Viable porcine arteriviruses with deletions proximal to the 3′ end of the genome

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In order to obtain attenuated live vaccine candidates of porcine reproductive and respiratory syndrome virus (PRRSV), a series of deletions was introduced at the 3′ end of the viral genome using an infectious cDNA clone of the Lelystad virus isolate. RNA transcripts from the full-length cDNA clones were transfected into BHK-21 cells. The culture supernatant of these cells was subsequently used to infect porcine alveolar macrophages to detect the production of progeny virus. It is shown that C-terminal truncation of the nucleocapsid (N) protein, encoded by ORF7, was tolerated for up to six amino acids without blocking the production of infectious virus. Mutants containing larger deletions produced neither virus nor virus-like particles containing viral RNA. Deletion analysis of the 3′ UTR immediately downstream of ORF7 showed that infectious virus was still produced after removal of seven nucleotides behind the stop codon of ORF7. Deletion of 32 nucleotides in this region abolished RNA replication and, consequently, no infectious virus was formed. Serial passage on porcine alveolar macrophages demonstrated that the viable deletion mutants were genetically stable at the site of mutation. In addition, the deletions did not affect the growth properties of the recombinant viruses in vitro, while their antigenic profiles were similar to that of wild-type virus. Immunoprecipitation experiments with the six-residue N protein-deletion mutant confirmed that the truncated protein was indeed smaller than the wild-type N protein. The deletion mutants produced in this study are interesting candidate vaccines to prevent PRRS disease in pigs.

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is an enveloped, positive-strand RNA virus that belongs to the family Arteriviridae, together with equine arteritis virus (EAV), lactate dehydrogenase-elevating virus (LDV) and simian haemorrhagic fever virus (SHFV) (Meulenberg et al., 1993). The family Arteriviridae has been grouped within the order Nidovirales together with the family Coronaviridae (Cavanagh, 1997). PRRSV has a single-stranded, polyadenylated RNA genome of about 15 kb that contains at least nine partially overlapping open reading frames (ORFs).

ORFs1a and 1b encode the viral replicase genes, which are expressed from the genomic RNA. The other, smaller ORFs are located at the 3′ end of the genome and are expressed from subgenomic (sg) RNAs. These are produced as a 3′-nested set during replication (de Vries et al., 1990; Meulenberg et al., 1993) and encode the structural proteins. ORF7 encodes the nucleocapsid protein, N, ORF6 encodes the membrane protein, M, ORF5 encodes the major envelope glycoprotein, GP, and ORFs 2–4 encode the minor envelope glycoproteins, GP, GP, and GP (Meulenberg et al., 1995). Recently, a novel structural protein, E, was described for EAV (Snijder et al., 1999). This protein is translated from sg mRNA2, which also encodes GP.

For vaccine purposes, we aim to generate viable deletion mutants of PRRSV. This raises basic questions about the regions of the viral genome in which deletions are tolerated. In this respect, two considerations are important. Firstly, PRRSV has a concise genome, like other RNA viruses. Since RNA viruses have evolved to optimal fitness, most of the genetic information is expected to be essential. Secondly, the ORFs that encode the structural proteins of the virus are partially...
overlapping. Deletions in overlapping regions would therefore
result in the mutation of two structural proteins, which would
almost inevitably lead to the production of a non-viable virus.
Earlier studies showed that deletions in many conserved
regions were lethal (M. H. Verheije, unpublished results).

In this study, we focused on the 3’ end of the PRRSV
genome, since this region does not contain sequences that
overlap other ORFs. Until now, deletions introduced in the
N-terminal and middle parts of the coding region of the N protein
have not resulted in viable virus (M. H. Verheije, unpublished
results). In the present study, we were more successful by
focusing on the region around the N gene stop codon.

Alignment of the N protein sequence and the 3’ UTR of
different PRRSV strains revealed heterogeneity at the C
terminus of the N protein and at the 5’ end of the 3’ UTR. A
deletion analysis of this region was therefore performed using
the available infectious cDNA clone (Meulenberg et al.,
1998) of Lelystad virus (LV) in order to determine the limits
of the sequences that can be removed while still resulting in the
production of a viable virus, but one with reduced patho-
genicity. This is the first publication to describe the generation
of viable arterivirus mutants that contain a deletion in the viral
genome that is maintained stably after multiple passages in
vitro.

**Methods**

**Cells and viruses.** BHK-21 cells were grown in BHK-21 medium
(Gibco BRL) supplemented with 5% FBS, 10% tryptose phosphate broth
(Gibco BRL), 20 mM HEPES–NaOH (Gibco BRL), pH 7.4, 200 mM
glutamine, 10 U/ml penicillin, 10 µg/ml streptomycin, 20 µg/ml kanamycin,
5 µg/ml polymyxin B and 0.2 µg/ml fungizone. Porcine alveolar
lung macrophages (PAMs) were maintained in MCA-RPMI-1640
medium containing 10% FBS, 100 µg/ml kanamycin, 50 U/ml penicillin,
50 µg/ml streptomycin, 25 µg/ml polymyxin B and 1 µg/ml fungizone.

**Construction of full-length genomic cDNA clones of LV.** PCR
mutagenesis was used to introduce sequences into the Pac mutant of the
genome-length cDNA clone of LV (pABV437) (Meulenberg et al.,
1998a). The primers used for PCR mutagenesis are listed in Table 1. PCR
fragments generated to introduce deletions into ORF 7 were digested with
HpaI and PacI and ligated into these sites of pABV437. PCR
fragments generated to introduce deletions into the 3’ UTR were
digested with PacI and XhoI and ligated into these sites of pABV437.
Standard cloning procedures were performed essentially as described
previously (Sambrook et al., 1989). Transformation conditions were
maintained as described previously (Meulenberg et al., 1998a). Sequence
analysis was performed to confirm the introduced mutations. The
constructs are drawn schematically in Fig. 2.

**Sequence analysis.** The regions of the full-length cDNA clones
that originated from the PCR products were analysed by nucleotide
sequencing. Sequences were determined with the PRISM Ready Dye
Deoxy Terminator cycle sequencing kit and the ABI PRISM 310 Genetic
Analyzer (Perkin Elmer).

**In vitro transcription and transfection of BHK-21 cells.** Full-
length genomic cDNA clones were transcribed in vitro and the resulting
RNA was transfected into BHK-21 cells either using Lipofectin (Gibco
BRL) or by electroporation (Meulenberg et al., 1998a).

**Table 1. Primers used to introduce deletions by PCR and to sequence the introduced mutations**

The orientation of the primers (+ and — for sense and antisense) and the location of each primer with respect to the nucleotide sequence of LV
(GenBank accession no. M90262) are indicated. Restriction sites are underlined.

<table>
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<tr>
<th>Primer</th>
<th>Sequence (5’→3’)</th>
<th>Orientation</th>
<th>Purpose*</th>
<th>Location</th>
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* Use of a primer in plasmid construction is indicated by the plasmid name.
Infection of PAMs. To rescue infectious virus, the culture supernatant of BHK-21 cells was harvested 24 h after transfection and 200 µl of this culture supernatant was used to inoculate PAMs. After 1 h, the inoculum was removed and fresh culture medium was added. Approximately 15 h after infection, the culture supernatant was harvested and PAMs were washed with PBS, dried and stored at −20 °C until the immunoperoxidase monolayer assay was performed.

Immunoperoxidase monolayer assay (IPMA). Immunostaining of BHK-21 cells and PAMs was performed by methods described previously (Wensvoort et al., 1986). MAbs against GP$_3$, GP$_4$ (122.14), GP$_5$ (122.1) and the M protein (126.3) (van Nieuwstadt et al., 1996) and against the different antigenic domains of the N protein (138.22 (domain A), 126.9 (domain B), 126.15 (domain C) and 122.17 (domain D); Meulenberg et al., 1998b) were used to detect expression of PRRSV proteins.

Genetic analysis of genomic RNA of recombinant viruses. In order to analyse the viral RNA in the culture supernatant of PAMs and in the fractions of the sucrose gradient, 200 µl of the culture supernatant or of the fraction was diluted with an equal volume of proteinase K buffer (100 mM Tris–HCl, pH 7.2, 25 mM EDTA, 300 mM NaCl, 2% w/v SDS) and 0.08 mg proteinase K was added. After incubation for 30 min at 37 °C, the RNA was extracted with phenol–chloroform and precipitated with ethanol. The RNA was reverse-transcribed with primer LV76 and PCR was performed with primers 119R218R and LV20, which flank the region of the viral genome that contained the deletions. Amplified fragments were analysed in 2% agarose gels and the PCR fragments were excised from the gel and purified with SpinX columns (Costar). Sequence analysis of the fragments was performed using the antisense primer of the PCR.

Radioimmunoprecipitation (RIP). Metabolic labelling and immunoprecipitation of proteins expressed in PAMs were performed essentially as described previously (Meulenberg & Petersen-den Besten, 1996). MAb 122.17 was used to immunoprecipitate the N protein. PAMs were infected with passage 5 of the viruses at an m.o.i. of 1 and were labelled for 4 h with Tran[35-S] label (ICN) at 15 h post-infection. Samples were analysed by SDS–PAGE using a 14% acrylamide gel.

Virus concentration and purification. In order to analyse the production of (non-infectious) virus particles, BHK-21 cells were electroporated with RNA transcripts from pABV747 and pABV437 and, 15 h after transfection, the cells were labelled metabolically with 75 µl of 10·5 µCi/ml Tran[35-S] label (ICN) for 24 h (Meulenberg & Petersen-den Besten, 1996). The particles in the supernatant were concentrated by centrifuging the supernatant through a 0–20% sucrose gradient (van Berlo et al., 1982). The sucrose gradient was centrifuged at 32000 r.p.m. for 19 h at 4 °C. Fractions of 0·5 ml were collected from bottom to top and 5 µl aliquots of the fractions were analysed by SDS–PAGE using a 14% acrylamide gel.

Results

Sequence comparison of the N proteins and 3′ UTRs of PRRSV strains LV and VR2332

Since PRRSV is an RNA virus with a very concise genome, most of its genetic information is expected to be essential. Therefore, genomic cDNA clones containing deletions, especially in the conserved regions, generally do not produce infectious transcripts (M. H. Verheije, unpublished results). In order to identify regions of heterogeneity, where deletions might be tolerated, sequence comparisons were performed. The ORF7 gene at the 3′ end of the LV genome was selected because this ORF does not overlap with other ORFs. Amino acid alignments of the N protein sequence encoded by ORF7 of the prototype European strain (Lelystad virus, LV) and the prototype North American strain (VR2332; Murtaugh et al., 1995) showed 60% overall identity (recently reviewed by Dea et al., 2000). At the C terminus of the N protein, the amino acid sequence is highly conserved up to residue 119 of LV. Downstream of this conserved region, a short stretch without amino acid conservation occurs. In addition, the C-terminal part of the N protein of LV is four amino acids longer than that of VR2332 (Fig. 1A). It was therefore anticipated that deletions in the heterogeneous C terminus of the N protein of LV might be tolerated, and this region was selected as a target to introduce deletions.

Further nucleotide sequence comparison of the 3′ UTR downstream of ORF7 also revealed interesting differences (Allende et al., 1999). The 11·5′-most nucleotides of the 3′ UTR of LV showed no identity to the first nucleotides of VR2332 (Fig. 1B). Directly downstream of these nucleotides, a stretch of 38 nucleotides is present in VR2332 that has no counterpart in LV. In contrast, high sequence conservation was observed further downstream. In view of this heterogeneity, the region directly downstream of the stop codon of ORF7 was also selected as a target site for deletion studies.

LV accepts C-terminal truncations of up to six amino acids of the N protein

cDNA clones with deletions in the sequence encoding the two (pABV639), four (pABV694) and nine (pABV695) C-terminal amino acids of the N protein were constructed by PCR mutagenesis and cloning of the PCR fragments into an infectious cDNA clone of LV containing a PacI site at the stop codon of ORF7 (Meulenberg et al., 1998a) (Fig. 2). RNA transcripts of these constructs were transfected into BHK-21 cells and tested for their ability to replicate by analysing the expression of the structural proteins in IPMA (Fig. 2). All transcripts expressed the viral proteins GP$_3$, GP$_4$ and M. In order to analyse the expression of the N protein, and in particular its antigenic domains (Meulenberg et al., 1998b), we used MAbs 138.22, against antigenic domain A, 126.9, against domain B, 126.15, against domain C, and 122.17, against domain D of the protein in IPMA. For all constructs, we found that the transfected cells could be stained with each of the MAbs. These results indicated that LV genomes containing deletions at the C terminus of the N protein still replicated and that the structural proteins were properly translated. In addition, these deletions did not disturb the antigenic domains of the N protein.

In order to investigate whether the LV mutants with a C-terminally truncated N protein produced infectious virus, we inoculated PAMs with culture supernatants of the transfected
BHK-21 cells, as PRRSV cannot infect BHK-21 cells. Twenty-four h later, the cells were fixed and stained with PRRSV-specific Mabs. PAMs inoculated with the supernatant of BHK-21 cells transfected with transcripts of pABV437, pABV639 or pABV694 stained positive. In contrast, no staining of PAMs was observed after inoculation with supernatant from BHK-21 cells transfected with RNA transcripts from pABV695. In conclusion, LV mutants producing an N protein with a C-terminal deletion of up to four amino acids produced infectious virus, whereas mutants producing an N protein with a C-terminal deletion of nine amino acids did not produce infectious virus at all.

In order to define further the acceptable limits of truncation of the N protein, we made stepwise deletions in the region encoding the five to eight most C-terminal amino acids. The fragments generated by PCR mutagenesis were again intro-
duced into pABV437, resulting in pABV745, 746, 747 and 748, encoding N proteins lacking five, six, seven and eight C-terminal amino acids (Fig. 2). Transfection of their RNA transcripts into BHK-21 cells resulted in the expression of the structural proteins for all constructs, as detected by IPMA. After infection of PAMs with the culture supernatant of the transfected BHK-21 cells, we only detected expression of the structural proteins for vABV745 and vABV746. For mutants lacking the region encoding the C-terminal seven amino acids or more, no staining was observed in IPMA. These results indicate that the maximum region that can be deleted at the 3' end of ORF7 without abolishing the production of infectious virus comprises 18 nucleotides, encoding the six C-terminal residues of the N protein. The virus produced by this deletion mutant (vABV746) was found to express the set of N protein epitopes, as demonstrated by using our panel of MAbs (data not shown).

Deletion of seven but not of 32 nucleotides at the 5' end of the 3' UTR of LV is tolerated

In view of the observed nucleotide sequence variation in the 3' UTR of the PRRSV genome downstream of ORF7 (Fig. 1B), we also investigated how deletion of these nucleotides would affect the infection process. Deletions were again introduced by PCR mutagenesis and the PCR fragments were introduced into pABV437, directly behind the PaeI site at the stop codon of ORF7. The first four nucleotides of the 3' UTR were left intact, as they are part of this PaeI site. This resulted in the plasmids pABV693, which has a deletion of seven nucleotides, and pABV729, in which a deletion of 32 nucleotides occurs at the 5' end of the 3' UTR. BHK-21 cells transfected with transcripts of pABV693 expressed the structural proteins. However, BHK-21 cells transfected with transcripts of pABV729 did not express these structural proteins to levels detectable by IPMA, suggesting that RNA replication and/or transcription did not occur. Subsequent infection of PAMs with the culture supernatant of the BHK-21 cells that had been transfected with pABV693 showed expression of the structural proteins in IPMA 24 h after infection. These results demonstrated that at least seven nucleotides at the 5' end of the 3' UTR are dispensable for the virus to remain infectious.

Analysis of the stability and growth characteristics of vABV746 and vABV693 in vitro

In order to investigate whether the deletions in the viruses generated from pABV746 and pABV693 were maintained stably in vitro, these viruses were serially passaged on PAMs. After five passages, viral RNA was isolated from the culture supernatant and studied by genetic analysis. The RNA was reverse-transcribed and the region flanking the introduced deletions was amplified by PCR. Sequence analysis of the fragments showed that the introduced deletion was still present in both cases (data not shown) and that no additional mutations had been introduced in the flanking regions. These results indicated that the deletions had been maintained stably during in vitro passaging on PAMs.

The growth characteristics of viruses vABV746 and vABV693 were investigated by determining their growth curves and comparing them with that of wild-type vABV437. PAMs were infected with viruses from passage 5 at an m.o.i. of 0.05 and virus was harvested at the indicated time-points. Virus titres were determined by end-point dilution on PAMs (Wensvoort et al., 1986).

Fig. 3. Growth curves in PAMs of wild-type virus vABV437 (●) and of mutant viruses vABV693 (♦) and vABV746 (×). PAMs were infected in duplicate with passage 5 of the indicated viruses at an m.o.i. of 0.05 and virus was harvested at the indicated time-points. Virus titres were determined by end-point dilution on PAMs (Wensvoort et al., 1986).

Fig. 4. Analysis of the N proteins expressed by the wild-type virus vABV437 and by the six-amino-acid N protein-deletion mutant vABV746. Proteins were immunoprecipitated from lysates of PAMs infected with passage 5 of vABV437 and vABV746. Labelling was performed for 4 h starting at 15 h after infection. The immunoprecipitated proteins were analysed by SDS-PAGE in a 14% acrylamide gel. Molecular masses of the marker proteins are indicated on the left.
differences in growth rates could be observed between recombinant viruses and wild-type virus.

**Analysis of the truncated N protein of vABV746**

In order to confirm the effect of the deletion at the protein level, the size of the N protein expressed by the recombinant virus vABV746 was analysed by immunoprecipitation. FAMs were infected and labelled metabolically with 35S-amino acids and cell lysates were prepared. The N protein in the lysates was precipitated with MAb 122.17, which is directed against the D domain of the protein, and analysed by SDS-PAGE. As expected, an N protein of wild-type size (15 kDa) was immunoprecipitated from lysates of cells transfected with vABV437 (Fig. 4), whereas a protein with an estimated size of 14 kDa was immunoprecipitated from vABV746-infected cell lysates (Fig. 4). The smaller N protein expressed by vABV746 is consistent with the six-residue truncation compared with wild-type vABV437.

**Analysis of particle assembly after N protein truncation**

The abrupt transition in viability upon deletion of more than six C-terminal amino acids may occur for various reasons. We analysed whether the life cycle of the virus was disturbed at the stage of virus assembly. To this end, BHK-21 cells were transfected with RNA transcripts from pABV747 and proteins were labelled for 24 h starting at 15 h after transfection. Virus particles released into the culture supernatant were concentrated and fractionated as described in Methods. (A) Proteins in the fractions were analysed by 14% SDS-PAGE. (B) RNA was isolated from the fractions and analysed by RT–PCR, for which the primers flanked the region in which the deletion was introduced. The sizes of the markers are indicated on the left. Lane +, positive PCR control.

![Image](https://via.placeholder.com/150)

(5 B). In each of these fractions, two PCR fragments were observed, in contrast to the lane in which we used cDNA of pABV437 as a template. The second band derived from the fractions could not be identified, however. It might have been derived from deleted, packaged RNAs, resulting in the production of heteroclite sg mRNAs (Yuan et al., 2000). The absence of virus particles or virus-like particles produced by transcripts of pABV747 suggests that virus assembly is disturbed in this mutant.

**Discussion**

With the eventual aim of obtaining live attenuated PRRSV vaccine candidates, this study describes the construction and analysis of several virus deletion mutants. In view of the genetic variability at the 3′ end of the PRRSV genome, we have tested the effect of deletions in this variable region. We report that constructs lacking the coding sequence for up to six C-terminal amino acids of the LV N protein still yielded infectious virus after transfection of their transcripts into BHK-21 cells. In contrast, further deletions were fully detrimental: the removal of just one additional residue abolished the production of viable virus completely. Furthermore, deletions were also tolerated directly downstream of the stop codon of ORF7. At least seven nucleotides in this region were dispensable for virus production; removal of 32 nucleotides was, however, fatal. Both the virus with a six amino acid truncation of the N protein and the virus with the seven nucleotide deletion in the 3′ UTR had in vitro growth characteristics and antigenic profiles similar to those of wild-type virus. Moreover, these viruses were both genetically stable.

The dramatic effect of truncation at the seventh residue of the LV N protein was quite surprising and was not predicted by the sequence. The sequence of the nine C-terminal residues of the LV N protein is very different from that of the VR2332 isolate except for its high content of hydroxy amino acids. In the LV and VR2332 N proteins, six of ten and three of six residues, respectively, at the very C terminus are serines or threonines. The functions of this domain and of these particular residues are unknown. Two other arteriviruses, LDV and...
SHFV, also contain hydroxy amino acids at the extreme C terminus of their N proteins, namely 3/10 and 4/10 amino acids, respectively. In contrast, hydroxy amino acids are completely absent from the last ten amino acids of the EAV N protein. While coronavirus N proteins do generally have a relatively high serine content (7–11%; Masters & Sturman, 1990), the proportion of serines and threonines at their C termini is quite insignificant; in these viruses, this region is markedly acidic. Obviously, these variable characteristics do not allow predictions about the role of the C terminus of the N protein in the virus life cycle. The truncated N protein had the same antigenic profile as that of the wild-type N protein, since it reacted with all MAb s directed against antigenic domains of the N protein. This is consistent with observations by Meulenbergen et al. (1998b), who identified that domain D, the most C-terminal domain of N, is a conformation-dependent or discontinuous epitope that involves amino acids 51–67 and 80–90. Virus particle production appeared to be blocked after truncation of the LV N protein by seven amino acids. This indicates strongly a defect at the level of virus assembly. For a Canadian PRRSV isolate, it has been demonstrated that non-covalent interactions between the C-terminal regions of N proteins are critical for formation of the isometric capsid protein (Wootton & Yoo, 1999). In a system expressing only the N protein, they showed that the last 11 amino acids were involved in these interactions. This might indicate that the C terminus of PRRSV is essential for nucleocapsid formation. Our study supports this idea. Other effects of C-terminal truncation of the N protein can, however, not be excluded, as the N protein has been implicated in various other processes, such as interaction with the viral RNA [Dea et al., 2000; for mouse hepatitis virus (MHV), see Cologna & Hogue, 1998; Molenkamp & Spaan, 1997] and interaction with other viral proteins (for MHV, see Narayan et al., 2000). Since it has been described for MHV, the best-studied coronavirus, that a 29 amino acid deletion in the putative spacer region preceding the C-terminal domain of the N protein resulted in temperature-sensitive and thermolabile viruses (Peng et al., 1995), we investigated whether our deletion mutants had similar characteristics; they appeared not to have these characteristics. Moreover, infectious virus was still not produced from the deletion mutants expressing truncated N proteins lacking seven amino acids or more after the incubation temperature was lowered to 30 °C. In an earlier study, we demonstrated that extension of the C terminus of the N protein by a nine amino acid sequence of the influenza virus HA protein significantly impaired virus growth (Groot Bramel-Verheije et al., 2000). We could not establish whether this was caused by disturbance of virus assembly or disassembly, however. Again, these observations are consistent with the C-terminal region of the LV N protein being involved in N–N interactions essential for the production of nucleocapsids during virus assembly. RNA viruses have at their termini non-coding sequences that play essential roles in RNA replication and sg mRNA transcription. Mutations in these domains are likely to affect the virus life cycle. Consistently, when we introduced deletions in the 5′-terminal region of the LV 3′ UTR, we found that removal of a small seven-nucleotide variable sequence was accepted, while removal of a somewhat larger, 32-nucleotide stretch was not. From the inability of the RNA transcripts to express the M and N proteins, we conclude that the defect probably resides in an effect on RNA replication or sg mRNA transcription. This suggests that this region of the 3′ UTR contains an essential RNA signal. Our results are in accordance with studies on coronaviruses, which showed that the 5′ terminus of the 3′ UTR is essential in the initial processes of the virus life cycle (Hsue et al., 2000). No host or virus protein was found to bind this region of the viral RNA specifically. However, the exact function of this region remains to be elucidated. Since deletion of the 3′-most 27 nucleotides of the N gene did not abolish RNA replication, this region apparently does not contain a replication signal similar to the one observed within the 3′-terminal region of the EAV N gene (Molenkamp et al., 2000).

In this study, we aimed to generate viable PRRSV mutants with maximal deletions at the target site. The viruses obtained were characterized in vitro and fulfilled the most important requirements; good growth and genetic stability. Because their growth characteristics in vitro on PAMs were identical to those of wild-type virus, virus production for in vivo studies can be accomplished easily. The growth characteristics in vitro do not necessarily correlate with or predict the behaviour of the virus in vivo. Thus, many vaccines used currently are attenuated in vivo, but show no differences in propagation in vitro (Yang et al., 1998). Therefore, only animal experiments will tell how these viruses behave in vivo, whether they are sufficiently attenuated and whether they induce immune responses that will protect against infection with virulent PRRSV.

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