Reversion of a live porcine reproductive and respiratory syndrome virus vaccine investigated by parallel mutations

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A live attenuated porcine reproductive and respiratory syndrome (PRRS) vaccine virus has been shown to revert to virulence under field conditions. In order to identify genetic virulence determinants, ORF1 from the attenuated vaccine virus and three Danish vaccine-derived field isolates was sequenced and compared with the parental strain of the vaccine virus (VR2332). This revealed five mutations that had occurred independently in all three vaccine-derived field isolates, indicating strong parallel selective pressure on these positions in the vaccine virus when used in swine herds. Two of these parallel mutations were direct reversions to the parental VR2332 sequence and were situated in a papain-like cysteine protease domain and in the helicase domain. The remaining parallel mutations might be seen as second-site compensatory mutations for one or more of the mutations that accumulated in the vaccine virus sequence during cell-culture adaptation. Evaluation of the remaining mutations in the ORF1 sequence revealed stronger selective pressure for amino acid conservation during spread in pigs than during vaccine production. Furthermore, it was found that the selective pressure did not change during the time period studied. The implications of these findings for PRRS vaccine attenuation and reversion are discussed.

Introduction

Live attenuated virus vaccines are used worldwide in both humans and animals. Although this kind of vaccine is generally considered safe, reported cases of reversion to virulence have highlighted the inherent safety problems in the use of traditional live attenuated vaccines (Cizman et al., 1989; Christensen et al., 1992; Minor, 1993; Xie et al., 1998; Huang et al., 1999).

A new vaccine, which seems to have reverted to virulence under farm conditions, is a live attenuated vaccine against porcine reproductive and respiratory syndrome (PRRS) (Bøtner et al., 1997; Madsen et al., 1998; Storgaard et al., 1999). This vaccine is based on a cell-culture adaptation of the pathogenic North American VR2332 isolate (Collins et al., 1992; Allende et al., 2000). In the autumn of 1996, this vaccine was used almost simultaneously in more than 1100 Danish swine herds to vaccinate 3- to 18-week-old pigs. None of these swine herds had PRRS-associated reproductive problems at the time of vaccination. However, within 1–2 months after vaccination, the majority of the herds experienced a significantly increased number of abortions and weak or stillborn piglets (Bøtner et al., 1997; Mortensen et al., 1998), but no PRRS-associated respiratory problems were observed in the vaccinated piglets. Porcine reproductive and respiratory syndrome virus (PRRSV) was isolated from such weak or stillborn piglets and was characterized by monoclonal antibody (MAb) typing, type-specific PCR and by sequencing of ORF5 and ORF7 as having originated from the live vaccine virus (Bøtner et al., 1997; Oleksiewicz et al., 1998; Storgaard et al., 1999). Furthermore, one such vaccine-derived field isolate was shown directly to cause reproductive disorders by experimental inoculation (Nielsen et al., 1998). Thus, the attenuated vaccine virus had spread to the non-vaccinated sows, changed genetically and reverted to virulence. Not only did the vaccine virus spread...
within the vaccinated swine herds, it also spread from the vaccinated to non-vaccinated herds. PRRSV is a positive-sense, single-stranded RNA virus with a genome of approximately 15 kb. The genome contains, in the 5’-to-3’ direction, a cap structure, a 5’-non-translated leader, two large open reading frames (ORF1a and ORF1b) encoding the non-structural polyproteins, six smaller ORFs (ORF2–ORF7) encoding the structural proteins, a 3’ non-translated region (3’-NTR) and a poly(A) tail (Meulenberg et al., 1993; Snijder & Meulenberg, 1998). The aim of the present work was to identify mutations potentially involved in the attenuation of the live PRRS vaccine virus and the subsequent reversion to virulence observed under field conditions. Previously, we have reported the sequences of the 5’-leader and ORF2–ORF7 of the attenuated vaccine virus and vaccine-derived field isolates (Madsen et al., 1998; Oleksiewicz et al., 1999; Storgaard et al., 1999). The present article describes the sequencing of ORF1 and the 3’-NTR of the vaccine virus and vaccine-derived field isolates and the subsequent identification of mutations. To obtain the most precise picture of genetic attenuation/virulence determinants, the complete genomic sequence of the vaccine virus was compared not only with its parental strain (VR2332), but also with several vaccine revertants.

Methods

- **Virus isolates.** The live attenuated Ingelvac PRRS Vet vaccine (Boehringer Ingelheim) is a cell culture-adapted, non-pathogenic derivative of the pathogenic North American VR2332 isolate (Mengeling et al., 1996; Allende et al., 2000). The vaccine virus (serial no. 245-183A) was used as supplied by the manufacturer without any further passage in cell culture.

  The vaccine-derived field isolates 17704A, 17733B, 18680, 19020 and 21317 originated from weak or stillborn piglets in five different Danish swine herds where the Ingelvac PRRS Vet vaccine had been used solely in weaned pigs. Isolates 17704A, 17733B, 18680 and 19020 were all made within 2–4 months after vaccination, while isolate 21317 was made 5 months later. Together, these five isolates represented the genetic changes incurred by the PRRS vaccine virus during spread within herds. Isolates 19407B and 19781 originated from stillborn piglets in two different non-vaccinated swine herds and were both isolated about 6 months after the Danish vaccination programme had started. Thus, these two isolates represented, in addition, the genetic changes incurred by the PRRS vaccine virus while spreading between herds. All seven field isolates have previously been characterized as originating from the vaccine virus by MAB typing as well as ORF5 and ORF7 sequencing (Bøtner et al., 1997; Storgaard et al., 1999). One of the isolates, 19407B, has been shown in addition to cause reproductive disorders in experimental infection in gilts (Nielsen et al., 1998). The vaccine-derived isolates were propagated for one to three passages on MARC-145 cells and all had titres above 10^9 TCID_{50}/ml.

- **RT–PCR.** RT–PCR was performed essentially as described previously (Oleksiewicz et al., 1998; Nielsen et al., 2000). To amplify and subsequently sequence the complete genomic sequence, 35 new PCR primer pairs were designed. Each primer pair amplified a PCR fragment of about 400 nt, overlapping each neighbouring fragment by about 200 nt. All PCR primers located in ORF1 were chosen in areas that are conserved between the sequences previously published for American-type PRRSV isolates (Nelsen et al., 1999; Allende et al., 1999). PCR primers covering ORF5 and ORF7 have been published previously (Oleksiewicz et al., 1998). PCR primers covering ORF2–ORF4 and ORF6 were designed according to the previously published vaccine virus sequence (Madsen et al., 1998). All PCR primer sequences are available from the authors on request. Throughout this study, nucleotide A of the ORF1 start codon of VR2332 (GenBank accession no. PRU87392) is considered nucleotide position 1.

  The complete ORF1 was amplified by PCR from the attenuated vaccine virus. The complete genomic sequence was amplified from three vaccine-derived field isolates, 17704A, 17733B and 19407B. The four vaccine-derived field isolates 18680, 19020, 19781 and 21317 were amplified by PCR only in selected areas of ORF1 in order to confirm or reject the presence of parallel selective pressure.

- **3’ RACE.** The 3’-NTRs of the vaccine virus and selected pathogenic vaccine-derived field isolates were amplified by 3’ RACE. Briefly, reverse transcription was performed on total RNA with Moloney murine virus reverse transcriptase as described above, but with oligo(dT)_{16} as the primer (Ambion). The subsequent PCR amplification was performed with a specific forward primer (5’-TTAATCAAGGCGCTGGGA 3’) situated in ORF7 and a poly(A) primer with a degenerate 3’ end (BA_{36}) as the reverse primer.

- **DNA sequencing and sequence analysis.** The PCR amplicons were gel-purified before cycle sequencing and sequenced in both directions by using the PCR primers, while the 3’-NTR fragments were sequenced in one direction using a nested forward primer (5’-CTCTCAGATTCAAGGGAGGA 3’). Cycle sequencing was performed with fluorescent BigDye chain terminators (Applied Biosystems) and sequencing products were electrophoresed on an ABI Prism 310 Genetic Analyser (Applied Biosystems).

  The sequences of the individual amplicons were assembled by using the computer program SeqMan II (DNASTAR). Secondary protein structure was predicted by using the Predict Protein software (Rost, 1996). Mapping of PRRSV mutations onto related proteins with solved tertiary structure was done with the Cn3D 2.5 software (Hogue, 1997).

- **Statistical analysis of mutation patterns.** In order to identify mutations associated with virulence/attenuation in PRRSV, the ORF1 sequences from the live attenuated vaccine and the pathogenic vaccine-derived field isolates were aligned. The alignment was searched for single nucleotide positions that were identical between the three field isolates but which differed from the vaccine virus. Such patterns were defined as ‘parallel’. In Fig. 1, all 48 theoretically possible parallel patterns are indicated. If they were found in the actual alignment (Fig. 2 and Table 1), such sites were considered as candidates to be involved in reversion towards virulence. In order to identify the most important candidates among the parallel substitutions, the previously published ORF1 sequence of VR2332 (GenBank accession no. PRU87392) was included in the alignment and the parallel substitutions were searched for those where the field isolates had the same sequence as VR2332. Such single nucleotide substitutions were defined as ‘parallel reversions’. In Fig. 1, the 12 theoretically possible parallel reversal patterns are indicated by asterisks.

In order to investigate whether the observed number of parallel substitutions was significantly higher than would be expected by chance, we used the framework of Goldman (1993). With the PAUP* program (Swofford, 2000), an HKY+Γ substitution model (Hasegawa et al., 1985; Yang, 1993) was fitted to the data by using the genealogy shown in Fig. 4 and the observed branch lengths. This models the process of nucleotide substitution under the assumption that evolution is in-
Reversion investigated by parallel mutations

<table>
<thead>
<tr>
<th>VR 2332</th>
<th>ACGT ACGT ACGT ACGT ACGT ACGT ACGT ACGT ACGT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaccine</td>
<td>AAAA AAAA AAAA CCCC CCCC CCCC GGGG GGGG GGGG TTTT TTTT TTTT</td>
</tr>
<tr>
<td>Vaccine revertant 1</td>
<td>CCCC GGGG TTTT AAAA GGGG TTTT AAAA CCCC TTTT AAAA CCCC GGGG</td>
</tr>
<tr>
<td>Vaccine revertant 2</td>
<td>CCCC GGGG TTTT AAAA GGGG TTTT AAAA CCCC TTTT AAAA CCCC GGGG</td>
</tr>
<tr>
<td>Vaccine revertant 3</td>
<td>CCCC GGGG TTTT AAAA GGGG TTTT AAAA CCCC TTTT AAAA CCCC GGGG</td>
</tr>
</tbody>
</table>

Fig. 1. The 48 theoretically possible patterns of parallel mutations that could potentially occur in an alignment of five model sequences are shown. The 12 theoretically possible parallel reversion patterns are indicated by asterisks. Five of the 48 theoretical parallel patterns (indicated by arrows) were actually observed by us in ORF1 (Table 1). Under the null model (HKY + Γ), the probability of observing a given number \( n_{pp} \) of parallel patterns (pp) in the alignment is given by the binomial distribution \( b(n_{pp}, A, P_{pp}) \), where \( A \) is the number of sites in the alignment and \( P_{pp} \) (the probability of observing a parallel pattern under the null model) is obtained by summing the probability of all the above 48 parallel patterns \( (x) \) according to the formula

\[
P_{pp} = \sum_{k=0}^{n_{pp}} P(x) = 7.1 \times 10^{-7}.\]

The probability under the null model of observing a value \( n_{pp} \) equal to or greater than the number of observed parallel patterns \( n_{obs} \) is then given by \( P(n_{pp} \geq n_{obs}) = 1 - \sum_{k=0}^{n_{obs}} P(n_{pp} = k) \leq 0.05 \), where \( P(n_{pp} = k) \) is given by \( b(k, A, P_{pp}) \). For the five patterns observed in the dataset (Table 1 and Fig. 2), this yielded \( P(n_{pp} \geq 5) \approx 10^{-5} \), indicating that an excess of parallel substitutions occurred due to similar evolutionary forces acting on the different revertant strains.

Dependent between different lineages in the genealogy; i.e. any selection pressure affects the evolution of all sequences equally and independently. Thus, the substitution model does not take into account the effect of evolution acting to produce patterns of parallel substitutions, but such patterns may nevertheless arise by chance. This provided a null model against which to compare the observed number of parallel patterns. Under this null model, we then calculated the probability \( (P) \) of getting an equal or larger number of parallel patterns than observed in the alignment. If this probability was small \( (P < 0.05) \), we concluded that there was an excess amount of parallel evolution in the sample that could not be ascribed to chance alone, but must be ascribed to similar evolutionary forces acting on the different virus strains (Fig. 1).

In order to investigate the type of selective pressure affecting the substitution process, the ratio between the rates of non-synonymous substitution (dN) and synonymous substitution (dS) was estimated using a codon-based model of substitution (Goldman & Yang, 1994) implemented in the codonml program of the PAML program package (Yang, 1997). For this analysis, four codons had to be excluded from the alignment due to a deletion in the 19407B sequence.

**Results**

**PCR amplification and alignment of ORF1**

The complete ORF1 and the 3'-NTR were sequenced from the live attenuated vaccine virus, as supplied by the manufacturer. In addition, the complete genomes were sequenced of three pathogenic vaccine-derived field isolates, all originating from weak or stillborn piglets. One of the isolates (19407B) was found previously to be pathogenic by experimental infection in late-term gilts (Nielsen et al., 1998). All of the sequences were obtained without any cloning in order to circumvent any sequence errors introduced by the PCR.

Isolates 17704A and 17738B, both originating from vaccinated herds, respectively had 26 and 27 nt changes in ORF1 when compared with the vaccine virus. Of these mutations, 11 resulted in amino acid changes (Fig. 2). Isolate 19407B, originating from a herd that had never been exposed to the vaccine, had 51 mutations in ORF1, of which 19 encoded amino acid changes (Fig. 2). Comparing the vaccine virus ORF1 sequence with VR2332 revealed 20 nt changes, of which 13 resulted in amino acid changes (Fig. 2). No mutations at all were found in the 3'-NTR sequence. Besides these point mutations, one deletion was found in isolate 19407B, at nt 3528–3539, which removed four amino acids without affecting the reading frame of ORF1a (Fig. 2).

**Parallel evolution**

Five of the above-mentioned point mutations were found in all three vaccine-derived field isolates, when compared with the vaccine virus ORF1 sequence. These five mutations were situated at codon positions 331, 760, 1505, 2052 and 3449 (Fig. 2 and Table 1). The three virus isolates were submitted to our laboratory from three different regions of Denmark (Ringsted, Nordborg and Silkeborg), all within 6 months after the vaccination was performed. The geographical distance and the short time-span effectively exclude virus transmission between these three swine herds. The five above-mentioned mutations might therefore indicate strong parallel selection on the vaccine virus when used in swine herds. Alternatively, this pattern of parallel mutations could theoretically have happened by chance. In order to evaluate whether the observed number of parallel mutations was significantly higher than that expected by chance alone, we calculated the probability of observing five or more parallel mutations in the alignment by using the estimated parameters of the chosen HKY + Γ model and the observed branch lengths in the phylogenetic tree. This probability was significantly less than \( 10^{-6} \) (see the legend to
Fig. 2. Summary of point mutations in PRRSV ORF1. The sequence of the attenuated vaccine virus was compared with the VR2332 sequence (the parental, pathogenic strain of the vaccine) and the sequences of individual vaccine-derived field isolates (17704A, 17738B and 19407B) were compared with the vaccine virus sequence. Vertical lines mark individual mutations. Non-synonymous mutations (NS) are above and synonymous mutations (S) are below the horizontal line. Five of the point mutations identified in ORF1 were found in all three vaccine-derived field isolates. Dashed lines indicate these parallel mutations and the resulting nucleotide changes are shown in capital letters. Two of the parallel mutations represent reversions to the VR2332 sequence. These were situated in the pcp domain of nsp1b (codon position 331) and the putative helicase domain (Hel) of nsp10 (codon position 3449). In isolate 19407B, a deletion of four amino acids situated in nsp2 was found. Filled arrowheads and numbers indicate predicted proteolytic cleavage sites in the ORF1 polyproteins.

Table 1. Parallel mutations identified in PRRSV ORF1 sequences

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Pathogenicity*</th>
<th>Codon position in ORF1</th>
</tr>
</thead>
<tbody>
<tr>
<td>VR2332</td>
<td>+</td>
<td>331: TCC (Ser) 760: CGG (Arg) 1505: AAG (Lys) 2052: CCA (Pro) 3449: TAC (Tyr)</td>
</tr>
<tr>
<td>Vaccine virus</td>
<td>-</td>
<td>331: TTC (Phe) 760: TGG (Trp) 1505: AAG (Lys) 2052: CCA (Pro) 3449: CAC (His)</td>
</tr>
<tr>
<td>17704A</td>
<td>(±)</td>
<td>331: TTC (Ser) 760: TGG (Trp) 1505: GAG (Glu) 2052: CCT (Pro) 3449: TAC (Tyr)</td>
</tr>
<tr>
<td>17738B</td>
<td>(±)</td>
<td>331: TTC (Ser) 760: TGG (Trp) 1505: GAG (Glu) 2052: CCT (Pro) 3449: TAC (Tyr)</td>
</tr>
<tr>
<td>19407B</td>
<td>+</td>
<td>331: TTC (Ser) 760: TGG (Trp) 1505: GAG (Glu) 2052: CCT (Pro) 3449: TAC (Tyr)</td>
</tr>
<tr>
<td>18680</td>
<td>(±)</td>
<td>331: TTC (Ser) 760: TGG (Trp) 1505: AAG (Lys) 2052: CCT (Pro) 3449: TAC (Tyr)</td>
</tr>
<tr>
<td>19020</td>
<td>(±)</td>
<td>331: TTC (Ser) 760: TGG (Trp) 1505: GAG (Glu) 2052: CCT (Pro) 3449: TAC (Tyr)</td>
</tr>
<tr>
<td>19781</td>
<td>(±)</td>
<td>331: TTC (Ser) 760: TGG (Trp) 1505: GAG (Glu) 2052: CCT (Pro) 3449: TAC (Tyr)</td>
</tr>
<tr>
<td>21317</td>
<td>(±)</td>
<td>331: TTC (Ser) 760: TGG (Trp) 1505: GAG (Glu) 2052: CCT (Pro) 3449: TAC (Tyr)</td>
</tr>
</tbody>
</table>

* Scored as: +, isolate has been shown to be pathogenic by experimental infection (Collins et al., 1992; Nielsen et al., 1998); (±), isolate originated from weak or stillborn piglets; -, isolate has been shown to be non-pathogenic (Mengeling et al., 1996).
Reversion investigated by parallel mutations

Fig. 3. The top schematic drawing represents a hypothetical superfamily 1 helicase with the canonical motifs 1, 1a, TxGx, 2, 3, 5 and 6 (Gorbalenya & Koonin, 1993; Kadare & Haenni, 1997). The putative helicase domains of PRRSV isolates were aligned against the E. coli Rep helicase, for which the tertiary structure has been solved. Based on this alignment, the reversion (asterisk) could be located to a beta-sheet in the non-conserved region between helicase motifs 3 and 5. Abbreviations: Rep, E. coli Rep helicase; VR2332, the parental strain of the vaccine; Vaccine, live attenuated Ingelvac PRRS Vet vaccine; Field isolates; Danish PRRSV vaccine-derived field isolates (17704A, 17738B, 18680, 19202, 19407B, 19781 and 21317). The symbols used to represent alpha-helices, beta-sheets, loops and deletions are indicated.

Fig. 1). As such, it could be concluded that there has been strong parallel selective pressure on the vaccine virus at these particular codon positions during passage in pigs under farm conditions. To investigate this even further, four additional vaccine-derived field isolates (18680, 19020, 19781 and 21317) from swine herds with reproductive problems after vaccination were sequenced across these five specific codon positions (Table 1). At codon positions 331, 2052 and 3449, all seven vaccine-derived field isolates had undergone exactly the same mutations. At codon position 1505, six of seven vaccine-derived field isolates had exactly the same mutation. In contrast, at codon position 760, the parallel mutation, and thus the strong selective pressure, could not be confirmed, since none of the four additional isolates investigated had mutated at all. The probability of observing a single parallel mutation in the alignment by chance alone was calculated to be greater than 5%. This particular mutation (C → T) at codon position 760 might therefore be seen as a coincidence, resulting from the very high transition bias observed in PRRSV (Oleksiewicz et al., 2000).

One obvious explanation for the observed strong parallel selection at codon positions 331, 2052, 1505 and 3449 would be reversion to the sequence of the parental VR2332 isolate. Indeed, when compared to the previously published VR2332 sequence, two of the parallel mutations (codon positions 331 and 3449) represented direct reversions to the VR2332 sequence. These observations were confirmed further by our own control sequencing of the VR2332 isolate across the five specific codon positions. The two parallel reversions were both situated in areas of ORF1 for which biological functions have been suggested (Meulenberg et al., 1993; Den Boon et al., 1995; Snijder & Meulenberg, 1998).

Parallel reversions

The parallel reversion at codon position 331 encoded a change from phenylalanine in the attenuated vaccine virus to serine in the pathogenic vaccine-derived field isolates. On the basis of amino acid composition, polarity and molecular volume, this is a radical amino acid change (Grantham, 1974). The reversion was situated in the papain-like cysteine protease domain (pcp) of non-structural protein (nsp) 1b (Fig. 2) (Den Boon et al., 1995).

The second parallel reversion at codon position 3449 was situated in the putative helicase domain of nsp10 (Fig. 2) (Meulenberg et al., 1993, 1995). This parallel reversion encoded a moderate conservative amino acid change (Grantham, 1974) from histidine to tyrosine in the vaccine-derived field isolates. For further characterization of this reversion, the PRRSV sequences were aligned to a related helicase motif (E. coli Rep helicase) belonging to the same superfamily as the PRRSV helicase (Fig. 3) (Gorbalenya & Koonin, 1993; Kadare & Haenni, 1997). The tertiary structure of the E. coli Rep helicase has been published.
previously (Korolev et al., 1997). Based on this alignment, the parallel reversion was situated in a beta-sheet in a non-conserved region between motifs 3 and 5 of the helicase domain (Fig. 3) (Gorbalenya & Koonin, 1993; Kadare & Haenni, 1997). This beta-sheet could, based on the tertiary structure of E. coli Rep helicase (data not shown), be located in the RNA-binding domain of the enzyme. The moderate conservative amino acid change identified at codon position 3449 is not predicted to alter this beta-sheet, however.

Even though neither of the parallel reversions could be localized to a functionally described motif within the protease and helicase domains, the fact that all seven pathogenic vaccine-derived field isolates had mutated back independently to the parental VR2332 sequence indicates strongly a biological effect of the mutations identified on protease and helicase function.

The dN/dS ratio

Having observed very strong parallel selection at four or five specific sites in ORF1, it was of interest to evaluate whether all the accumulated point mutations found in the pathogenic vaccine-derived field isolates had mutated back independently to the parental VR2332 sequence indicates strongly a biological effect of the mutations identified on protease and helicase function.

Table 2. Different models of dN/dS ratios

<table>
<thead>
<tr>
<th>Model</th>
<th>ln L*</th>
<th>d.f.</th>
<th>2Δ†</th>
<th>χ²</th>
</tr>
</thead>
<tbody>
<tr>
<td>One dN/dS ratio</td>
<td>-17015·14</td>
<td>6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Two dN/dS ratios</td>
<td>-17012·73</td>
<td>7</td>
<td>4·8</td>
<td>P = 0·03#</td>
</tr>
<tr>
<td>Free dN/dS ratios</td>
<td>-17012·50</td>
<td>9</td>
<td>0·46</td>
<td>P = 0·8</td>
</tr>
</tbody>
</table>

* Likelihood value, the probability of observing the indicated number of dN/dS ratios in the phylogenetic tree.
† The difference in likelihood (ln L) multiplied by two.
‡ Significant (P < 0·05).

derived field isolate 19407B was about twice as great (Fig. 4), which corresponded with an isolation date about 3 months later in this non-vaccinated herd.

By using a codon-based maximum-likelihood method, it is possible to estimate the dN/dS ratio at individual branches in the tree and also to estimate whether the same dN/dS ratio had been in effect at the different branches (Fig. 4) (Yang & Nielsen, 1998; Yang, 1998; Oleksiewicz et al., 2000). The significantly most-likely model to explain the observed mutations assumes different selective pressures on the parental VR2332 strain during attenuation in cell culture and during the later spread of the vaccine virus in pigs (two dN/dS ratios) (Table 2). A
dN/dS ratio of 0.9 was found during attenuation in cell culture (Fig. 4), while a dN/dS ratio of 0.3 was found when the vaccine virus spread in pigs (Fig. 4). As such, there had been significantly stronger selective pressure for amino acid conservation on the vaccine virus sequence during passage in pigs compared with during production of the vaccine. Even though the three vaccine-derived field isolates had different genetic distances from the vaccine virus (Fig. 4), the dN/dS ratios of the branches that connected the individual isolates to the vaccine virus were equal (Fig. 4). This indicated that the selective pressure of the vaccine virus during passage in pigs did not change over the time period studied.

Discussion

Summary of mutations in ORF1 potentially involved in attenuation/virulence

Shortly after a live attenuated PRRS vaccine was used almost simultaneously on 3- to 18-week-old pigs in more than 1100 Danish swine herds, a large proportion of these herds experienced PRRSV-associated reproductive problems such as abortions and weak or stillborn piglets (Botner et al., 1997; Mortensen et al., 1998). Virus isolation and characterization revealed that the live attenuated PRRS vaccine had changed genetically and reverted to virulence in the field, thereby causing reproductive problems (Botner et al., 1997; Madsen et al., 1998; Storgaard et al., 1999).

With the purpose of identifying the genetic determinants involved in the reversion to virulence, the 5'-leader and ORF2–ORF7 of the attenuated PRRS vaccine virus and several vaccine-derived field isolates have been sequenced previously (Madsen et al., 1998; Oleksiewicz et al., 1999; Storgaard et al., 1999). Furthermore, during the preparation of this manuscript, Allende et al. (2000) published the ORF1 sequence from the vaccine virus and compared it with the North American isolate 16244B. The latter isolate is assumed to be a vaccine virus revertant, judged by its high genetic similarity to the vaccine virus and its parental strain (VR2332) (Nelsen et al., 1999). Having only one vaccine virus revertant, however, makes it difficult to discriminate mutations caused by selection from mutations generated at random. Furthermore, the North American isolate 16244B has apparently been circulating for a relatively long time and thus has accumulated many mutations when compared with the vaccine virus. This resulted in a high level of noise when trying to identify mutations responsible for the attenuation of the vaccine virus and the subsequent reversion to virulence. In the current study, these complications were avoided by comparing several independent vaccine virus revertants (vaccine-derived field isolates) that were all isolated relatively shortly after use of the vaccine. Sequencing three such vaccine virus revertants revealed between 20 and 51 point mutations in ORF1, when compared with our own sequencing of the vaccine virus. Five of the mutations were characterized as parallel mutations and were further substantiated by sequencing four additional vaccine virus revertant isolates across these specific sites. Two of the parallel mutations represented direct reversions to the parental VR2332 sequence.

A possible role of the parallel reversions in attenuation/virulence

The two parallel reversions found in all seven vaccine virus revertants have previously been observed in the pathogenic North American vaccine-like isolate, 16244B (Allende et al., 1999). These two parallel reversions are located in areas encoding a papain-like cysteine protease (codon position 331) and the putative viral helicase (codon position 3449) (Meulenberg et al., 1993; Den Boon et al., 1995; Snijder & Meulenberg, 1998). The presence of these two reversions in all eight vaccine-like isolates from two different continents suggests strongly that these are involved in the attenuation of the vaccine virus and the subsequent reversion to virulence. All eight isolates originated from field cases of PRRS and two of the isolates have additionally been proven to be pathogenic in experimental infections (Nielsen et al., 1998; Allende et al., 2000). Despite the fact that neither of these two mutations affects amino acids with known function directly, several mechanisms could be envisaged.

Mutations in a viral protease have been suggested previously to be involved in the attenuation of a live vaccine against Japanese encephalitis (Ni et al., 1995) and might therefore also play a role in the attenuation of the PRRS vaccine. This is supported by the finding that the parallel reversion in the PRRSV papain-like cysteine protease at codon position 331 (phenylalanine in the vaccine virus is changed to serine in the vaccine-derived isolates) is a radical change (Grantham, 1974), which increases the probability that it might affect protein structure and function. Furthermore, the parental strain, VR2332, and all the vaccine revertants can replicate in both MARC cells and primary porcine alveolar macrophages. In contrast, the attenuated vaccine virus has lost its ability to replicate in the natural target cell, the alveolar macrophage (Botner et al., 1999). This observed difference in host-cell specificity could potentially be explained by, for example, amino acid differences in viral non-structural proteins that interact with different cellular partners in pigs and monkeys.

Besides the two parallel ORF1 reversions described above, an additional reversion situated in nsp2 (codon position 668) has been described in the 16244B isolate as a potential genetic determinant for attenuation/virulence (Allende et al., 2000). However, this reversion was not observed in any of the Danish vaccine-derived field isolates. It is probably a random and non-essential point mutation in the 16244B isolate. Allende et al. (2000) also suggested an additional ORF1 reversion, at codon position 952. However, we did not find any difference between the VR2332 and vaccine virus sequences at this site. Allende et al. (2000) cloned their PCR amplicons from the vaccine virus
prior to sequencing and their result might therefore reflect a PCR-generated mutation or the quasispecies nature of the vaccine virus.

Parallel reversions have been identified previously in ORF5 and ORF6 (Madsen et al., 1998; Storgaard et al., 1999) and ORF3 (Madsen et al., 1998). However, neither the 16244B isolate (Allende et al., 1999) nor the three vaccine virus revertants sequenced in the present study could confirm the ORF3 reversion. Most likely, the reported ORF3 reversion was an artifact due to the investigation of isolates that were epidemiologically linked (Madsen et al., 1998). The recent vaccine virus sequencing by Allende et al. (2000) identified two nucleotide differences in ORF2 (confirmed by us) when compared with the vaccine virus sequence published previously by Madsen et al. (1998). These nucleotide differences resulted in two additional parallel reversions, which were both confirmed in the three isolates sequenced in the present study. However, only one of the parallel reversions in ORF2 was observed in the 16244B isolate (Allende et al., 1999). Thus, the combined data from this and previous studies have identified six parallel reversions in the complete vaccine virus genome. All six reversions might have happened de novo and independently due to strong parallel selective pressure. Alternatively, the same strong parallel selective pressure might also have resulted in selection of a trace amount of VR2332-like pathogenic virus potentially remaining in the vaccine formulation. Such trace amounts of VR2332-like virus might be expected if the vaccine virus was not plaque-purified during the original attenuation process. In such a scenario, the parallel selection may have operated on a single parallel site and all other parallel sites could merely be the result of genetic linkage. On the other hand, a very high level of recombination is in effect during PRRSV replication (Yuan et al., 1999), which would effectively break any linkage over longer distances. It seems most likely, therefore, that positive selection has been involved in each of the parallel mutations observed.

A possible role of the parallel mutations

Sequencing several vaccine virus revertants enabled us to identify mutations that were identical between the field isolates but differed from the vaccine virus and the VR2332 isolate. Two such parallel mutations (codon positions 1505 and 2052) were identified in six or seven of seven Danish vaccine-derived field isolates. The parallel mutation at codon position 1505, but not that at codon position 2052, is also present in the 16244B isolate (Allende et al., 2000). The parallel mutation at codon position 2052 is unique in the sense that it does not affect the amino acid sequence of ORF1. Such synonymous changes could play a critical role by, for example, affecting the binding of viral or cellular proteins to the viral RNA genome. Both parallel mutations might therefore be explained as second-site compensatory mutations (Crill et al., 2000) for one or more of the mutations that accumulated in the vaccine virus ORF1 sequence during cell-culture attenuation. Potentially, such parallel mutations in ORF1 might even compensate for the mutations previously identified in the sequence of the vaccine virus ORF2–ORF7 (Madsen et al., 1998; Storgaard et al., 1999).

Mutations in the virus termini

As seen previously for the 5'-leader sequence (Oleksiewicz et al., 1999), no mutations were identified in the 3'-NTR of the sequenced vaccine-derived field isolates. Both the 5'-leader and the 3'-NTR are relatively well-conserved and are probably critically involved in viral RNA replication. Studies of poliovirus vaccine and of a vaccine against Venezuelan equine encephalitis have identified mutations in the 5'-leader as important determinants for the attenuation of the vaccines (Minor et al., 1990; Chumakov et al., 1992; Kinney et al., 1993). However, in PRRSV, these well-conserved termini might be too critical for virus replication and mutations may therefore not be tolerated during attenuation in cell culture.

Implications for vaccine development

In addition to the selective pressure at the four or five parallel ORF1 sites, selective pressure on the complete ORF1 was investigated by estimating the dN/dS ratios by using a maximum-likelihood model. Much stronger selective pressure for amino acid conservation was found during spread of the vaccine virus in pigs (dN/dS = 0.3) than during attenuation of the parental VR2332 strain (dN/dS = 0.9).

The live PRRS vaccine was produced by extended passage of the pathogenic VR2332 isolate on MARC-145 cells. This attenuation is expected to result in strong selective pressure for amino acid changes that make the virus more fit for replication in monkey cells. This adaptation will probably happen during the first few passages and is probably restricted to very few positions in the virus genome. During the many subsequent passages in cell culture, multiple, predominately random, mutations may accumulate, especially in regions of the genome with no function in monkey cells. The very high dN/dS ratio of 0.9 in effect during the attenuation may therefore most likely be explained by a lack of selective pressure to conserve amino acids in cell culture, instead of being seen as direct selection for amino acid changes. However, during spread in pigs, strong selective pressure for amino acid conservation will be present on the majority of the amino acids, since these are probably critical for virus replication and spread in pigs. Strong selective pressure for change was probably restricted to the few positions where parallel mutations were observed, resulting in the overall relatively low dN/dS ratio of 0.3 during virus spread in pigs.

In total, seven to nine mutations have been identified in the complete genomic sequence of the PRRS vaccine virus as potentially involved in attenuation and the subsequent re-
version to virulence. However, to determine the precise contribution of each of these mutations, individually or in combination, construction of an infectious clone of the PRRS vaccine virus will be needed. This work is currently in progress.

Inge Nielsen and Preben Normann are thanked for their excellent technical assistance. This study was partly supported by a grant from the Foundation of Norma and Frode Jacobsen and by a grant (960926) from the Danish Agricultural and Veterinary Research council to T.S.

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Received 11 September 2000; Accepted 23 February 2001