATTTTCCTCC	12710–12734
P15393RpolyA	5'GTGTCAGTCGACGCGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT TTTT TTTTTTTTTTTTAATTTTCGGCCGCATGGTTCTCGC	15391–15454
For genetic tag		
P24F	5'TGCCTCGGCATTTGTATTGTC	24–44
P1763R	5'CACATTCAAGGGGGAGCAGAGAAG	1741–1764

<sup>a</sup> T7 RNA polymerase promoter sequence is italicized. Underlined sequences represent restriction enzyme recognition sites utilized in full-length cDNA assembly. PRRSV-specific sequences are in bold-face type.

<sup>b</sup> Nucleotide position within the NVSL#97-7895 PRRSV isolates consensus full-length genome.

clones were identified for sequencing in their entirety. The full-length cDNA clones contained serine and proline residues at amino acid positions 1028 and 2783 in ORF1ab polyprotein, respectively, because these amino acids were predicted in the consensus sequence of the viral genome. Sequence of all three full-length clones matched completely with the consensus parental viral sequence at the amino acid level. One clone, pFL12 (Fig. 1B), was used in the studies reported here. Also, polymerase-defective mutant template, pFL12Pol<sup>-</sup>, was generated by alteration of the catalytic domain of the polymerase from SDD to AAA. This template served as the negative control in our studies. During the cloning process, we introduced a *Bsr*GI site at nt1170, which served as the genetic marker (Fig. 1B), as it was not present in the parental virus genome at that position.

In a previous report (Meulenberg et al., 1998), certain variants of the full-length cDNA clone of PRRSV were found to be unstable when propagated in *E. coli* DH5 $\alpha$ . Although all of our subclones, as well as the full-length cDNA clone in pBR322 vector, were stable during initial passages in *E. coli* DH5 $\alpha$ , we wanted to ensure that our plasmid (pFL12) containing the full-length cDNA of PRRSV genome was stable during further passages. The original bacterial stock carrying pFL12 was serially passaged six times. The plasmid from the original culture and the sixth passage culture was recovered and subjected to restriction digestion with *Mfe*I, which recognizes seven sites within the viral genomic cDNA in the plasmid. Electrophoretic analysis of the digestion products in an agarose gel shows identical DNA banding patterns (Fig. 1C) for plasmids obtained from the two passages. This result demonstrates that the pFL12 plasmid is stable during propagation in *E. coli* DH5 $\alpha$  cells.

#### Rescue of cloned PRRSV from in vitro transcripts

The plasmids, pFL12, and pFL12Pol<sup>-</sup> were linearized by restriction with *Ac*II and used for in vitro transcription by T7 RNA polymerase to synthesize capped RNAs. Synthesized full-length transcripts possessed two nonviral guanosine residues at the 5' terminus of the transcripts. In addition, the 3' terminus would be expected to contain a poly(A) tail of 43 adenosine residues: 41 from the RT-PCR amplicon and 2 from the restricted *Ac*II site.

Previous studies with the LV and VR-2332 infectious cDNA clones utilized BHK-21 cells for transfection of genome length capped RNAs and virus recovery (Meulenberg et al., 1998; Nielsen et al., 2003). Our preliminary studies indicated that MARC-145 cells could be electroporated with RNA for recovery of infectious PRRSV as efficiently as BHK-21 cells. In subsequent studies, we have used MARC-145 cells for the rescue of PRRSV by transfection of in vitro transcripts and also for virus propagation.

Two days following transfection of MARC-145 cells with in vitro transcripts or RNA from virus-infected cells, cells were examined by fluorescence microscopy for expression

of nucleocapsid (N) protein. Results (Fig. 2A) show that about 5% of cells transfected with FL12 RNA expressed the N protein. Although overall transfection efficiency of intact genome length transcripts was low, the detection of N protein indicated that MARC-145 cells were indeed transfected and that these transcripts were translated and replicated leading to the synthesis of sgRNAs, particularly, ORF7 N sgmRNA. Immunofluorescent analysis of transfected cells suggested that on average, 20000 infectious centers per microgram of cDNA-derived full-length in vitro transcripts were seen. Cells transfected with FL12Pol<sup>-</sup> RNA did not express the N protein as evidenced by the absence of fluorescent cells. Approximately 2% of cells transfected with parental viral RNA expressed the N protein. Expression of N protein in parental viral RNA transfected cells likely originated from both input ORF7 sgmRNA and from replication of genomic RNA followed by de novo transcription of ORF7 sgmRNA.

At 48 h posttransfection, supernatants from parallel cultures of transfected cells were collected and used to infect naïve MARC-145 cells. Immunofluorescence examination of infected cells at 2 dpi (Fig. 2B) showed high levels of expression of N protein indicating that viable and infectious PRRSV was rescued from the cells transfected with in vitro transcripts. Cells infected with supernatant

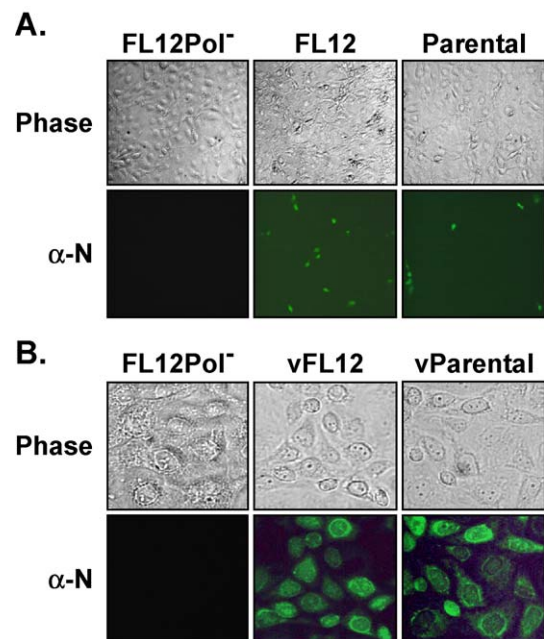


Fig. 2. Rescue and passage of PRRSV. (A) MARC-145 cells were transfected with in vitro transcripts derived from pFL12Pol<sup>-</sup> or pFL12, or with total RNA from cells infected with parental virus. At 2 days posttransfection, cells were examined for expression of N protein by immunofluorescence. (B) Supernatants from parallel cultures of transfected cells were collected 2 days posttransfection, clarified, and passaged onto naïve MARC-145 cells. Forty-eight hpi, the cell monolayer was examined for expression of N protein as before. Cells in panel B were photographed at higher magnification than in panel A.



from FL12Pol<sup>-</sup> RNA transfected cells did not show N protein expression. In addition, cells inoculated with supernatant, derived from parental and FL12 RNA transfected cells, displayed cytopathic effects (CPE) within 2–3 dpi. No CPE was observed in cells inoculated with supernatant from FL12Pol<sup>-</sup> RNA transfected cells. Titration of PRRSV in supernatants collected from transfected and passage one cells showed average titers of  $10^5$  and  $10^6$  PFU/ml, respectively. One additional passage of the culture supernatant resulted in growth of vFL12 to titers reaching about  $4 \times 10^7$  PFU/ml, which is similar to that obtained with the parental virus. We also observed that vFL12 could infect PAMs (Fig. 3B), indicating that the recombinant virus possessed biological characteristics of the parental virus. Thus, our results show that fully viable and infectious virus was recovered from MARC-145 cells transfected with capped in vitro transcripts derived from a PRRSV full-length cDNA clone.

#### Characterization of the cloned virus

During assembly of the genome length cDNA, we engineered a genetic marker, a *Bsr*GI site at nt 1170, to distinguish vFL12 from the parental virus. To determine if the rescued virus was derived from in vitro transcripts, we amplified a 1740 bp DNA fragment, encompassing the genetic marker, from RNA of infected cells and examined for the presence of the *Bsr*GI site. Results show that the amplified DNA from vFL12-infected sample contained the *Bsr*GI site as it was digested with the enzyme to generate two fragments (Fig. 3A). As expected, amplified DNA from the parental virus-infected sample did not contain the *Bsr*GI site. These results indicate that virus rescued from FL12 RNA transfected cells was indeed derived from FL12 RNA and not from contamination by parental virus.

To compare the growth of vFL12 and parental virus in MARC-145 cells, we performed growth kinetics studies on confluent monolayers containing a total of  $2.5 \times 10^6$  cells. Results (Fig. 3B) show that growth kinetics of vFL12 and parental virus was not significantly different from each other and that titers peaked at 48 hpi for both viruses. The maximum titers of vFL12 reached  $4 \times 10^7$  PFU/ml, whereas the parental virus grew to a peak titer of about  $8 \times 10^7$  PFU/ml. These studies indicate that vFL12 possesses growth characteristics similar to the parental virus. Infection of PAMs (Fig. 3B) also indicated that vFL12 replicated with similar growth kinetics as the parental virus, although peak titers for both viruses were lower than those obtained with MARC-145 cells. The lower peak titer of both viruses in PAMs is most likely because we used significantly fewer numbers of PAMs in these experiments as compared with the MARC-145 cells. Furthermore, PAMs exhibited significant cytopathic effects and cell death by 24 hpi and therefore, culture supernatants beyond this time point were not used for growth kinetics studies.

#### Primer extension analysis of the cloned virus genome

The in vitro transcripts used to rescue infectious vFL12 contained two nonviral guanosine residues at the 5' terminus. Previous studies with infectious cDNA clones of RNA virus genomes (Boyer and Haenni, 1994) suggest that 5' terminal nonviral nucleotides sometimes are not tolerated by viral polymerases and lead to severe attenuation or obliteration of viral replication. To analyze whether PRRSV polymerase would correct the nonviral nucleotides during replication, we infected MARC-145 cells with vFL12 or parental virus, and primer extension was performed using RNA from infected cells or using in vitro transcripts as

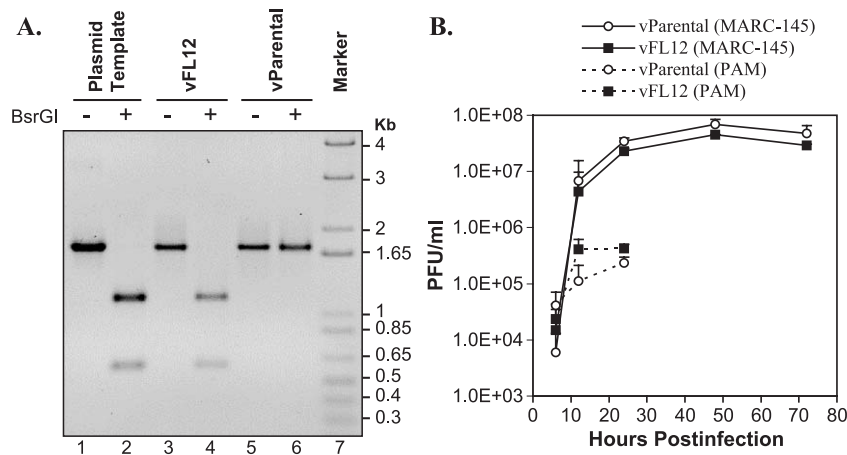


Fig. 3. Characterization of rescued virus. (A) An amplicon of 1740 bp, spanning the genetic marker, was amplified by RT-PCR using total RNA from cells infected with either vFL12 (lanes 3 and 4) or parental virus (lanes 5 and 6). pFL12 DNA also was used in PCR amplification as a positive control (lanes 1 and 2). The amplicons were digested with *Bsr*GI. DNA was electrophoresed on 1% agarose gel and an inverted image was acquired. (B) Growth kinetics of cloned and parental virus. MARC-145 cells ( $2.5 \times 10^6$ ) were infected with either vFL12 or parental virus. At 6, 12, 24, 48, and 72 hpi, supernatant was collected and titrated by plaque assay. PAMs were infected and cultures were collected at 6, 12, and 24 hpi for titration of infectivity. Error bars represent standard deviation from replica experiments.

templates. Results show that the in vitro transcripts, as expected, predominantly contained two guanosines and the cap, which is extended by reverse transcriptase during primer extension (Fig. 4, lane 6). The products from vFL12 RNA (lane 7) were two nucleotides shorter than the in vitro transcripts but were of the same size as those obtained with the parental virus (lane 8). These results indicate that the PRRSV RNA polymerase corrected the additional residues at the 5' terminus during replication of the transfected in vitro transcripts.

*Experimental infection of young pigs with the cloned virus results in vigorous replication in vivo and induction of frank clinical symptoms*

Upon inoculation, both vFL12- and parental virus-inoculated groups developed symptoms of PRRS similar to those typically described when young pigs are inoculated with a virulent strain of PRRSV (Rossow et al., 1995). These symptoms were expressed in our experiment by lethargy, lack of appetite, roughness of hair coat, occasional sneezing, some coughing, and some diarrhea most of which started within 48 h postinoculation. The intensity and duration of the symptoms seemed to correlate closely with the kinetics of the development and the intensity of hyperthermia (Fig. 5A). The vFL12-inoculated group exhibited a hyperthermic peak that was significantly higher ( $P < 0.05$ ) than the temperature baseline in the sham-inoculated control group, specifically from days 3 to 6 postinoculation. Interestingly, in the parental virus-inoculated group, which also developed some degree of hyperthermia, the thermal peak was less promi-

nent, modestly rising above the normal temperature curve registered for the sham-inoculated group. The symptoms exhibited by the vFL12-inoculated group were the more prominent of the two groups as observed by the amount of food left unconsumed or the degree of alertness of the animals. In addition, the higher severity of the disease observed in the vFL12-inoculated group was further evidenced by the fact that one of the animals died of typical secondary complications to PRRSV infection at 23 dpi. A necropsy and diagnostic workup on this animal showed immunohistochemical detection of PRRSV in several tissues and histopathological lesions suggestive of colonization by *Streptococcus suis* or *Salmonella*. Infectious vFL12 was isolated from multiple tissues, including lymph nodes, lung, spleen, and kidney. The parental virus-inoculated group exhibited milder symptoms of infection although the sham-inoculated control group remained healthy during the study period.

Based on the time points postinoculation that we used to follow the viremia profile, it can be inferred that the peak of infectious PRRSV titer in blood must have occurred during the first week postinoculation, with an ensuing sustained and slow decay afterwards, which is typical of infection with virulent strains of PRRSV (Fig. 5B) (Yoon et al., 1996). By the end of the observation period at 22 days postinoculation, a detectable viremia, a titer above the  $10^{1.5}$  TCID<sub>50</sub>/ml detection threshold of the assay, was still evident in two of the animals of the vFL12-inoculated group and in three animals of the parental virus-inoculated groups. Further evidence of in vivo replication was obtained by analysis of the PRRSV specific seroconversion, as indicated by the curve of S/P ratios from the commercial Idexx ELISA kit. Although both antibody curves (Fig. 5C) indicate occurrence of a robust PRRSV replication, it should be noted that the response in clone inoculated group was significantly higher ( $P < 0.05$ ).

*The cloned virus is genetically stable and transmitted horizontally to uninfected animals*

From the viremic serum samples of the two PRRSV inoculated groups at 14 days postinoculation, RNA was extracted and RT-PCR was performed to amplify the 1740 bp amplicon to ascertain whether or not the *BsrGI* genetic marker was present. Results show that the amplicon contained the *BsrGI* site in the vFL12-inoculated group (Fig. 5D, left panel) that should be expected if the virus' genotype was stably maintained throughout the multiple rounds of replication that occur in vivo. Virus isolated from the parental virus-inoculated pig did not contain the *BsrGI* site. When two sentinels were introduced at day 19 postinoculation into the group infected with vFL12, rapid horizontal transmission occurred. Virus was detected in one sentinel within 3 days of contact although both sentinels were viremic by day 8. On the other hand, the parental virus was transmitted only after 3 weeks of

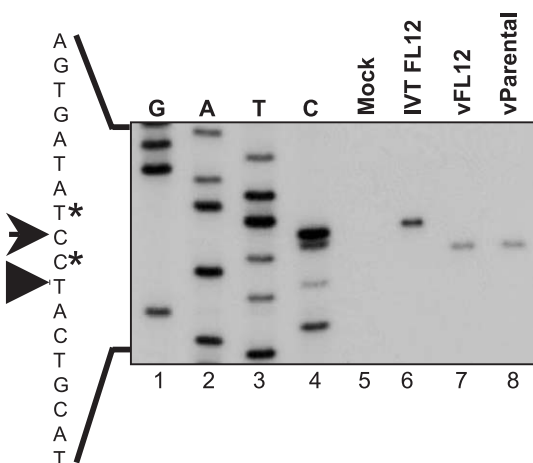


Fig. 4. Nonviral 5' guanosine residues in the in vitro transcripts from pFL12 are corrected by PRRSV polymerase during RNA replication. Primer extension analysis of RNA, isolated from cells mock-infected (lane 5) or infected with vFL12 (lane 7) or parental virus (lane 8) or capped in vitro transcripts alone (lane 6), was performed using a negative-sense oligonucleotide. Lanes 1–4 show the sequence ladder obtained with the same oligonucleotide using pFL12 DNA template. Nucleotides marked by \* correspond to migration positions of extended cDNAs due to addition of cap. Arrow and arrowhead show the positions of 5' ends of in vitro transcripts and replicating viral RNAs, respectively.

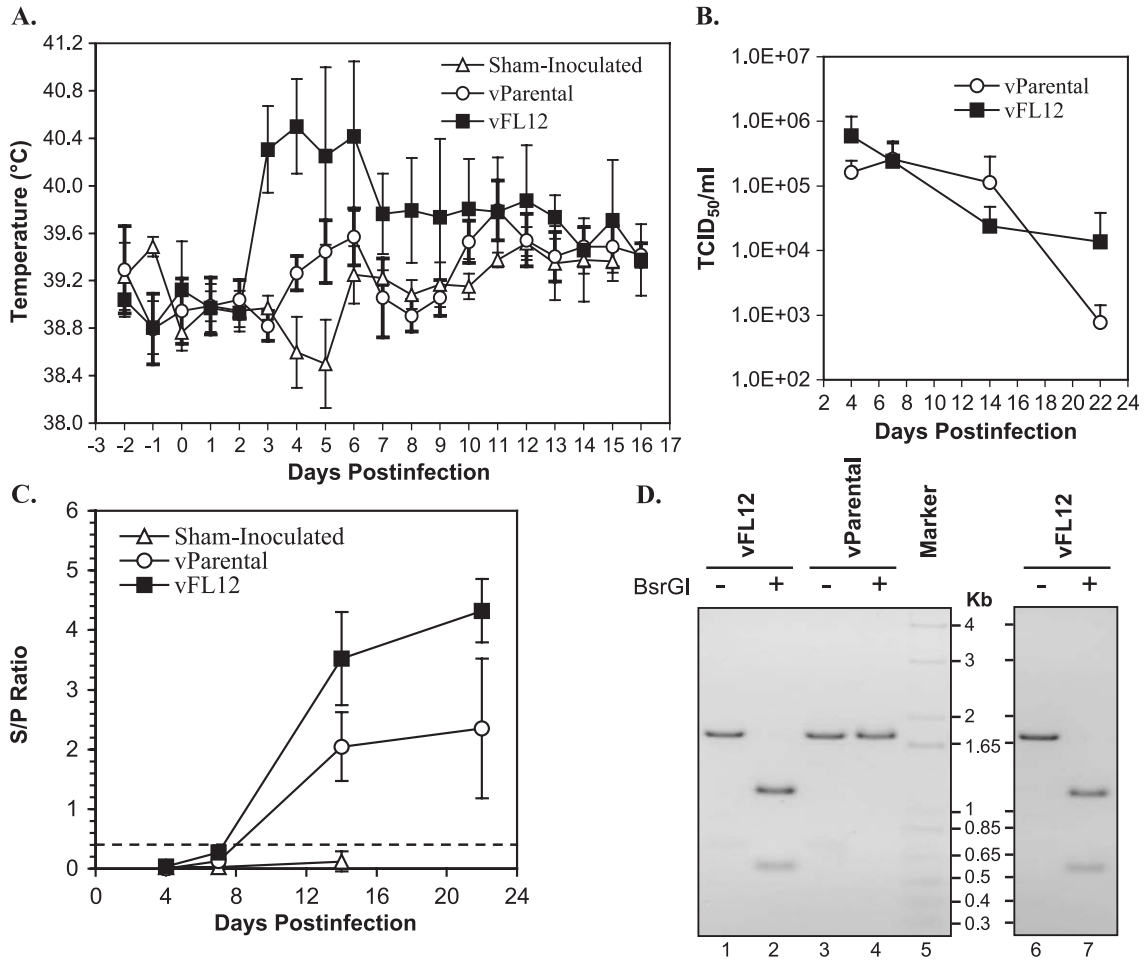


Fig. 5. The cloned virus retains in vivo markers of virulence and is genetically stable. Three groups of pigs (four pigs per group) were sham-inoculated or inoculated with either vParental or vFL12 at TCID<sub>50</sub>/ml of 10<sup>5.2</sup> and 10<sup>4.8</sup>, respectively. (A) Temperatures were recorded daily from 2 days before inoculation to 16 dpi. Average temperatures from four different animals in each group are shown. (B) Serum samples were collected at 4, 7, 14, and 22 dpi and virus titers were determined and expressed as TCID<sub>50</sub>/ml. Values represent average titers from four animals. (C) Serum antibody titers were determined with the Idexx ELISA kit. Values represent average titers from four animals. Dashed line at 0.4 S/P ratio designates threshold value above which titers are considered positive for anti-PRRSV antibodies. Error bars in panels A, B, and C represent standard deviation. (D) Serum from 14 dpi, recovered from inoculated animals, was used to distinguish between vFL12 and vParental by examining for the presence of the *BsrGI* genetic tag (left panel). Viral RNA extracted from sera was amplified by RT-PCR and the products were digested with *BsrGI*. Similar analysis (lane 7) was performed with virus in serum from a sentinel pig at 8 days postcohabitation with pigs infected with vFL12 (right panel). Gel image is inverted. Error bars represent standard deviation.

cohabitation. Importantly, the identity of the transmitted virus as being vFL12 was unequivocally demonstrated by the presence of the genetic marker (Fig. 5D, right panel), thus confirming that the genetic stability of the cloned virus is a feature maintained both within and between animals.

**Discussion**

In this report, we describe the complete nucleotide sequence of the viral genome and the construction of infectious cDNA clone, FL12, for a highly pathogenic, North American isolate of PRRSV. Transfection of capped in vitro transcripts, derived from this clone, directly into MARC-145 cells led to efficient recovery of infectious

virus from cultured cells. Indeed, at day 5 posttransfection, CPE was observed in transfected cells. Robust recovery of the virus was further evident by detection of CPE in cells infected with culture supernatant from transfected cells. Cloned virus, vFL12, displayed similar growth properties to its parent virus in MARC-145 cells, in PAMs, and in the animal host. More significantly, animal studies showed that vFL12 and the parental virus rescued from cultured cells induced similar patterns of viremia and seroconversion. Furthermore, we observed horizontal transmission of vFL12 from infected to sentinel pigs by as early as day 3 after cohabitation. Detection of the *BsrGI* genetic marker indicates that vFL12 remained genetically stable during passage in cells, multiple rounds of replication in inoculated pigs, and after secondary transmission to sentinel pigs.



Since its initial isolation in the early 1990s in North America and Europe, PRRSV has become endemic in virtually every swine farming nation and has emerged as a significant economic menace within the swine industry. The most feasible approach to thwarting the virus exists in vaccination. Production of first generation vaccines has resulted in wide immunizations on both continents. However, it is becoming evident that currently available vaccine strains are unstable and sometimes revert to virulent phenotypes in vaccinated animals (Key et al., 2003; Mengeling et al., 1999; Nielsen et al., 2002; Opriessnig et al., 2002). Thus, development of vaccine strains from multiple passages in tissue culture may not be effective. Rather development of attenuated strains through reverse genetics manipulation of viral full-length recombinant cDNA clones would provide greater predictability in phenotype outcome and host immunization efficacy. With the advent of infectious cDNA clones for Lelystad virus and VR-2332, such studies will be possible. Here, we have reported the development of an infectious cDNA clone for a highly pathogenic North American strain of PRRSV that appears to resemble its parent strain, closely, in terms of its growth and pathogenic properties. This reagent will play an important role in our endeavor toward understanding the determinants of virulence of this significant swine pathogen.

Sequence determination showed a 91% identity between NVSL #97-7895 and VR-2332, indicating that NVSL #97-7895 is a distinct isolate. Interestingly, the consensus sequence of the full-length genome contained a two-base insertion in the 5' UTR at nt 90 and nt 129 in comparison to North American isolates VR-2332, PA-8, and 16244B. It is highly unlikely that these insertions resulted from error during reverse transcription or from sequencing because they were present in all sequenced clones derived from two independent RT-PCR amplifications. Whether such insertions in the 5' UTR of the viral genome influence the growth and pathogenic characteristics or not remain to be established.

In the report by Nielsen et al. (2003), recovery of virus was achieved after multiple passages and CPE was first observed in passage 3, whereas virus derived from parental viral RNA displayed CPE as early as passage 1. In addition, titers recorded for the cloned virus were consistently several fold lower than that for the parental virus. The authors speculated that it was possible that incorporation of nonviral guanosine residues at the 5' terminus of the viral genome may have contributed to diminished growth capacity of the cloned virus. However, our primer extension analysis suggests that the viral RNA polymerase possesses inherent property to correct the 5'-terminal nonviral residues during replication. The fact that our studies, reported here, demonstrate robust recovery of infectious PRRSV indicates that the full-length cDNA with the consensus sequence containing the combination of amino acids at positions 1028 and 2783 is optimal for growth of the virus.

The cloned virus, vFL12, transmits from host-to-host with high efficiency, therefore, providing the background for studying the role that individual sequences or genes may have in transmissibility, a fundamental property of viral virulence. Such applications of reverse genetics have already proven highly successful in studying the molecular basis of transmission in plant (Andrejeva et al., 1999; McLean et al., 1993), nematode (Vassilakos et al., 2001), and animal (Luciw et al., 1999) RNA viruses. Likewise, the properties of vFL12 provide the experimental basis for a better, more precise discrimination between the attributes of shedding and attenuation of virulence in future vaccines, which would help to correct a defect currently affecting commercial vaccines. Current classically attenuated PRRSV vaccines are known to shed from the recipient animal extensively to a level equivalent to that of wild-type PRRSV (Nielsen et al., 2002; Verheije et al., 2003).

A remarkable property of vFL12 resides in its robust immunogenicity, which translates into a vigorous seroconversion exceeding that of the parental virus. We interpret that this enhanced serologic titer of vFL12 is due to higher levels of replication of vFL12 *in vivo*. This aspect of vFL12 biology, however, should be of cardinal importance to identify possible dispensable B-cell epitopes that may serve as significant serological markers for future vaccines. Examples of potentially deletable sequences that could serve for the development of marker vaccines were recently reported to map throughout ORF1a in nsp2, ORF3, and ORF4 (Oleksiewicz et al., 2001).

The apparent enhancement of infectivity and virulence in vFL12 over the parental virus is remarkable. It has been known for some time, from other cDNA clone systems, that some infectious transcripts appear to be more infectious than the corresponding parent virus (Hamilton and Baulcombe, 1989; Hayes and Buck, 1990). In these cases, the occurrence of this enhanced virulence was associated with possible mutations favoring infectivity. However, a remarkable aspect of our experiment is that vFL12 precisely corresponds to the prevailing consensus sequence. Our *in vitro* and *in vivo* data, specifically enhanced growth of vFL12 in MARC-145 cells and macrophages, clinical signs (i.e. hyperthermia), enhanced ability to excrete through mucous membranes and transmit to uninfected sentinels, and immune response kinetics, suggest that vFL12 may represent one of the most virulent populations within quasispecies of the parental virus.

Overall, vFL12 replicates efficiently in cell culture, in macrophages, and in the natural host. Therefore, we believe that this provides a significant tool, through a systematic site-specific mutagenesis approach, to examine the determinants of virulence and attenuation based on differential replication in cell lines, on the one hand, and in cultured PAMs and the natural host, on the other hand. This will have significance in development of safer, more efficacious replicating vaccines against PRRSV.

## Materials and methods

### *Virus and cells*

PRRSV NVSL #97-7895 was isolated in 1997 in south-eastern Iowa from a vaccinated herd on a farm that was experiencing severe reproductive failure in pregnant sows. The virus was passaged up to five times on MARC-145 cells, which were maintained in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 units/ml penicillin, 20 µg/ml streptomycin, 20 µg/ml kanamycin). In vitro transcripts were electroporated into MARC-145 cells, which were also used for virus rescue and titration experiments. BHK-21 cells were maintained in Earle's MEM with 5% FBS and antibiotics and were used in initial transfection studies for recovery of virus from in vitro transcripts. Porcine alveolar macrophages (PAM) were extracted by lung lavage of 4-week-old piglets free of PRRSV, following a published protocol (Mengeling et al., 1996). Isolated PAMs were maintained in RPMI medium with 10% FBS at 37 °C and 5% CO<sub>2</sub>.

### *Extraction of viral RNA*

Fifth passage virus grown on MARC-145 cells was collected from culture supernatant and pelleted by ultracentrifugation at 100 000 × *g* through a 30% sucrose cushion. The virus pellet was resuspended in phosphate-buffered saline (PBS) and viral RNA was extracted with TRIzol LS (Invitrogen) using the manufacturer's protocol. The final RNA pellet was resuspended in water and stored at –30 °C in small aliquots.

### *Sequence analyses*

Five amplicons, designated A, B, 5C, 3C, and D, spanning the full-length genome, were generated by reverse transcription (RT) with Superscript III (Invitrogen) and PCR amplification with Expand HiFidelity Taq (Roche) or Triple Master Mix (Eppendorf). The amplicons were individually inserted into pBR322. The primers used in RT and subsequent PCR amplification are shown in Table 1. Fragment D was amplified with primer P15393RpolyA, which incorporated 41 adenosines at the 3' terminus. Six to 12 clones of each fragment were sequenced. Using dideoxy-chain terminator sequencing chemistries (Sanger et al., 1977) and an Applied Biosystems 3730 automated DNA sequencer (PE Biosystems, Foster City, CA), DNA templates were sequenced bidirectionally with forward and reverse primers. Bases were called using Phred (version 0.020425.c) (Ewing et al., 1998), which also produced a quality file containing a predicted probability of error at each base position. DNA sequences were assembled with Phrap (version 0.990329) (Ewing and Green, 1998) using the quality files and default settings to produce a consensus sequence with some manual

editing using Consed (version 13) sequence editor (Gordon et al., 1998).

The 5' terminus of the viral genome was determined by RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) using the GeneRACER kit (Invitrogen). Briefly, an RNA oligonucleotide of known sequence was ligated to viral genomic RNA that had been decapped by treatment with tobacco acid pyrophosphatase. RT-PCR was performed with primers that hybridize to known sequences to generate a fragment, which was cloned in pBR322. Six clones of this fragment were sequenced on both strands to establish the consensus.

The consensus sequence of the full-length viral genome has been deposited in Genbank under accession number AY545985.

### *Assembly of full-length cDNA clone*

Clones containing the consensus sequences were used for assembly of the full-length cDNA. Plasmids containing fragments A and D, representing the genomic terminal sequences, were combined using a BsrGI site to form the plasmid pAD. The 5' terminus of the viral genome was repaired to integrate the bacteriophage T7 RNA polymerase promoter and the authentic 5'-terminal nucleotides determined by RLM-RACE. The 3' terminus was modified to introduce an *AcII* restriction enzyme site for linearization of the template before in vitro transcription. Ligation and transformation was performed following the standard procedures described by Sambrook et al. (1989). All plasmids were propagated in *E. coli* DH5α. Plasmids containing fragments 5C and 3C were combined via an *XbaI* site and the new recombinant was fused with the plasmid containing fragment B using a shared *Bsu36I* site. This intermediate recombinant plasmid was digested with *BsrGI* to release a 12.3-kb fragment, which was cloned into the *BsrGI* site of the repaired pAD. The resulting plasmid, pFL, contained the sequences of the entire viral genome under the control of T7 RNA polymerase promoter in the vector pBR322. Both strands of three full-length clones were sequenced for accuracy. All three clones contained the consensus sequences and in vitro transcripts from all three clones were shown to produce infectious virus. Clone #12, pFL12, was used in the studies reported here.

Coincidentally, the cloned *BsrGI* at nt1170, which was introduced in the primer for initial PCR amplification, was not present in the determined consensus sequence for the parental virus at that position and thus, served as a genetic marker. A replication defective construct, pFL12Pol<sup>-</sup>, was generated from pFL12 by nucleotidyl mutation of the putative catalytic domain of the viral polymerase from 5'TCGGACGAC to 5'GCGGCCGCC, resulting in an SDD to AAA amino acid change. This construct served as a negative control in our studies with rescue of PRRSV from in vitro transcripts.

### *Transfection of in vitro transcripts and rescue of PRRSV*

pFL12 and pFL12Pol<sup>-</sup> plasmids were restricted with *AcI*. Purified, linearized DNAs served as templates for in vitro transcription of capped RNA using the mMESSAGE mMACHINE Ultra T7 kit (Ambion) with cap analog. The reaction mixture was digested with DNaseI to remove template DNA and subsequently was extracted with phenol–chloroform. RNA was precipitated with an equal volume of isopropanol and recovered by centrifugation. The RNA pellet was resuspended in water and stored at –30 °C in small aliquots. Size, integrity, and concentration of the capped in vitro transcripts were determined by electrophoresis under denaturing conditions on a glyoxal agarose gel.

Initial transfections were performed on BHK-21 and MARC-145 cells. Cells were harvested, washed, and resuspended in DMEM containing 1.25% dimethyl sulfoxide at a concentration of  $5 \times 10^6$  cells/ml. Per pulse, 0.4 ml of cell suspension was used. Ten micrograms of total cellular RNA from MARC-145 cells infected with parental virus or 5 µg of in vitro transcripts along with 5 µg of uninfected cellular RNA were added to cells and electroporated at 250 V and 950 µF (Gene Pulser Xcell, Bio-Rad). After electroporation, the cells were diluted in cell growth medium and 90% of the cells were seeded in 60-mm plates. The remaining fraction of cells was seeded in 24-well plates to monitor PRRSV N protein expression by immunofluorescence. At 16–18 h posttransfection, the cells were washed and incubated in DMEM with 5% FBS. At 48 h posttransfection, supernatant was collected and clarified by centrifugation. Ten percent of the clarified supernatant was used to infect naïve MARC-145 cells in 35-mm wells. Because virus was rescued from both cell types, all subsequent experiments were performed using MARC-145 cells.

Rescue of infectious virus was confirmed by immunofluorescence microscopy for the expression of N protein in infected cells at 48 h. Cells were fixed in methanol/acetone (1:1) for 30 min at –20 °C. Fixed cells were incubated with anti-N monoclonal antibody SDOW17 (National Veterinary Services Laboratories, Ames, IA) (Nelson et al., 1993) at 1:500 dilution in phosphate-buffered saline (PBS) at 37 °C for 30 min. After three washes with PBS, cells were incubated with fluorescein isothiocyanate-conjugated secondary anti-mouse goat antibody at 37 °C for 30 min. Finally, cells were washed three times with PBS and visualized with a Nikon inverted fluorescence microscope fitted with a camera (Optronics). Transfected cells were examined for expression of the viral N protein, as described above.

### *Detection of genetic marker*

MARC-145 cells were infected with cloned, vFL12, or parental virus at a multiplicity of infection (MOI) of 1. Cells were harvested with TRIzol (Invitrogen) at 60 h postinfection (hpi) and total cellular RNA was extracted. Recovered

RNA was reverse transcribed, as described above, using the primer P1763R, and the cDNA was amplified using the same RT primer along with a second primer, P24F (Table 1). When digested with *Bsr*GI, the 1740 bp amplicon, encompassing the cloned *Bsr*GI genetic marker, should produce two fragments of 1146 and 594 bp.

### *Growth kinetics and characterization*

Growth kinetics was examined by infecting MARC-145 cells with vFL12 and parental virus at an MOI of 1. After 1 h of virus adsorption, cells were washed and incubated at 37 °C in virus growth medium. Small aliquots of infected cell supernatant were collected at various times postinfection, and virus titers were determined by plaque assay on MARC-145 cells and expressed as plaque forming unit per milliliter (PFU/ml). Growth kinetics in PAMs, infected at an MOI of 10, was determined similarly and the viral titers were expressed as 50% tissue culture infectious dose per milliliter (TCID<sub>50</sub>/ml) and converted to PFU/ml for graphical analysis, using an empirically derived ratio of 1.11 PFU/ml to one TCID<sub>50</sub>/ml.

### *Primer extension*

Total RNA, from cells mock-infected or infected with vFL12 or parental virus, and in vitro transcribed capped RNAs were used for primer extension. A <sup>32</sup>P end-labeled oligonucleotide of negative-sense polarity, starting at nt 78 of the viral genome, was used in the extension reaction with Superscript III, as described before. A sequencing ladder was obtained with pFL12 as template and the same radio-labeled primer. The primer extension products were analyzed alongside the sequencing reaction products in a 6% polyacrylamide gel containing 7 M urea.

### *Animal experiments*

Mixed breed (Duroc × Landrace), recently weaned pigs, averaging 12 lb weight and 18 days of age, were obtained from a PRRSV-free farm. The pigs were consistent with negative serology for PRRSV and absent in clinical history of the disease. Three groups of four animals were used. One group was inoculated with a total dose of 10<sup>5.2</sup> TCID<sub>50</sub>/2 ml of PRRSV NVSL #97-7895 (parental virus) instilled intranasally (1 ml in each nostril). Another group was inoculated in a similar manner using a similar dose of vFL12 (second passage on MARC-145 cells). A third group of animals was sham-inoculated with cell culture medium (DMEM with 10% FBS). The three groups of animals were housed in strict isolation from each other using animal housing facilities compatible with biosafety level-2 conditions, including negative pressure environment to prevent any possible cross-infection between groups. On a daily basis, the animals were clinically inspected and their body temperatures were recorded from



2 days pre- until 16 days postinfection (dpi). Sequential blood samples were collected from all the animals at 4, 7, 14, and 22 dpi and used to verify levels of viremia and kinetics of PRRSV-specific antibody development using a validated commercial ELISA kit (Idexx Labs, Portland, ME). Blood samples were used to detect PRRSV by conventional viral isolation on MARC-145 cells, as previously described (Bastos et al., 2004). Likewise, virus obtained from viremic sera, from both cloned and parental PRRSV-inoculated groups, were used for RT-PCR amplification and analyzed for the presence of *Bsr*GI site that differentiates the clone from the parental virus. Virus transmissibility studies were undertaken with each of the two PRRSV-inoculated groups following a previously published protocol (Wills et al., 2002). Briefly, at 19 dpi, two additional PRRSV-free animals, homogeneous with the rest of the animals in size and origin, were introduced into the two PRRSV-inoculated groups to act as sentinels of contagion. These animals were sampled together with the rest of the group for evidence of animal-to-animal transmission by viremia and PRRSV serology. Also, in the case of viremia, serum samples were used for molecular analysis of the genetic marker, as described above.

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